Citofluorimetria

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MULTICOLOR ANALYSIS OF HEMATOLOGIC MALIGNANCIES

Mature lymphoid neoplasms

- Neoplasms of mature lymphoid cells include
  - the chronic leukemia lymphoid neoplasms
  - non-Hodgkin lymphomas.

- This group of diseases is recognized by
  - an immunophenotype that is similar to normal mature lymphoid cells (eg, surface immunoglobulin on mature B cells)
  - lack of antigenic features of immaturity, such as expression of TdT, CD34, or weak intensity staining for CD45.

- Through identification of lineage-associated antigens, neoplasms of mature lymphoid cells can be divided into those of
  - B-cell lineage,
  - T-cell lineage
  - NK-cell lineage.

Mature B-cell lymphoid neoplasms

- FC immunophenotyping studies are indispensable for the diagnosis of mature B-cell lymphoid neoplasms through the identification of
  - phenotypically abnormal cells belonging to the B-cell lineage
  - recognition of phenotypes characteristic of separate disease entities.

- FC can also be used to
  1. identify expression of targets for potential antibody-directed therapy (i.e. CD20)
  2. provide some additional prognostic information such as CD38 and ZAP-70 in (CLL/SLL).
  3. To evaluate, following therapy, minimal residual disease.

Reagents of clinical utility in the evaluation of mature B-cell lymphoid neoplasms

initial evaluation

Reagent	Normal distribution of staining	Clinical utility in mature B-cell lymphoid malignancy	Comments
CD5	T cells and minor B-cell subset.	Expression on B cells: CLL, MCL.	-
CD10	Immature T cells and B cells, subset of mature T cells and B cells, and neutrophils.	Germinal center–like phenotype: FL, DLBCL, BL. Frequently in ALL.	-
CD19	All B cells, including lymphoblasts, mature B lymphoid cells, and most plasma cells.	Indicates B-cell lineage. May demonstrate abnormal intensity in B-cell neoplasms. Usually absent in PC neoplasms.	Aberrant expression on myeloid cells in AML or MDS.
CD20	Acquired during maturation of precursor B cells (hematogones). Mature B-lymphoid cells positive. Absent on most BM PC. Minor T-cell subset.	Supports B-cell lineage. Intensity often differs between subtypes: CLL/SLL dim, FL brighter. Aberrant expression on ALL or PCN.	Present on T-cell lymphoid neoplasms.
CD45	All B cells (weaker intensity on precursors and PC), all T cells (weaker intensity on precursors).	Useful in distinguishing mature lymphoid neoplasms (bright intensity) from ALL and PCN (weak intensity to negative).	-
sK and sλ	Mature B cells.	Ig light chain restriction.	-

Craig FE, Foon KA. Blood 2008;111:3941-3967

Reagents of clinical utility in the evaluation of mature B-cell lymphoid neoplasms

secondary evaluation

Reagent	Normal distribution of staining	Clinical utility in mature B-cell lymphoid malignancy	Comments
CD9	Precursor B cells, activated T cells, platelets.	Precursor B-cell ALL.	—
CD11c	Some B cells, some T cells.	Hairy cell leukemia CD11c (+ br.)	Frequent weaker expression on CLL, MCL and others.
CD15	Myeloid and monocytic cells.	May be aberrantly expressed in B-cell neoplasia.	More frequently seen in ALL than in mature neoplasm.
CD22	Cytoplasmic expression in early B cells. Surface expression acquired during maturation of precursor B cells.	Indicates B-cell lineage in ALL and mature lymphoid neoplasms. Intensity often differs between subtypes of mature B-cell neoplasm: CLL/SLL dim.	Cross reactivity of some clones with monocytes and basophils.
CD23	Weak intensity expression on resting B cells and increased with activation.	Distinguish CD5+B-cell lymphoid neoplasms: CLL/SLL (+ br).	-
CD25	Activated B cells and T cells.	Hairy cell leukemia in combination with CD11c and CD103.	-

Craig FE, Foon KA. Blood 2008;111:3941-3967

Reagents of clinical utility in the evaluation of mature B-cell lymphoid neoplasms

secondary evaluation

Reagent	Normal distribution of staining	Clinical utility in mature B-cell lymphoid malignancy	Comments
CD79a & b	Cyt staining in precursor B cells, PCs positive, variable expression mature B cells.	Indicates B-cell lineage in ALL and mature lymphoid neoplasms. Intensity often differs between subtypes of mature B-cell neoplasm: CLL/SLL dim CD79b.	CD79a staining has been reported in some T-ALL and rare mature T-cell lymphoid neoplasms
CD103	B-cell subset, intramucosal T cells.	Hairy cell leukemia and some MZL.	Also EATCL.
FMC7	B cells.	Distinguish CD5 lymphoid neoplasm: CLL, MCL often positive. Also HCL.	
Bcl-2	T cells, some B cells; negative normal germinal center cells.	Distinguish CD10 lymphoid neoplasms: FL, BL.	Variable staining in DLBCL.
cyK and cyl	Plasma cells.	Light chain restriction in cells with plasmacyticdifferentiation.	Most FC assays detect sIg and cyIg.
Zap-70	T cells, NK cells, precursor B cells.		
TdT	B-cell and T-cell precursors.	ALL	Also some AML
cIgM	First Ig component in precursor B cells. Expressed by subset of PC and mature B cells.	IgM producing neoplasms that might be associated with Waldenstrom macroglobulinemia	

Craig FE, Foon KA. Blood 2008;111:3941-3967

Identification of abnormal mature B-lymphoid cells

- **Neoplastic mature B-lymphoid cells can be distinguished from normal cells by the identification of 2 main types of phenotypic abnormalities:**
  - **immunoglobulin light chain class restriction**
  - **aberrant antigen expression.**

Ig light chain class restriction

- In contrast to most normal and reactive populations, neoplasms of mature B cells usually represent a single clone of cells that express only one class of Ig light chain (ie, kappa or lambda).

- It should not be assumed that Ig light chain class restriction is synonymous with monoclonality or is by itself diagnostic of neoplasia.

- The results of FC immunophenotyping should be interpreted in conjunction with other clinical, morphologic, and sometimes genotypic data.

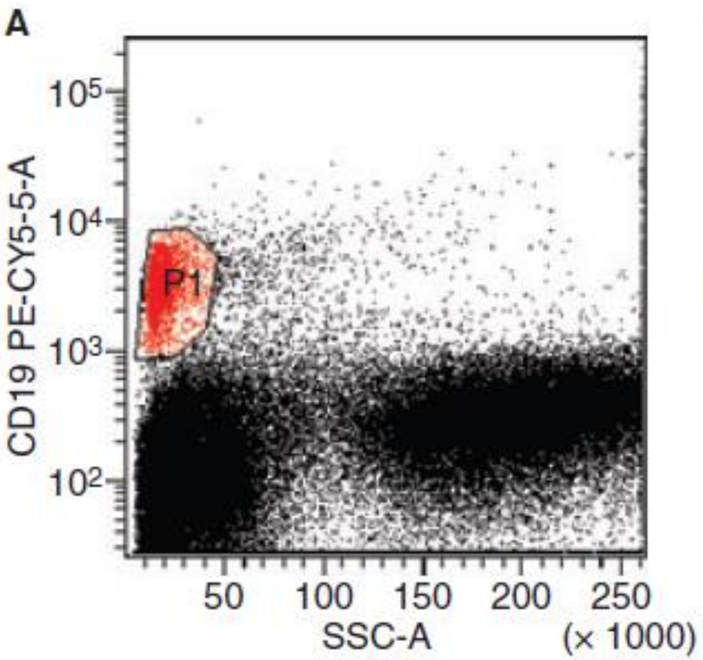
B-Cell Lymphoproliferative Disorders

- **Normal/reactive B-cells in PB, BM, and lymphatic tissue are polyclonal with an average k/l ratio of 1.5 (range 0.9–3).**
  - **An increase of polyclonal B-cells in blood, called the persistent polyclonal B-cell lymphocytosis (PPBL) is characterized by a chronic, stable, persistent, and polyclonal increase of B-cells (median 5 × 109/L), the presence of binucleated lymphocytes in the PB, and a polyclonal increase in serum immunoglobulin-M (IgM).**
  - **Most patients are asymptomatic but isochromosome 3q and development of malignant lymphoma has been described in some cases.**

- **B-cell malignancies are clonal expansions of B-cells that express only one type of Ig light chain (k or l).**

- **Analysis of light chain expression in total B-cell population and in CD5/CD19 or CD10/CD19 positive cells forms the basis for B-cell lymphoma diagnosis.**

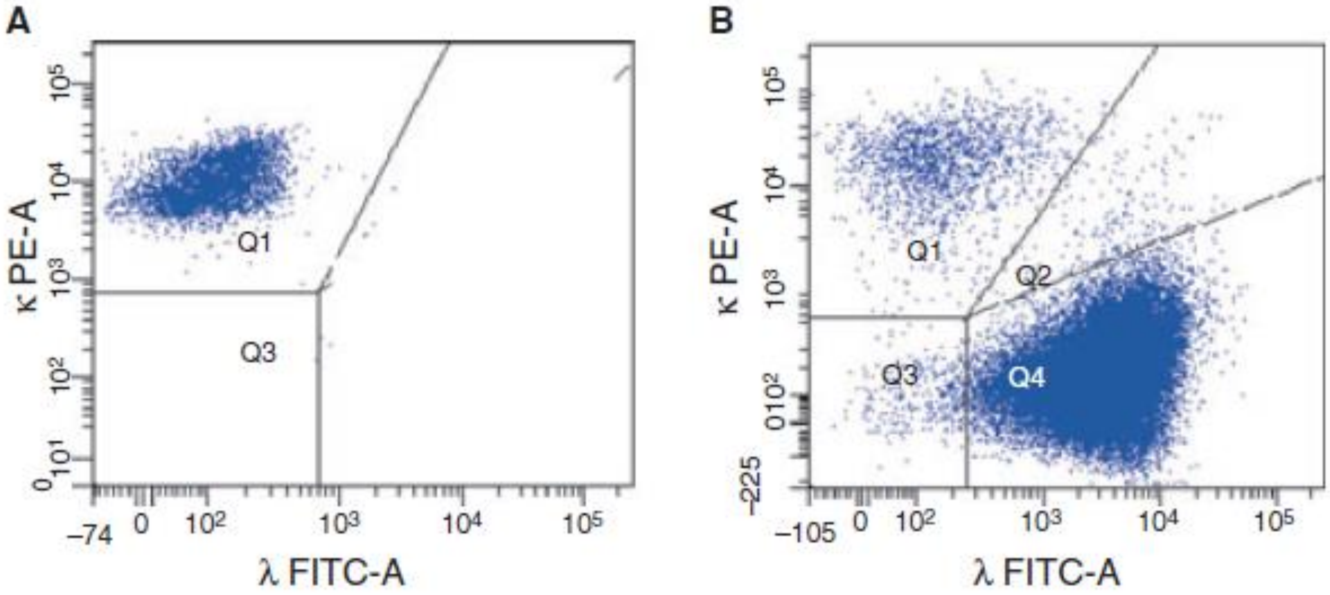
B-cells identified by gating the CD19+ events



CD19 is expressed at all stages of B-cell development from progenitor to plasma cell. Plot B shows that the gated cells do indeed consist of a mixture of Kappa positive and Lambda positive mature B-cells and surface immunoglobulin negative B-cell progenitors.

De Tute RM. Histopathology 2011, 58, 90–105

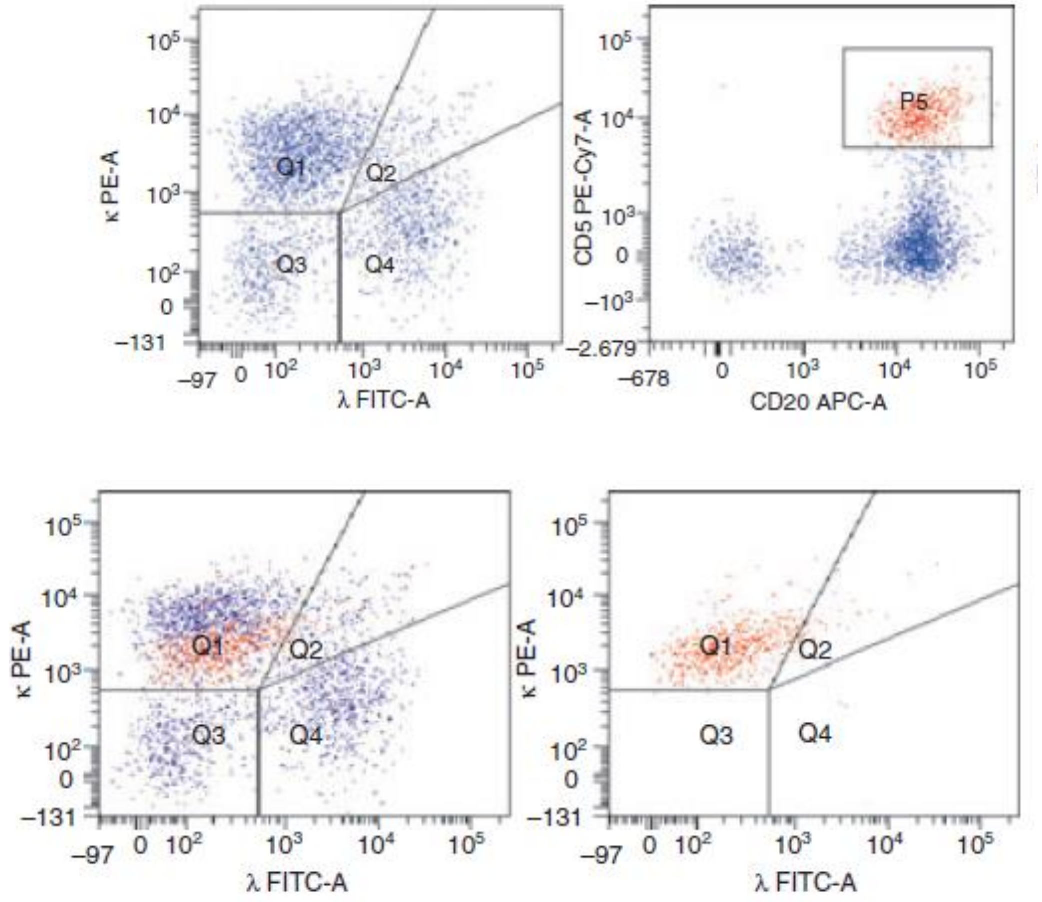
Monoclonal populations in samples of lymphomas



Plot A: a kappa positive neoplasm and no normal B-cells remain.
Plot B: although there are some normal polyclonal cells still present, there is still an obvious lambda positive population.

De Tute RM. Histopathology 2011, 58, 90–105

Small monoclonal populations can be hidden in a normal polyclonal background.



De Tute RM. Histopathology 2011, 58, 90–105

Ig light chain class restriction

- Interpretation of staining for kappa and lambda Ig light chains can be made more difficult by the presence of nonspecific staining.
  - Nonspecific (cytophilic) binding of antibodies can occur through association with Fc receptors and adherence of antibody to “sticky” cells, including damaged or dying cells.
- Binding of antibodies to non–B cells can be excluded by evaluating only cells that express one or more B-lineage–associated antigens:
  - for example, by gating on CD19 or CD20 cells.
- Nonspecific staining can also be minimized by incubation of cells with a blocking reagent such as immune sera prior to staining with anti–light chain antibodies.
- Blocking can be used if nonspecific staining is encountered using conventional staining techniques or in situations where nonspecific staining is frequently encountered, for instance in the evaluation for hairy cell leukemia (HCL).

Ig light chain class restriction

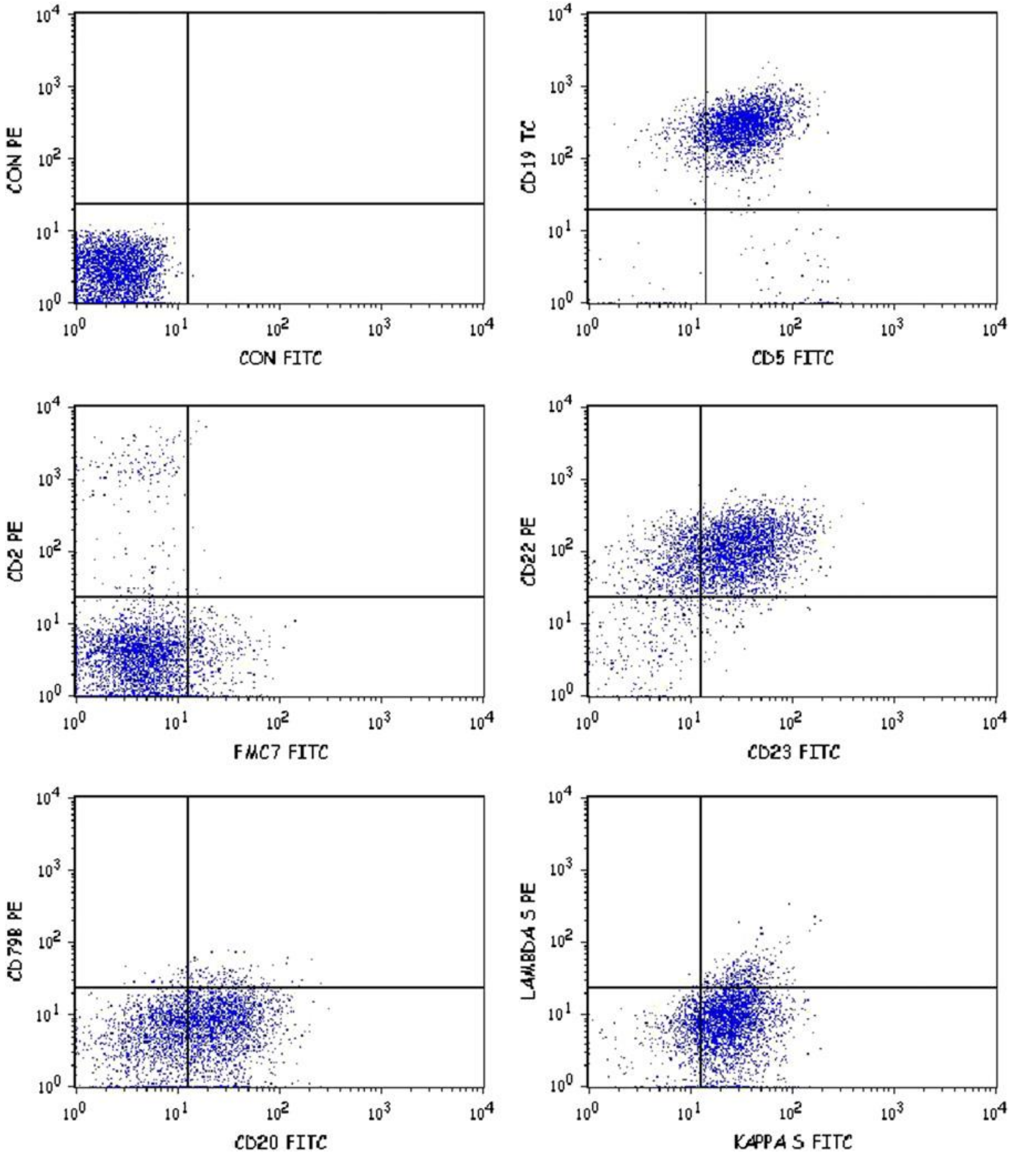
- **Another issue in the FC laboratory is apparent lack of staining for sIg.**

- **To avoid false-negative results due to soluble antibody interfering with the binding of detection antibody, it is important to include an initial wash step for Fc tubes containing anti-Ig antibodies.**

B-cell Chronic Lymphatic Leukemia

- **The characteristic immunophenotype of chronic lymphatic leukemia (CLL) includes positivity for CD19, CD5, CD23, and CD200, weak expression of CD20 and Ig light chains, and often expression of IgM with or without IgD.**
- **FMC7 (antibody recognizing one of CD20 epitopes) is negative or only partially expressed in most cases; CD79b and CD22 are absent or weakly expressed in the cell membrane.**
- **CD11c, CD25, and other markers that recognize adhesion molecules are variably positive in CLL.**

CLL



Matutes et al Best Practice & Research Clinical Haematology 20, 367–384, 2007

B-Cell Lymphoproliferative Disorders

- An issue that may cause diagnostic problems is the demonstration of small monoclonal B-cell populations in the BM samples taken during investigations for staging of lymphoma.

- As FCM sensitivity increases, it becomes more likely that small abnormal populations are detected; how these relate to the neoplastic cells found in other organs is not clear. In some cases a clonal relationship to the diagnostic lymphoma sample has been demonstrated.

- However, if the histopathologic signs of lymphoma involvement are missing, these cells may represent sc. monoclonal B-cell lymphocytosis (MBL).

Monoclonal B-cell lymphocytosis

- **Chronic lymphocytic leukemia (CLL) and the other low-grade non-Hodgkin lymphomas are among the most common lymphoid malignancies.**
- **Recent studies suggest that more than 4% of the general population over age 40 harbor a population of clonal B cells with the phenotype of either CLL or another B-cell malignancy, a condition now designated monoclonal B-cell lymphocytosis (MBL).**
- **Although all cases of CLL appear to be preceded by MBL, the majority of individuals with MBL will not develop a hematologic malignancy.**
- **The biologic characteristics and clinical implications of MBL appear to differ based on whether it is identified during the diagnostic evaluation of lymphocytosis or incidentally discovered through screening of individuals with normal lymphocyte counts as part of research studies using highly sensitive detection methods.**

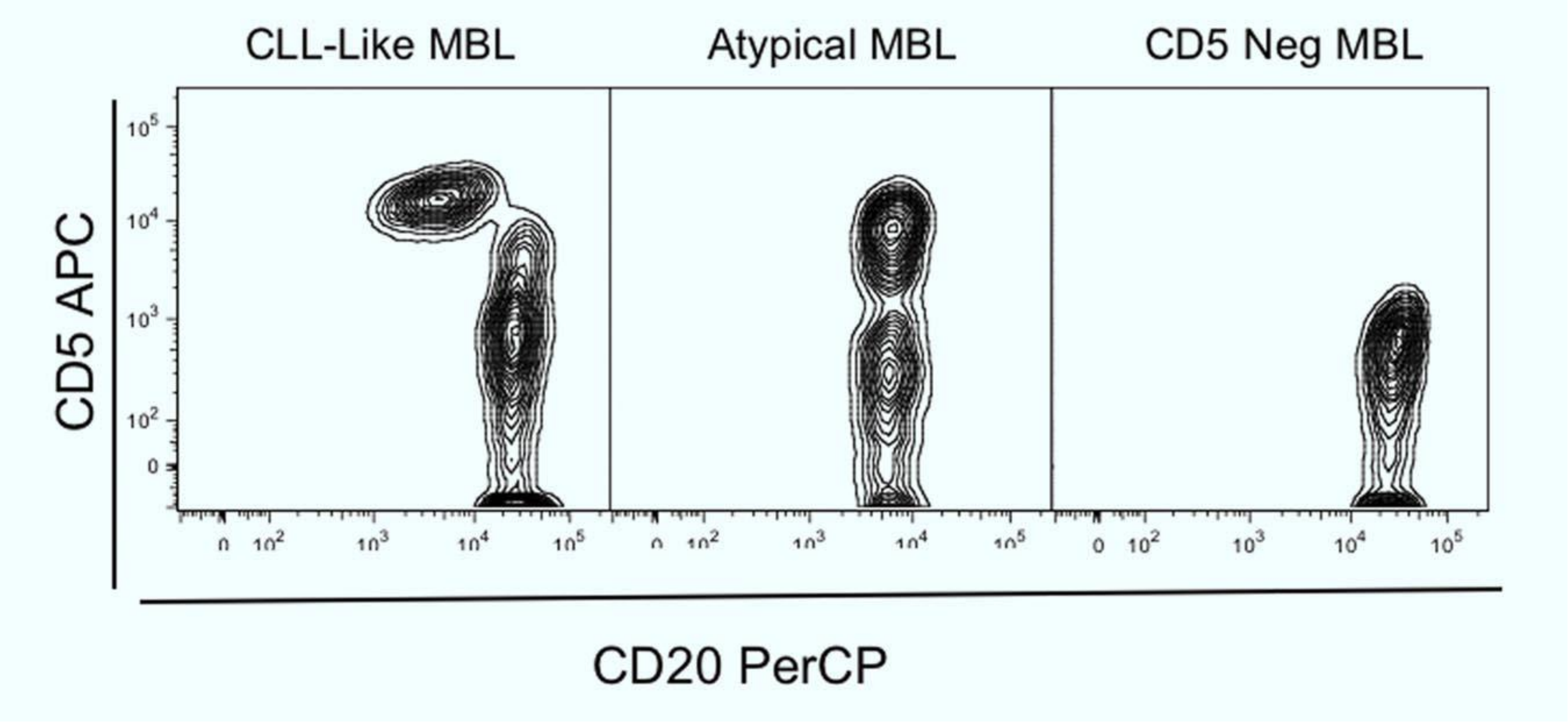
Monoclonal B-cell Lymphocytosis

- Monoclonal B-cell lymphocytosis (MBL) is an asymptomatic hematologic condition defined by the presence of monoclonal B-lymphocytes detected in PB of persons who do not have CLL, other B-lymphoproliferative disorders, or underlying conditions such as infectious and autoimmune diseases.

- Initial criteria have been based on detection of a monoclonal B-cell population in the PB with an overall k:l ratio >3:1 or 0.3:1, or >25% of B-cells lacking or expressing low-level surface Ig in conjunction with a specific phenotype.

- Three different types of MBL have been described, defined on the basis of CD19 positivity, CD5 presence or absence, and CD20 intensity.
  - The most common MBL type is the CLL-like MBL that co-express CD19 and CD5, and CD23 with dim expression of CD20.
  - The second type is similar to CLL but shows bright CD20 expression.
  - B-cells in the third type of MBL do not express CD5; these are classified as CD5-MBL or non-CLL-like MBL.

Role of absolute B-cell count in MBL and immunophenotype patterns



The 3 major immunophenotypic patterns seen in MBL.

Blood 2011;118:6480

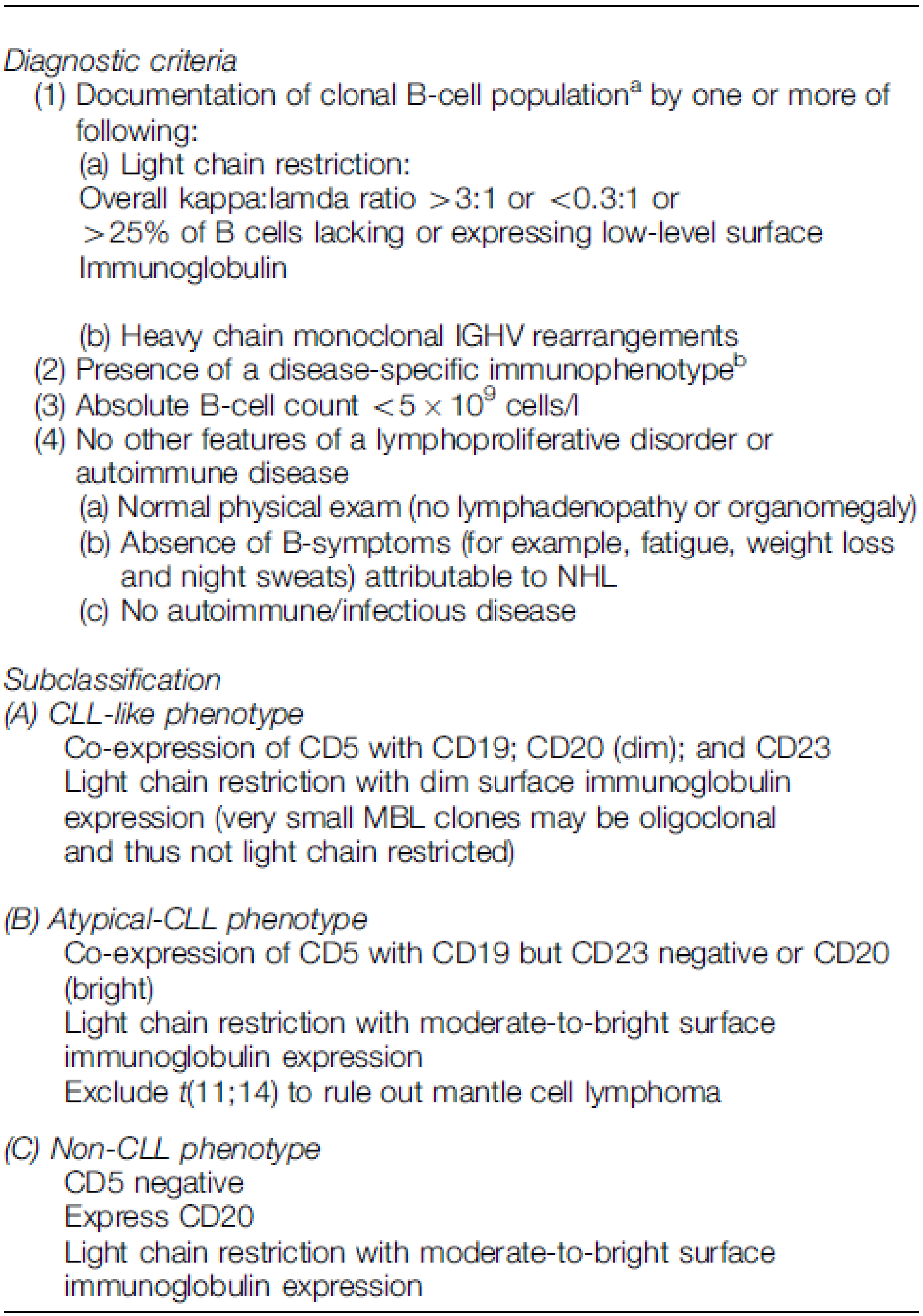
Monoclonal B-cell Lymphocytosis

- Studies performed using four-color FCM with a sensitivity of detection commonly used for detection of MRD in patients with CLL (1 clonal cell per 1 × 10^5 events) showed a 5% prevalence of CLL-like MBL in adults aged over 60.

- A more recent study, using a much higher sensitivity of FCM, analyzed 5 × 10^6 PB cells per individual and identified CLL-like MBL in 12% of all tested subjects and in 20% of adults over 60 years old.

- Finding of peripheral MBL should always be correlated with clinical data and interpreted in the absence of peripheral lymphadenopathy, splenomegaly, and extensive lymphatic bone marrow infiltrates.

MBL



Diagnostic criteria

(1) Documentation of clonal B-cell population[a] by one or more of following:
  - (a) Light chain restriction:
    Overall kappa:lamda ratio $>3:1$ or $<0.3:1$ or
    $>25\%$ of B cells lacking or expressing low-level surface Immunoglobulin
  - (b) Heavy chain monoclonal IGHV rearrangements

(2) Presence of a disease-specific immunophenotype[b]

(3) Absolute B-cell count $<5\times10^{9}$ cells/l

(4) No other features of a lymphoproliferative disorder or autoimmune disease
  - (a) Normal physical exam (no lymphadenopathy or organomegaly)
  - (b) Absence of B-symptoms (for example, fatigue, weight loss and night sweats) attributable to NHL
  - (c) No autoimmune/infectious disease

Subclassification

(A) CLL-like phenotype

Co-expression of CD5 with CD19; CD20 (dim); and CD23
Light chain restriction with dim surface immunoglobulin expression (very small MBL clones may be oligoclonal and thus not light chain restricted)

(B) Atypical-CLL phenotype

Co-expression of CD5 with CD19 but CD23 negative or CD20 (bright)
Light chain restriction with moderate-to-bright surface immunoglobulin expression
Exclude *t*(11;14) to rule out mantle cell lymphoma

(C) Non-CLL phenotype

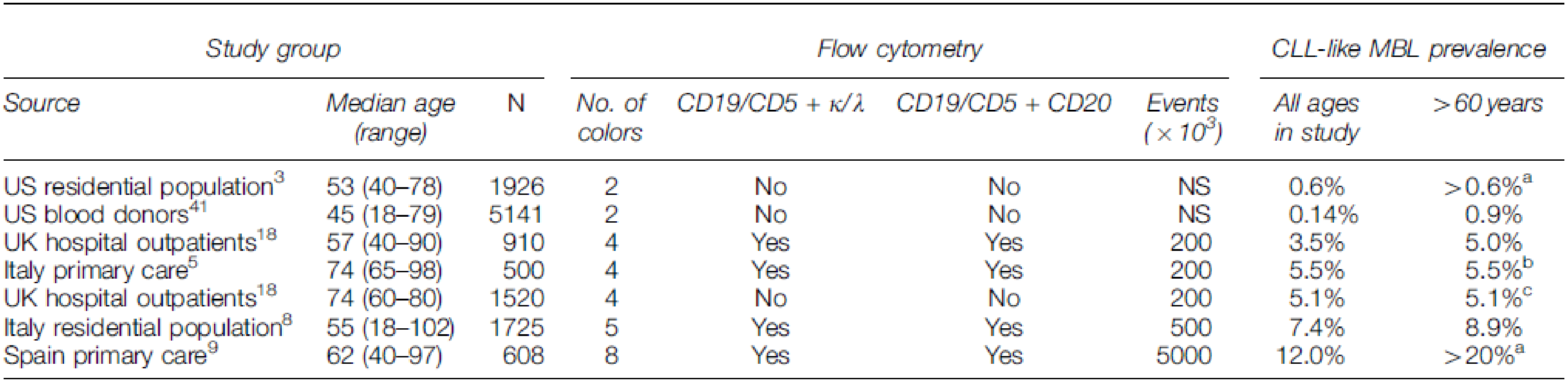
CD5 negative
Express CD20
Light chain restriction with moderate-to-bright surface immunoglobulin expression

a. **Where possible, repeat assessment should show the monoclonal B-cell population is stable over 3-month period.**

b. **In the absence of a disease-specific immunophenotype, a highly skewed kappa:lamda can be the result of a reactive process.**

TD Shanafelt, Leukemia (2010) 24, 512–520

Prevalence of CLL-like MBL in population studies



Study group			*Flow cytometry*				*CLL-like MBL prevalence*	
Source	*Median age (range)*	*N*	*No. of colors*	*CD19/CD5 + κ/λ*	*CD19/CD5 + CD20*	*Events ($\times 10^3$)*	*All ages in study*	*> 60 years*
US residential population[3]	53 (40–78)	1926	2	No	No	NS	0.6%	>0.6%[a]
US blood donors[41]	45 (18–79)	5141	2	No	No	NS	0.14%	0.9%
UK hospital outpatients[18]	57 (40–90)	910	4	Yes	Yes	200	3.5%	5.0%
Italy primary care[5]	74 (65–98)	500	4	Yes	Yes	200	5.5%	5.5%[b]
UK hospital outpatients[18]	74 (60–80)	1520	4	No	No	200	5.1%	5.1%[c]
Italy residential population[8]	55 (18–102)	1725	5	Yes	Yes	500	7.4%	8.9%
Spain primary care[9]	62 (40–97)	608	8	Yes	Yes	5000	12.0%	>20%[a]

from a biological point of view,
MBL detected in clinical practice **('clinical MBL')** may be more similar to frank CLL than MBL detected on population screening **('population screening MBL').**

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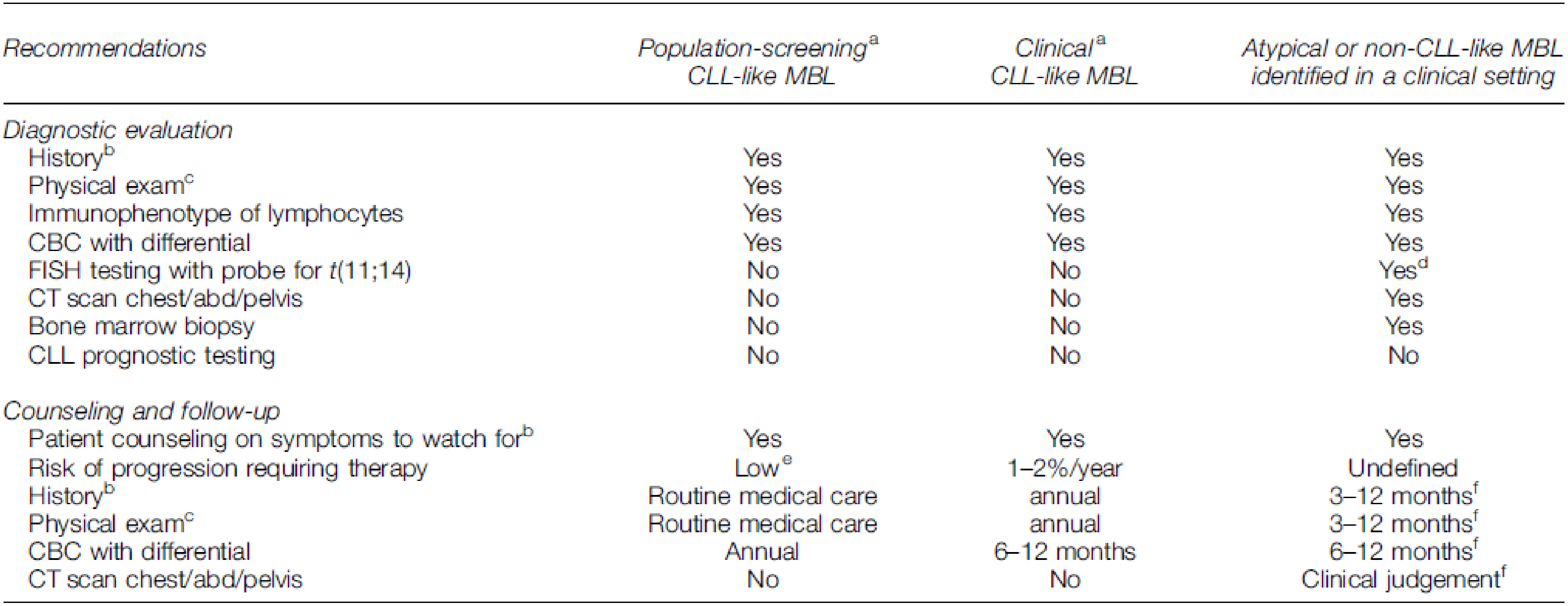
CLL and clinical MBL

- In spite of the similarities between CLL and clinical MBL, several biologic differences between clinical MBL and population-screening MBL have been identified.

  - First, although MBL was originally thought to represent exclusively monoclonal lymphocytes, it has become evident that biclonal, oligoclonal and polyclonal cases of MBL can be identified.
    - a CLL-like phenotype (CD5+, CD20dim) is not necessarily related to the acquisition of monoclonality.

  - Concomitant clones of CLL-like B lymphocytes appear and persist in many otherwise healthy individuals in which one clone may expand and, in a small subset of individuals, become predominant with time.

Recommendations for evaluation and follow-up of MBL in routine practice



Recommendations	*Population-screening[a]* *CLL-like MBL*	*Clinical[a]* *CLL-like MBL*	*Atypical or non-CLL-like MBL* *identified in a clinical setting*
Diagnostic evaluation			
History[b]	Yes	Yes	Yes
Physical exam[c]	Yes	Yes	Yes
Immunophenotype of lymphocytes	Yes	Yes	Yes
CBC with differential	Yes	Yes	Yes
FISH testing with probe for *t*(11;14)	No	No	Yes[d]
CT scan chest/abd/pelvis	No	No	Yes
Bone marrow biopsy	No	No	Yes
CLL prognostic testing	No	No	No
Counseling and follow-up			
Patient counseling on symptoms to watch for[b]	Yes	Yes	Yes
Risk of progression requiring therapy	Low[e]	1–2%/year	Undefined
History[b]	Routine medical care	annual	3–12 months[f]
Physical exam[c]	Routine medical care	annual	3–12 months[f]
CBC with differential	Annual	6–12 months	6–12 months[f]
CT scan chest/abd/pelvis	No	No	Clinical judgement[f]

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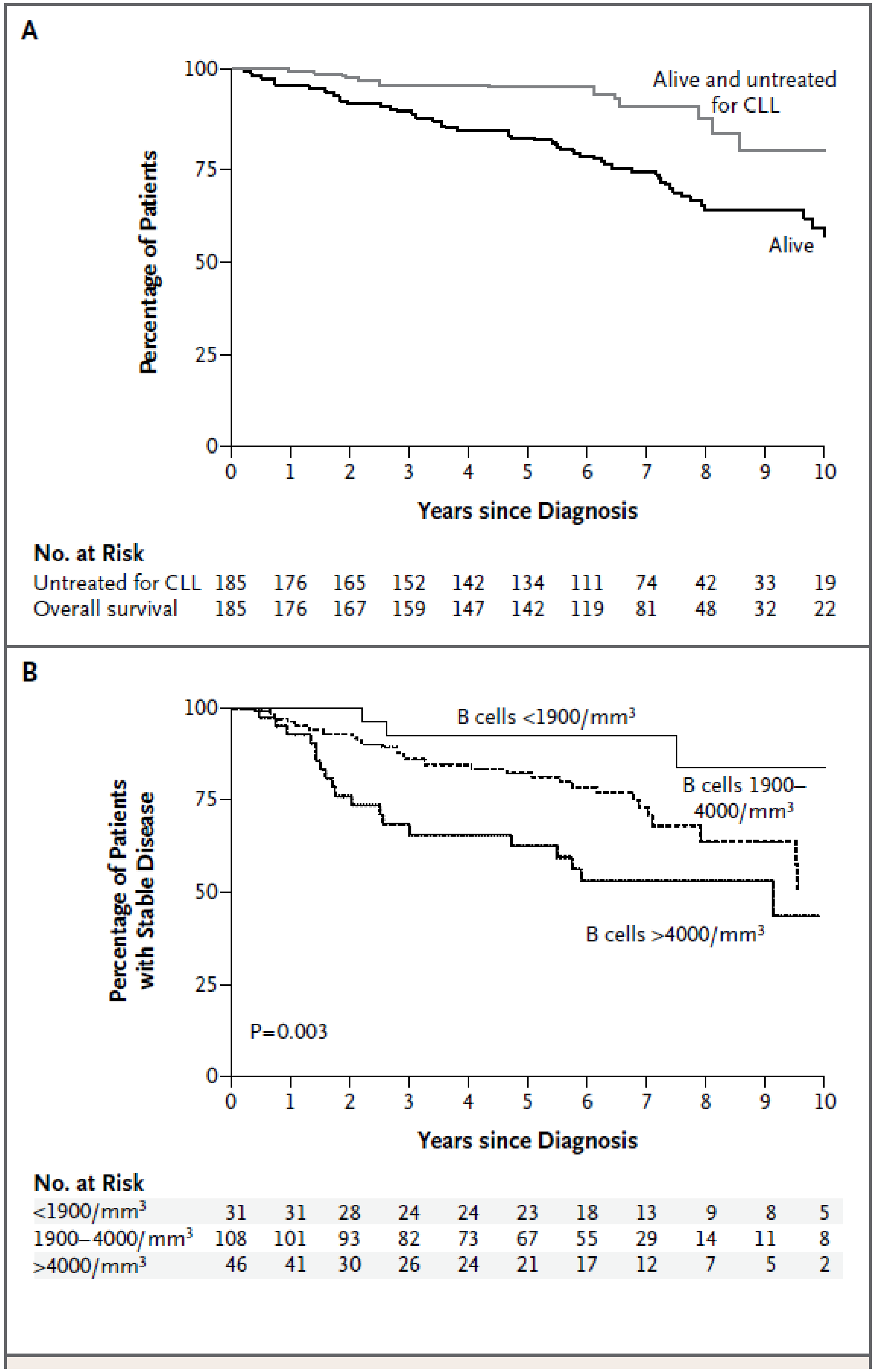


No. at Risk

	0	1	2	3	4	5	6	7	8	9	10
Untreated for CLL	185	176	165	152	142	134	111	74	42	33	19
Overall survival	185	176	167	159	147	142	119	81	48	32	22

No. at Risk

	0	1	2	3	4	5	6	7	8	9	10
<1900/mm³	31	31	28	24	24	23	18	13	9	8	5
1900–4000/mm³	108	101	93	82	73	67	55	29	14	11	8
>4000/mm³	46	41	30	26	24	21	17	12	7	5	2

Figure 1. Kaplan–Meier Estimates of Outcomes among Subjects with CLL-Phenotype MBL and Lymphocytosis.

Panel A shows the proportion of subjects remaining alive and the proportion who were alive and remained free from treatment for CLL. Panel B shows the proportion of subjects with stable CLL-phenotype MBL, defined as the absence of symptoms or features of CLL and the maintenance of a stable lymphocyte count (a count less than twice that at presentation).

Rawstron et al N Engl J Med 2008;359:575-83.

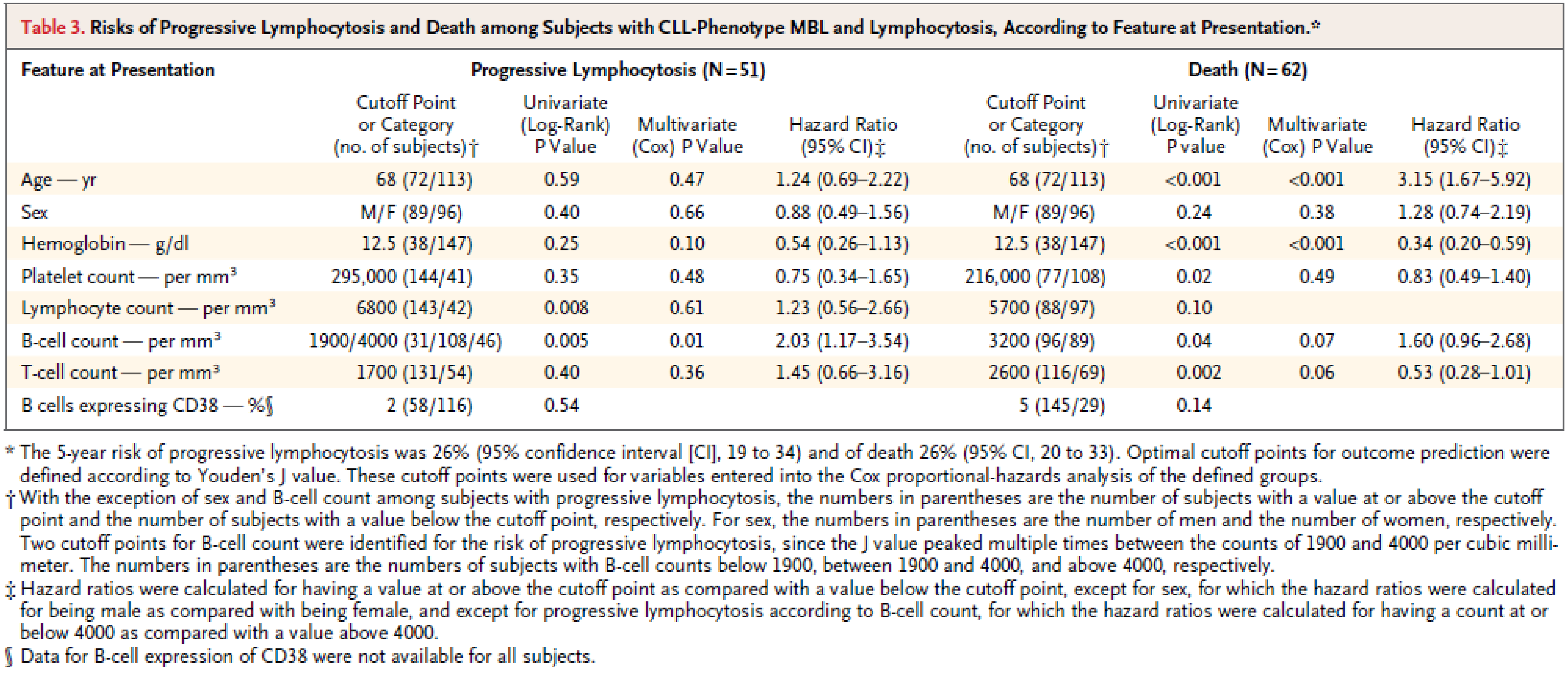


Table 3. Risks of Progressive Lymphocytosis and Death among Subjects with CLL-Phenotype MBL and Lymphocytosis, According to Feature at Presentation.*

Feature at Presentation	Progressive Lymphocytosis (N=51)				Death (N=62)			
	Cutoff Point or Category (no. of subjects)†	Univariate (Log-Rank) P Value	Multivariate (Cox) P Value	Hazard Ratio (95% CI)‡	Cutoff Point or Category (no. of subjects)†	Univariate (Log-Rank) P value	Multivariate (Cox) P Value	Hazard Ratio (95% CI)‡
Age — yr	68 (72/113)	0.59	0.47	1.24 (0.69–2.22)	68 (72/113)	<0.001	<0.001	3.15 (1.67–5.92)
Sex	M/F (89/96)	0.40	0.66	0.88 (0.49–1.56)	M/F (89/96)	0.24	0.38	1.28 (0.74–2.19)
Hemoglobin — g/dl	12.5 (38/147)	0.25	0.10	0.54 (0.26–1.13)	12.5 (38/147)	<0.001	<0.001	0.34 (0.20–0.59)
Platelet count — per mm^3	295,000 (144/41)	0.35	0.48	0.75 (0.34–1.65)	216,000 (77/108)	0.02	0.49	0.83 (0.49–1.40)
Lymphocyte count — per mm^3	6800 (143/42)	0.008	0.61	1.23 (0.56–2.66)	5700 (88/97)	0.10		
B-cell count — per mm^3	1900/4000 (31/108/46)	0.005	0.01	2.03 (1.17–3.54)	3200 (96/89)	0.04	0.07	1.60 (0.96–2.68)
T-cell count — per mm^3	1700 (131/54)	0.40	0.36	1.45 (0.66–3.16)	2600 (116/69)	0.002	0.06	0.53 (0.28–1.01)
B cells expressing CD38 — %§	2 (58/116)	0.54			5 (145/29)	0.14		

* The 5-year risk of progressive lymphocytosis was 26% (95% confidence interval [CI], 19 to 34) and of death 26% (95% CI, 20 to 33). Optimal cutoff points for outcome prediction were defined according to Youden's J value. These cutoff points were used for variables entered into the Cox proportional-hazards analysis of the defined groups.

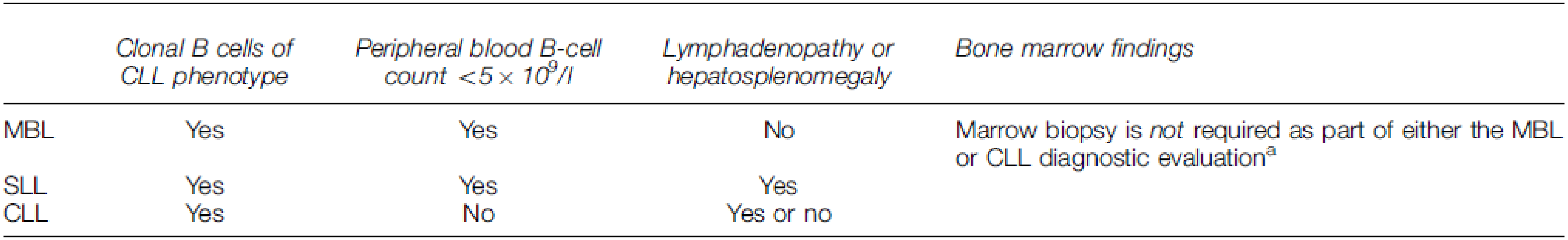
† With the exception of sex and B-cell count among subjects with progressive lymphocytosis, the numbers in parentheses are the number of subjects with a value at or above the cutoff point and the number of subjects with a value below the cutoff point, respectively. For sex, the numbers in parentheses are the number of men and the number of women, respectively. Two cutoff points for B-cell count were identified for the risk of progressive lymphocytosis, since the J value peaked multiple times between the counts of 1900 and 4000 per cubic millimeter. The numbers in parentheses are the numbers of subjects with B-cell counts below 1900, between 1900 and 4000, and above 4000, respectively.

‡ Hazard ratios were calculated for having a value at or above the cutoff point as compared with a value below the cutoff point, except for sex, for which the hazard ratios were calculated for being male as compared with being female, and except for progressive lymphocytosis according to B-cell count, for which the hazard ratios were calculated for having a count at or below 4000 as compared with a value above 4000.

§ Data for B-cell expression of CD38 were not available for all subjects.

Rawstron et al N Engl J Med 2008;359:575-83.

Distinguishing between MBL, CLL and SLL



	Clonal B cells of CLL phenotype	*Peripheral blood B-cell count $<5 \times 10^9/l$*	*Lymphadenopathy or hepatosplenomegaly*	*Bone marrow findings*
MBL	Yes	Yes	No	Marrow biopsy is *not* required as part of either the MBL or CLL diagnostic evaluation[a]
SLL	Yes	Yes	Yes	
CLL	Yes	No	Yes or no	

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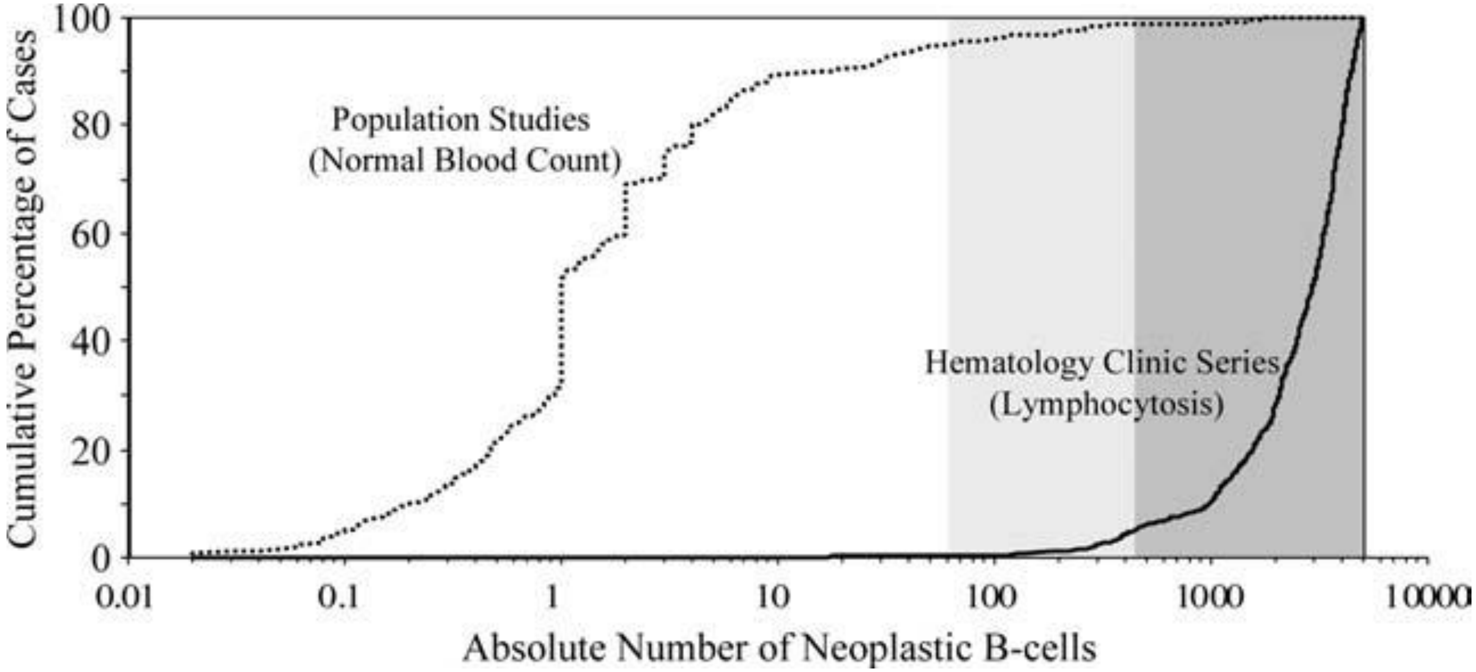
MBL and clinical MBL



The cumulative percentage of cases according to the absolute number of clonal B-cells for studies of individuals from the general population with a normal blood count (dotted line) and from series of individuals referred for clinical hematology investigations usually with a current or prior lymphocytosis (solid line).
The clonal B-cell count in CLL-type MBL shows a marked difference in cases from population studies (median 1 clonal B-cell per μL with 95% of cases having less than 56 clonal B-cells per μL highlighted by the white background) compared to clinical hematology series (median 2,939 clonal B-cells per μL with 95% of cases having more than 447 clonal B-cells per lL highlighted by the dark grey background). Very few cases from either series have a clonal B-cell count within the same range as polyclonal B-cell levels in individuals with no detectable abnormal B-cells (light grey background).

Aberrant B-cell antigen expression.

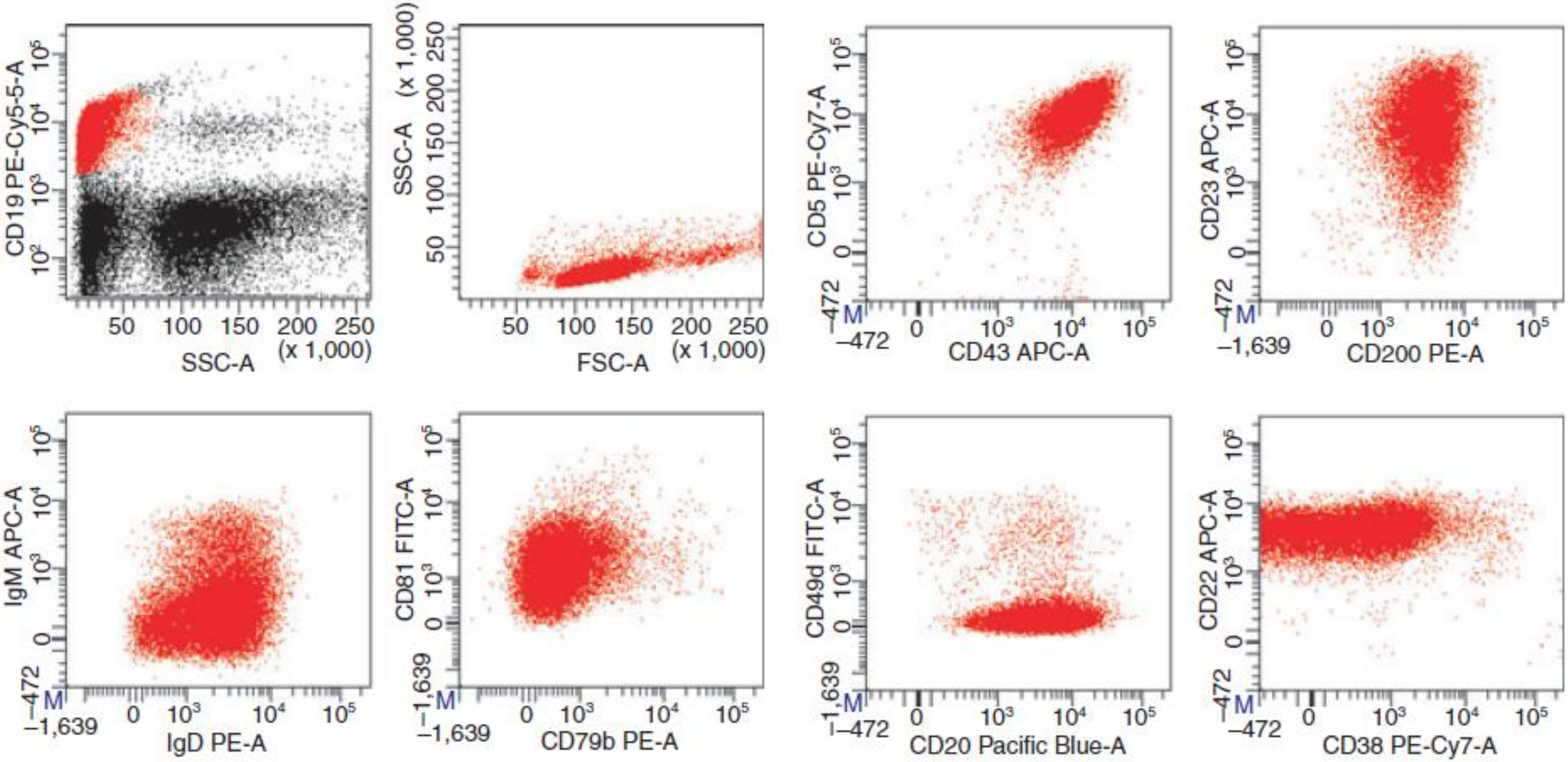
- FC immunophenotyping can be used to identify deviations from the normal pattern of B-cell antigen expression.

- One type of phenotypic aberrancy is the presence of antigens not normally expressed by B cells (eg, myeloid antigens CD13 or CD33).
    - Aberrant expression of myeloid antigens is found less frequently in mature B-cell lymphoid neoplasms than in ALL.
    - Although it has been reported infrequently in mature lymphoid neoplasm, aberrant myeloid antigen expression is perhaps most often found in lymphoplasmacytic lymphoma (LPL).

Aberrant B-cell antigen expression.

- **CD5 expression on B cells is often referred to as an aberrant phenotype, but small populations of normal, mature CD5 B cells exist.**
  - **Nonneoplastic CD5 B cells are found most often in the PB, but may also be seen in lymph node specimens, especially in pts with autoimmune disease.**
  - **CD5 expression has also been reported in a subset of normal BM B-cell precursors (hematogones).**
  - **interpretation of CD5 expression by B cells requires evaluation for other abnormalities, including Ig light chain restriction and altered intensity staining for CD20, CD22, and CD79b.**

Typical immunophenotype of CLL.



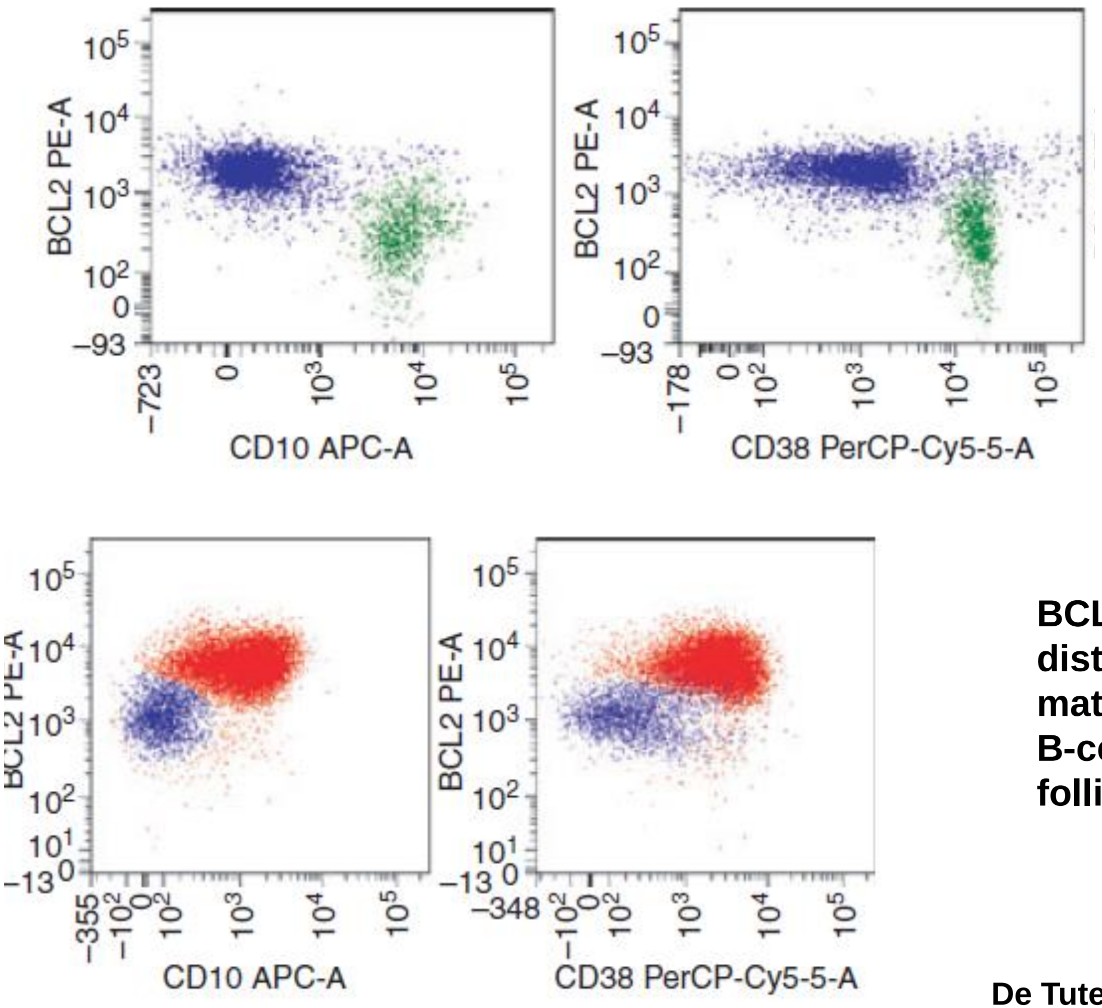
De Tute RM. Histopathology 2011, 58, 90–105

Aberrant B-cell antigen expression

- **Another type of phenotypic aberrancy is abnormal expression of antigens not typically present in a subset of B cells belonging to a distinct biologic compartment (eg, detectable bcl-2 expression on CD10 B cells).**
  - **Normal germinal center B cells and hematogones are both CD10pos and bcl-2neg, whereas bcl-2 is expressed by most other B-cell subsets.**
  - **Abnormally increased bcl-2 expression can be found in most FL, some DLBCL, and some B-lineage ALL.**
  - **Burkitt lymphoma is usually CD10+ and bcl-2-.**

- **More subtle phenotypic aberrancies include alteration in intensity of staining for B-lineage–associated antigens.**
  - **FL often demonstrates decreased intensity staining for CD19 and brighter intensity for CD10, which can help in the distinction from normal follicular germinal center cells.**

Immunophenotyping of follicular lymphoma.



BCL2 expression can be used to distinguish between normal mature B-cells (shown in blue), B-cell progenitors (in green) and follicular lymphoma cells (in red).

De Tute RM. Histopathology 2011, 58, 90–105

False-negative flow cytometric evaluation

- **Occasionally, flow cytometric evaluation fails to detect an abnormal population of B cells in a specimen involved by a B-cell lymphoid neoplasm.**

- **There are several possible explanations.**

1. ***Sampling error.***
2. ***Cell loss during processing***
3. ***Paucity of neoplastic cells.***
4. ***Difficult-to-identify cell populations***

Sampling error

- Allocation of appropriate material for flow cytometric studies is rarely an issue in liquid specimens, but becomes essential for tissue samples because the infiltrate of interest might not involve the entire specimen.

- Therefore, fresh tissue should be evaluated, such as with touch preparations, to identify representative areas to allocate for flow cytometric and other testing.

Cell loss during processing

- The frequency of cell loss during processing for flow cytometric studies varies with the type of cells present and the procedure used to process the specimen.

- Large lymphoid cells and plasma cells appear to be more easily lost during processing, particularly following manual disaggregation of tissue specimens.

- Comparison of smears or touch imprints prepared from the fresh specimen with a cytospin prepared from the cell suspension after processing can help to confirm the presence of the cells of interest.

Paucity of neoplastic cells

- Some tumors contain relatively few neoplastic cells, such as the T cell/histiocyte–rich variant of DLBCL, or include many admixed reactive B cells, such as MZL.

- Although, it is important to acquire enough events to detect small populations of abnormal cells, most clinical laboratories have not routinely acquired the 500 000 to 1 million events usually required for MRD detection, primarily because of time constraints.

- More frequently, clinical laboratories acquire 30 000 to 100 000 events with acknowledgment of the limitations of routine clinical flow cytometric testing.

Difficult-to-identify cell populations

- Populations of abnormal B cells may be present but not recognized on FC immunophenotypic studies.
- Examples of populations that are easily overlooked include
  - B cells that are negative for CD20, such as may be seen following therapy with rituximab anti-CD20 MoAb therapy,
  - B cells lacking demonstrable sIg.
- The following strategies can be used to avoid overlooking elusive populations:
  - perform a basic evaluation of all cell types present in the specimen, not just those that are CD20;
  - evaluate more than one B cell–associated antigen such as CD19, CD20, CD22, or CD79;
  - thoroughly assess all B-cell populations for phenotypic aberrancies, including cells lacking staining for sIg

FLOW CYTOMETRY IMMUNOPHENOTYPIC FEATURES OF MAJOR B-LINEAGE LYMPHOPROLIFERATIVE DISORDERS

WHO 2008 Category[a]	CD19	CD20	CD22	CD23	CD10	CD5	CD11c	CD103	CD25	CD123	sIg
CLL	+#	+d/−	d/−	+	−	+	±	−	±	−	d
HCL	+	+b	+	−	−/(+)	−	+	+	+	+	b
HCLv	+	+	+	−	−/(+)	−	±	+/(−)	−	−(+)	b
SMZL	+	+	+	−	−	−	+	−/(+)	±	−	+
MCL	+	+b	+	−/(+d)	−	+	−	−	−	−	b
FL	+	+	+	±	+	−	−	−	−	−	+
DLBCL	+	+/(−)	+	±	±	±	±	−/(+)	±	−	±
BL	+	+	+	−	+	−	−	−	−	−	+/(−)

[a] BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large cell B-lymphoma; FL, follicular lymphoma; HCL; HCLv, hairy cell leukemia (v) variant; MCL, mantle cell lymphoma; SMZL/SLVL, splenic marginal zone lymphoma, splenic lymphoma with villous lymphocytes. # + = most cases positive, − = most cases negative, ± = can be positive or negative, +/(−) = usually positive, rarely negative, −/(+) = usually negative, rarely positive, d, "dim", b, "bright."

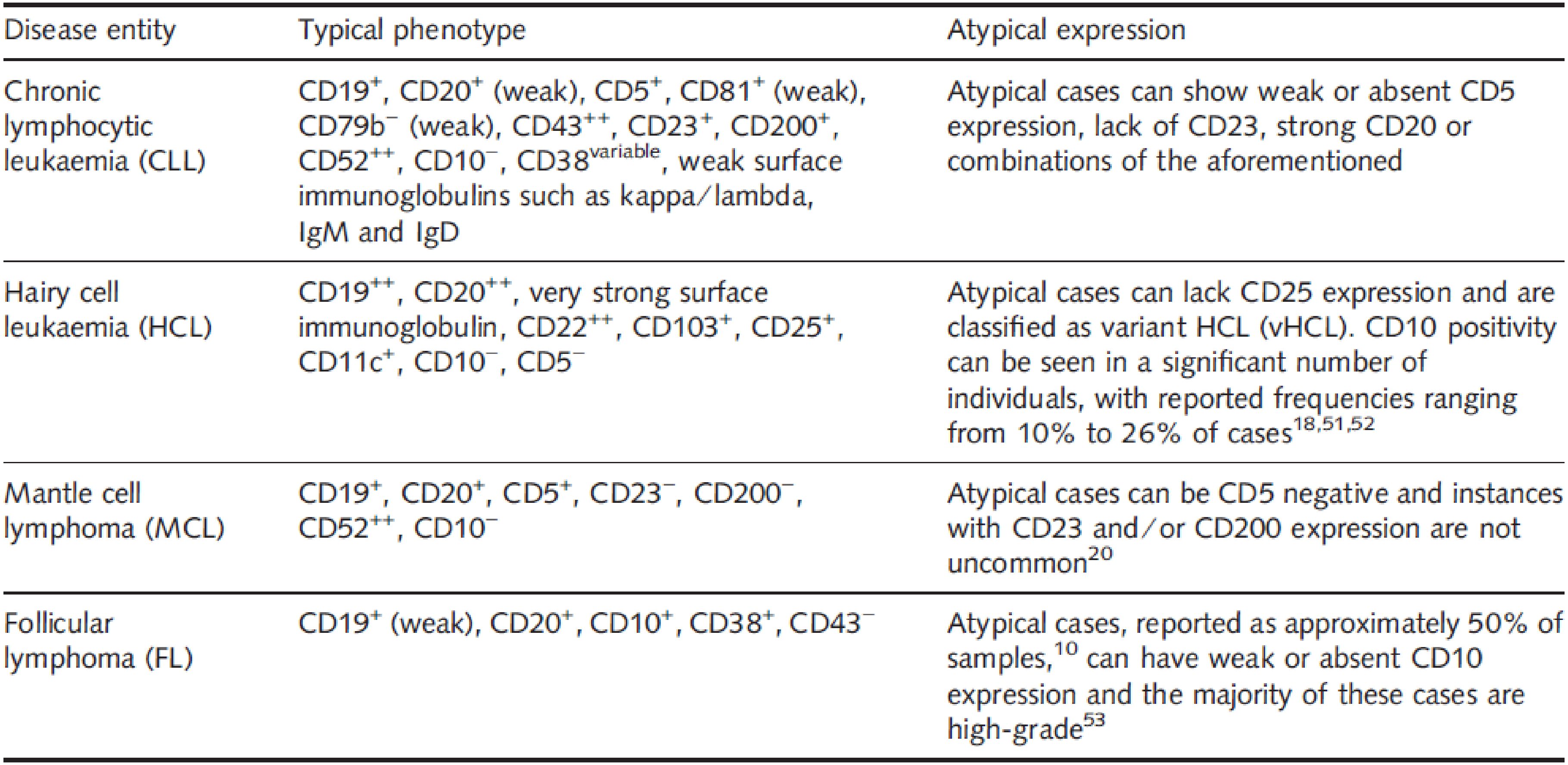
FLOW CYTOMETRY IMMUNOPHENOTYPIC FINDINGS IN MAJOR CATEGORIES OF MATURE T/NK-CELL NON-HODGKIN LEUKEMIA/LYMPHOMA

WHO Category[a]	mCD3	Cyt CD3	CD4	CD8	CD2	CD5	CD7	CD10	HLA-DR	CD25	CD56	CD57	CD16
T-PLL	+/−	+	+/−	− (+)	+	+	+ (−)	−	− (+)	−	−	−	−
ATLL	+	+	+	−	+	+	−/d	−	+	+	−	−	−
SS	+/d	+	+	−	+ (−)	+	− (+)	−	+/−	− (+)	−	−	−
AILT	+/−	+	+	−	+/d	+/d	+/d	+	+/−	−	−	−	−
ALC	+/−	+/−	+/−	− (+)	+/−	−	−	−	+/−	−	+	+	
LGL	+	+ (−)	−	+	+	−/+	−/+	−	+	−	− (+)	+	+/−
ANKL	−	−	−	−/+	+	−	−	−	−/+	−	+b	−/−	+
HSTCL	+/−	+	−	−/d/+	+	− (+)	+ (−)	−	+	−	−/+	− (+)	−/+
PTCL	+/−	−	+/−	+/−	+	−/+	+/−	−	+/−	− (+)	− (+)	−	−

[a] Diagnostic categories of WHO 2008 classification. AILT, angioimmunoblastic T-cell lymphoma; ALC, anaplastic large cell lymphoma; ANKL, aggressive NK-cell leukemia; ATLL, adult T-cell leukemia/lymphoma; HSTCL, hepatosplenic T-cell lymphoma; LGL; T-cell large granular lymphocyte leukemia; PTCL, peripheral T-cell lymphoma; SS, Sézary syndrome; T-PLL, T-cell prolymphocytic leukemia.

d (dim), weak positive staining; b (bright) strong positive staining; (+) or (−) some cases positive or negative.

Examples of immunophenotypes for some of the most commonly seen mature B cell neoplasms



Disease entity	Typical phenotype	Atypical expression
Chronic lymphocytic leukaemia (CLL)	CD19^{+}, CD20^{+} (weak), CD5^{+}, CD81^{+} (weak), CD79b^{-} (weak), CD43^{++}, CD23^{+}, CD200^{+}, CD52^{++}, CD10^{-}, CD38$^{\text{variable}}$, weak surface immunoglobulins such as kappa/lambda, IgM and IgD	Atypical cases can show weak or absent CD5 expression, lack of CD23, strong CD20 or combinations of the aforementioned
Hairy cell leukaemia (HCL)	CD19^{++}, CD20^{++}, very strong surface immunoglobulin, CD22^{++}, CD103^{+}, CD25^{+}, CD11c^{+}, CD10^{-}, CD5^{-}	Atypical cases can lack CD25 expression and are classified as variant HCL (vHCL). CD10 positivity can be seen in a significant number of individuals, with reported frequencies ranging from 10% to 26% of cases[18,51,52]
Mantle cell lymphoma (MCL)	CD19^{+}, CD20^{+}, CD5^{+}, CD23^{-}, CD200^{-}, CD52^{++}, CD10^{-}	Atypical cases can be CD5 negative and instances with CD23 and/or CD200 expression are not uncommon[20]
Follicular lymphoma (FL)	CD19^{+} (weak), CD20^{+}, CD10^{+}, CD38^{+}, CD43^{-}	Atypical cases, reported as approximately 50% of samples,[10] can have weak or absent CD10 expression and the majority of these cases are high-grade[53]

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Role of flow cytometric immunophenotyping in the classification of mature B-cell lymphoid neoplasms

- it is useful to consider 4 broad groups as determined by their expression of CD5 and CD10:
  - CD5+/CD10-
  - CD5-/CD10+
  - CD5+/CD10+
  - CD5-/CD10-

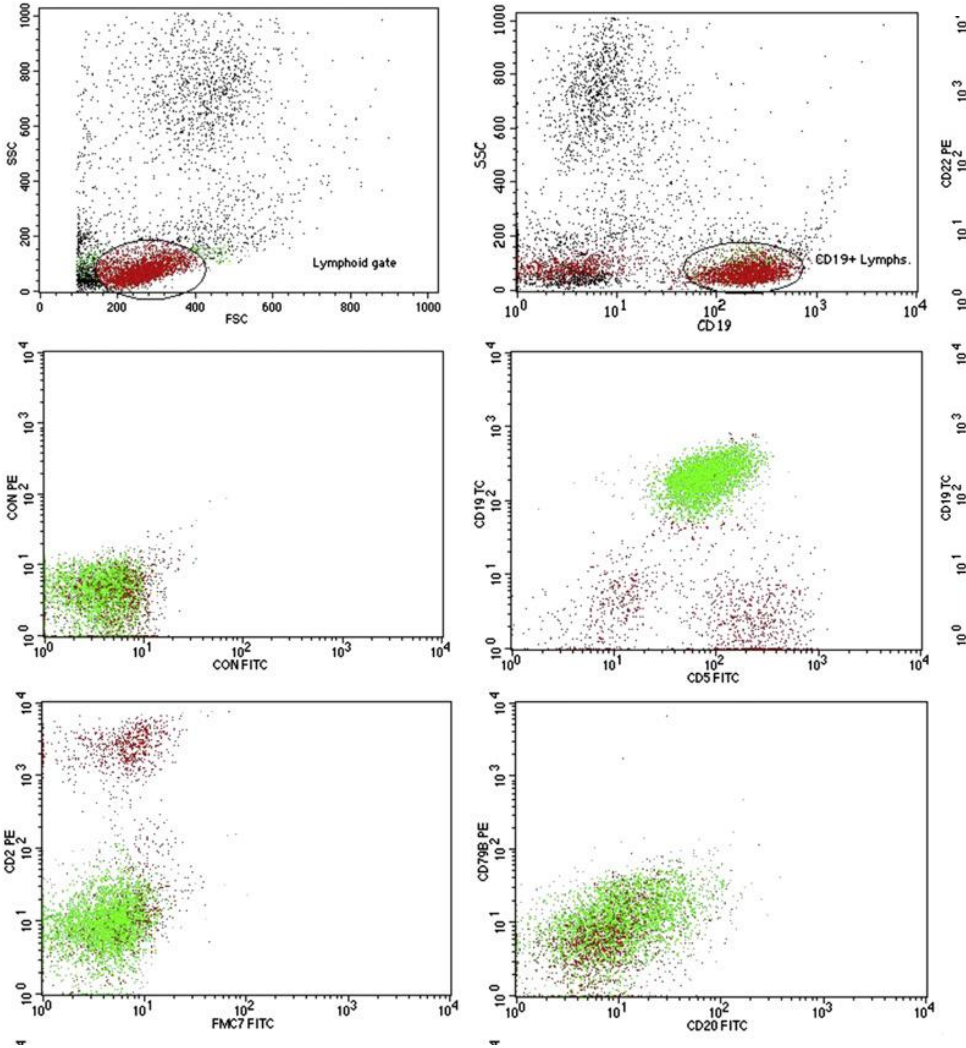
- For each group, additional flow cytometric data in combination with the morphology can narrow down the diagnostic possibilities and direct the use of additional ancillary studies

FC approach to the diagnosis and classification of B-cell lymphoid neoplasms: CD5pos CD10neg

DISEASE	Distinguishing phenotypic features	Additional diagnostic information
Chronic Lymphocytic Lymphoma	Typical phenotype: CD20(d), CD22(d), sIg(d), CD23+, FMC-7-	Characteristic morphology
Mantle Cell lymphoma	Variable phenotype not typical for CLL; often CD20(i), sIg(i), CD23+/-, FMC-7+/-	Cyclin-D1 IHC, t(11;14)/CCND rearrangement
Prolymphocytic leukemia	Variable phenotype, may overlap with CLL and MCL CD20(i), sIg(i), FMC-7+/-, CD5+/-	Large cells prominent nucleoli; exclude blastic MCL
Marginal Zone B-cell Lymphoma	Variable phenotype, not typical for CLL: CD23+; often CD11c+/-, CD103+/- but not typical for HCL, sometimes cIg only	Growth around and into follicles, may demonstrate plasmacytic differentiation, t(11;18), t(1;14), t(14;18)/MALT-1 rearrangement
Difuse Large B-cell Lymphoma	Variable phenotype	Large cells, diffuse growth pattern; consider Richter transformation CLL and MCL
Lymphoplasmacytic lymphoma	Phenotype not typical for CLL, often CD23(-/d), sometimes sIg- but cIg+	Small cells, subset with plasmacytic differentiation Primarily PB and BM

Craig FE Foon KA. Blood 2008;111:3941-3967

FC in CLL



FC dot plots from a CLL gating on the CD19+ cell population. The majority of CD19þ cells are CD5+, CD23+, CD22+ and dim CD20, weak kappa+, and are negative with FMC7, CD79b and T-cell markers (CD2, CD4 and CD8).
CD38 is strongly expressed in the CLL cells.

Matutes E, et al Best Practice & Research Clinical Haematology 23 (2010) 3–20

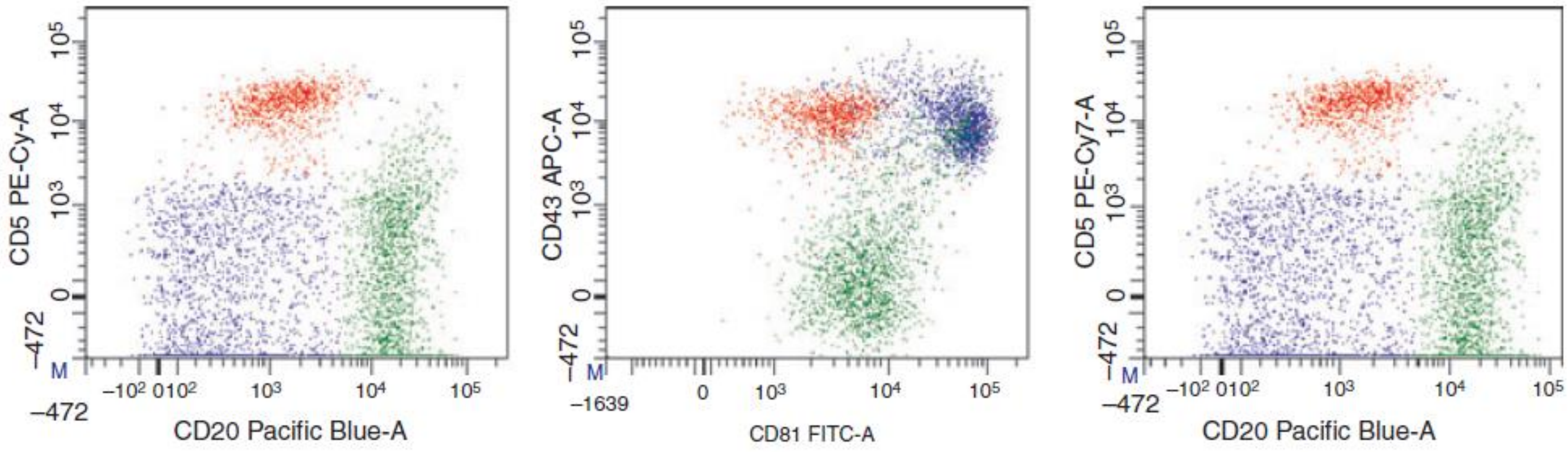
Immunophenotypic score.

marker	Points	
	1	0
CD5	Positive	Negative
CD23	Positive	Negative
FMC7	Negative	Positive
sIg	Weak	Moderate/strong
CD22/CD79b	Weak/negative	Moderate/strong

Scores in CLL range from 3 to 5 while in the other B-cell disorders are 0-2

Matutes E, et al Best Practice & Research Clinical Haematology 23 (2010) 3–20

MRD analysis in CLL.



The plots above show analysis of a bone marrow with a minimal CLL population and demonstrate how the use of a few carefully selected antigens can discriminate between CLL cells (red), normal mature B-cells (green) and B-cell progenitors (blue). B-cells have been gated in a CD19 versus SSC plot and are 1.6% of total leucocytes. CLL cells represent 0.32% of total leucocytes.

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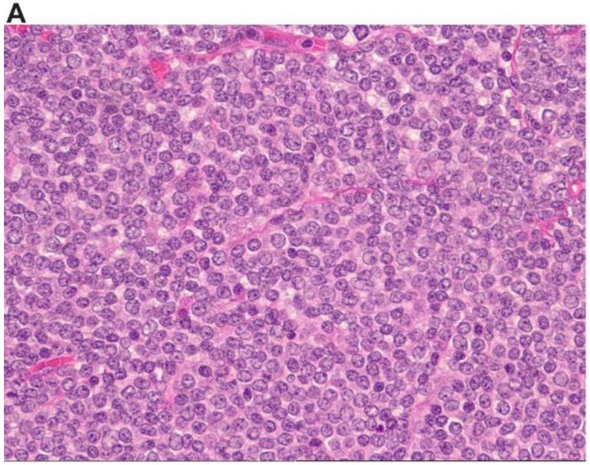
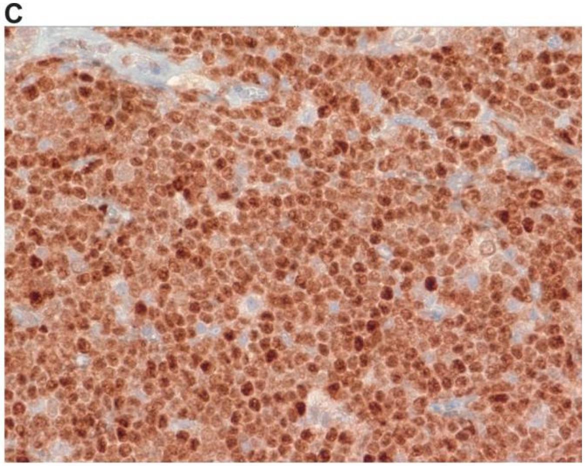
Mantle Cell Lymphoma

- Mantle cell lymphoma (MCL) cells typically express bright CD20, CD5, FMC7, and bright to moderate sIg but lack CD23 and CD200.
- However, MCL cases positive for CD23 and negative for FMC7 as well as rare CD5 negative cases have been found.
- Therefore, confirmation of MCL diagnosis by FISH for t(11,14) is recommended.
- Cyclin D1 expression can also be detected by FCM 89 but this method is not routinely applied in most diagnostic laboratories.

Mantle cell lymphoma.



Histologic section from a submandibular gland biopsy specimen demonstrating an abnormal diffuse infiltrate of small to intermediate-size lymphoid cells. Several mitotic figures are present. Hematoxylin & eosin stain, magnification x 40.

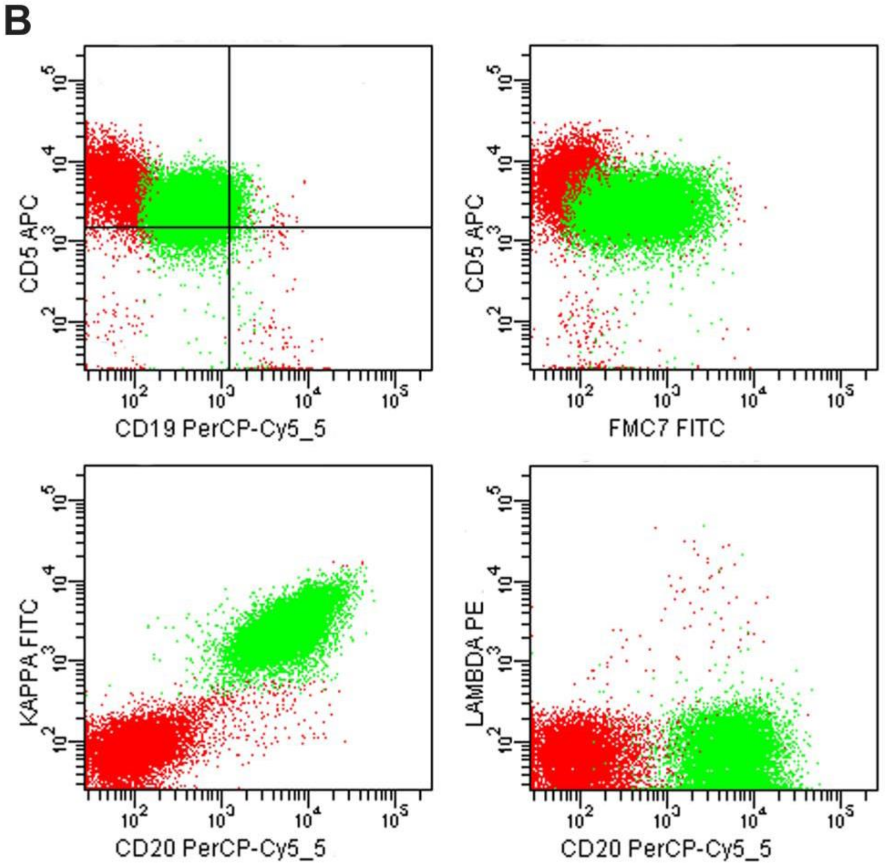


Cyclin-D1 paraffin section immunohistochemical stain, demonstrating many positive cells with characteristic nuclear staining; magnification x 40.

Craig FE Foon KA. Blood 2008;111:3941-3967

Mantle cell lymphoma.

Representative FC dot plots with population of interest highlighted in green: CD19 versus CD5 demonstrates CD5 B-cell population with weak intensity staining for CD19;
FMC-7 versus CD5 demonstrates positivity for FMC-7;
CD20 versus kappa and CD20 versus lambda demonstrate moderate intensity staining for CD20 and kappa immunoglobulin light chain restriction. In addition, B cells were CD10- and CD23-.

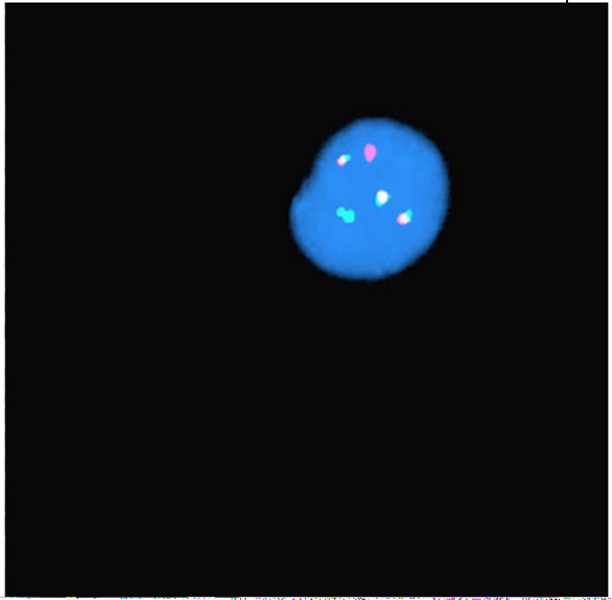


Craig FE Foon KA. Blood 2008;111:3941-3967

Mantle cell lymphoma.

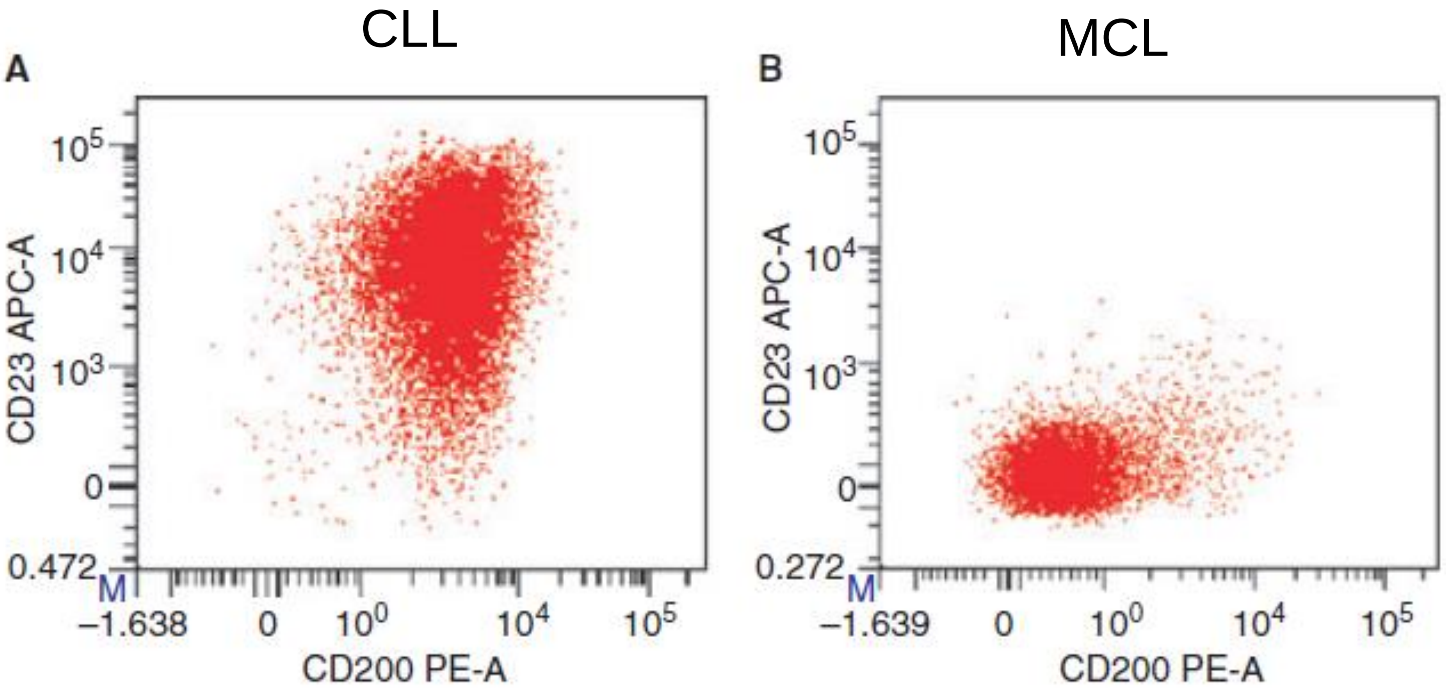
FISH demonstrating the *IGH/CCND1* [t(11,14)(q13;q32)] rearrangement. Hybridization with the LSI IGH/CCND1-XT dual color, dual fusion DNA probe demonstrates

- one green signal from the unrearranged chrom. 14q32,
- one red signal from the unrearranged 11q13,
- 3 fusion signals:
  - one from the derivative chrom 11,
  - one from the derivative chrom 14, and
  - an extra signal suggesting the presence of an additional copy of all or part of one of the derivative chromosomes involved in the *IGH/CCND1* rearrangement.



Craig FE Foon KA. Blood 2008;111:3941-3967

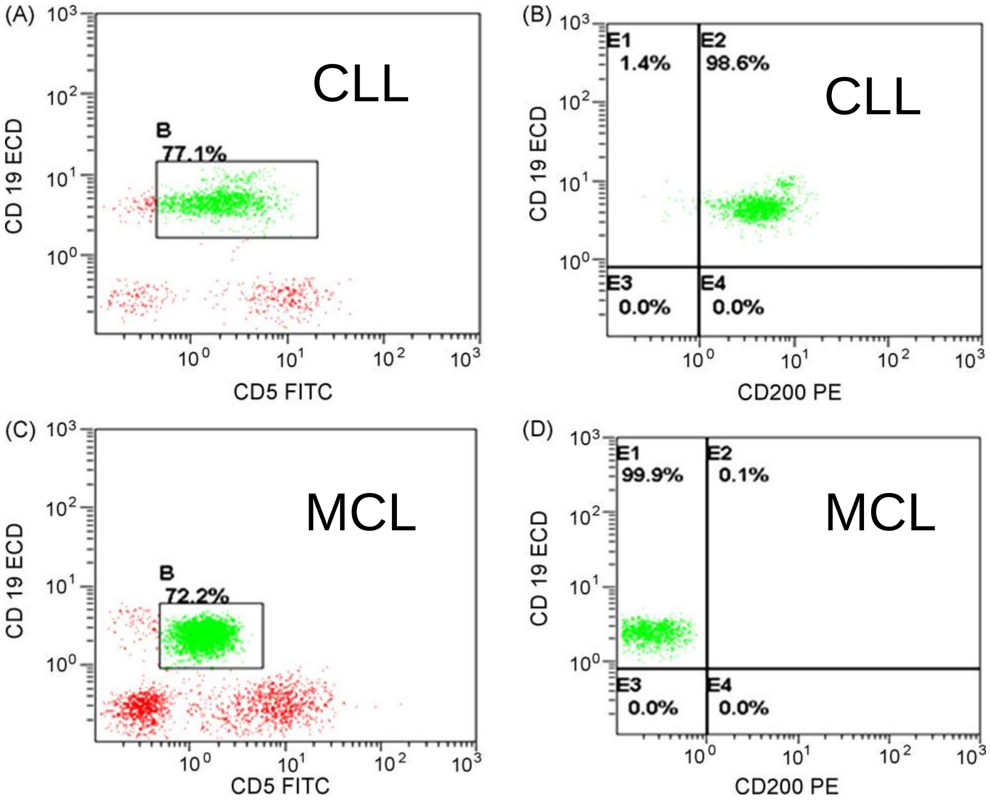
Use of CD200 to discriminate CLL from MCL

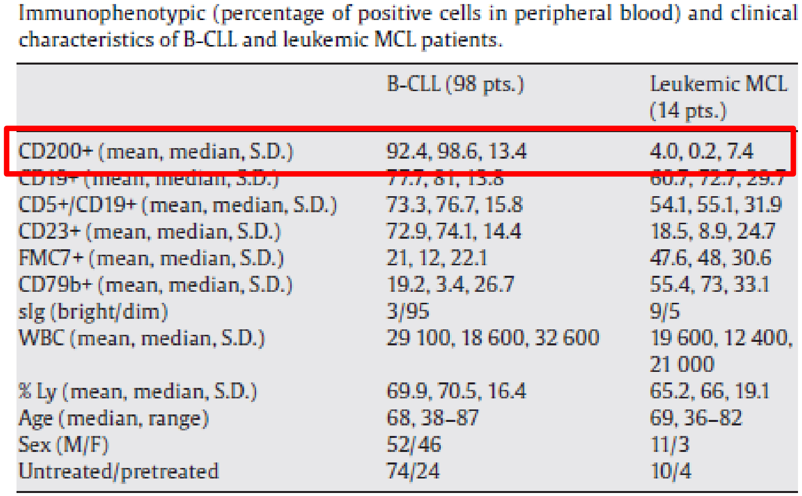


Plot A shows gated B-cells from a BMA sample involved with CLL. The cells demonstrate expression of CD23 and CD200. Plot B displays B-cells from a patient with MCL. The cells have a typical MCL phenotype and are negative for both markers. CD200 is extremely useful in cases of MCL which exhibit atypical CD23 expression.

De Tute RM. Histopathology 2011, 58, 90–105

CD200: CLL vs MCL





Immunophenotypic (percentage of positive cells in peripheral blood) and clinical characteristics of B-CLL and leukemic MCL patients.

	B-CLL (98 pts.)	Leukemic MCL (14 pts.)
CD200+ (mean, median, S.D.)	92.4, 98.6, 13.4	4.0, 0.2, 7.4
CD19+ (mean, median, S.D.)	77.7, 81, 13.8	60.7, 72.7, 29.7
CD5+/CD19+ (mean, median, S.D.)	73.3, 76.7, 15.8	54.1, 55.1, 31.9
CD23+ (mean, median, S.D.)	72.9, 74.1, 14.4	18.5, 8.9, 24.7
FMC7+ (mean, median, S.D.)	21, 12, 22.1	47.6, 48, 30.6
CD79b+ (mean, median, S.D.)	19.2, 3.4, 26.7	55.4, 73, 33.1
sIg (bright/dim)	3/95	9/5
WBC (mean, median, S.D.)	29 100, 18 600, 32 600	19 600, 12 400, 21 000
% Ly (mean, median, S.D.)	69.9, 70.5, 16.4	65.2, 66, 19.1
Age (median, range)	68, 38–87	69, 36–82
Sex (M/F)	52/46	11/3
Untreated/pretreated	74/24	10/4

Palumbo et al. Leukemia Research 33 (2009) 1212–1216

FC approach to the diagnosis and classification of B-cell lymphoid neoplasms: CD5neg CD10pos

DISEASE	Distinguishing phenotypic features	Additional diagnostic information
Follicular lymphoma	Usually bcl-2+, CD43-	Some follicular growth, t(14;18)/*BCL-2 r*earrangement
Diffuse large B-cell lymphoma	Variable phenotype, bcl-2+/-, CD43+/-	Large cells, diffuse growth pattern
Burkitt lymphoma	Usually bcl-2-,CD10(+b), CD43+	Uniform intermediate size cells; *MYC* rearrangement, Ki-67 approximately 100%
Hairy cell leukemia	Typical phenotype: CD20(b), CD22(b), CD11c(b), CD25+, CD103+, sIg(i), CD123+	Characteristic morphology; Annexin-A1+

Craig FE Foon KA. Blood 2008;111:3941-3967

Follicular Lymphoma

- The follicular lymphoma (FL) cells usually express sIg, more frequently IgM +/−IgD than IgG or rarely IgA, together with B-cell–associated antigens (CD19, CD20, CD22, CD79a, and CD79b), and in most cases CD10. Expression of CD19 and CD22 is often weaker than in normal B-cells.

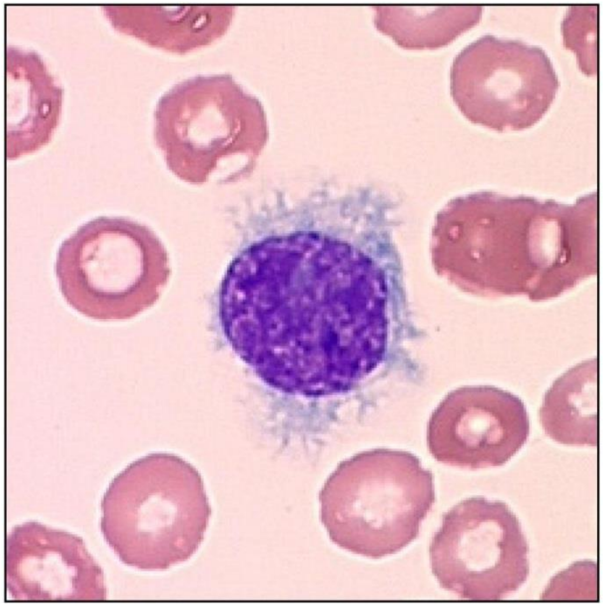
- FL cells are usually CD5−, CD43–, and CD23−/+, CD11c−/+. The weaker expression of CD38 helps to differentiate FL cells from CD10 positive B-cell precursors.

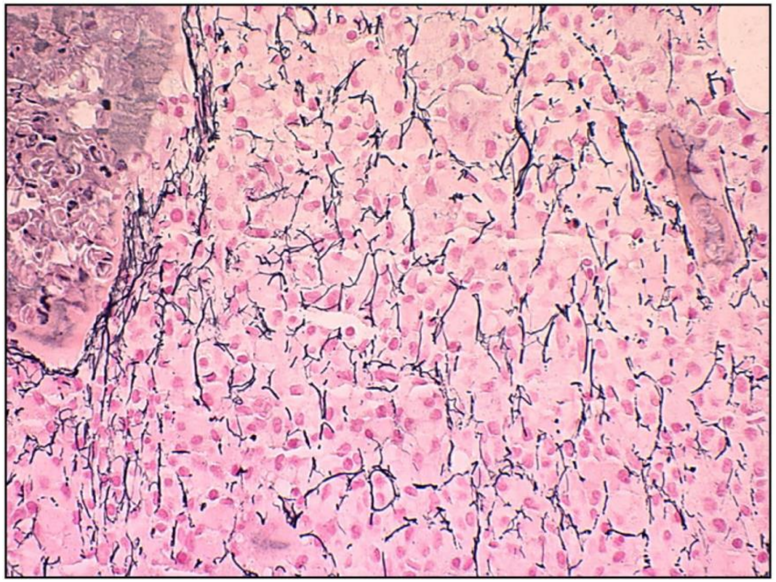
Hairy Cell Leukemia and Hairy Cell Leukemia Variant

- Both hairy cell leukemia (HCL) and HCLv strongly express CD103, CD11c, CD20, CD22, CD19, and are negative for CD5, CD23, and in most cases negative for CD38.
-
- HCL cells are often large (can be found in the monocyte region) and are positive for CD25 and CD123 in contrast to HCLv cells that are smaller and CD25 negative.

Differential diagnosis for HCL

Disease	Immunophenotype	Other features
HCL	CD11c, CD25, CD103, CD123, annexin A1^{+}, CD20$^{\text{bright}}$	Monocytopenia, frequent leukopenia
Hairy cell variant	CD11c, CD103, CD25^{-}	No monocytopenia, high leukemic cell count
SMZL/SLVL	CD11c, CD25, CD24, CD79b	
Chronic lymphocytic leukemia	CD5, CD19, CD23	
B-prolymphocytic leukemia	CD19, FMC7, CD79b, CD20, and CD22$^{\text{bright}}$	High leukocyte count

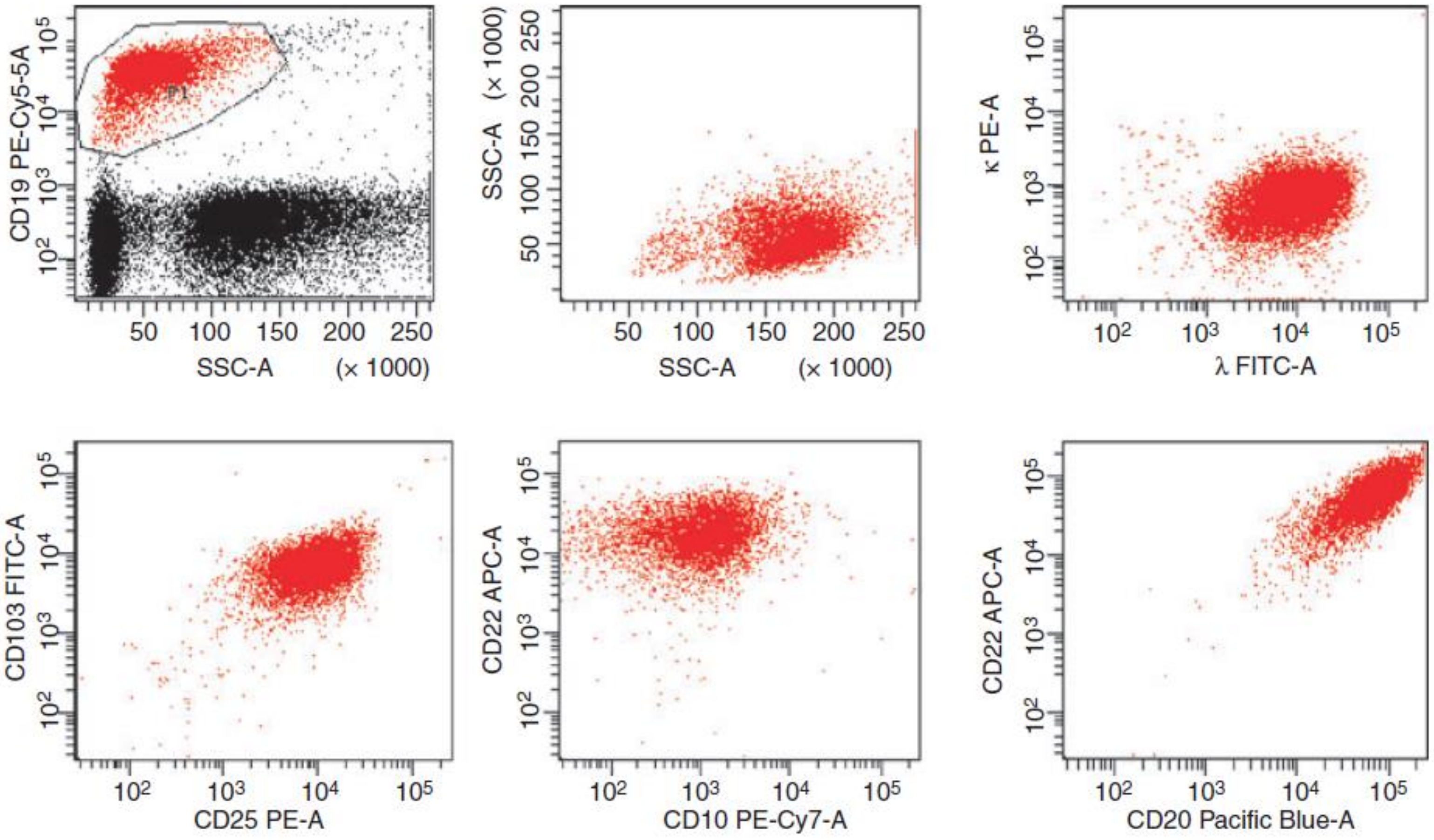




Reticulin staining

Grever Blood. 2010;115:21-28)

Immunophenotype of typical HCL



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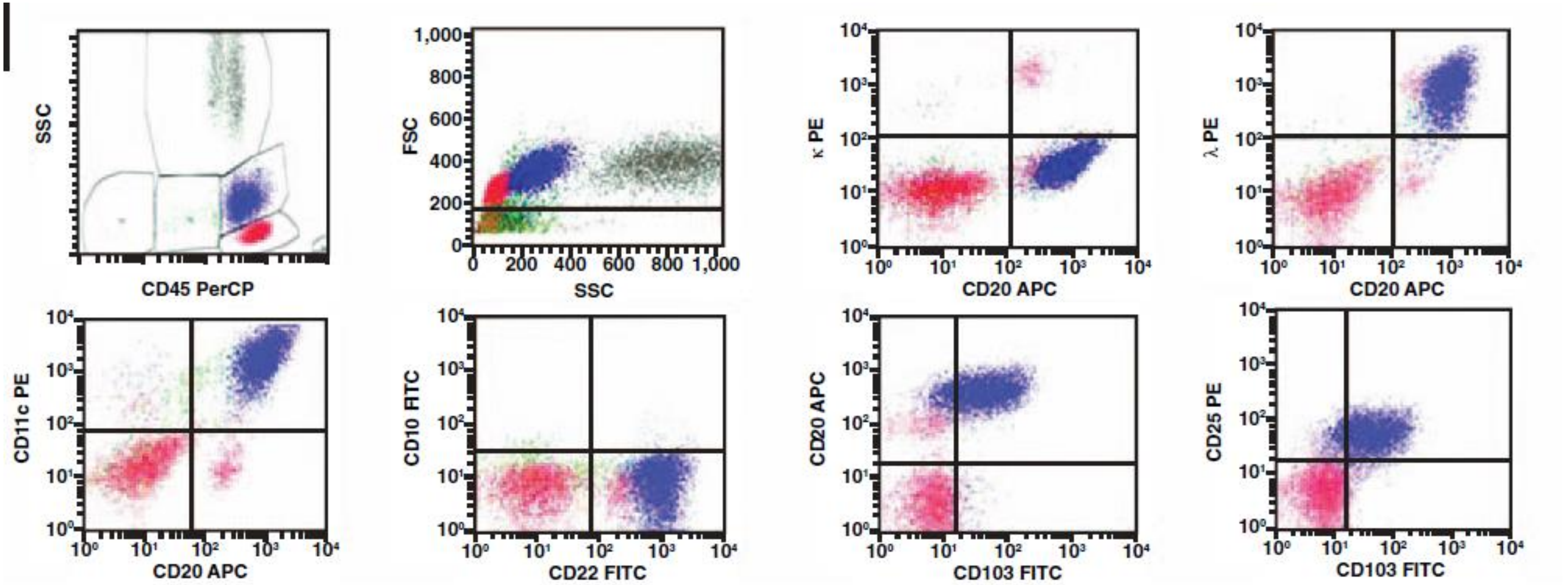
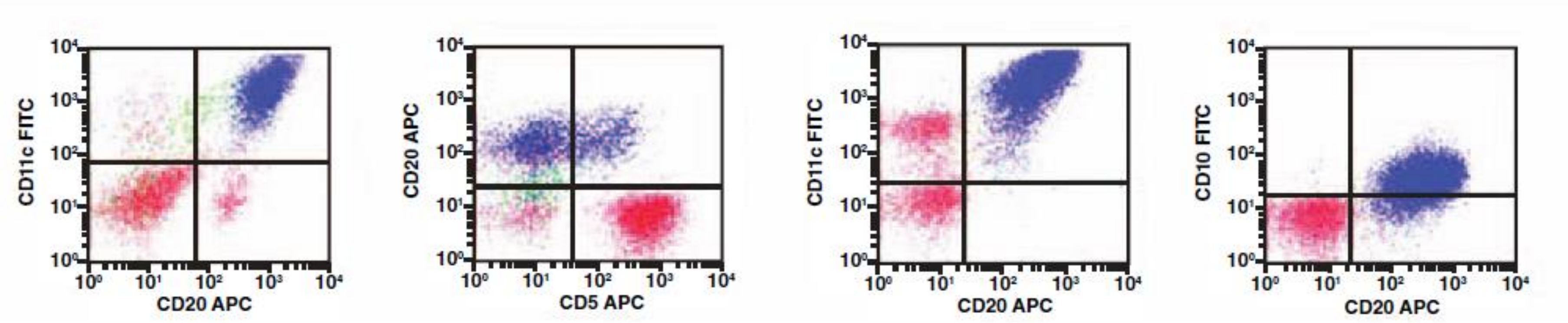
HCL



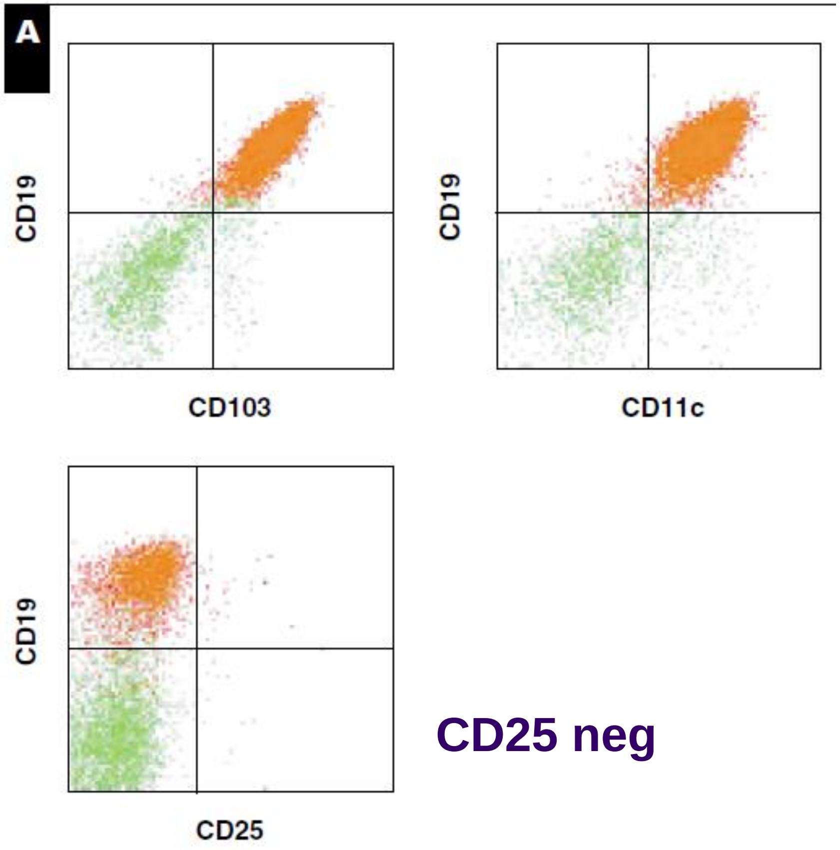
Am J Clin Pathol 2009;131:586-595

HCL CD5 weak

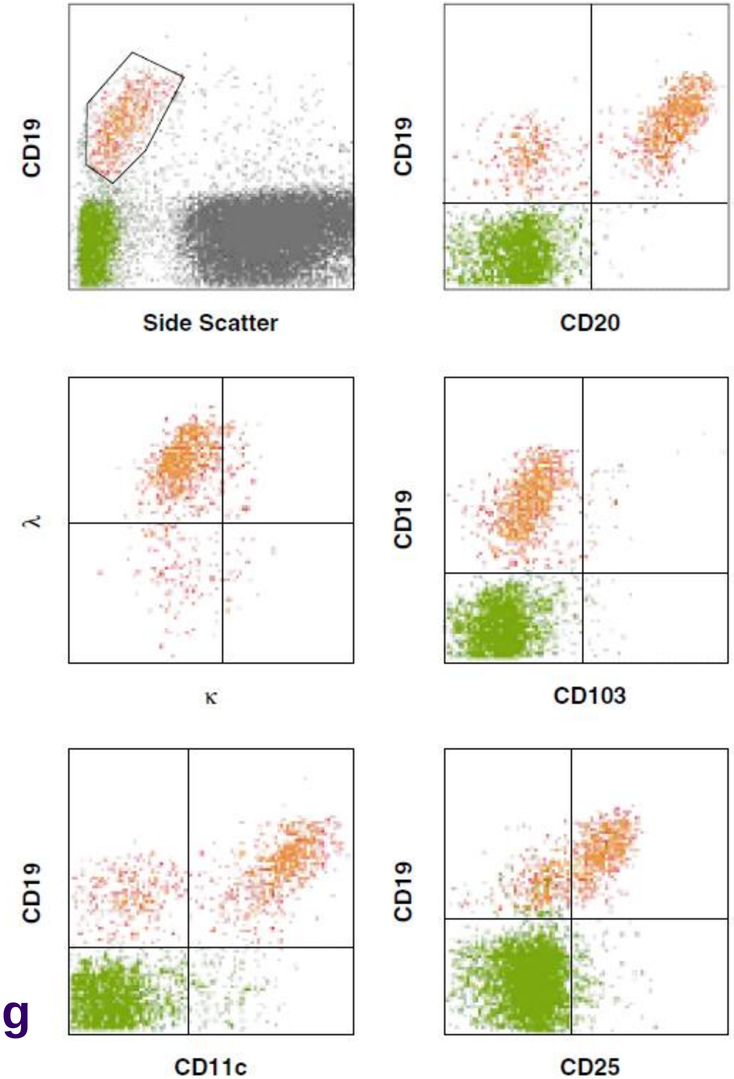


Am J Clin Pathol 2009;131:586-595

HCL immunological variants



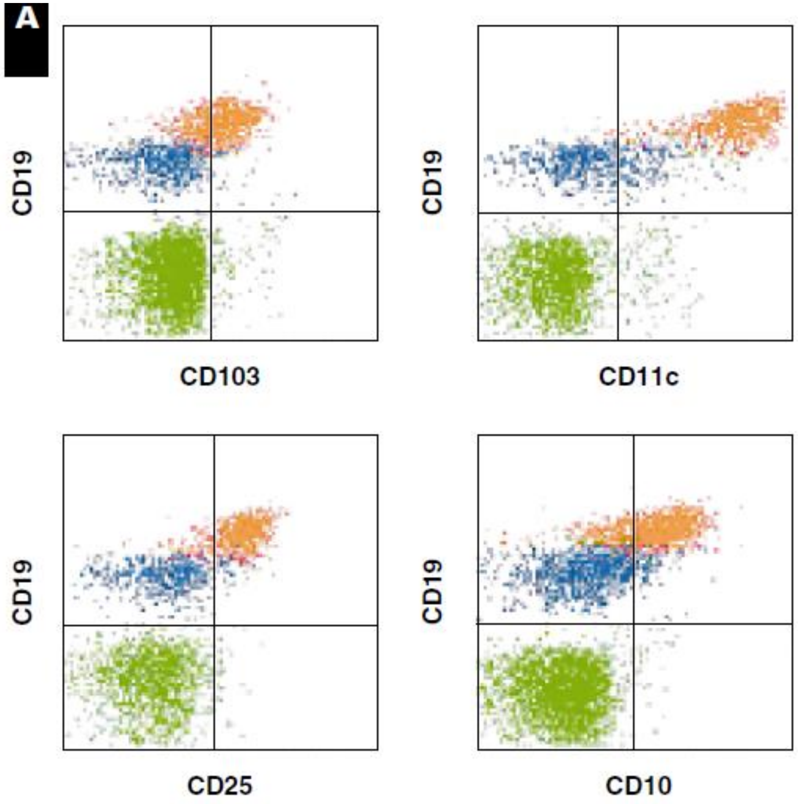
CD25 neg



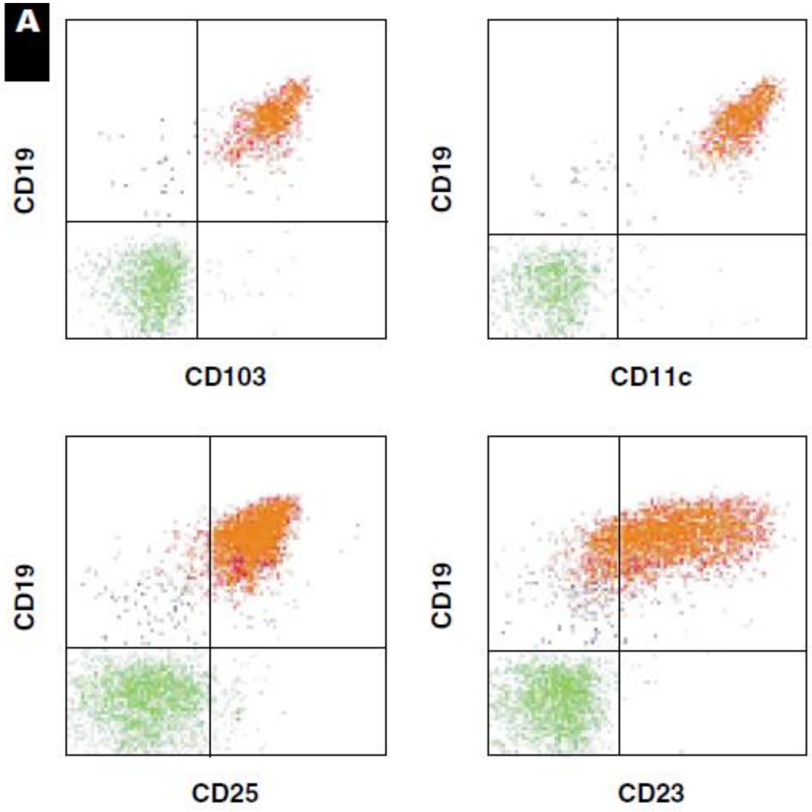
CD103 neg

Am J Clin Pathol 2006;125:251-259

HCL immunological variants



CD10 pos



CD23 pos

Am J Clin Pathol 2006;125:251-259

BRAF Mutations in Hairy-Cell Leukemia

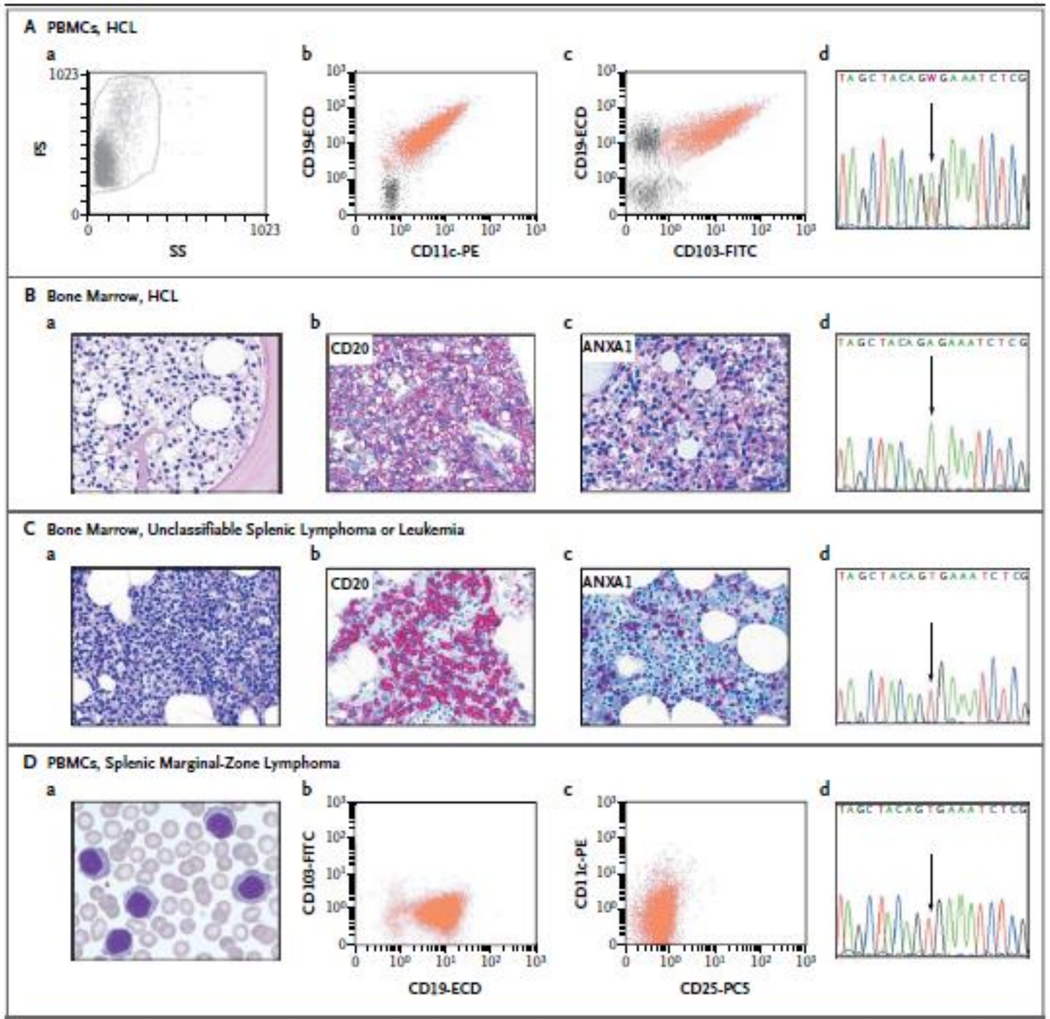


Figure 2. *BRAF* V600E Mutation in Hairy-Cell Leukemia (HCL), as Compared with Other Peripheral B-Cell Neoplasms.

N Engl J Med 2011;364:2305-15.

FC approach to the diagnosis and classification of B-cell lymphoid neoplasms: CD5pos CD10pos

DISEASE	Distinguishing phenotypic features	Additional diagnostic information
Follicular lymphoma	Usually bcl-2+, CD43-	Some follicular growth, t(14;18)/*BCL-2* rearrangement
Diffuse large B-cell lymphoma	Variable phenotype, bcl-2+/-, CD43+/-	Large cells, diffuse growth pattern
Mantle cell lymphoma	Variable phenotype not typical for CLL; often CD20(i), sIg(i), CD23+/-, FMC-7+/-	Cyclin-D1 IHC, t(11;14)/CCND rearrangement
Burkitt lymphoma	Usually bcl-2-, CD10(b), CD43+	Uniform intermediate size cells; *MYC* rearrangement, Ki-67 approximately 100%

Craig FE Foon KA. Blood 2008;111:3941-3967

FC approach to the diagnosis and classification of B-cell lymphoid neoplasms: CD5neg CD10neg

DISEASE	Distinguishing phenotypic features	Additional diagnostic information
Hairy cell leukemia	Typical pheotype: CD20(b), CD22(b), CD11c(b), CD25+, CD103+, sIg(i)	Confirm characteristic morphology
Marginal zone B-cell lymphoma	Often CD11c+/-, CD103+/- but not typical for HCL, sometimes sIg- but cIg+	Growth around and into follicles, maybe plasmacytic t(11;18), t(1;14), t(14;18)/MALT-1 rearrangement
Diffuse large B-cell lymphoma	Variable phenotype	Large cells, diffuse growth pattern
Follicular lymphoma CD10-	Variable phenotype	Some follicular growth, t(14;18)/*BCL-2* rearrangement
Mantle cell lymphoma CD5-	Variable phenotype	Cyclin-D1 IHC, t(11;14)/CCND rearrangement

Craig FE Foon KA. Blood 2008;111:3941-3967

Immunophenotypic information of additional prognostic value in mature B-cell lymphoid neoplasms

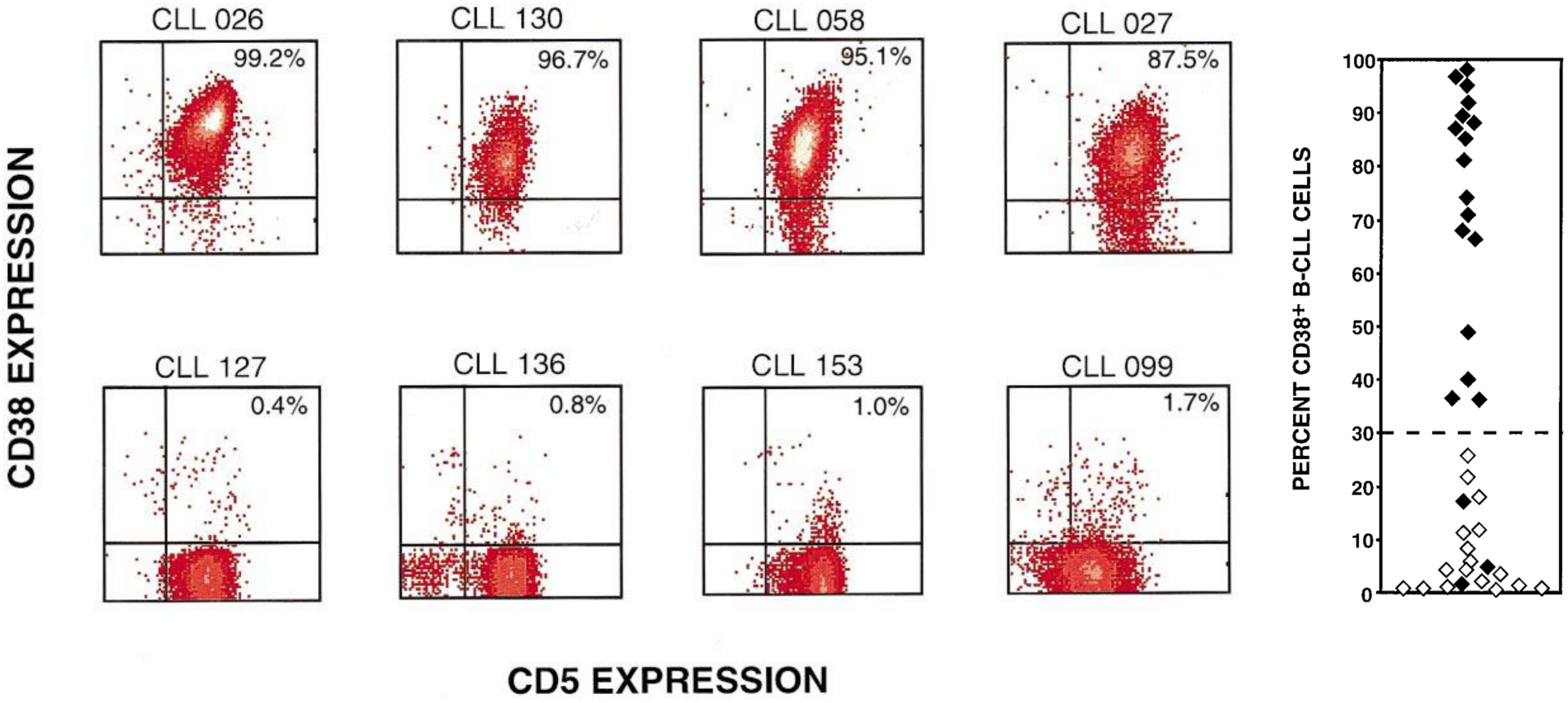
- Expression of CD38 and ZAP-70, as determined by FC, has been reported to have prognostic significance in CLL/SLL.

- Although CD38 expression was initially thought to correlate with unmutated status of the Ig heavy-chain variable region gene (IgVH), subsequent studies demonstrated a significant number of discordant results.

CD38 in CLL

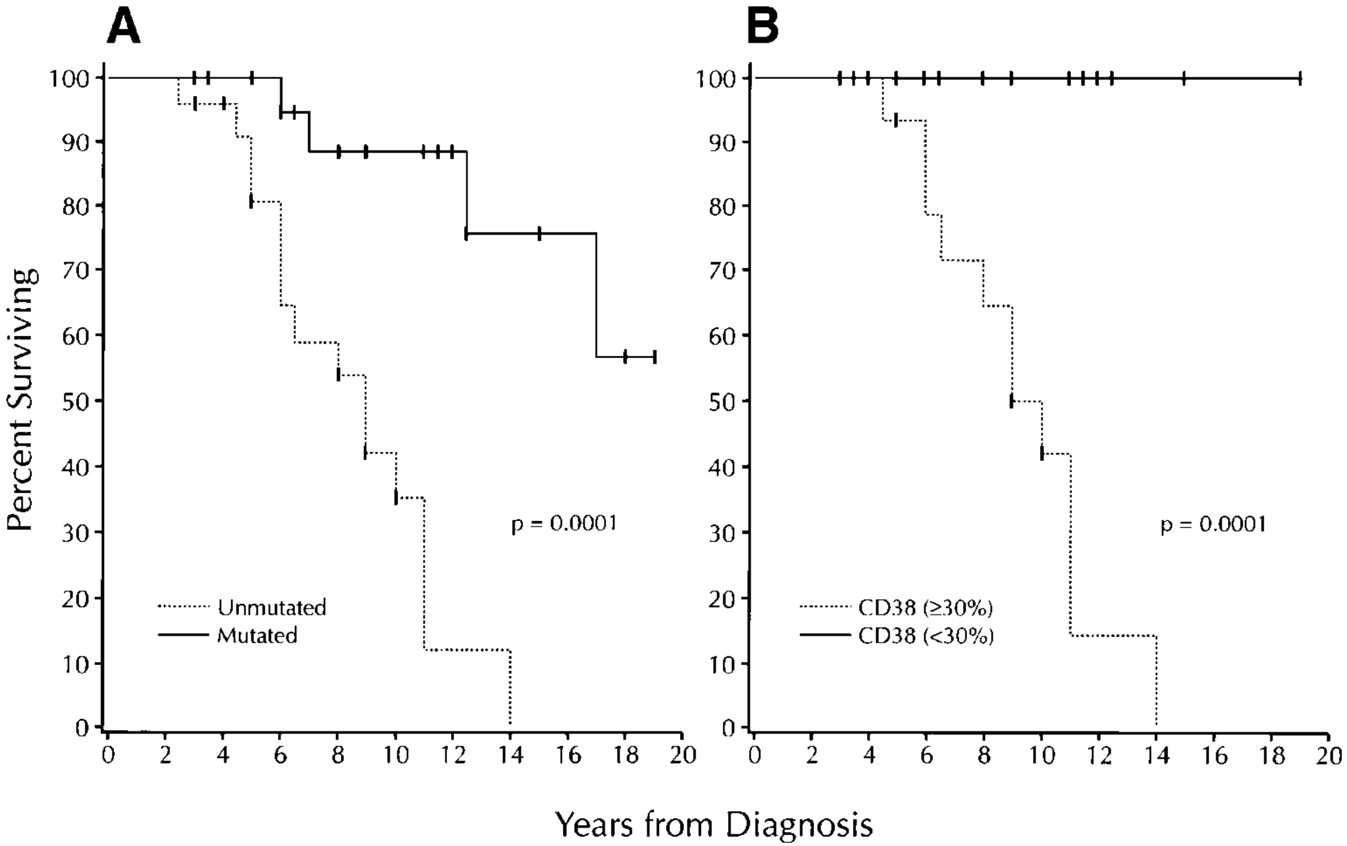
- CD38 expression is an independent marker of a poor prognosis in CLL/SLL.
- Most studies use 30% as cut-off for positivity (in some studies 20%)
- The following factors can make determination of the percentage of CD38 cells difficult:
  - a spectrum of intensity for CD38 staining without clear distinction between positive and negative populations,
  - differences in intensity that derive from the fluorochrome,
  - bimodal staining with the presence of positive and negative cells in the same sample,
  - differences in staining between tissue sites such as PB and BM,
  - changes in CD38 expression during the course of the disease and with therapy.

CLL and CD38



Damle RN et al, Blood 1999:94:1840

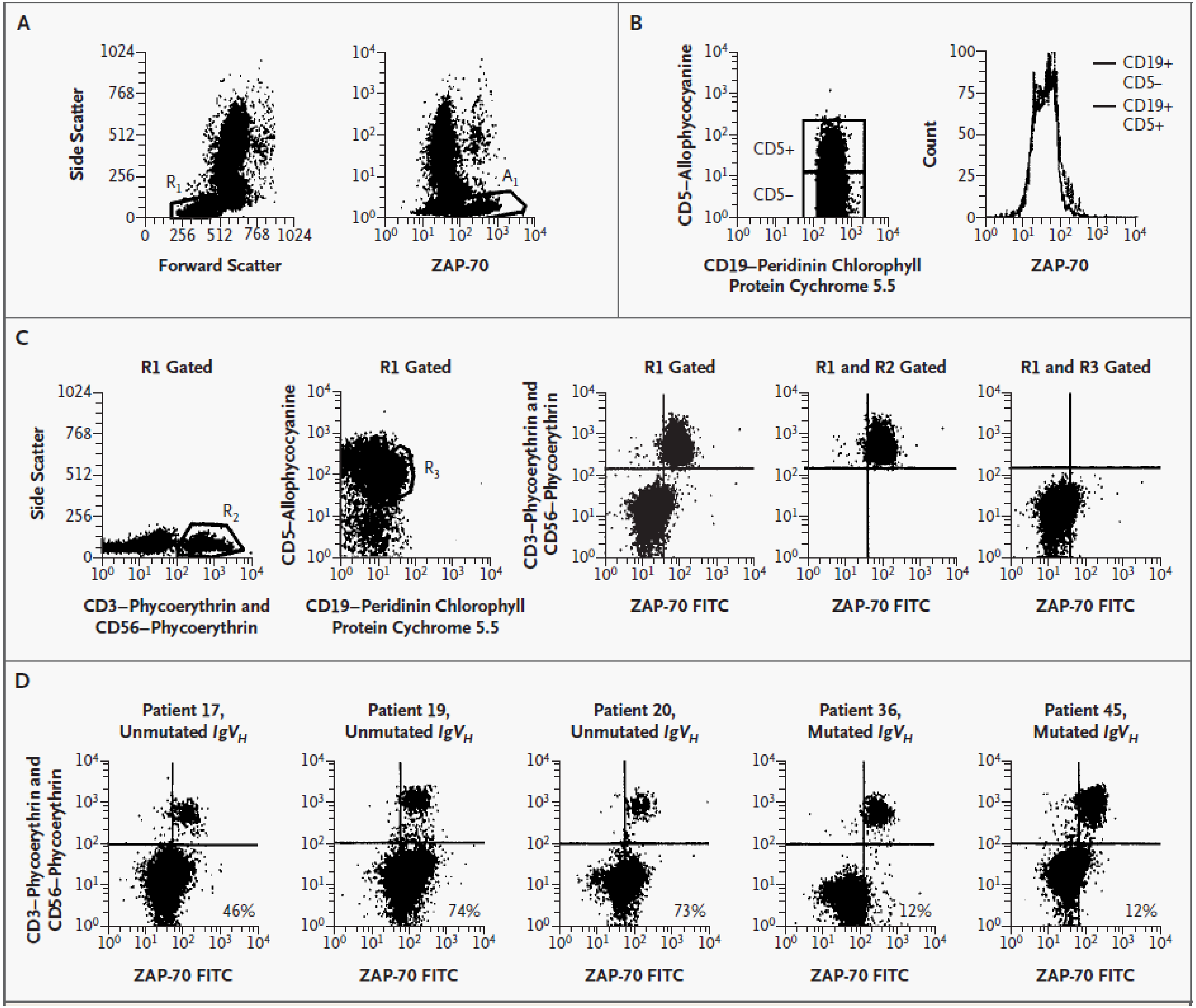
CLL: Survival according to CD38 and IGHV status



Damle RN et al, Blood 1999:94:1840

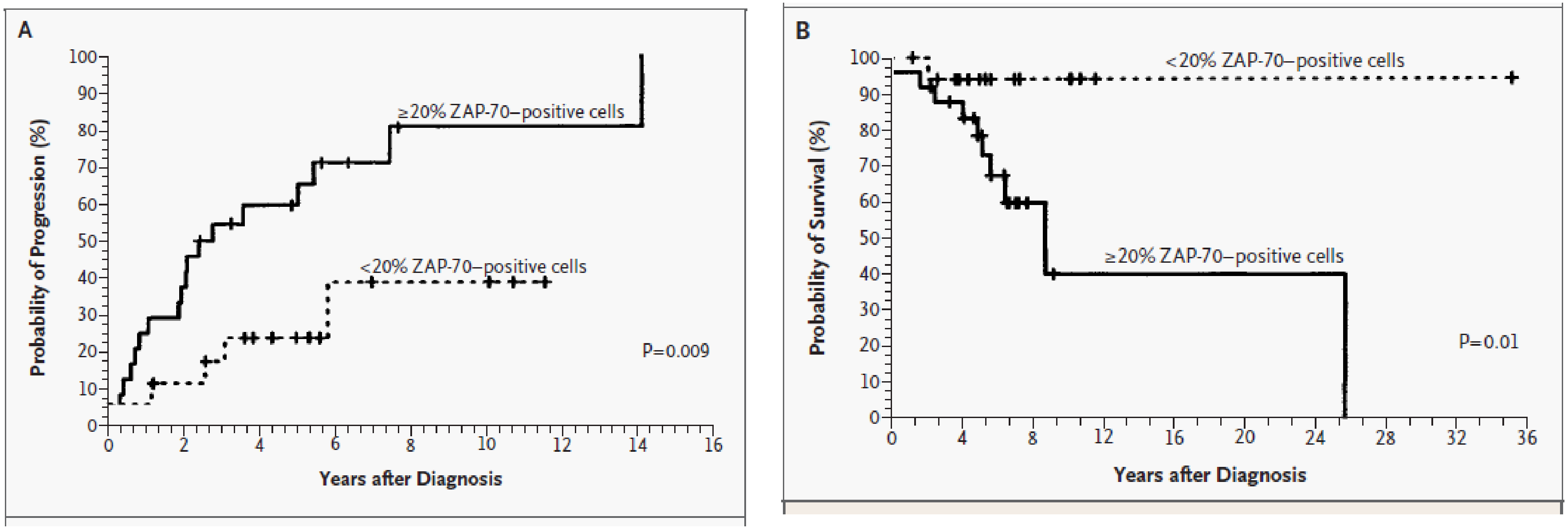
ZAP-70

- ZAP-70 was identified in a search for genes that are differentially expressed in CLL/SLL with mutated and unmutated IgVH.
  - Although the initial FC study of ZAP-70 expression in CLL/SLL demonstrated a strong association of ZAP-70 expression on greater than 20% of CLL/SLL cells with unmutated IgVH, subsequent studies have demonstrated a higher number of discordant pts.
- Some studies have suggested that in these discordant patients, ZAP-70 staining is the best indicator of prognosis in CLL/SLL.



Crespo NEJM 2003:348:1764

Disease progression and overall survival according to ZAP70

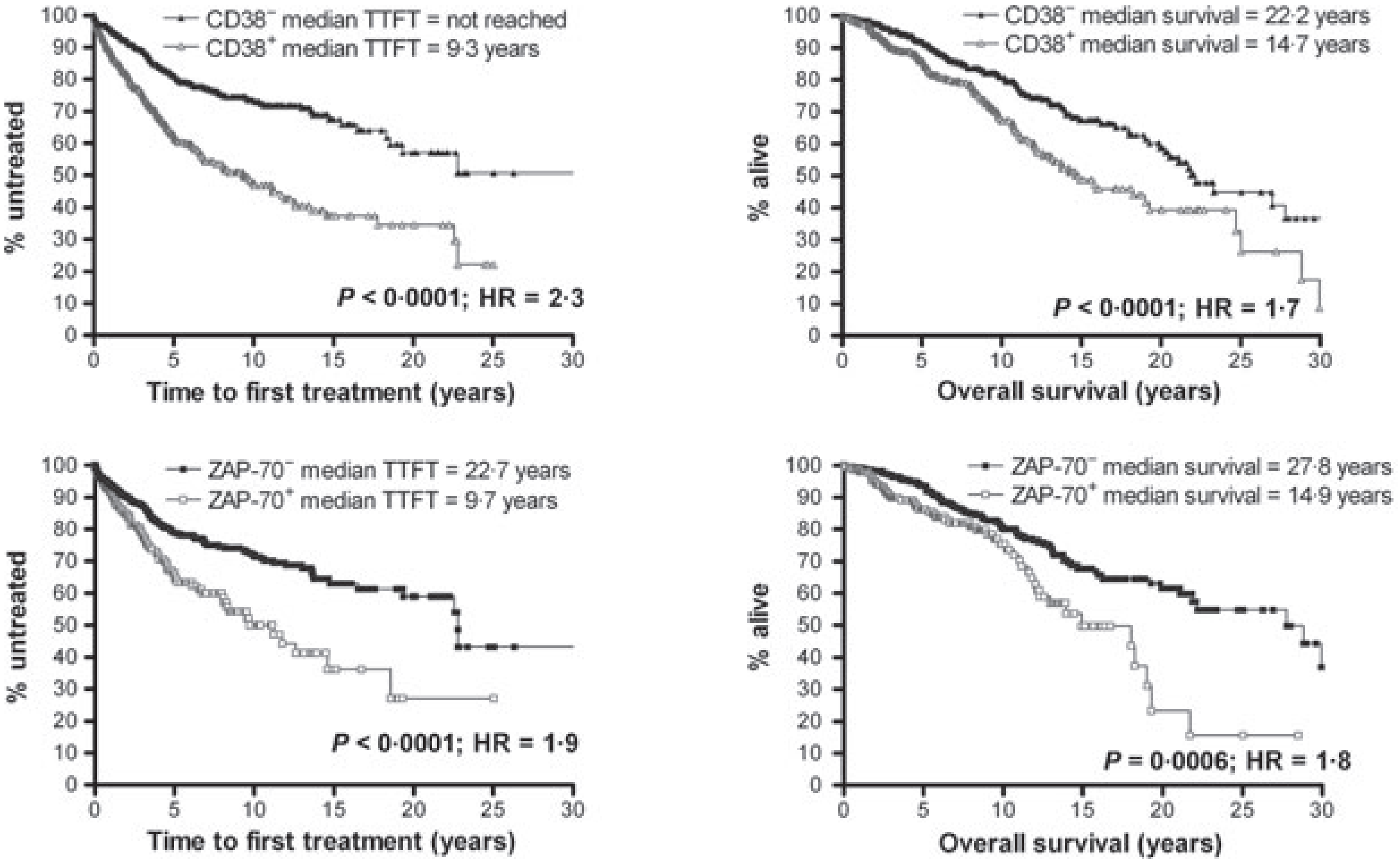


Crespo NEJM 2003:348:1764

ZAP-70: technical difficulties

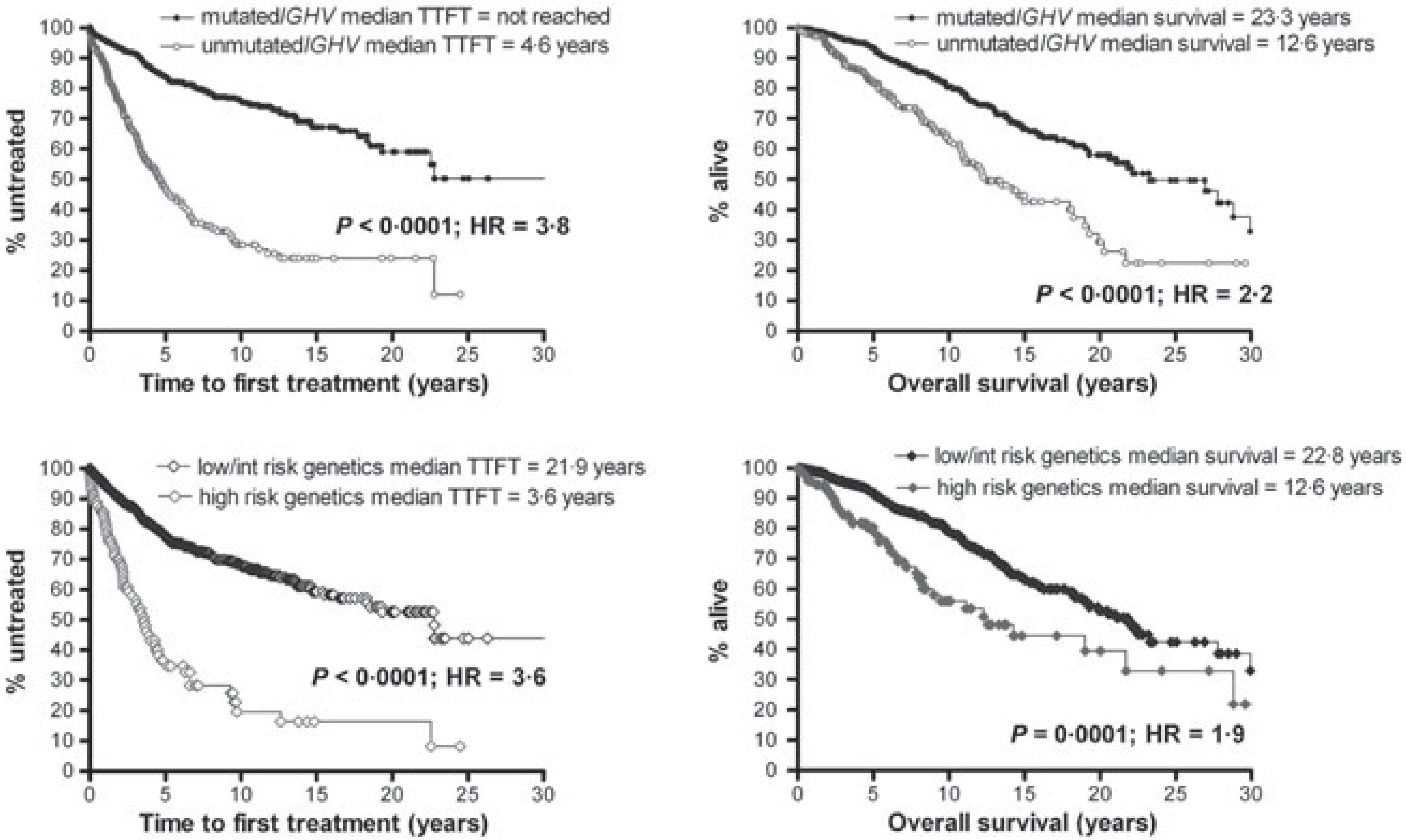
- ***Weak intensity staining for ZAP-70.***
  - The intensity of staining is relatively weak, making it difficult to distinguish positive and negative cells.
  - ZAP-70 is localized in the cytoplasm, and therefore detection requires cell permeabilization techniques.
- ***Nonspecific staining related to the Ab and permeabilization procedure.***
- ***Decrease in staining over time.***
  - ZAP-70 expression appears to be labile and sensitive to different anticoagulants (EDTA < 24h).
- ***What to call positive.***
  - Consensus has not been reached on the optimal method for ZAP-70.
  - The original FC study of ZAP-70 staining in CLL/SLL used the normal staining of T cells within the specimen to determine the lower limit for positive ZAP-70 staining. However, T cells demonstrate some variability in staining intensity for ZAP-70 within a specimen and between samples.
  - Because CLL/SLL cells often demonstrate a narrow range of staining for ZAP-70, small differences in the position of the cursor used to divide cells designated as positive and negative can make a large difference in the percent of ZAP-70 CLL/SLL cells.

CD38 and ZAP 70 in early CLL



Pepper BJH 2011;156:499

IGHV and genetics in early CLL



Pepper BJH 2011;156:499

Reagents of clinical utility in the evaluation of myeloid and monocytic neoplasms

initial evaluation

Reagent	Normal distribution of staining	Clinical utility	Comments
CD11b	Maturing neutrophils and monocytes, some lymphoid cells.	May be aberrantly expressed in AML, MDS, and MPD.	
CD13	Neutrophilic and monocytic cells.	Indicator of neutrophilic and monocytic lineage in AL. May be aberrantly expressed in AML, MDS, and MPD.	
CD14	Monocytes.	Indicator of monocytic differentiation.	Not a sensitive marker of immature monocytes.
CD15	Maturing neutrophilic cells and monocytes.	May be aberrantly expressed in AML, MDS, and MPD.	
CD16	Maturing neutrophilic cells, monocytes and NK cells.	May be aberrantly expressed in AML, MDS, and MPD.	

Craig FE Foon KA. Blood 2008;111:3941-3967

Reagents of clinical utility in the evaluation of myeloid and monocytic neoplasms

initial evaluation

Reagent	Normal distribution of staining	Clinical utility	Comments
CD33	Neutrophilic and monocytic cells	May be aberrantly expressed in AML, MDS, and MPD.	Some normal variability in intensity of expression.
CD34	B-cell and T-cell precursors and myeloblasts.	Identification and enumeration of blasts	Not all blasts are CD34
CD45	All B cells (weaker intensity on precursors and PC), all T cells (weaker intensity on precursors)	Identification of blasts (CD45 gating often with low orthogonal (side) light scatter).	
CD56	CD56 NK cells and NK-like T cells	May be aberrantly expressed in AML, MDS, and MPD.	Low level of expression on regenerating normal neutrophilic and monocytic cells and with growth factor stimulation.
CD117	Immature neutrophilic cells and mast cells.	Identification myeloblasts and mast cells.	May be present in MM and some T-cell neoplasms.
HLA-DR	Myeloblasts, monocytes, all B cells, activated T cells.	Identification of promyelocytes, such as in APL. May be aberrantly expressed in AML, MDS, MPD.	Non-APL AML may also be negative.

Craig FE Foon KA. Blood 2008;111:3941-3967

Reagents of clinical utility in the evaluation of myeloid and monocytic neoplasms

secondary evaluation

Reagent	Normal distribution of staining	Clinical utility	Comments
CD2	T cells, NK cells.	May be aberrantly expressed in AML (some association with inv16) and in systemic mastocytosis.	
CD4	T-cell subset, monocytic.	Often positive in AML, particularly with monocytic differentiation.	Also mature T-cell neoplasms and hematodermic neoplasms
CD7	T cells and NK cells.	May be aberrantly expressed in AML, MDS, and MPD.	
CD25	Activated B cells and T cells.	May be aberrantly expressed in systemic mastocytosis.	Reported association with *BCR/ABL* ALL.
CD36	Monocytes, erythroid cells, megakaryocytes and platelets.	When combined with CD64 is a more sensitive marker of monocytic differentiation than CD14.	
CD38	Precursor B cells (hematogones), normal follicle center B cells, immature and activated T cells, PC (bright intensity), myeloid and monocytic cells, and erythroid precursors.	Identification of early BM progenitor cell populations for further evaluation of phenotypic abnormalities	

Craig FE Foon KA. Blood 2008;111:3941-3967

Reagents of clinical utility in the evaluation of myeloid and monocytic neoplasms

secondary evaluation

Reagent	Normal distribution of staining	Clinical utility	Comments
CD41	Megakaryocytes and platelets.	Megakaryocytic differentiation.	May detect nonspecific binding of plt proteins to other cells such as monocytes.
CD61	Megakaryocytes and platelets.	Megakaryocytic differentiation.	May detect nonspecific binding of plt proteins to other cells such as monos. Sometimes combined with CD42b to distinguish pls from bl
cCD61	Megakaryocytes and platelets.	Megakaryocytic differentiation.	May demonstrate fewer problems with adherence of plt proteins.
CD64	Monocytes and intermediate neutrophilic precursors.	Identification of monocytic differentiation. May be aberrantly expressed in AML, MDS, and MPD.	Gained on mature neutrophils with sepsis.
CD71	Erythroid precursors (bright), myeloid, activated lymphoid, proliferating cells.	Identification of immature rythroid cells. Possibly expressed in MDS.	
cMPO	Neutrophilic and monocytic cells.	Indicator of myeloid differentiation.	In contrast to cytochemical stain, measures the presence of antigen, not enzyme activity.

Craig FE Foon KA. Blood 2008;111:3941-3967

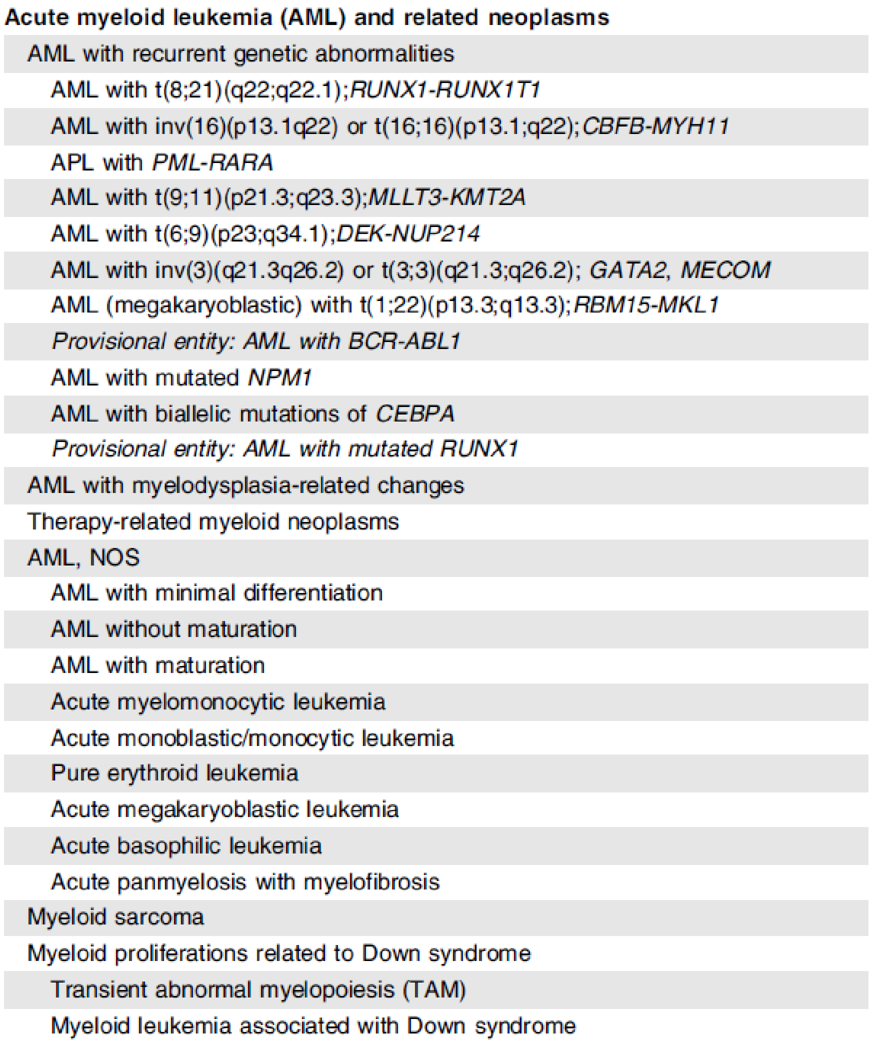
Reagents of clinical utility in the evaluation of myeloid and monocytic neoplasms

secondary evaluation

Reagent	Normal distribution of staining	Clinical utility	Comments
CD117	Immature neutrophilic cells and mast cells.	Identification myeloblasts.	May be expressed by cells more mature cells than blasts.
CD123	Monos, neutrophils, basophils, megakaryocytes,plasmacytoid dendritic cells (bright).	Identification of hematodermic neoplasms. Positive some AML, especially with monocytic differentiation.	Plasmacytoid DC may be increased in some reactive conditions such as Castleman disease and Kikuchi lymphadenitis and in association with MPD.
CD163	Monocyte, macrophage.	Indicator of monocytic differentiation	
CD235a	Erythroid precursors.	Indicator of erythroid maturation.	Not present on some immature erythroid precursors.

Craig FE Foon KA. Blood 2008;111:3941-3967

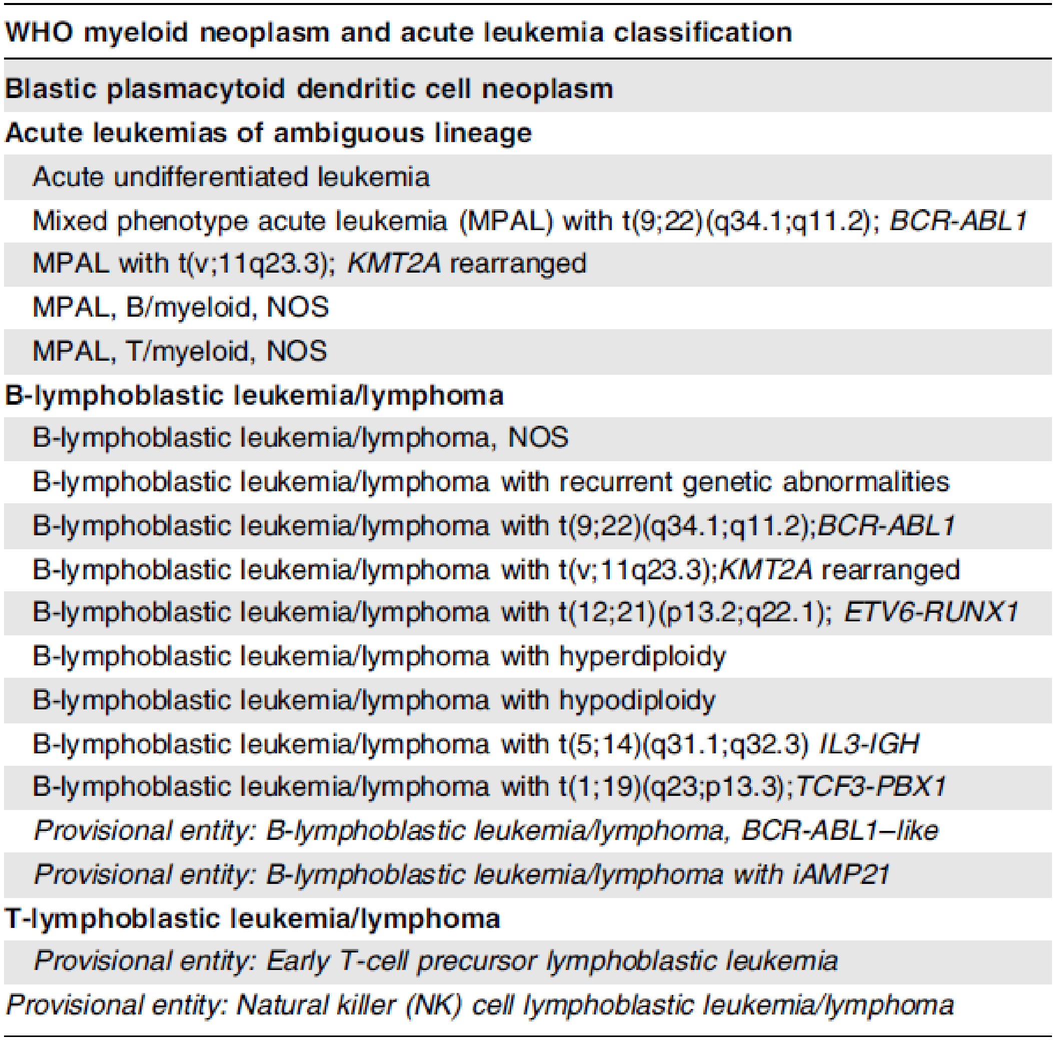
Classificazione WHO 2016 delle leucemie acute mieloidi e delle neoplasie correlate



Acute myeloid leukemia (AML) and related neoplasms

- AML with recurrent genetic abnormalities
  - AML with t(8;21)(q22;q22.1);*RUNX1-RUNX1T1*
  - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB-MYH11*
  - APL with *PML-RARA*
  - AML with t(9;11)(p21.3;q23.3);*MLLT3-KMT2A*
  - AML with t(6;9)(p23;q34.1);*DEK-NUP214*
  - AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM*
  - AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);*RBM15-MKL1*
  - *Provisional entity: AML with BCR-ABL1*
  - AML with mutated *NPM1*
  - AML with biallelic mutations of *CEBPA*
  - *Provisional entity: AML with mutated RUNX1*
- AML with myelodysplasia-related changes
- Therapy-related myeloid neoplasms
- AML, NOS
  - AML with minimal differentiation
  - AML without maturation
  - AML with maturation
  - Acute myelomonocytic leukemia
  - Acute monoblastic/monocytic leukemia
  - Pure erythroid leukemia
  - Acute megakaryoblastic leukemia
  - Acute basophilic leukemia
  - Acute panmyelosis with myelofibrosis
- Myeloid sarcoma
- Myeloid proliferations related to Down syndrome
  - Transient abnormal myelopoiesis (TAM)
  - Myeloid leukemia associated with Down syndrome

WHO classification of myeloid neoplasms and acute leukemia



WHO myeloid neoplasm and acute leukemia classification
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2); *BCR-ABL1*
MPAL with t(v;11q23.3); *KMT2A* rearranged
MPAL, B/myeloid, NOS
MPAL, T/myeloid, NOS
B-lymphoblastic leukemia/lymphoma
B-lymphoblastic leukemia/lymphoma, NOS
B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities
B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2);*BCR-ABL1*
B-lymphoblastic leukemia/lymphoma with t(v;11q23.3);*KMT2A* rearranged
B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); *ETV6-RUNX1*
B-lymphoblastic leukemia/lymphoma with hyperdiploidy
B-lymphoblastic leukemia/lymphoma with hypodiploidy
B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.3) *IL3-IGH*
B-lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3);*TCF3-PBX1*
Provisional entity: B-lymphoblastic leukemia/lymphoma, BCR-ABL1–like
Provisional entity: B-lymphoblastic leukemia/lymphoma with iAMP21
T-lymphoblastic leukemia/lymphoma
Provisional entity: Early T-cell precursor lymphoblastic leukemia
Provisional entity: Natural killer (NK) cell lymphoblastic leukemia/lymphoma

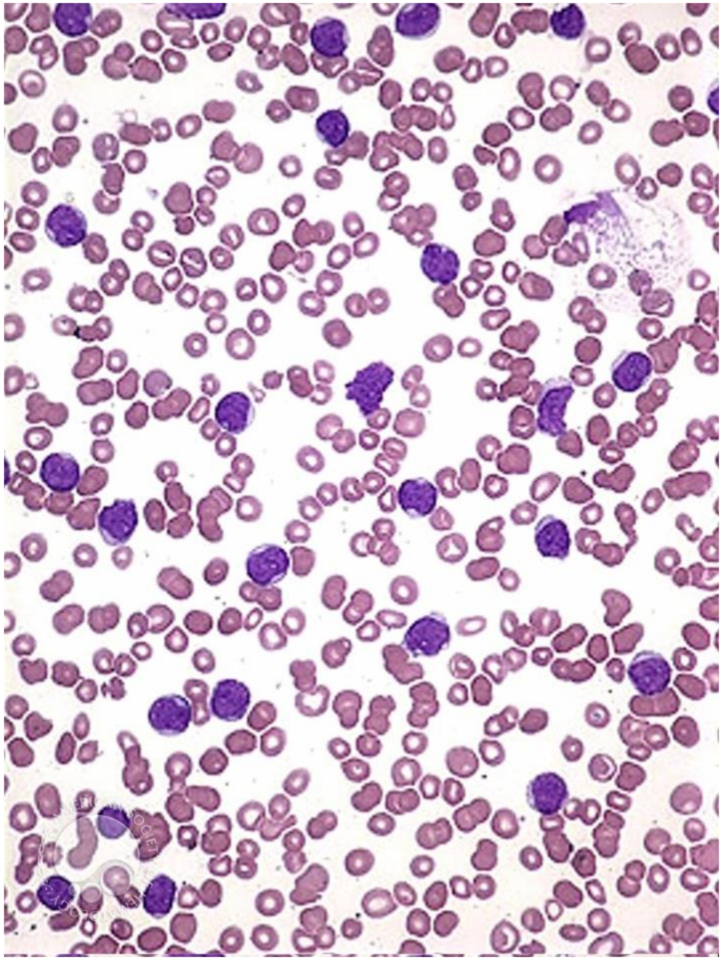
Diagnosi di LAM

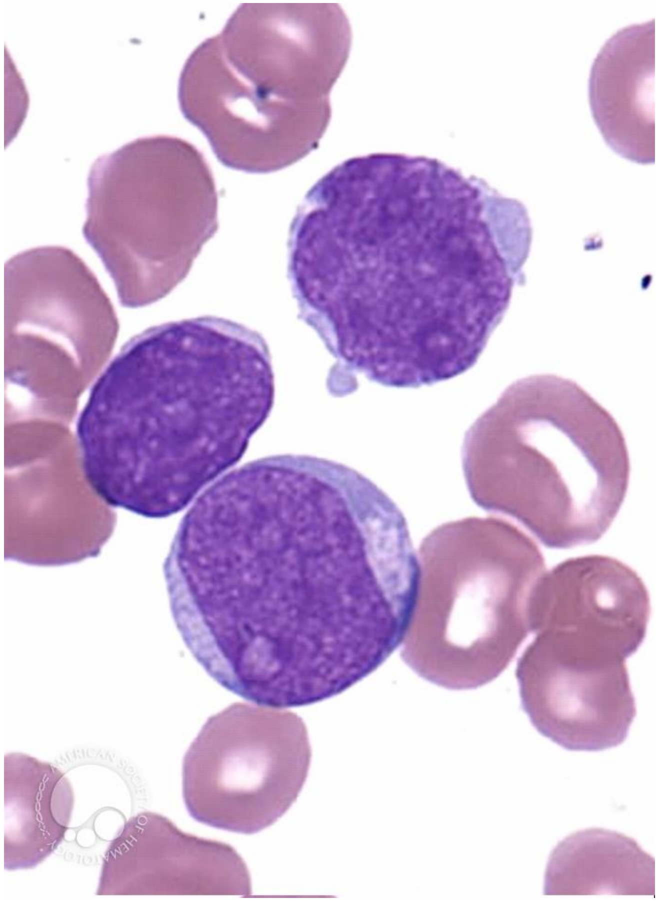
- La dimostrazione di un accumulo di blasti a livello midollare a causa di un blocco differenziativo è la principale caratteristica richiesta per la diagnosi di LAM.

- La diagnosi di LAM nella classificazione FAB, basata sulla citomorfologia e la citochimica, veniva posta in presenza di una percentuale di blasti midollari maggiore del 30%.

- L’attuale classificazione WHO ha abbassato il livello di blasti per la diagnosi di LAM al 20% includendo pertanto molti casi che precedentemente erano stati classificati come MDS.

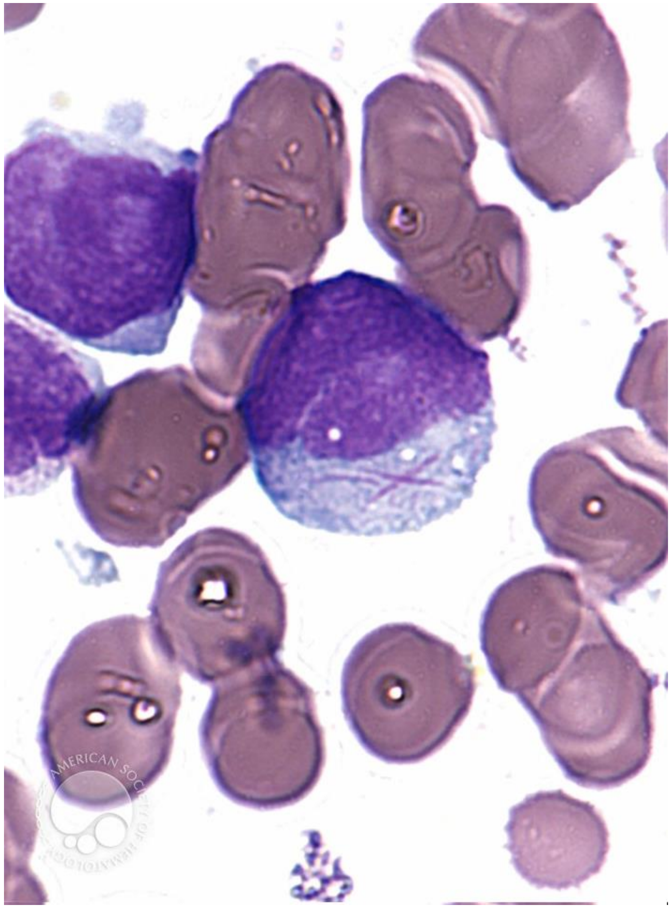
LAM: sangue periferico

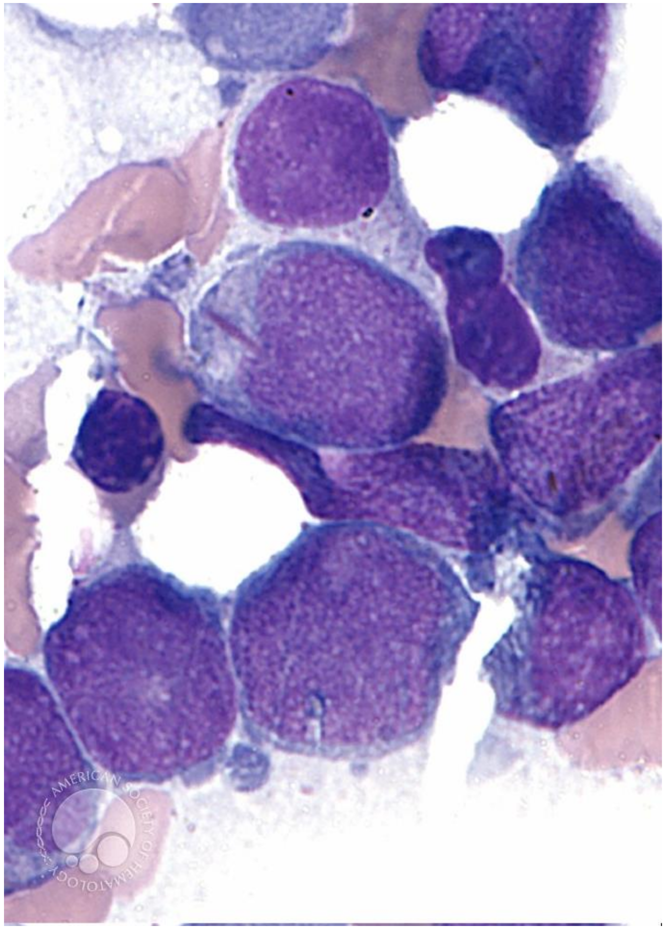




ASH image bank

LAM: corpi di Auer





ASH image bank

Campioni richiesti

- Per poter porre diagnosi di LAM la WHO suggerisce di analizzare i seguenti materiali biologici:
  - un campione di sangue periferico e di aspirato midollare cellulato per la valutazione morfologica con colorazione di May Grumwald Giemsa o similari.
  - una biopsia ossea di almeno 1,5 cm di lunghezza in tutti i casi possibili.
  - campioni di sangue midollare per lo studio citogenetico, citofluorimetrico, e molecolare da condurre in base alle informazioni fornite dai dati clinici, morfologici, immunofenotipici e citogenetici.

Valutazione dei blasti

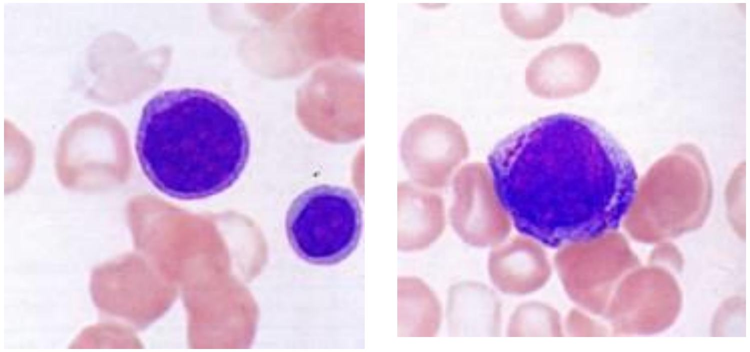
- La determinazione della % di blasti nel SP e BM viene fatta mediante valutazione visiva.
  - La % di blasti deve essere definita valutando, se possibile, 200 cellule nello striscio di SP e 500 cellule nucleate nell'aspirato midollare.
  - Devono essere conteggiati come blasti i mieloblasti, i monoblasti, i promonociti, i megacarioblasti (ma non i megacariociti displastici); i promielociti anomali sono da considerarsi come "blasti equivalenti" nella leucemia acuta promielocitica.
  - I proeritroblasti non devono essere considerati blasti tranne che nella eritroleucemia acuta "pura".
- La valutazione citofluorimetrica del CD34 non deve essere considerate come un sostituto del conteggio visivo dei blasti in quanto non tutti i blasti esprimono il CD34.
- Se l'aspirato midollare è povero e/o se è presente fibrosi midollare è necessario effettuare una valutazione immunoistochimica sulla biopsia ossea per il CD34 perché in questi casi i blasti sono CD34+.

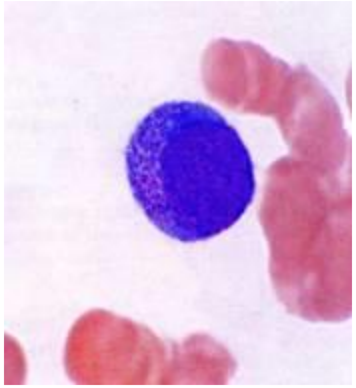
blasti e promielociti

Aspetti cellulari	Blasto non granulato	Blasto granulato	Promielocito normale	Promielocito displastico
Nucleo	Centrale di forma variabile	Centrale di forma variabile	Ovale, rotondo, indentato Centrale od eccentrico	Ovale, rotondo, indentato in posizione eccentrica
Cromatina	fine	fine	Fine od intermedia	Fine o grossolana
Nucleolo	1-2	1.2	Ben riconoscibile	Ben visibile
Zona Golgi	Non evidente	Non evidente	Ben visibile	Presente ma poco sviluppata
Granuli	Non visibili	Presenti (talora corpi di Auer)	Azzurrofili uniformemente dispersi	irregolare presenza e distribuzione
Citoplasmaa	basofilo	basofilo	basofilo	Basofilia ridotta ed irregolare

Blasto non granulato

Blasti granulati





promielocito

Haematologica 2008; 93:1712-1717.

Valutazione della filiera di appartenenza dei blasti

- La citofluorimetria multiparametrica (almeno a 3 o più colori) è raccomandata per determinare la filiera di appartenenza come pure per definire il profilo antigenico anomalo per lo studio della malattia minima residua.
- I marcatori di filiera sono:
  - mieloide: anti-MPO,
  - monocitaria (almeno 2 dei seguenti): esterasi non specifiche, CD11c, CD14, CD64, lisozima
  - linfoide B: cμ, cCD22 e CD79a,
  - linfoide T cCD3, CD3 e anti-TCR.
- La citochimica, MPO o esterasi non specifiche, possono essere di aiuto ma non sono essenziali in tutti i casi.
- L'immunoistochimica sulla biopsia può essere di aiuto nel riconoscimento di antigeni mieloidi e linfodi.

Assignment of blast lineage

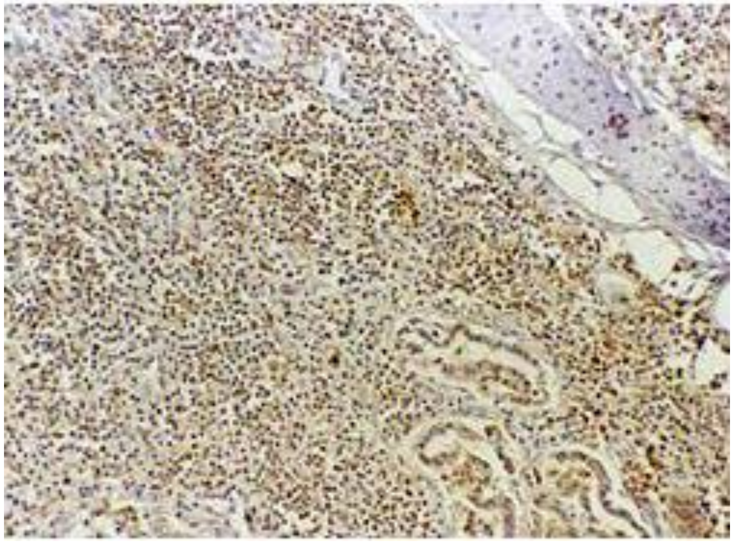
- Reliable distinction between AML and ALL is important for the selection of appropriate therapy.
- AML usually expresses
  - antigens characteristic of neutrophilic or monocytic differentiation such as CD13, CD15, CD33, CD64, CD117,
  - myeloperoxidase.
- B ALL expresses
  - CD19 has the highest sensitivity and specificity for the detection of B-cell lineage and
  - CytCD22 is also a sensitive and specific B-lineage marker,
  - surface CD22 staining is often weak
- T ALL expresses
  - cyt CD3.

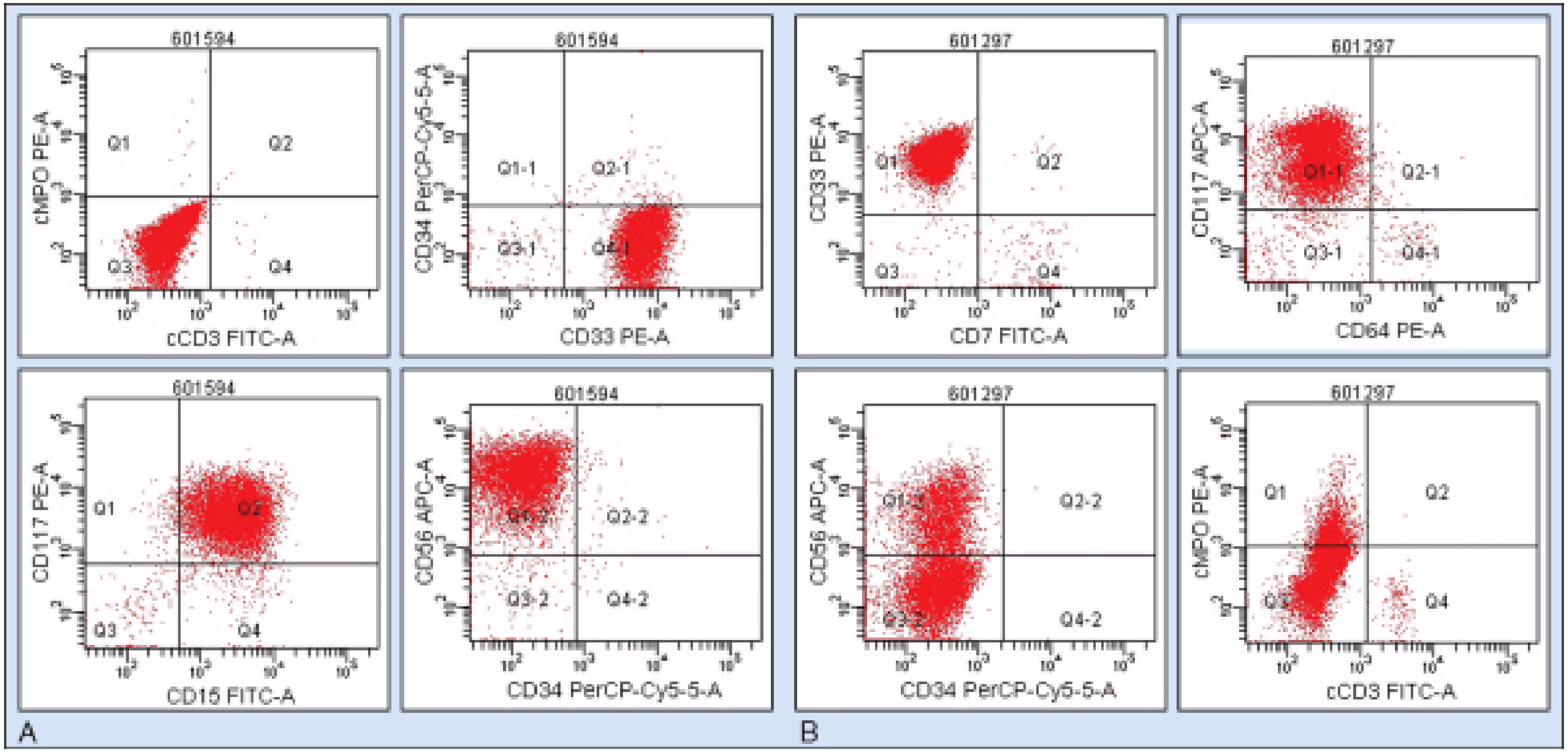
Classificazione immunologia delle leucemie acute mieloidi (EGIL 1995)

1. filiera mielomonocitaria*: anti MPO+, CD13+, CD33+, CD65+, CD117+
2. filiera eitroide (eritroide pura M6):
   - precoce/immatura: non classificabile con marcatori immunologici
   - tardiva/matura: antiglicoforina A+
3. Filiera megacariocitaria (M7): CD41+ e/o CD61+ (di membrana o citoplasmatici)
4. mieloide precoce (M0)(definito solo sulla base di marcatori immunologici): fenotipo come per le LAM mielomonocitiche ma con citochimica e marcatori linfoidi specifici negativi: CD3, CD79a, CD22)
5. LAM TdT+
6. LAM con espressione di antigeni linfoidi (LAM Ly+)

*positiva per almeno 2 marcatori mieloidi

MPO




601594
cMPO PE-A
cCD3 FITC-A
Q1
Q2
Q3
Q4
601594
CD34 PerCP-Cy5-5-A
CD33 PE-A
Q1-1
Q2-1
Q3-1
Q4-1
601297
CD33 PE-A
CD7 FITC-A
Q1
Q2
Q3
Q4
601297
CD117 APC-A
CD64 PE-A
Q1-1
Q2-1
Q3-1
Q4-1
601594
CD117 PE-A
CD15 FITC-A
Q1
Q2
Q3
Q4
601594
CD56 APC-A
CD34 PerCP-Cy5-5-A
Q1-2
Q2-2
Q3-2
Q4-2
601297
CD56 APC-A
CD34 PerCP-Cy5-5-A
Q1-2
Q2-2
Q3-2
Q4-2
601297
cMPO PE-A
cCD3 FITC-A
Q1
Q2
Q3
Q4
10^2
10^3
10^4
10^5
A
B

Classificazione immunologia della LAL (EGIL 1995)

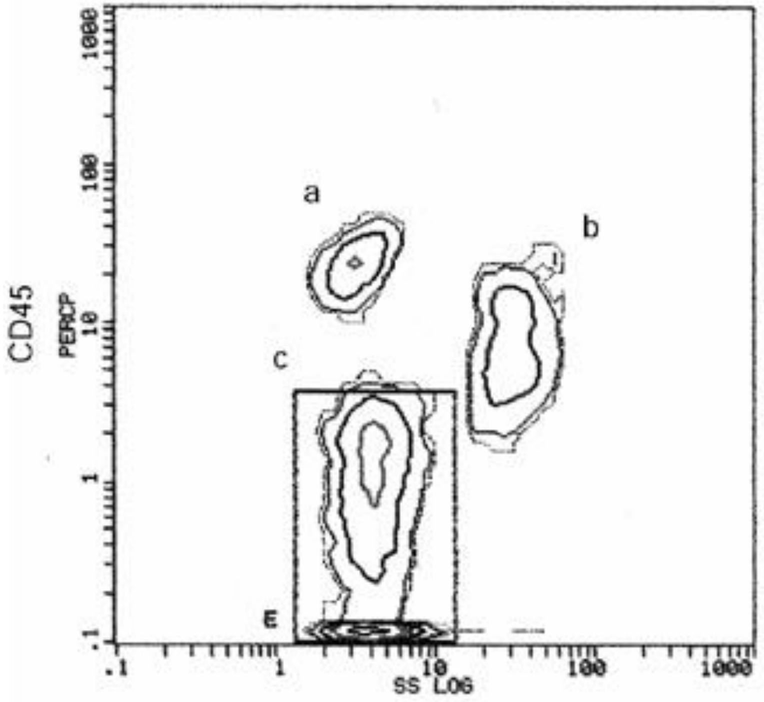
		Marcatori							
LAL filiera B	**FAB**	**CD34**	**CD19**	**cCD22**	**CD79α**	**HLA-DR**	**CD10**	**cμ**	**Sig**
Pro-B (B1)	L1, L2	+	+	+	+	+	-	-	-
B-comune (B2)	L1, L2	+/-	+	+	+	+	+	-	-
Pre-B (B3)	L1	-	+	+	+	+	+/-	+	-
B-matura (B4)	L3	-	+	+	+	+	-	-	+
LAL filiera T	**FAB**	**cCD3**	**CD3**	**CD2**	**CD7**	**TdT**	**TcR**	**CD1a**	**CD4**
Pro-T (T1)	L1, L2	+	-/+	-	+	+	+	-	-
Pre-T (T2)	L1, L2	+/-	+	+/-	+	+/-	+	-	-
T-corticale (T3)	L1, L2	-	+	+	+	-/+	+	+	+
T-matura (T4)	L1, L2	-	+	+	+	-	+	-	+

Identification and enumeration of blasts

- A plot of CD45 versus orthogonal (side) light scatter is very useful in identifying blasts by their low side light scatter and weak intensity expression of CD45.

- This representation can help to distinguish blasts from
  - lymphocytes (bright CD45),
  - erythroid precursors (essentially negative CD45),
  - neutrophilic precursors and eosinophils (higher side light scatter),
  - monocytes (higher side light scatter and brighter CD45).

A two-parameter histogram [CD45 vs. SSC] shows the presence of cells with low CD45 expression and low SS, which represent immature cells in acute leukemia.



The ungated histogram clearly shows three main populations: (***a***) small numbers of cells with strong CD45 expression and low SS (lymphocytes); (***b***) high to intermediate CD45 expression and high SS (granulocytes); and (***c***) low SS with decreasing expression of CD45. These are immature cells, and gating can be used to determine their phenotype.

Identification and enumeration of blasts

- Blasts often differ from more mature cells by expressing markers of immaturity and lacking antigens expressed by more mature cells.
  - Myeloblasts can be distinguished from maturing myeloid cells if they display
    - low orthogonal (side) light scatter,
    - markers of immaturity such as CD34 and CD117,
    - lack markers of maturation such as CD11b, CD15, and CD16.

Identification and enumeration of blasts

- Immature B-lymphoid cells can be distinguished from mature B-lymphoid cells if
  - they express CD34 and TdT,
  - lack surface immunoglobulin and CD20.

- Immature T-lymphoid cells can be distinguished from mature T-lymphoid cells if
  - they express CD34, TdT, or CD1a, or lack surface expression of CD3.

Identification of abnormal blasts

- Neoplastic blasts often have an abnormal phenotype that permits their distinction from normal immature cells.

- Phenotypic abnormalities include
  - expression of markers not normally present on cells of that lineage, such as
    - myeloid markers on lymphoblasts or
    - lymphoid markers on myeloblasts,
  - deviations from the well-coordinated gain and loss of antigens seen with normal maturation.

Role of FC in the diagnosis and classification of AML

- The identification of recurrent genetic abnormalities has assumed priority in the classification of AML.
- FC immunophenotypic studies remain of value in the
  - distinction from ALL.
  - identification of megakaryocytic differentiation with expression of CD41, CD61
  - identification of pure erythroid leukemia with expression of CD235a (glycophorin A) or CD36 in the absence of CD64, myeloperoxidase, and other myeloid-associated antigens.

Role of FC in the diagnosis and classification of AML

- Although FC studies can also evaluate for monocytic differentiation, cytochemical stains remain part of the current WHO classification scheme.
  - FC evaluation for CD14 lacks sensitivity for the detection of monocytic differentiation.

- the sensitivity of the FC assay can be improved by evaluation of other monocyte-associated antigens such as
  - coexpression of CD36 and CD64 bright,
  - intermediate CD15 plus bright CD33

Phenotype and recurrent genotypic abnormalities

- Some phenotypes in AML are associated with the presence of recurrent genotypic abnormalities.
  - AML with t(8;21)(q22;q22) is associated with aberrant expression of CD19, CD56, and sometimes TdT.
  - APL with t(15;17)(q22;q12) often has the following phenotype:
    - CD34 neg or only partially positive,
    - HLA-DR neg or only partially positive,
    - CD11b, CD13 heterogeneous,
    - CD117, CD33 (homogeneous bright staining),
    - CD15 or weak intensity staining.

Role of FC in the diagnosis and classification of ALL

- FC immunophenotyping is important for
  - the distinction between ALL and AML,
  - identification of B-cell or T-cell lineage,
  - assessing response to treatment, including the identification of early responders and the detection of MRD

Role of FC in the diagnosis and classification of ALL

- Some phenotypes in ALL are associated with the presence of prognostically significant cytogenetic and molecular abnormalities.
  - B-cell ALL with a CD9+, CD10+, CD19+, CD20- or only partial, CD34- phenotype is a sensitive marker for t(1;19)(q23;p13), but lacks specificity.
  - B-cell ALL with a CD10-, CD15+, CD24- or partial phenotype is associated with t(4;11)(q21;q23).

- However, FC immunophenotyping does not provide a suitable surrogate tool for detection of these subtypes of ALL.

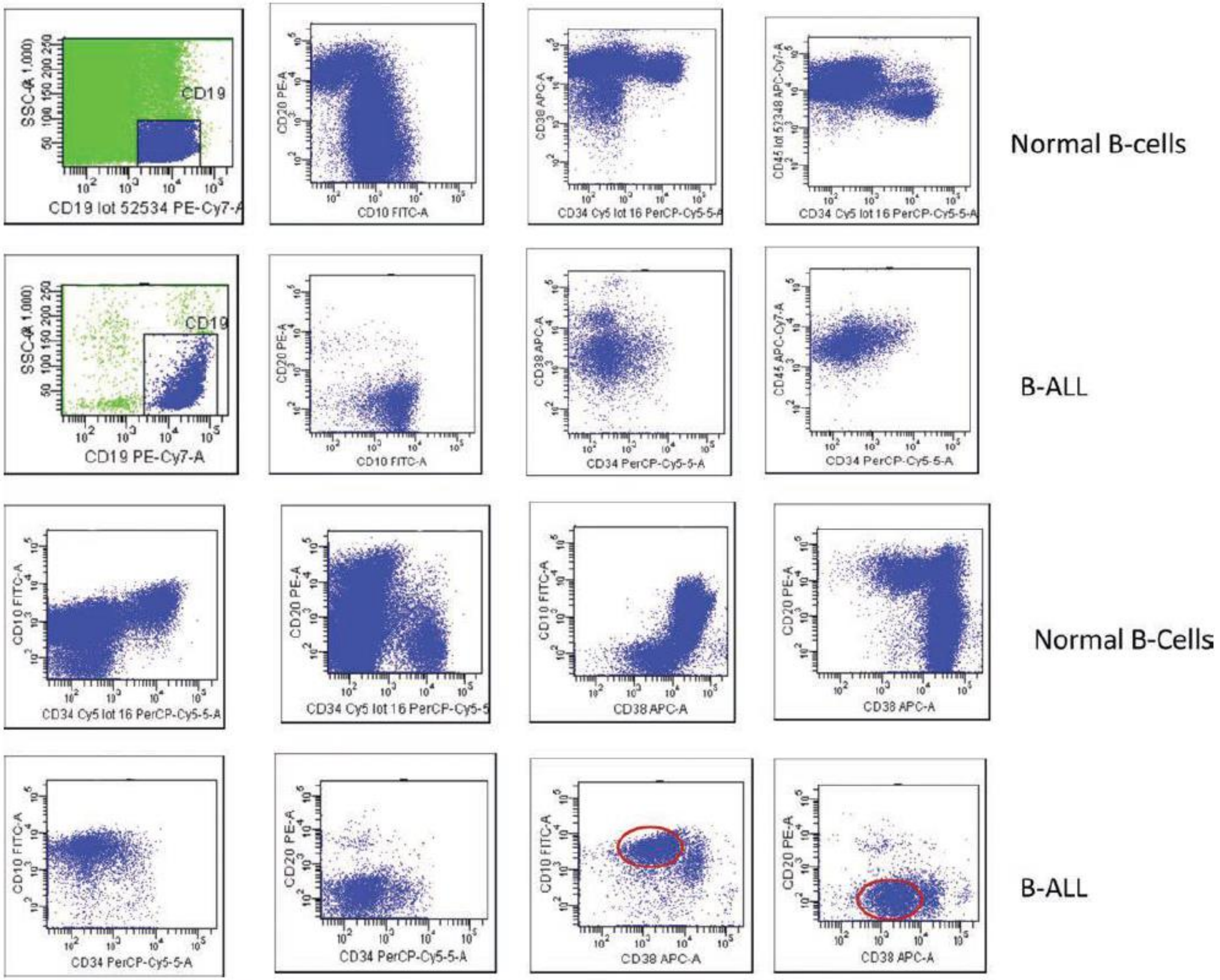


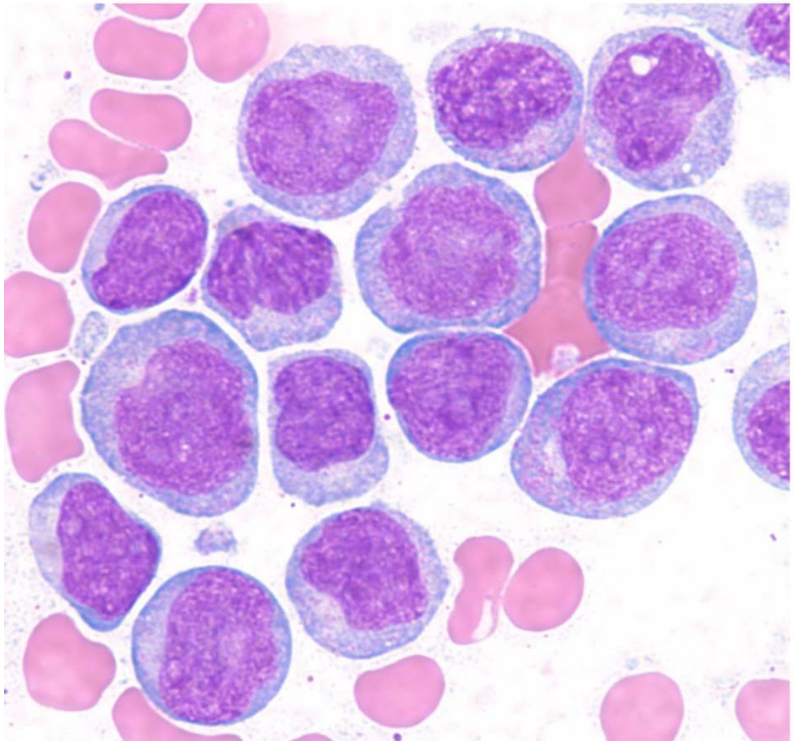
FIGURE 2.7. **Example of antigen expression in a case of B-precursor lymphoblastic leukemia/lymphoma in comparison to normal bone marrow B-cells in a child.** Bone marrow samples were analyzed with a six-color MAb panel, FACS-CANTOII flow cytometer, and Diva software (BD Bioscience). The panel consisted of CD10 FITC/CD20–PE/CD34 PerCp–Cy5.5/CD38–APC/CD19–PE–CY7/CD45 AmCyan (not shown). To acquire a sufficient number of B-cells, 500,000 events were analyzed on a normal bone marrow sample and 30,000 cells were acquired on the B-ALL sample. CD19+ B-cells were gated on the CD19/SS plot. Normal antigen expression patterns are shown in the upper and third row and corresponding plots for the B-ALL case are shown below (in the second and lower rows). In two lower right plots leukemic cells are found in "empty spaces," areas where normal cells are not found (red circles).

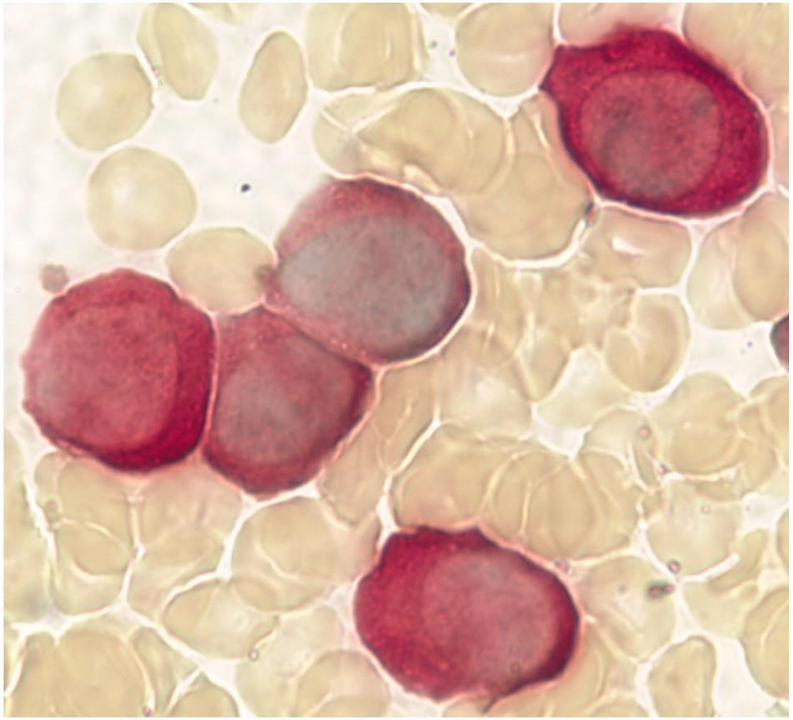
Phenotype and recurrent genotypic abnormalities

- Although FC immunophenotypic studies may be used as a screening tool, they lack specificity and sensitivity for the detection of genotypic abnormalities.

- FC immunophenotyping of AML is also of value in patients being considered for gemtuzumab ozogamicin therapy by demonstrating expression of the target antigen CD33.

AML with *MLL* rearrangement and monocytic differentiation.





butyrate esterase cytochemical stain

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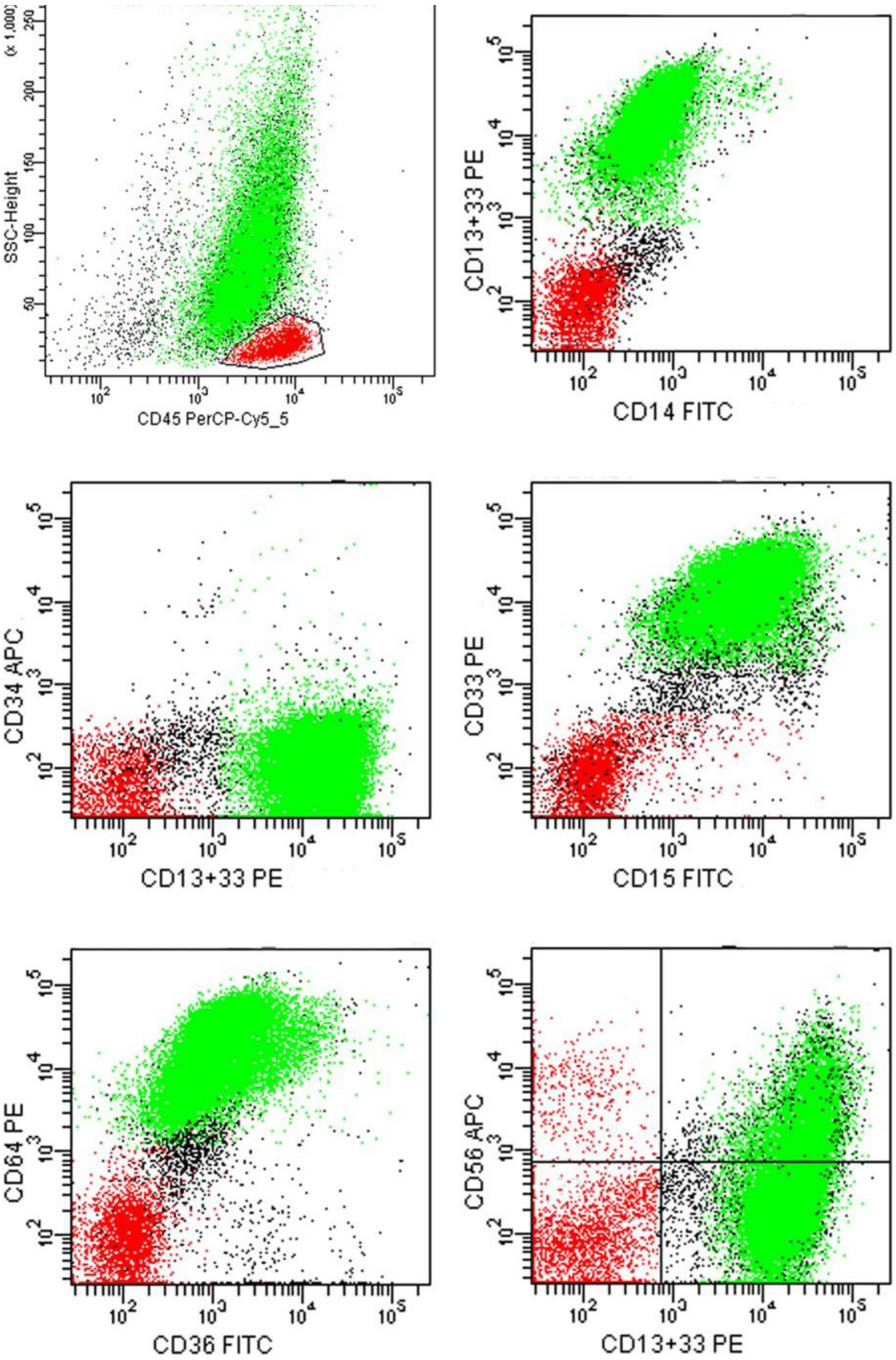

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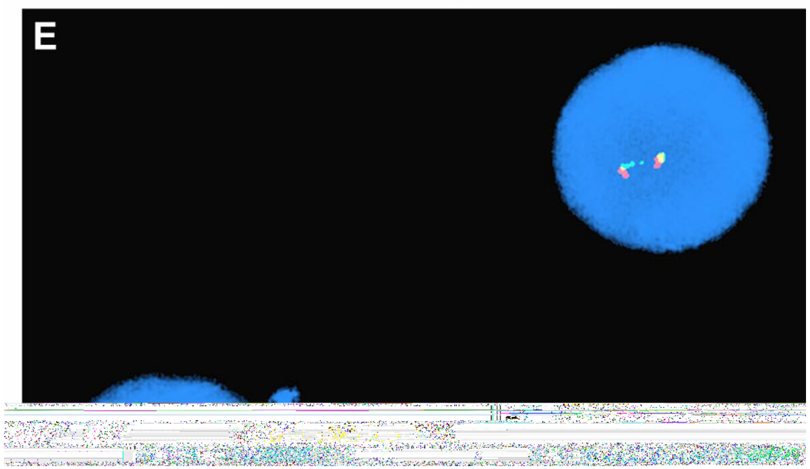
AML with *MLL* rearrangement and monocytic differentiation.



FISH demonstrating an MLL gene rearrangement. Hybridization with the LSI MLL dual color DNA probe demonstrates one cell (lower left) with one fusion signal (corresponding to the unrearranged chromosome 11 at band 11q23) and separate green and red signals corresponding to the split MLL gene, and one normal cell (top right) with 2 fusion signals.

47,XX, 8,t(11,19)(q23;p13.3).

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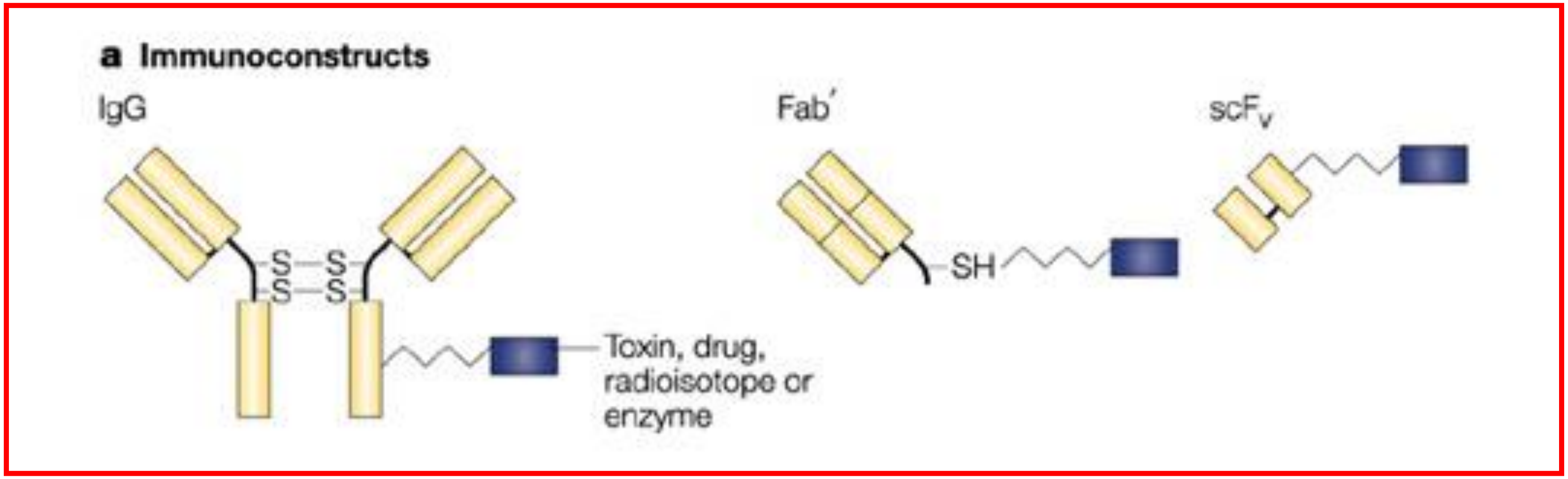
IMMUNOPHENOTYPIC PATTERNS ASSOCIATED WITH RECURRENT SPECIFIC CYTOGENETIC ABNORMALITIES IN LEUKEMIA

	Cytogenetic Abnormality	Characteristic Flow Cytometry Findings
AML	t(8;21) (q22;q22), *RUNX1-RUNX1T1*	At least a fraction of blasts with CD34$^{\text{bright}}$, often co-expressing CD19 and TdT but not CD10 Granulocytic differentiation (CD13, CD33, MPO and CD15), aberrant expression of CD56 common No monocytic differentiation
AML	Inv.(16)(p13.1q22) or t(16;16)(p13.1(q22) *CBF-MYH11*	Distinct populations of blasts, granulocytic and monocytic (CD14, CD4, CD64) precursors Co-expression of CD34 and CD64 common Eosinophils can be delineated by high SS and low FS than neutrophils and CD16 neg Often CD2 on blasts and precursors
AML	t(9;11)(p22;q23) *MLLT3-MLL*	MAb 7.1 positivity Monocytic differentiation (HLA-DR, CD4dim, CD11b, CD13, CD15, CD36, CD33, and CD64)
AML	*NPM1* mutated	Most often blasts CD34–, often HLA-DR–, CD117+, CD123+, CD33 $^{\text{bright}}$, CD110+ Show granulocytic differentiation (CD15+) Monocytic differentiation in 30% of cases Some cases only CD33 $^{\text{bright}}$ and MPO $^{\text{bright}}$ with no differentiation
AML	Inv.(3)(q21;q26.2) or t(3;3)(g21;q26.2) *RPN1-EVI1*	Positive for CD34, CD117, CD13, CD33, HLA-DR, and MPO
AML	t(6,9)(p23;q34) *DEK-NUP214*	CD9+, CD13+, CD33+, CD117+, and HLA-DR+, May be CD34– at presentation but CD34+ at relapse Basophils are often increased (CD123++, HLA-DR–)
AMkL	t(1;22)(p13;q13) *RBM15-MKL1*	Megakaryocytic differentiation CD41+, CD61+ often together with CD34, HLA-DR
APL	t(15;17)(q22;q12) *PML-RARα*	Hypergranular: most cases CD34–, HLA-DR–, CD11b–, CD11c–, CD117+, MPO+, CD33 $^{\text{bright}}$, CD13 heterogenous,CD15–/$^{\text{dim}}$, Hypogranular: often CD2+, subsets positive for CD34 and/or HLA-DR present
ALL (B)	t(4;11)(q21;q23) *AF4-MLL*	CD34+, CD19+, CD10−, CD20−, CD13 and/or CD33 may be positive, often CD15 and/or CD65+, 7.1+ , cyt.IgM−
ALL (B)	t(9;22)(q34;q11.2) *BCR-ABL1*	CD34++, CD19+, CD10+, CD20−/+, CD13, CD33, CD66c often positive, CD15−, CD65−, 7.1–, cyt.IgM-
ALL (B)	t(12;21)(p12;q22) *TEL-AML1*	CD34+ or −, CD19+, CD10+, CD20−/+, CD13, and/or CD33 often positive, CD66c−, CD15−, CD65−, 7.1−, cyt.IgM−
ALL (B)	hyperdiploid	CD34+ or subset, CD19+, CD10+++, CD123++, CD20−/+, CD13−, CD33−, CD66c−/+, CD15−, CD65−, 7.1–, cyt.IgM−
ALL (B)	t(1;19)(q23;p13.3) *TCF3-PBX1*	CD34− or subset, CD19+, CD10− or subset, CD20+, CD13−,CD33− CD66c−/+, CD15−, CD65−, 7.1–, cyt.IgM+
ALL (T)	*FLT3* activating mutation	Expression of CD117

Gemtuzumab Ozogamicin (GO): Mylotarg

- Anticorpo monoclonale umanizzato anti-CD33 legato covalentemente con la caliceamicina
- Caliceamicina: un derivato semisintetico di un potente antibiotico antitumorale che si inserisce nella struttura del DNA causando rotture nella struttura a doppia elica e determinando così la morte cellulare



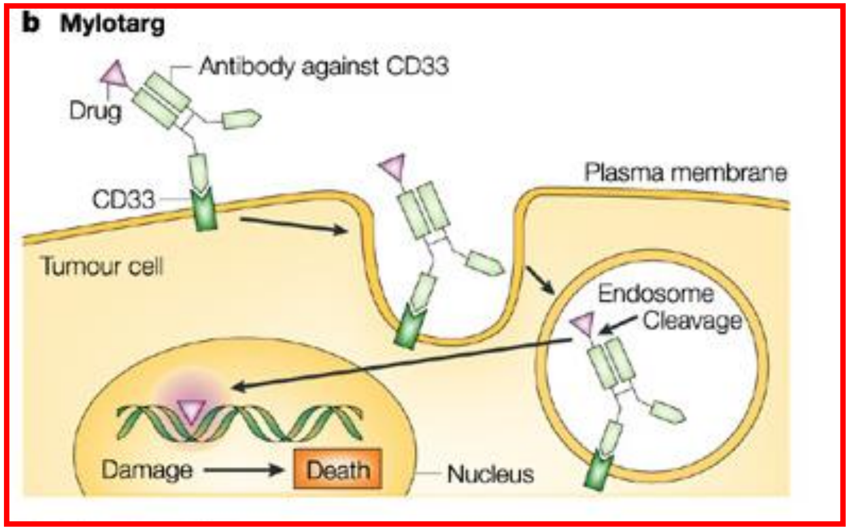
GO: Target

Target: CD33

- L’antigene CD33 è una proteina glicosilata transmembranaria (funzione sconosciuta) espressa:
  - sulle cellule mieloidi mature ed immature
  - sulle cellule eritroidi e megacariocitarie
  - sulla maggior parte delle cellule staminali emopoietiche ma non su quelle più immature
  - è poco espresso al di fuori il sistema emopoietico
- L’antigene CD33 è espresso in più del 90% delle LAM e delle sindromi mielodisplastiche

GO: modalità di azione

- Dopo il legame con l'antigene, GO è internalizzato mediante endocitosi.
- Il legame tra l'AtcMo e la caliceamicina viene scisso all'interno dei lisosomi dalle idrolasi acide, con conseguente rilascio della caliceamicina
- La caliceamicina liberata esercita la propria azione a livello del DNA con attivazione della apoptosi mdiata dalla p53



Leucemie di “lineage” ambiguo

- La WHO nel 2008 ha proposto di raggruppare le leucemie acute bifenotipiche e quelle bilineari in un’unica subentità definita “Leucemie acute a fenotipo misto (MFAL)”.
- I casi di leucemia acuta BCR-ABL1–positivi e MLL-positivi possono talora sodddifsare i criteri per MFAL; in presenza di patologie BCR-ABL1–positive, occorre escludere una crisi blastica di LMC.

Requisiti per l'attribuzione a più di una filiera di una singola popolazione blastica in corso di leucemia acuta a fenotipo misto (MPAL).

Filiera	**positività**
mieloide	mieloperossidasi (ctometria a flusso, immunoistochica, o citochimica) o differenziazione monocitica (almeno 2 tra: esterasi non specifiche, CD11c, CD14, CD64, lisozima
linfoide T	CD3 citoplasmatico (citometria a flusso con anticorpi contro la catena ipsilon del CD3). o CD3 di superficie (raro nella MFAL)
linfoide B	Forte positività per il CD19 con associata almeno una forte positività tra: CD79a, CD22 citoplasmatico, CD10 o Debole positività per il D19 con associate almeno 2 forti positività tra: CD79a, CD22 citoplasmatico, CD10

Assignment of blast lineage

- Leukemic blasts may aberrantly express antigens from another lineage.

- In AML lymphoid antigens frequently expressed include CD7, CD56, CD2, and CD19.

- ALL frequently demonstrates expression of one or more myeloid antigen.
  - Although the detection of 1 or 2 myeloid antigens on lymphoblasts can assist in the identification of the abnormal cells, it has not an independent prognostic significance.

MPAL: morphology

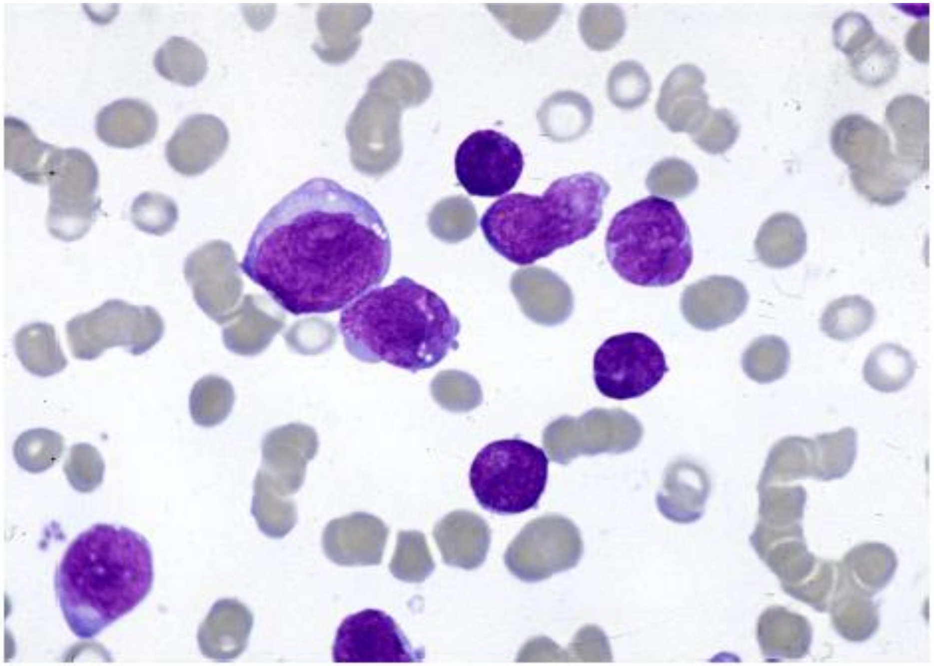


Figure 1. May-Grunwald-Giemsa–stained BM smear showing a mixed-cell population of large and small blasts.

Morphology consistent with

- **ALL; 43%,**
- **AML; 42%, or**
- **Inconclusive; 15%.**

Matutes et al Blood. 2011; 117(11):3163-3171

MPAL

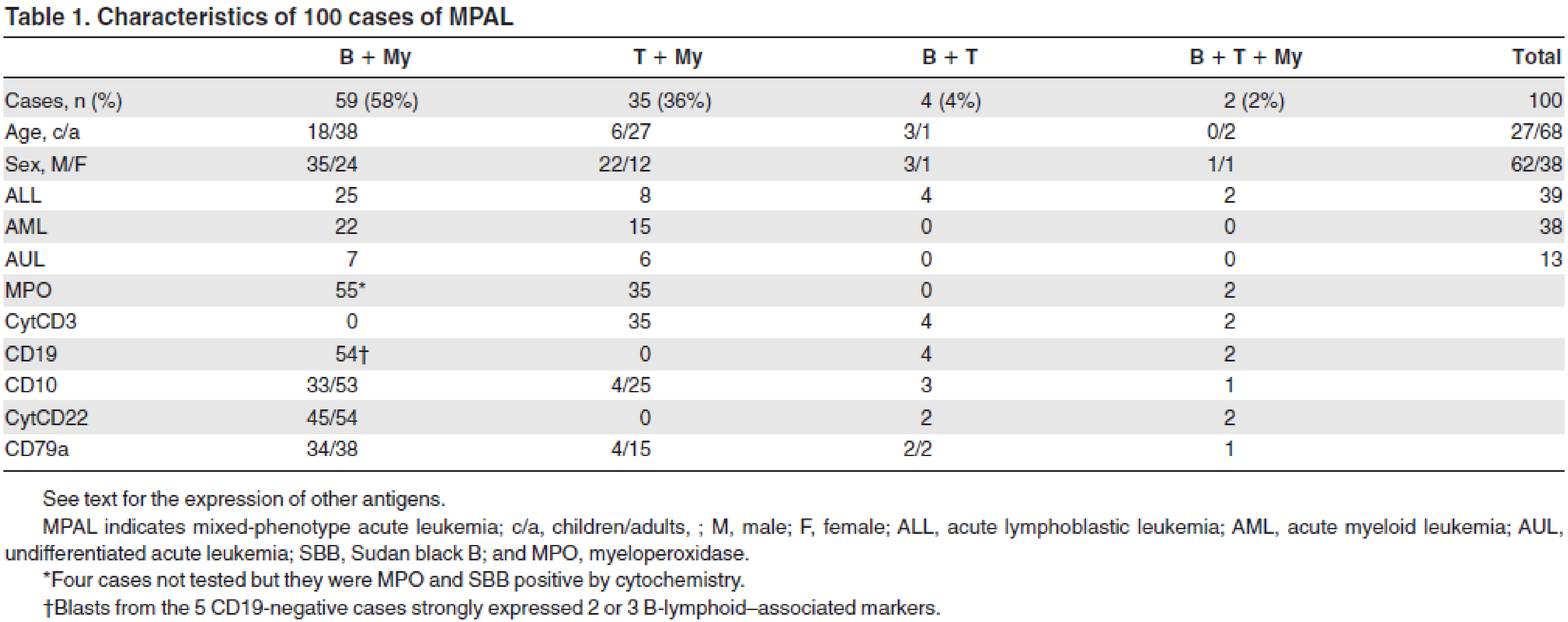


Table 1. Characteristics of 100 cases of MPAL

	B + My	T + My	B + T	B + T + My	Total
Cases, n (%)	59 (58%)	35 (36%)	4 (4%)	2 (2%)	100
Age, c/a	18/38	6/27	3/1	0/2	27/68
Sex, M/F	35/24	22/12	3/1	1/1	62/38
ALL	25	8	4	2	39
AML	22	15	0	0	38
AUL	7	6	0	0	13
MPO	55*	35	0	2	
CytCD3	0	35	4	2	
CD19	54†	0	4	2	
CD10	33/53	4/25	3	1	
CytCD22	45/54	0	2	2	
CD79a	34/38	4/15	2/2	1	

See text for the expression of other antigens.

MPAL indicates mixed-phenotype acute leukemia; c/a, children/adults, ; M, male; F, female; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AUL, undifferentiated acute leukemia; SBB, Sudan black B; and MPO, myeloperoxidase.

*Four cases not tested but they were MPO and SBB positive by cytochemistry.

†Blasts from the 5 CD19-negative cases strongly expressed 2 or 3 B-lymphoid–associated markers.

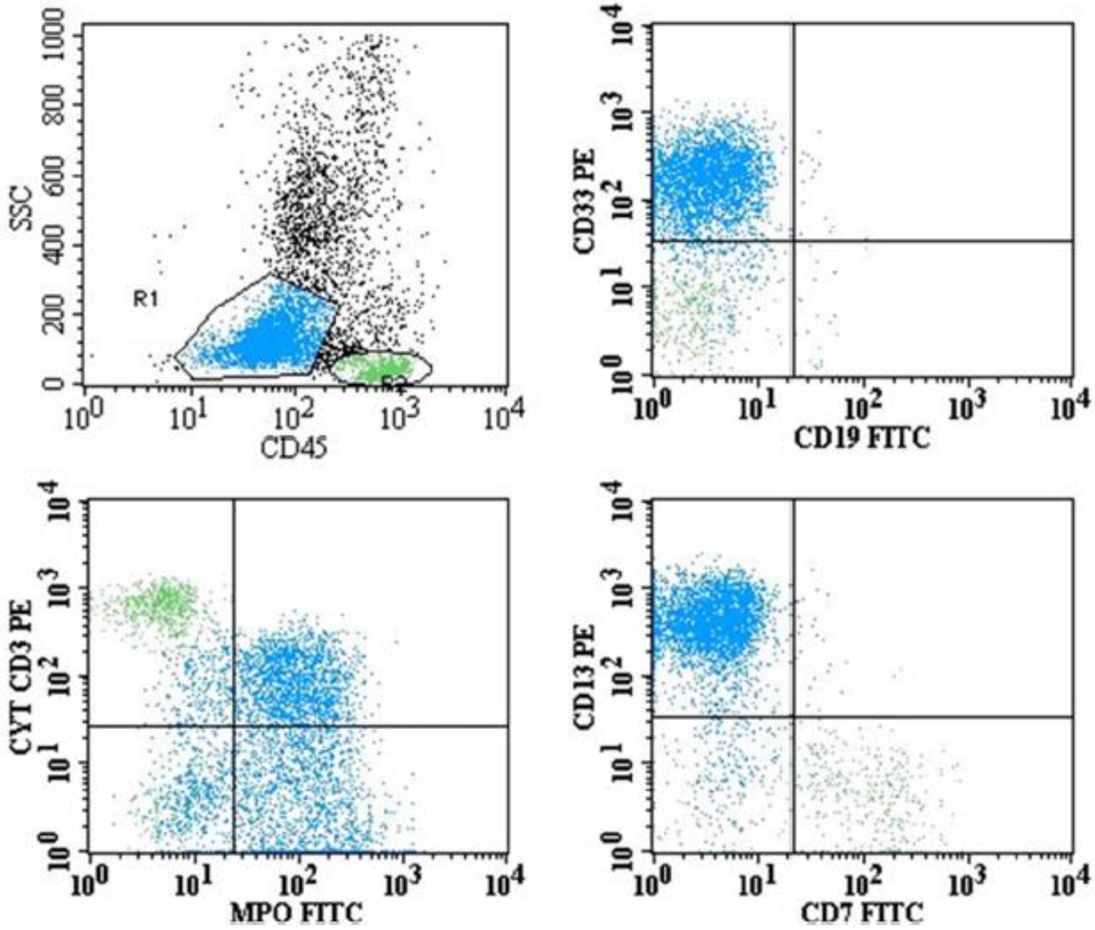
Immunophenotyping showed

- **B myeloid (59%),**
- **T myeloid (35%),**
- **B T (4%), or**
- **trilineage (2%).**

Matutes et al Blood. 2011; 117(11):3163-3171

MPAL

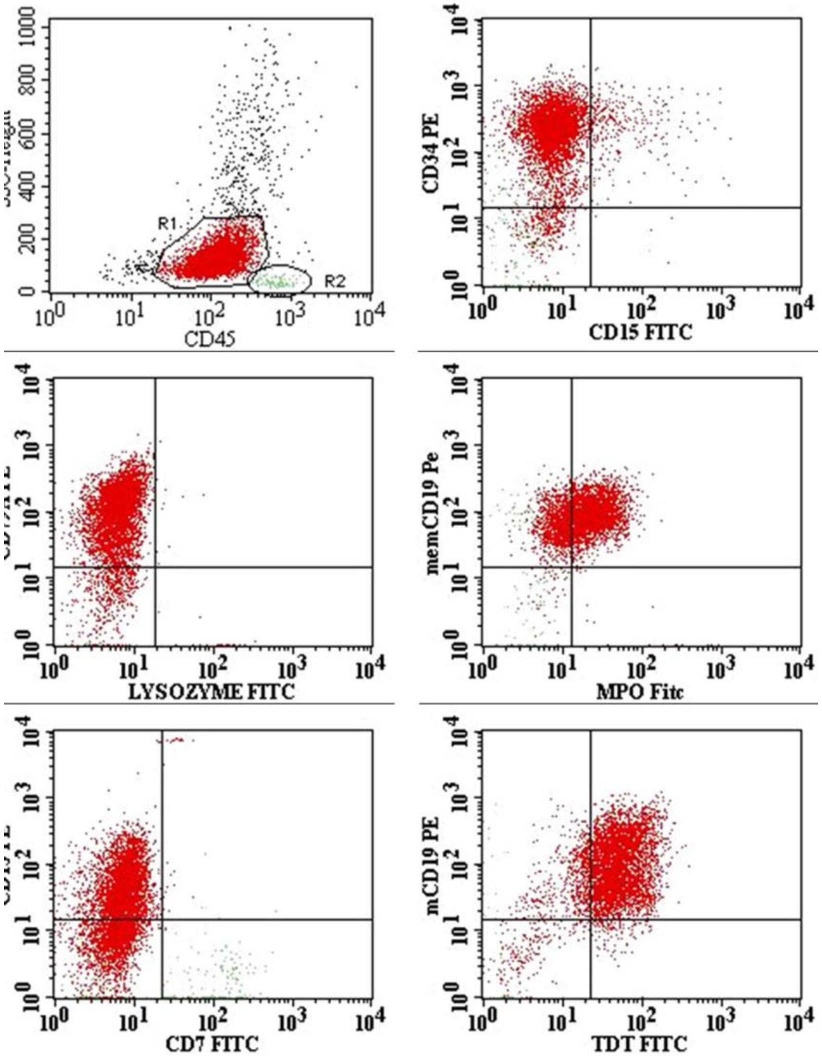
Dot plots with the blast population highlighted in blue (R1) and lymphocyte population in green (R2). These dot plots demonstrate the expression of CD13 and CD33 and coexpression of MPO with cytCD3. Residual T lymphocytes are positive for cyt.CD3 but MPO-neg. CD19 and CD7 are negative.



Matutes et al Blood. 2011; 117(11):3163-3171

MPAL

Dot plots with the blast population highlighted in red (R1) and lymphocyte population in green (R2).
These dot plots demonstrate the expression of CD13 and coexpression of MPO with CD19.
Other positive markers are CD34, CD79a, and TdT. CD7, CD15, and lysozyme are negative



Matutes et al Blood. 2011; 117(11):3163-3171

MPAL

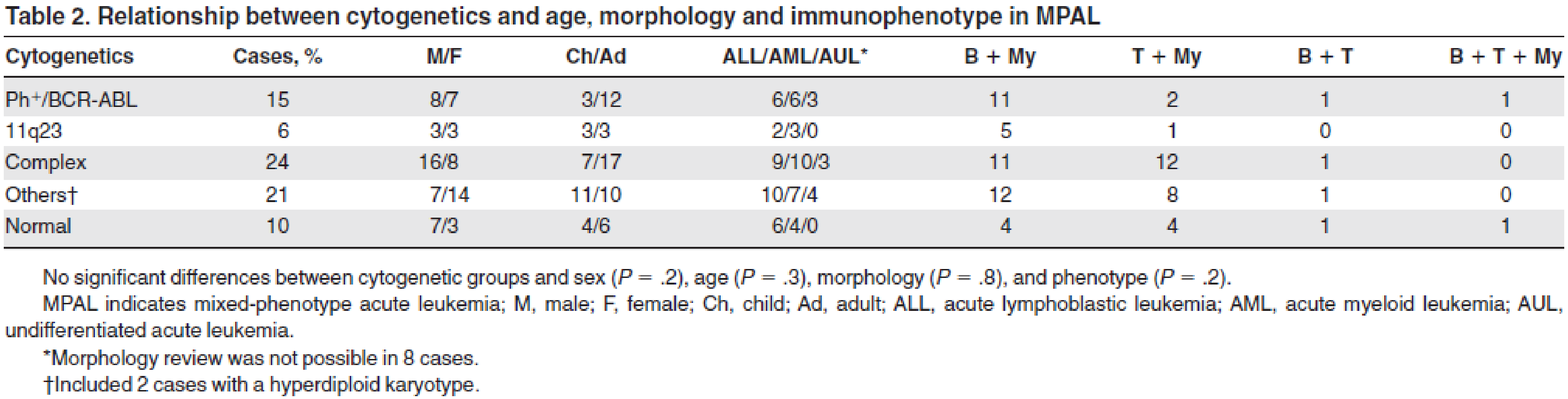


Table 2. Relationship between cytogenetics and age, morphology and immunophenotype in MPAL

Cytogenetics	Cases, %	M/F	Ch/Ad	ALL/AML/AUL*	B + My	T + My	B + T	B + T + My
Ph^+/BCR-ABL	15	8/7	3/12	6/6/3	11	2	1	1
11q23	6	3/3	3/3	2/3/0	5	1	0	0
Complex	24	16/8	7/17	9/10/3	11	12	1	0
Others†	21	7/14	11/10	10/7/4	12	8	1	0
Normal	10	7/3	4/6	6/4/0	4	4	1	1

No significant differences between cytogenetic groups and sex ($P = .2$), age ($P = .3$), morphology ($P = .8$), and phenotype ($P = .2$).

MPAL indicates mixed-phenotype acute leukemia; M, male; F, female; Ch, child; Ad, adult; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AUL, undifferentiated acute leukemia.

*Morphology review was not possible in 8 cases.

†Included 2 cases with a hyperdiploid karyotype.

Matutes et al Blood. 2011; 117(11):3163-3171

Plasma cell disorders

- Plasma cell disorders are often identified through increased serum or urine gamma globulins and can be divided into
  - polyclonal/reactive proliferations
  - those producing monoclonal gammopathy.

- The monoclonal gammopathies can be further divided into
  - monoclonal gammopathy of undetermined significance (MGUS)
  - overt PCN, including plasmacytoma, plasma cell myeloma and variants, plasma cell leukemia, amyloidosis, and immunoglobulin light and heavy chain diseases.

Plasma cell disorders

- The diagnosis of a PCN usually requires
  - identification of increased plasma cells (greater than 10% of marrow cells),
  - demonstration of an abnormal phenotype and/or clonality,
  - further classification using a combination of morphologic, laboratory, radiologic, and other clinical findings.

- Flow cytometric immunophenotyping is a useful tool
  - for the identification of abnormal plasma cells
  - for the distinction between lymphoid and plasma cell neoplasms.
  - may provide additional prognostic information

Reagents of clinical utility in the evaluation of plasma cell disorders

initial evaluation

Reagent	Normal distribution of staining	Clinical utility	Comments
CD19	All B cells including lymphoblasts, mature B lymphoid cells, and most PCs.	PCN often CD19-, mature B-cell lymphoid neoplasms with plasmacytic differentiation+	Aberrant expression on myeloid cells.
CD38	Precursor B cells (hematogones), normal follicle center B cells, immature and activated T cells, PCs (bright intensity), myeloid and monocytic cells, and erythroid precursors.	Identification of PCs with bright intensity staining, often in combination with dim to negative CD45.	Not specific for PCs.
CD45	All B cells (weaker intensity on precursors and some PCs), all T cells (weaker intensity on precursors).	Identification of PCs often in combination with CD38.	-

Craig FE Foon KA. Blood 2008;111:3941-3967

Reagents of clinical utility in the evaluation of plasma cell disorders

secondary evaluation

Reagent	Normal distribution of staining	Clinical utility	Comments
CD56	NK cells, NK-like T cells	May be aberrantly expressed in PCN	CD56- PCN more often leukemic.
CD10	Immature T cells and B cells, subset of mature T cells and B cells, and neutrophils	May be aberrantly expressed in PCN	-
CD117	Immature myeloid, mast cells.	May be aberrantly expressed in PCN	Also described in MGUS.
CD138	Plasma cells	Identification of plasma cells.	More specific than CD38, but not as sensitive.
Cyt κ & λ,	Plasma cells	Light chain restriction in cells with plasmacytic differentiation.	Most FC assays detect sIg and cytIg.

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Identification of abnormal plasma cells

- Two antigens are commonly used to identify PCs:
  - CD38 (bright intensity)
  - CD138.
- In contrast to the bright CD38 staining characteristic of PCs, other cell types typically express CD38 at a lower intensity:
  - hematogones, some mature B cells, activated T cells, and myeloid cells.

- CD138 expression is restricted to PCs and some carcinoma cells, but is less sensitive for the detection of PCs than CD38.
- Evaluation for both CD38 and CD138 provides the most sensitive and specific means of detecting PCs.

Identification of abnormal plasma cells

- **CD38 and CD138 are often used in combination with CD45.**
  - **Normal tonsillar and peripheral blood PCs are CD45+.**
  - **Normal BM usually contains 2 subsets of PCs:**
    - **a CD45 subset that appears to be proliferative,**
    - **a small subset that is negative or only expresses CD45 at a low intensity.**
  - **The phenotype of neoplastic PCs usually differs from that of reactive PCs**
    - **Neoplastic PCs often demonstrate**
      - **lower levels of expression of CD38 and CD138,**
      - **include a larger CD45 negative to low-intensity population with only a small CD45+ population.**

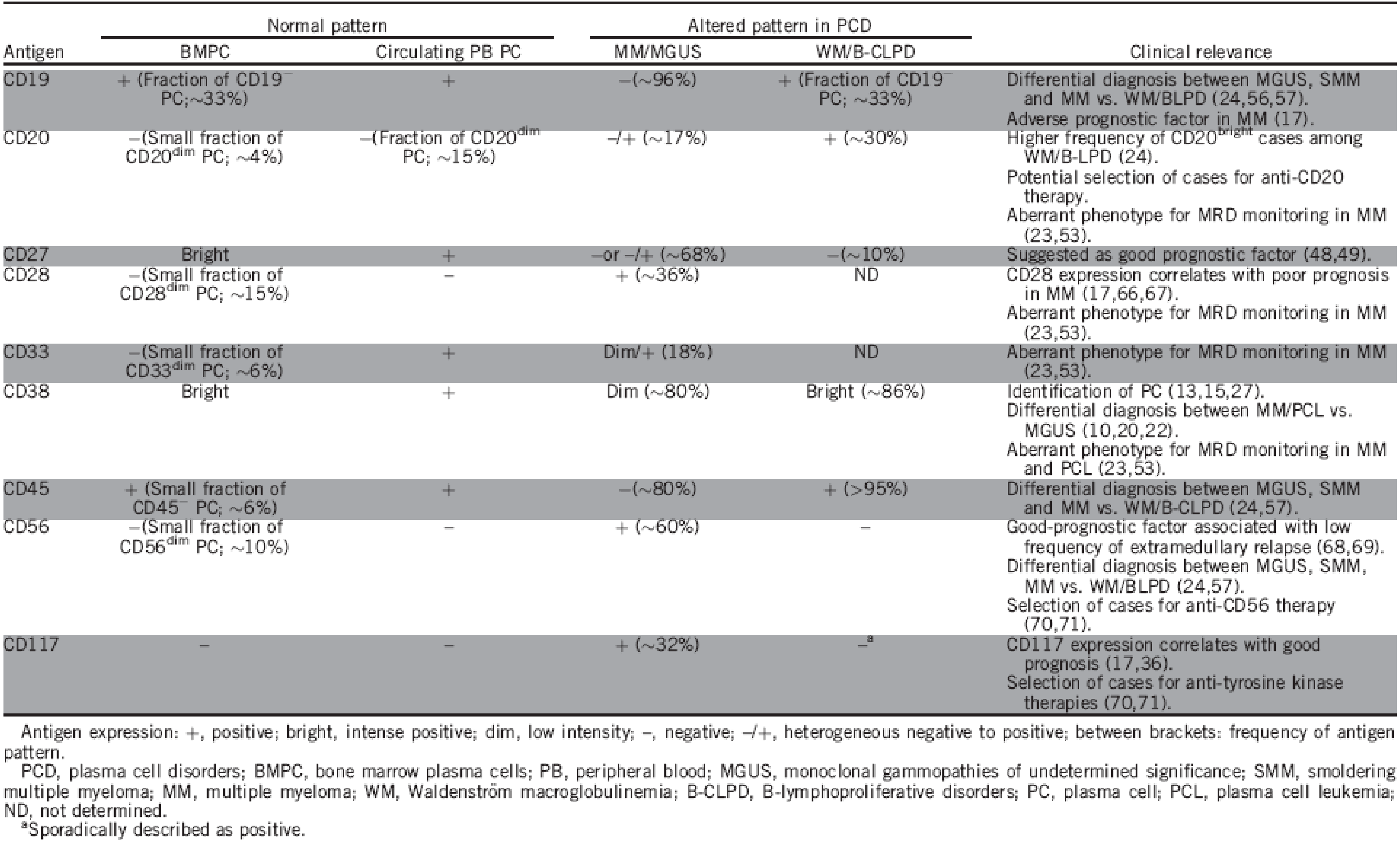
Identification of abnormal plasma cells

- **PCNs usually demonstrate**
  - **an abnormal CD19-, CD20- phenotype that differs from most normal CD19+, CD20- BM PCs and CD19+, CD20+ B-cell lymphoid neoplasms.**

- **Although approximately 10% of PCNs express CD20, and a smaller subset are CD19+, expression of both CD19 and CD20 is rare and should raise the possibility of a mature lymphoid B-cell neoplasm.**

- **CD20 expression in myeloma has been associated with a**
  - **more “lymphoid” appearance,**
  - **presence of the translocation t(11;14),**
  - **cyclin-D1 protein staining by paraffin section IHC.**

Most Relevant Antigens and Their Most Frequent Pattern of Expression in Normal vs. Clonal PCs



	Normal pattern		Altered pattern in PCD		
Antigen	BMPC	Circulating PB PC	MM/MGUS	WM/B-CLPD	Clinical relevance
CD19	+ (Fraction of CD19$^-$ PC;~33%)	+	−(~96%)	+ (Fraction of CD19$^-$ PC; ~33%)	Differential diagnosis between MGUS, SMM and MM vs. WM/BLPD (24,56,57). Adverse prognostic factor in MM (17).
CD20	−(Small fraction of CD20^{dim} PC; ~4%)	−(Fraction of CD20^{dim} PC; ~15%)	−/+ (~17%)	+ (~30%)	Higher frequency of CD20^{bright} cases among WM/B-LPD (24). Potential selection of cases for anti-CD20 therapy. Aberrant phenotype for MRD monitoring in MM (23,53).
CD27	Bright	+	−or −/+ (~68%)	−(~10%)	Suggested as good prognostic factor (48,49).
CD28	−(Small fraction of CD28^{dim} PC; ~15%)	−	+ (~36%)	ND	CD28 expression correlates with poor prognosis in MM (17,66,67). Aberrant phenotype for MRD monitoring in MM (23,53).
CD33	−(Small fraction of CD33^{dim} PC; ~6%)	+	Dim/+ (18%)	ND	Aberrant phenotype for MRD monitoring in MM (23,53).
CD38	Bright	+	Dim (~80%)	Bright (~86%)	Identification of PC (13,15,27). Differential diagnosis between MM/PCL vs. MGUS (10,20,22). Aberrant phenotype for MRD monitoring in MM and PCL (23,53).
CD45	+ (Small fraction of CD45$^-$ PC; ~6%)	+	−(~80%)	+ (>95%)	Differential diagnosis between MGUS, SMM and MM vs. WM/B-CLPD (24,57).
CD56	−(Small fraction of CD56^{dim} PC; ~10%)	−	+ (~60%)	−	Good-prognostic factor associated with low frequency of extramedullary relapse (68,69). Differential diagnosis between MGUS, SMM, MM vs. WM/BLPD (24,57). Selection of cases for anti-CD56 therapy (70,71).
CD117	−	−	+ (~32%)	−[a]	CD117 expression correlates with good prognosis (17,36). Selection of cases for anti-tyrosine kinase therapies (70,71).

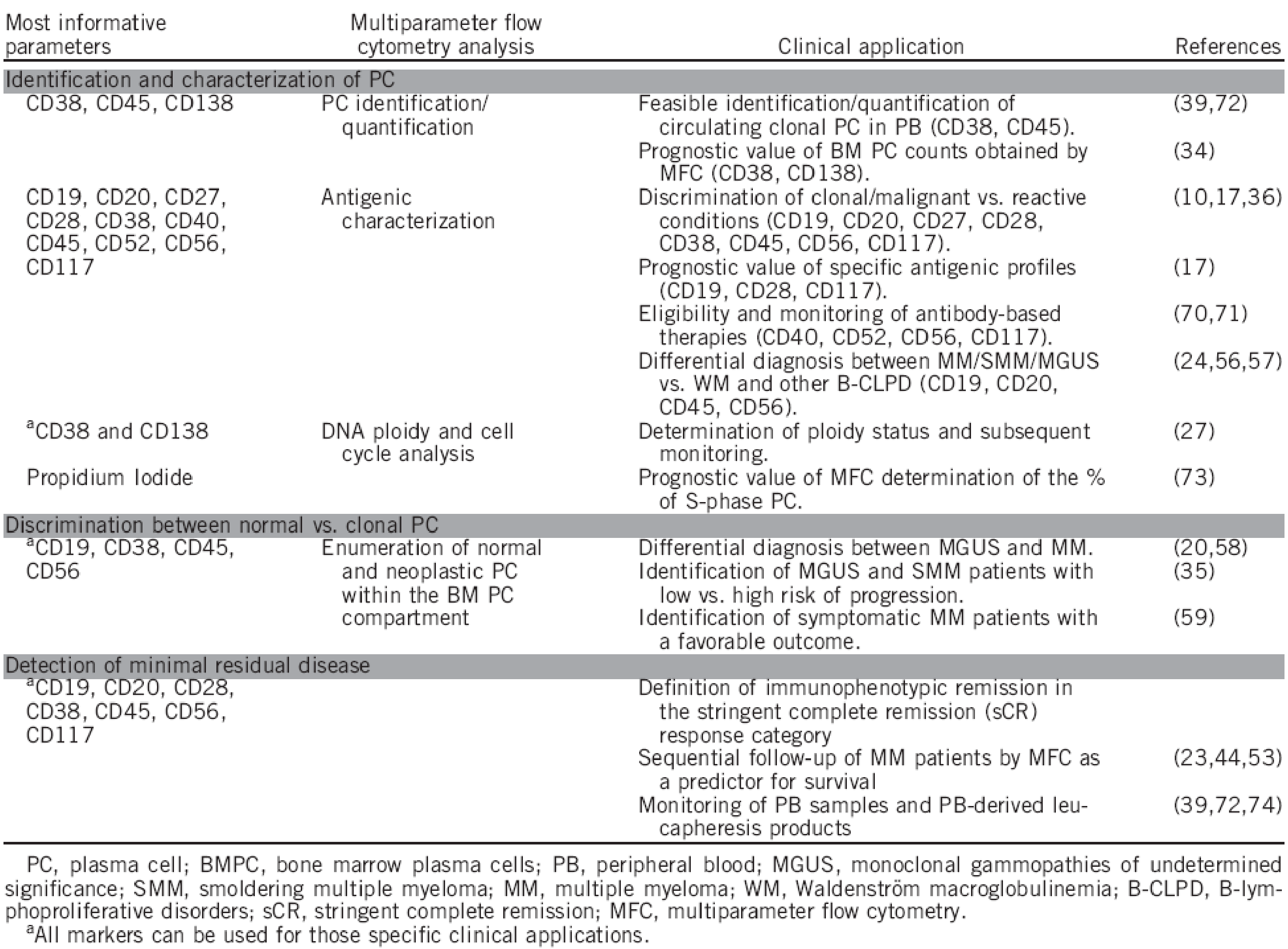
Antigen expression: +, positive; bright, intense positive; dim, low intensity; −, negative; −/+, heterogeneous negative to positive; between brackets: frequency of antigen pattern.

PCD, plasma cell disorders; BMPC, bone marrow plasma cells; PB, peripheral blood; MGUS, monoclonal gammopathies of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma; WM, Waldenström macroglobulinemia; B-CLPD, B-lymphoproliferative disorders; PC, plasma cell; PCL, plasma cell leukemia; ND, not determined.

[a] Sporadically described as positive.

Cytometry Part B (Clinical Cytometry) 78B:239–252 (2010)

Most Relevant Clinical Applications of Multiparameter FC Immunophenotyping of PC-related Disorders



Most informative parameters	Multiparameter flow cytometry analysis	Clinical application	References
Identification and characterization of PC			
CD38, CD45, CD138	PC identification/ quantification	Feasible identification/quantification of circulating clonal PC in PB (CD38, CD45).	(39,72)
		Prognostic value of BM PC counts obtained by MFC (CD38, CD138).	(34)
CD19, CD20, CD27, CD28, CD38, CD40, CD45, CD52, CD56, CD117	Antigenic characterization	Discrimination of clonal/malignant vs. reactive conditions (CD19, CD20, CD27, CD28, CD38, CD45, CD56, CD117).	(10,17,36)
		Prognostic value of specific antigenic profiles (CD19, CD28, CD117).	(17)
		Eligibility and monitoring of antibody-based therapies (CD40, CD52, CD56, CD117).	(70,71)
		Differential diagnosis between MM/SMM/MGUS vs. WM and other B-CLPD (CD19, CD20, CD45, CD56).	(24,56,57)
[a]CD38 and CD138	DNA ploidy and cell cycle analysis	Determination of ploidy status and subsequent monitoring.	(27)
Propidium Iodide		Prognostic value of MFC determination of the % of S-phase PC.	(73)
Discrimination between normal vs. clonal PC			
[a]CD19, CD38, CD45, CD56	Enumeration of normal and neoplastic PC within the BM PC compartment	Differential diagnosis between MGUS and MM.	(20,58)
		Identification of MGUS and SMM patients with low vs. high risk of progression.	(35)
		Identification of symptomatic MM patients with a favorable outcome.	(59)
Detection of minimal residual disease			
[a]CD19, CD20, CD28, CD38, CD45, CD56, CD117		Definition of immunophenotypic remission in the stringent complete remission (sCR) response category	
		Sequential follow-up of MM patients by MFC as a predictor for survival	(23,44,53)
		Monitoring of PB samples and PB-derived leucapheresis products	(39,72,74)

PC, plasma cell; BMPC, bone marrow plasma cells; PB, peripheral blood; MGUS, monoclonal gammopathies of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma; WM, Waldenström macroglobulinemia; B-CLPD, B-lymphoproliferative disorders; sCR, stringent complete remission; MFC, multiparameter flow cytometry.

[a]All markers can be used for those specific clinical applications.

Cytometry Part B (Clinical Cytometry) 78B:239–252 (2010)

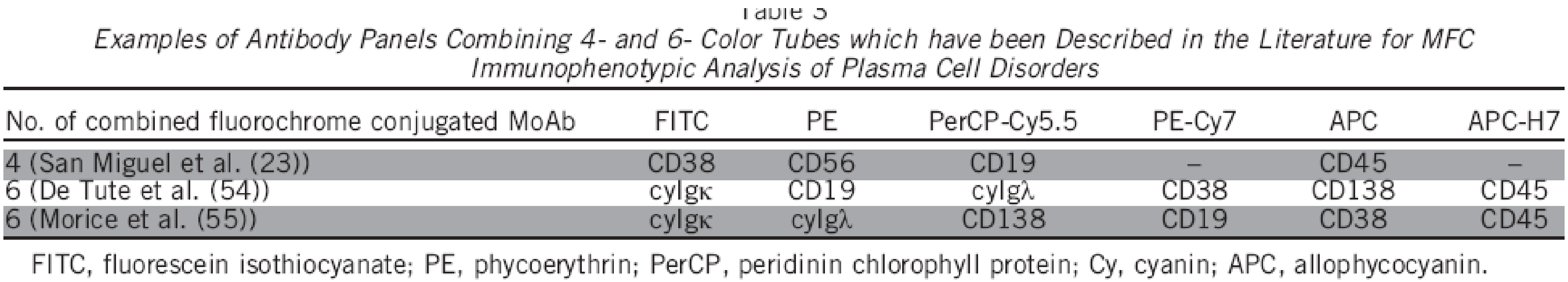


Table 3

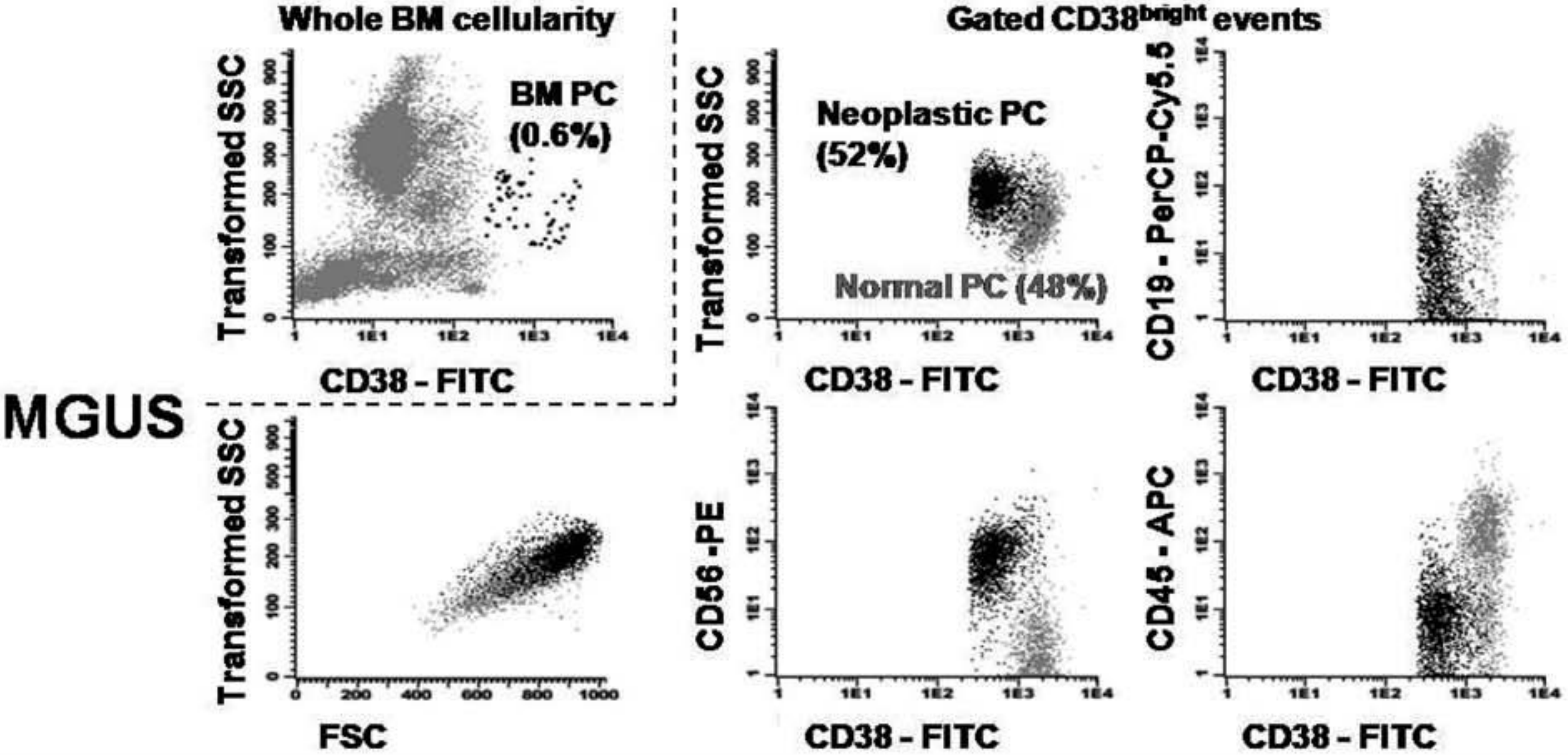
Examples of Antibody Panels Combining 4- and 6- Color Tubes which have been Described in the Literature for MFC Immunophenotypic Analysis of Plasma Cell Disorders

No. of combined fluorochrome conjugated MoAb	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
4 (San Miguel et al. (23))	CD38	CD56	CD19	–	CD45	–
6 (De Tute et al. (54))	cyIgκ	CD19	cyIgλ	CD38	CD138	CD45
6 (Morice et al. (55))	cyIgκ	cyIgλ	CD138	CD19	CD38	CD45

FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; Cy, cyanin; APC, allophycocyanin.

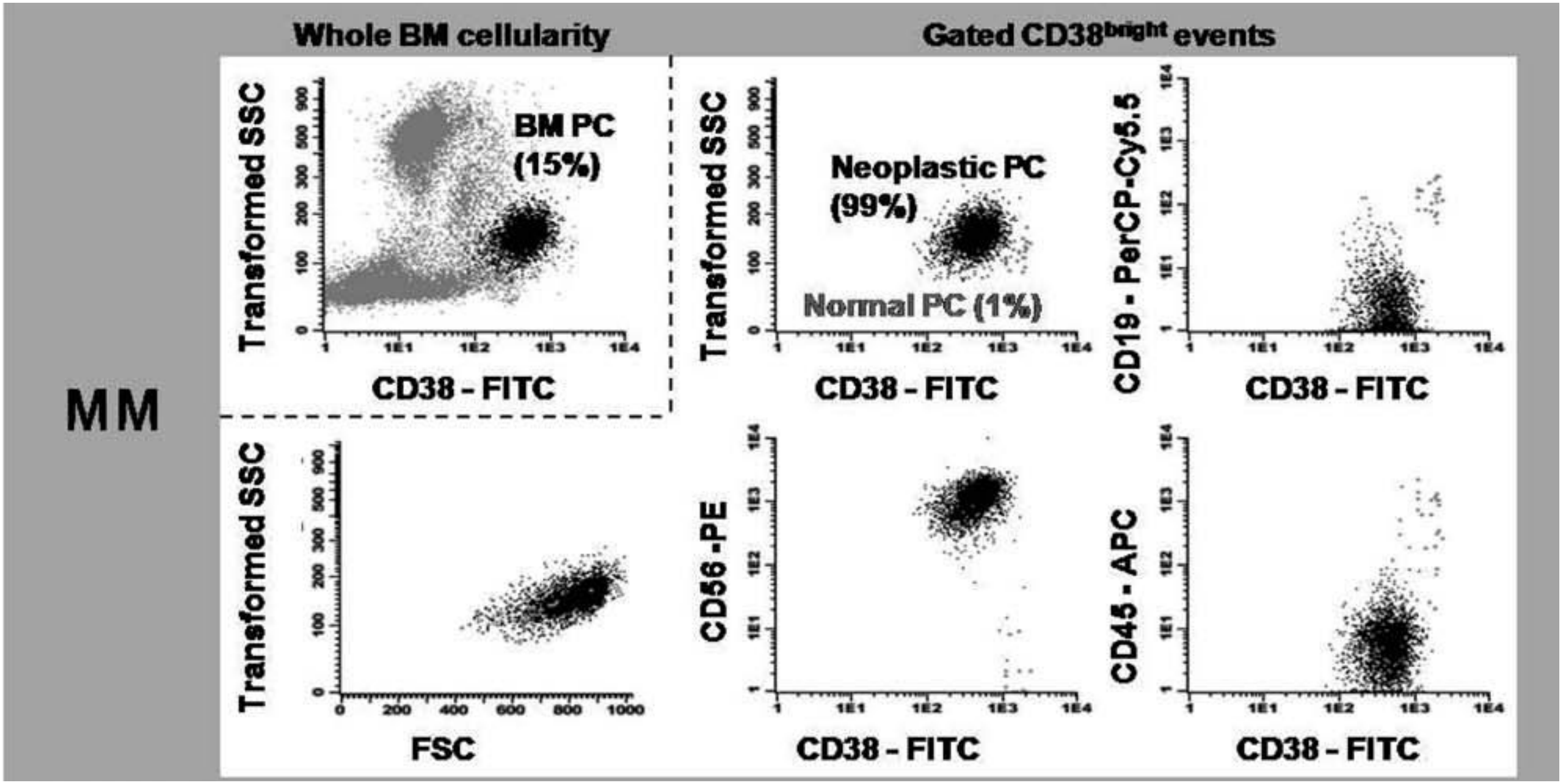
Cytometry Part B (Clinical Cytometry) 78B:239–252 (2010)

FC Immunophenotyping in MM and Other Clonal PC-Related Disorders



Cytometry Part B (Clinical Cytometry) 78B:239–252 (2010)

FC Immunophenotyping in MM and Other Clonal PC-Related Disorders



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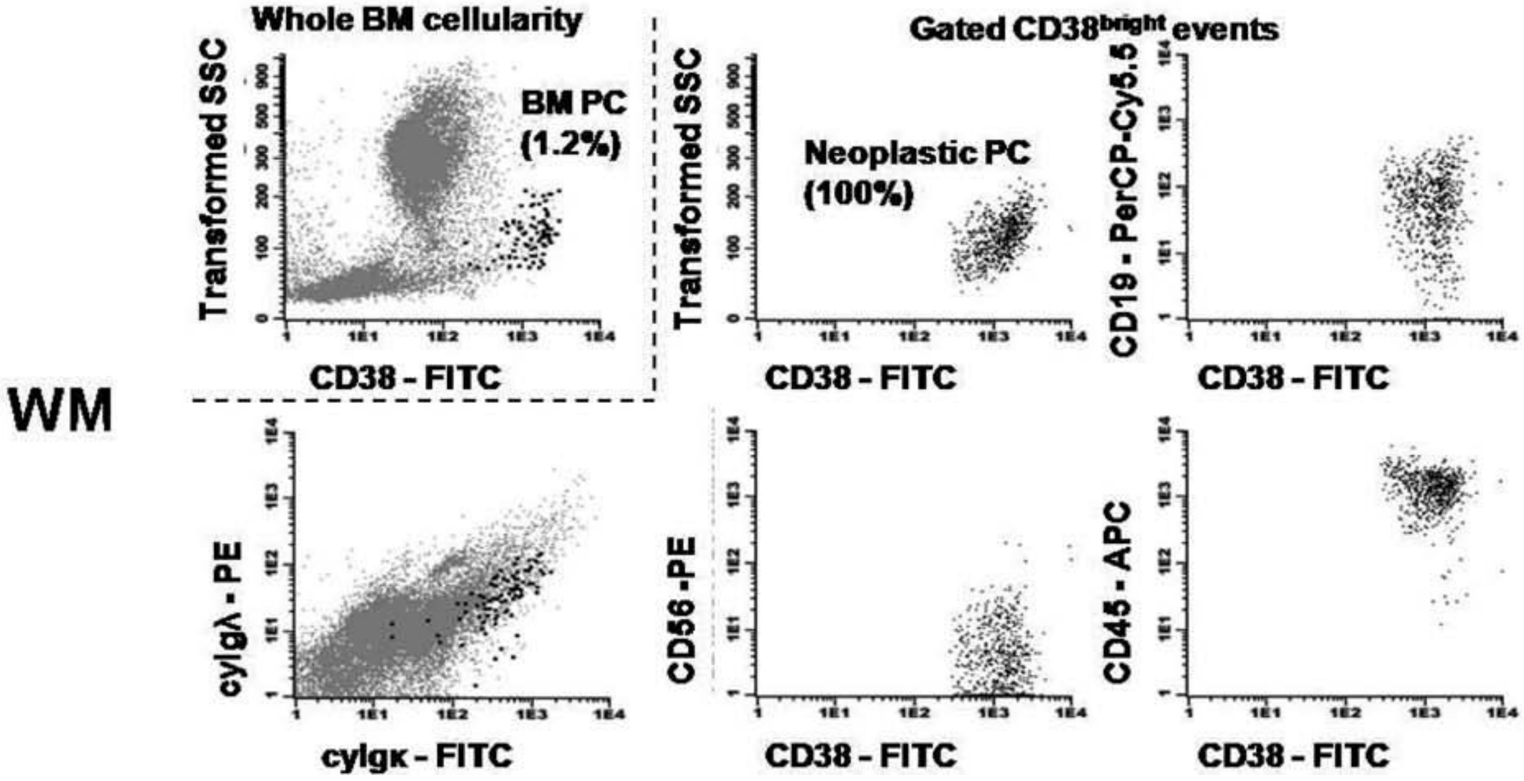

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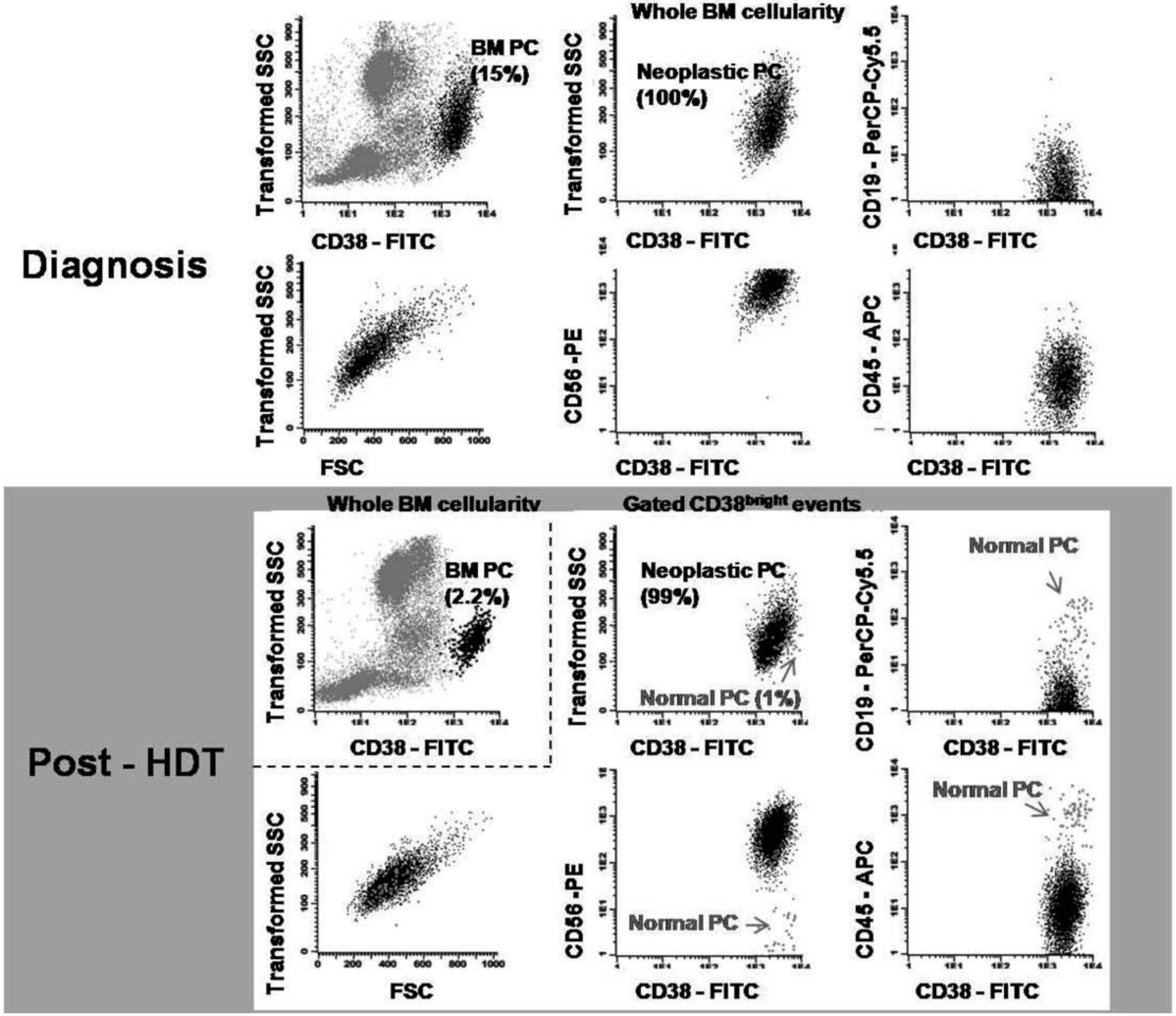

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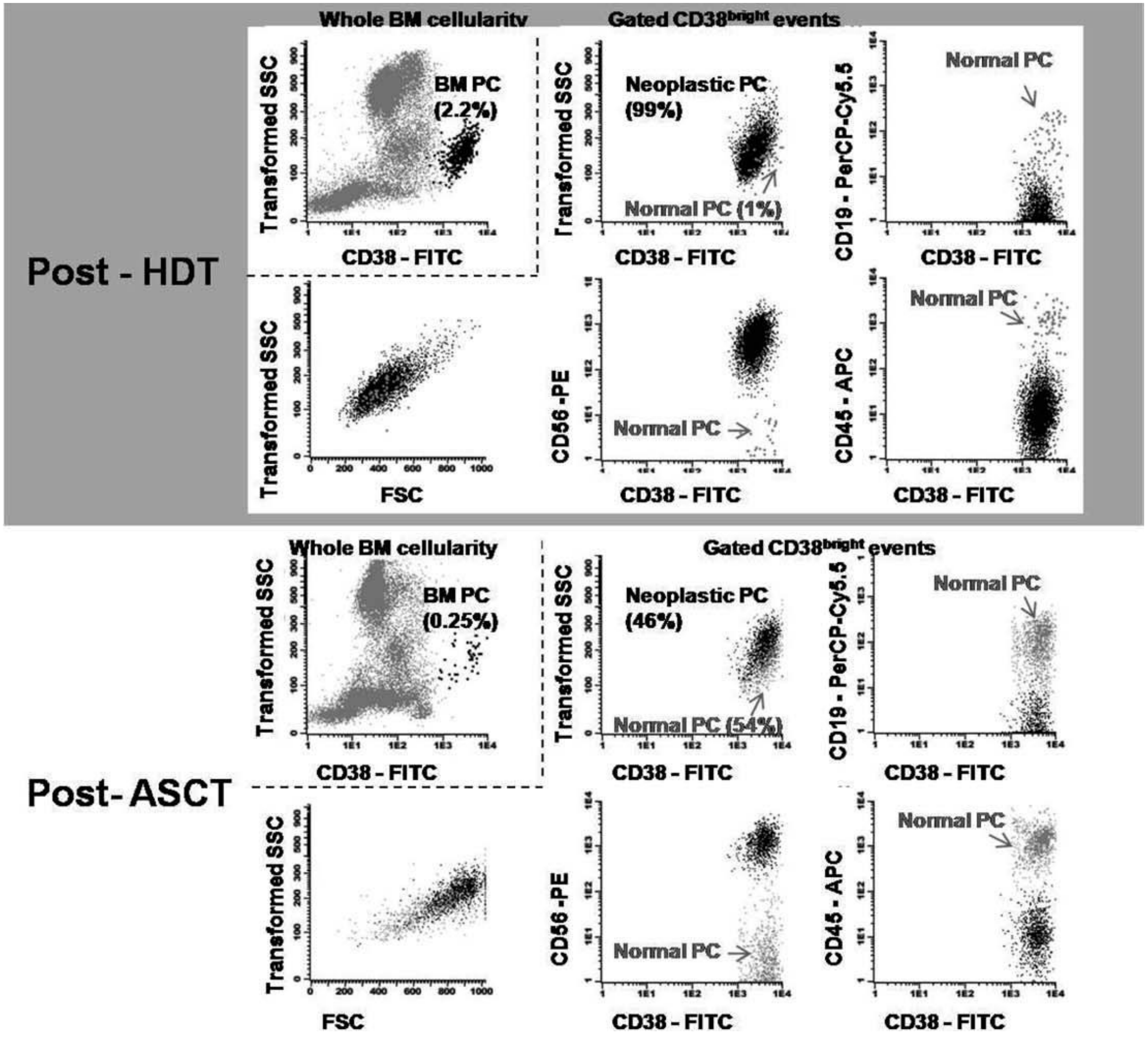


Cytometry Part B (Clinical Cytometry) 78B:239–252 (2010)

Role of FC immunophenotyping in the diagnosis and classification of plasma cell disorders

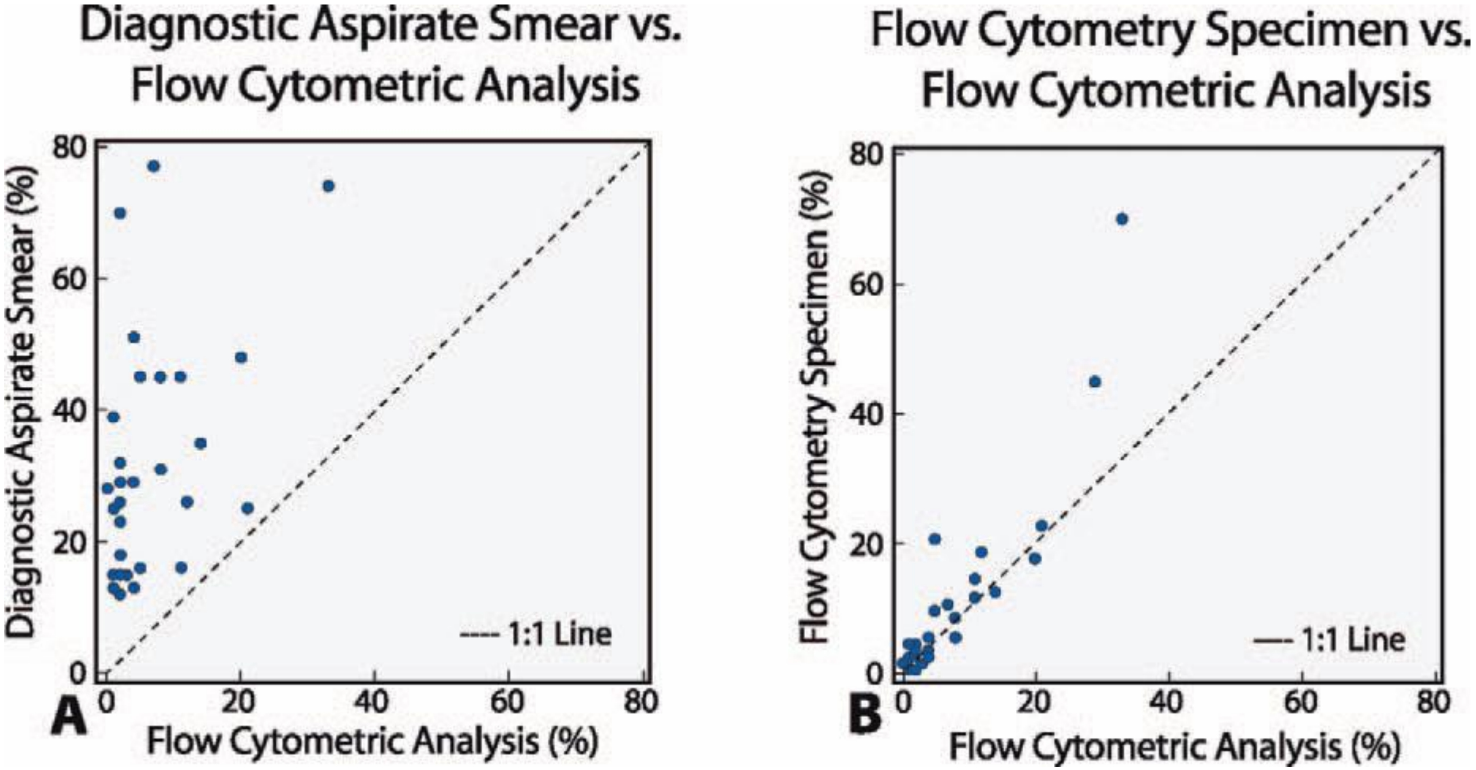
- FC immunophenotyping diagnostic utility is limited by difficulties encountered in enumerating PCs.

- Even using sensitive techniques, **FC immunophenotyping usually identifies fewer PCs than paraffin section IHC of biopsy sections**.

- This discrepancy appears to reflect a combination of
  - sampling differences,
  - loss of plasma cells during processing for FC studies,
  - difficulty encountered in including all surviving PCs in the FC analysis.

- Because of the relative paucity of PCs identified using FC, many of the studies characterizing PCN and MGUS have used sensitive techniques that permit accurate detection of less than 1% PCs, and often down to 0.01%.

Comparisons of morphologic and FC–determined plasma cell percentages.



Smock KJ et al Arch Pathol Lab Med. 2007;131:951–955

Role of FC immunophenotyping in the diagnosis and classification of plasma cell disorders

- The phenotype of MGUS overlaps that of overt PCN.
- Sensitive FC studies can usually identify a population of abnormal Ig light chain–restricted PCs in MGUS, and there are even reports of CD56 and CD117 expression.
- In addition, because of loss of PCs during processing, the identification of less than 10% bone marrow PCs using FC cannot be used to distinguish MGUS from an overt PCN.
- The most useful distinguishing feature between MGUS and PCN is the identification of a significant proportion of phenotypically normal PCs admixed with the abnormal cells.
- They represented less than 3% of all PCs in the majority of patients.

CONCLUDING REMARKS

- In MDS, FC is regarded as a new forthcoming standard.
- The FC data may enable to categorize bone marrow FC as normal or possibly consistent with MDS.
- FC in MDS can only be used as a part of an integrated diagnosis.
- Repeated FC assessments are highly recommended, not only in inconclusive cases, but also to monitor the course of the disease in untreated, mainly low-risk MDS patients, and during treatment with current available drugs.
- A report from the FC analysis should include descriptions of validated FC abnormalities and lineage-infidelity marker expression.
- Coincidence of MDS with other disorders should be indicated, such as clonal lymphoproliferative disease, mastocytosis, autoimmune diseases and PNH-associated GPI-deficient cells.
- The ultimate goal will be to refine and improve diagnosis and prognostic scoring systems.

Westers TM Leukemia. 2012 Feb 6. doi: 10.1038/leu.2012.30. [Epub ahead of print]

Role of flow cytometric immunophenotyping in the detection of MRD

- In recent years, enhancements in instrumentation have made it feasible for clinical laboratories to consider acquiring enough events for MRD detection and use 4 or more colors to reliably identify populations of phenotypically abnormal cells.

- Clinical flow cytometric assay have been developed to reliably detect populations representing 0.01% of events (1 cell in 10^4) and therefore can complete with PCR-based methods.

- Although in many situations there is concordance between the 2 techniques, FC methods have the advantage over PCR of discriminating viable and dead cells and directly measuring the proportion of positive cells, rather than using an amplification method.

Role of flow cytometric immunophenotyping in the detection of MRD (1/2)

- Several factors need to be taken into consideration in developing a successful FC assay for the detection of MRD.
  1. Number of events
     - although increasing the number of acquired events improves sensitivity, it is limited by the speed of acquisition. At present, most MRD assays aim to detect 1 cell in 10^4 through the analysis of 500000 to 1 million cells and the goal of detecting at least 50 to 100 events of interest.
  2. Limit of detection
     - if no disease is identified, the limit of detection should be determined to take into account the number of cells analyzed, presence of contaminating cells, and background noise.
  3. Carryover between tubes should be eliminated though the addition of wash steps.

Role of flow cytometric immunophenotyping in the detection of MRD (2/2)

- Several factors need to be taken into consideration in developing a successful FC assay for the detection of MRD.

4. The analysis should be designed to identify phenotypic features characteristic of the disease of interest and facilitate distinction from other cell populations in the specimen.
    - Phenotypes often change over time and with treatment, and therefore the MRD assay should not rely on an exact match between the phenotype of the residual disease and the original diagnostic specimen.
    - Therefore the antibody combinations should be chosen to maximize detection of disease, limit the impact of phenotypic variation, and permit detection of disease following antibody directed therapy.

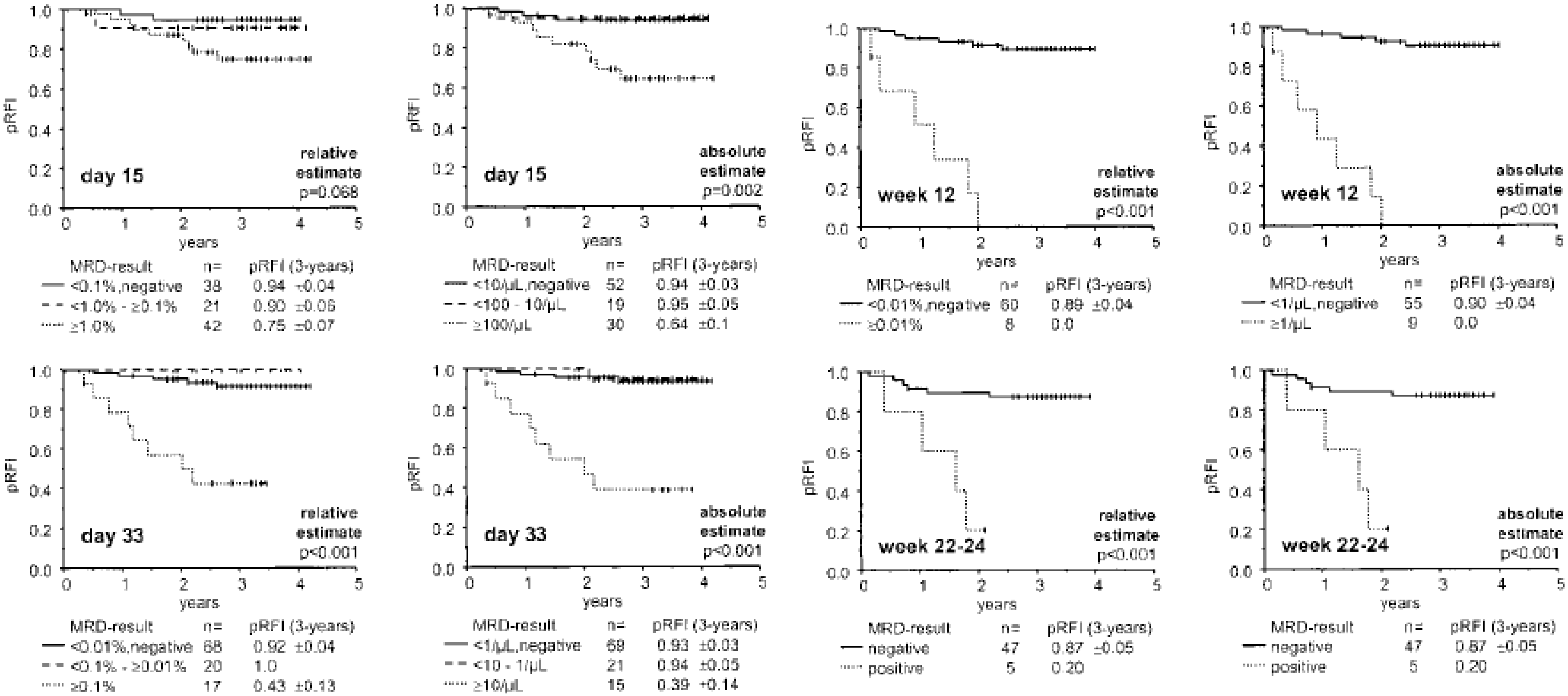
Role of flow cytometric immunophenotyping in the detection of MRD

- Flow cytometric immunophenotyping has
  - an established role in the detection of minimal residual ALL,
  - an emerging role CLL/SLL,
  - potential role in some other hematopoietic and lymphoid malignancies,
    - AML
    - PCN

Role of flow cytometric immunophenotyping in the detection of minimal residual ALL

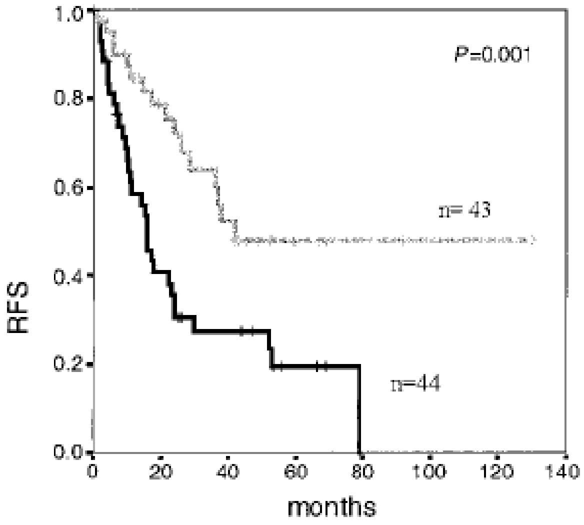
- Several studies have demonstrated that MRD detected by FC is an independent adverse prognostic factor in pediatric ALL.
  - The presence of MRD in BM samples with no morphologic evidence of disease is associated with a greater risk of relapse, and this risk increases with the level of disease detected.
- Although there is less data for adult ALL, the detection of MRD appears to be an independent risk factor for relapse.
- In theory, this information could assist in identifying high-risk patients who might benefit from additional therapy, or those low-risk patients who could be treated with a less intense regimen with lower toxicity.

Probability of sustained remission according to FC MRD results, relative and absolute estimate, in childhood ALL

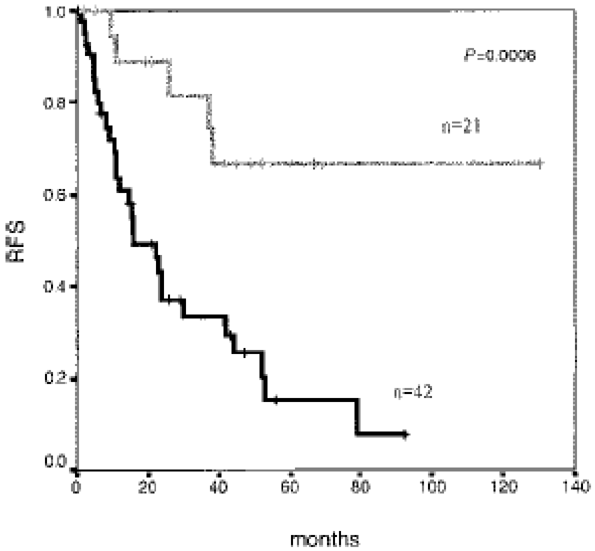


Blood. 2002;99:1952-1958)

Relapse-free survival in adolescent and adult patients with ALL according to immunophenotypic MRD level at day 14 and 35 of induction therapy



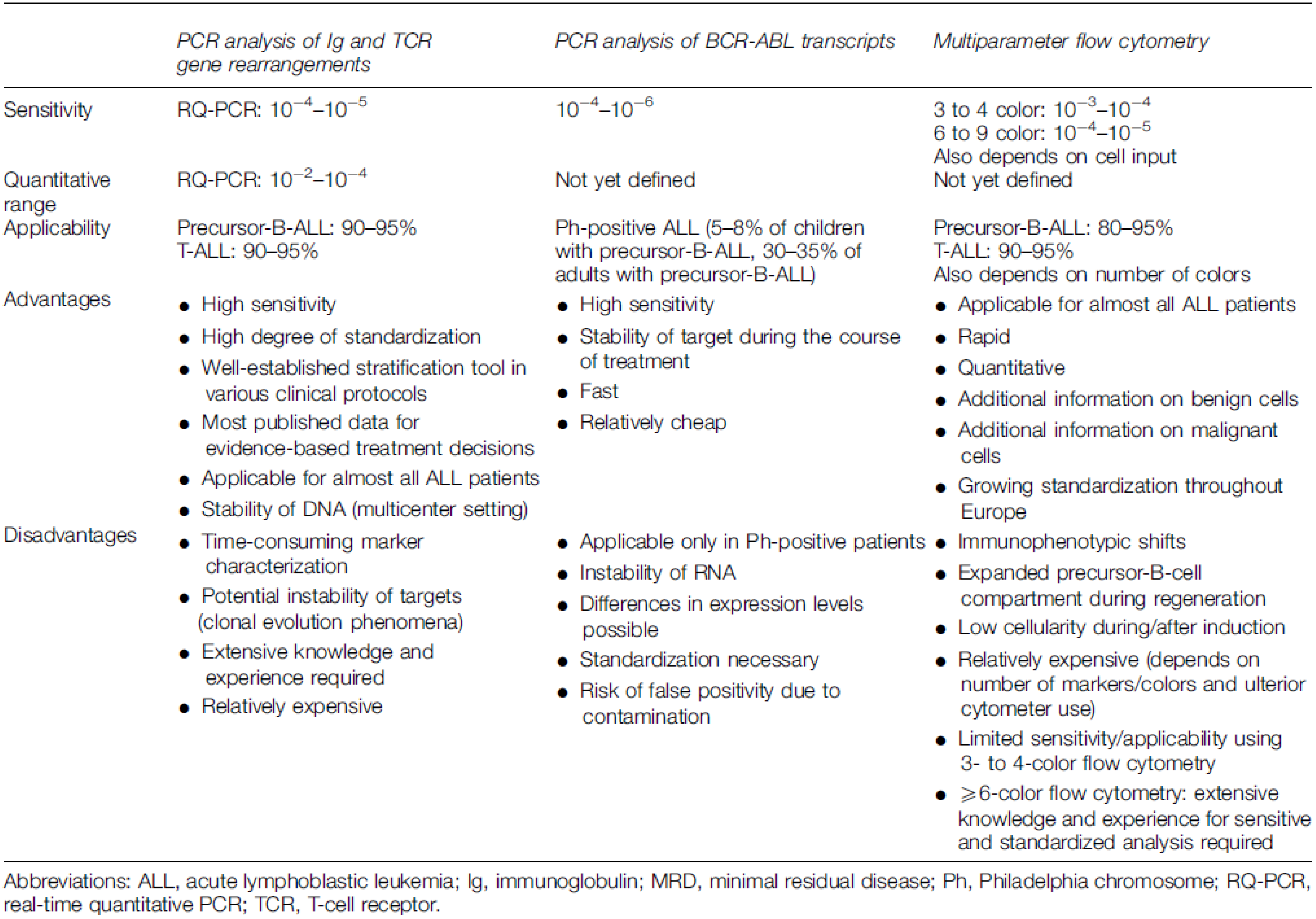
Median RFS of 42 months for patients with low MRD levels (<0.05%; n=43; gray line) versus 16 months for patients with high MRD levels (>0.05%; n=44; black line) (*P* .001).



Median RFS not reached for patients with low MRD levels (<0.5%; n=21; gray line) versus 16 months for patients with high MRD levels (>0.5%; n=42; black line) (*P* .0008).

Blood. 2003; 101:4695-4700

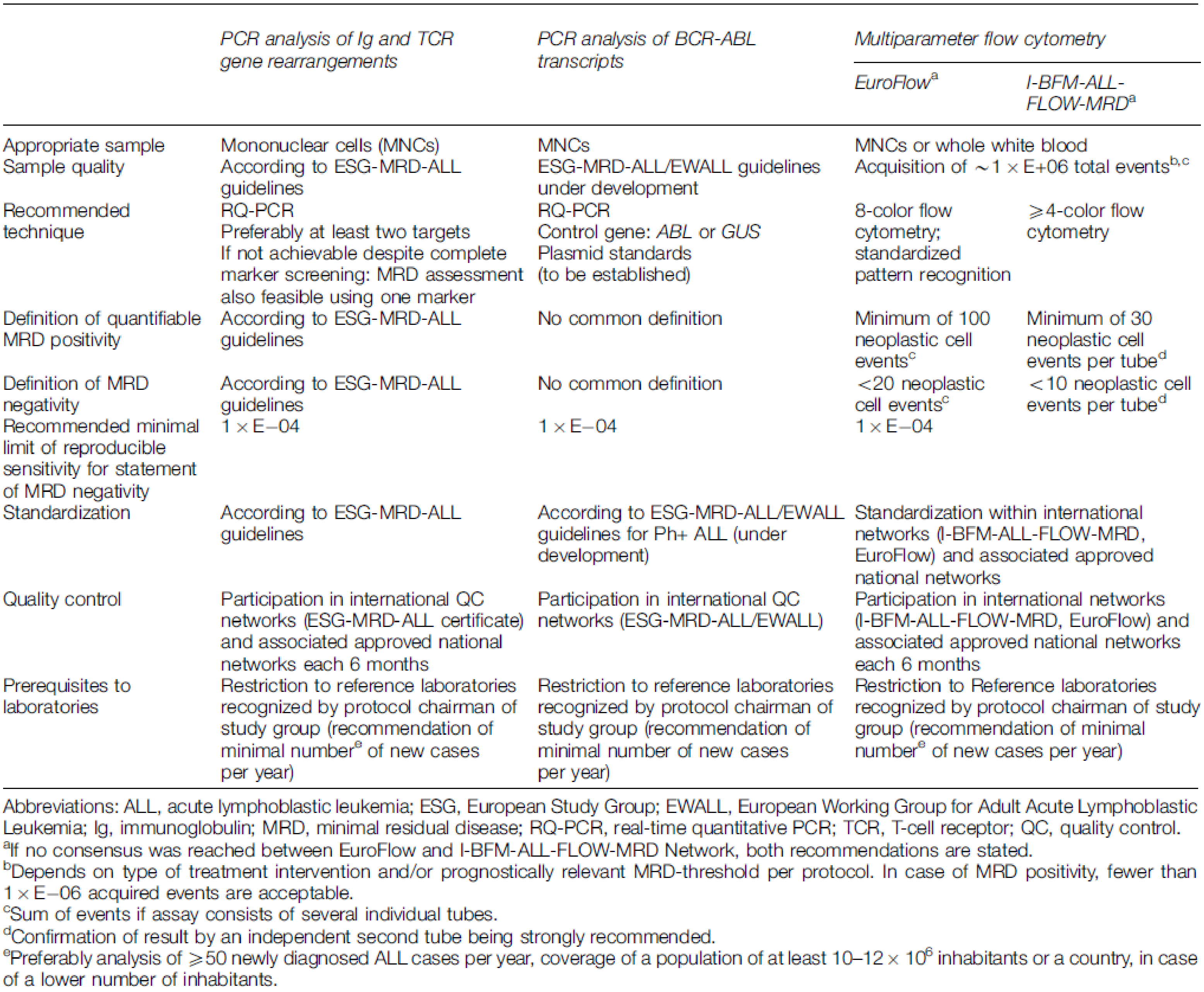
Characteristics of the techniques currently used for MRD detection in ALL



	PCR analysis of Ig and TCR gene rearrangements	*PCR analysis of BCR-ABL transcripts*	*Multiparameter flow cytometry*
Sensitivity	RQ-PCR: 10^{-4}–10^{-5}	10^{-4}–10^{-6}	3 to 4 color: 10^{-3}–10^{-4} 6 to 9 color: 10^{-4}–10^{-5} Also depends on cell input
Quantitative range	RQ-PCR: 10^{-2}–10^{-4}	Not yet defined	Not yet defined
Applicability	Precursor-B-ALL: 90–95% T-ALL: 90–95%	Ph-positive ALL (5–8% of children with precursor-B-ALL, 30–35% of adults with precursor-B-ALL)	Precursor-B-ALL: 80–95% T-ALL: 90–95% Also depends on number of colors
Advantages	● High sensitivity ● High degree of standardization ● Well-established stratification tool in various clinical protocols ● Most published data for evidence-based treatment decisions ● Applicable for almost all ALL patients ● Stability of DNA (multicenter setting)	● High sensitivity ● Stability of target during the course of treatment ● Fast ● Relatively cheap	● Applicable for almost all ALL patients ● Rapid ● Quantitative ● Additional information on benign cells ● Additional information on malignant cells ● Growing standardization throughout Europe
Disadvantages	● Time-consuming marker characterization ● Potential instability of targets (clonal evolution phenomena) ● Extensive knowledge and experience required ● Relatively expensive	● Applicable only in Ph-positive patients ● Instability of RNA ● Differences in expression levels possible ● Standardization necessary ● Risk of false positivity due to contamination	● Immunophenotypic shifts ● Expanded precursor-B-cell compartment during regeneration ● Low cellularity during/after induction ● Relatively expensive (depends on number of markers/colors and ulterior cytometer use) ● Limited sensitivity/applicability using 3- to 4-color flow cytometry ● ⩾6-color flow cytometry: extensive knowledge and experience for sensitive and standardized analysis required

Abbreviations: ALL, acute lymphoblastic leukemia; Ig, immunoglobulin; MRD, minimal residual disease; Ph, Philadelphia chromosome; RQ-PCR, real-time quantitative PCR; TCR, T-cell receptor.

Table 5 Recommendations regarding minimal technical requirements for MRD assessment within clinical trials using PCR analysis of Ig and TCR gene rearrangements, PCR analysis of *BCR-ABL* transcripts or multiparameter flow cytometry



	PCR analysis of Ig and TCR gene rearrangements	*PCR analysis of BCR-ABL transcripts*	*Multiparameter flow cytometry*	
			EuroFlow[a]	*I-BFM-ALL-FLOW-MRD*[a]
Appropriate sample	Mononuclear cells (MNCs)	MNCs	MNCs or whole white blood	
Sample quality	According to ESG-MRD-ALL guidelines	ESG-MRD-ALL/EWALL guidelines under development	Acquisition of ∼1 × E+06 total events[b,c]	
Recommended technique	RQ-PCR Preferably at least two targets If not achievable despite complete marker screening: MRD assessment also feasible using one marker	RQ-PCR Control gene: *ABL* or *GUS* Plasmid standards (to be established)	8-color flow cytometry; standardized pattern recognition	⩾4-color flow cytometry
Definition of quantifiable MRD positivity	According to ESG-MRD-ALL guidelines	No common definition	Minimum of 100 neoplastic cell events[c]	Minimum of 30 neoplastic cell events per tube[d]
Definition of MRD negativity	According to ESG-MRD-ALL guidelines	No common definition	<20 neoplastic cell events[c]	<10 neoplastic cell events per tube[d]
Recommended minimal limit of reproducible sensitivity for statement of MRD negativity	1 × E−04	1 × E−04	1 × E−04	
Standardization	According to ESG-MRD-ALL guidelines	According to ESG-MRD-ALL/EWALL guidelines for Ph+ ALL (under development)	Standardization within international networks (I-BFM-ALL-FLOW-MRD, EuroFlow) and associated approved national networks	
Quality control	Participation in international QC networks (ESG-MRD-ALL certificate) and associated approved national networks each 6 months	Participation in international QC networks (ESG-MRD-ALL/EWALL)	Participation in international networks (I-BFM-ALL-FLOW-MRD, EuroFlow) and associated approved national networks each 6 months	
Prerequisites to laboratories	Restriction to reference laboratories recognized by protocol chairman of study group (recommendation of minimal number[e] of new cases per year)	Restriction to reference laboratories recognized by protocol chairman of study group (recommendation of minimal number of new cases per year)	Restriction to Reference laboratories recognized by protocol chairman of study group (recommendation of minimal number[e] of new cases per year)	

Abbreviations: ALL, acute lymphoblastic leukemia; ESG, European Study Group; EWALL, European Working Group for Adult Acute Lymphoblastic Leukemia; Ig, immunoglobulin; MRD, minimal residual disease; RQ-PCR, real-time quantitative PCR; TCR, T-cell receptor; QC, quality control.

[a] If no consensus was reached between EuroFlow and I-BFM-ALL-FLOW-MRD Network, both recommendations are stated.

[b] Depends on type of treatment intervention and/or prognostically relevant MRD-threshold per protocol. In case of MRD positivity, fewer than 1 × E−06 acquired events are acceptable.

[c] Sum of events if assay consists of several individual tubes.

[d] Confirmation of result by an independent second tube being strongly recommended.

[e] Preferably analysis of ⩾50 newly diagnosed ALL cases per year, coverage of a population of at least 10–12 × 10^6 inhabitants or a country, in case of a lower number of inhabitants.

Leukemia (2010) 24, 521–535

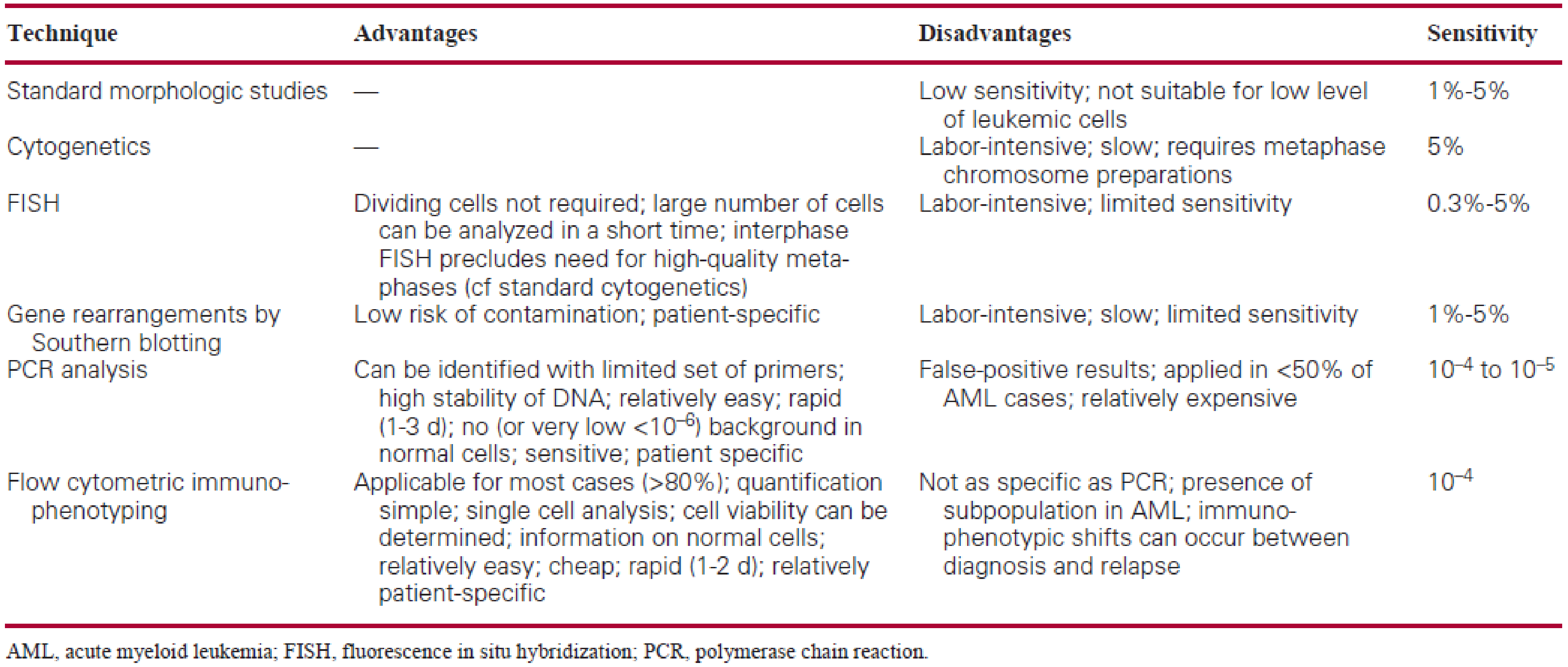
Role of flow cytometric immunophenotyping in the detection of minimal residual AML

- Although less well-established than for ALL, FC evaluation for MRD in AML is becoming more widespread.
- As for ALL, MRD detection in AML involves the identification of phenotypically abnormal populations.
- Because of frequent changes in phenotype over time and with therapy, it is not recommended to restrict evaluation to detection of abnormalities identified at diagnosis.
- A further complication is recognition that regenerating marrow may contain populations of cells with an unusual phenotype, such as a low level of CD56 expression on myeloid precursors.
- However, using multicolor flow cytometric immunophenotyping it is possible to detect residual AML at levels of 0.1% to 0.01%.

Role of flow cytometric immunophenotyping in the detection of minimal residual AML

- In most studies, the documentation of residual AML has been associated with a poor prognosis.
- one of the main challenges is identifying effective therapy for these high-risk patients.

Methods for Detection of MRD in AML

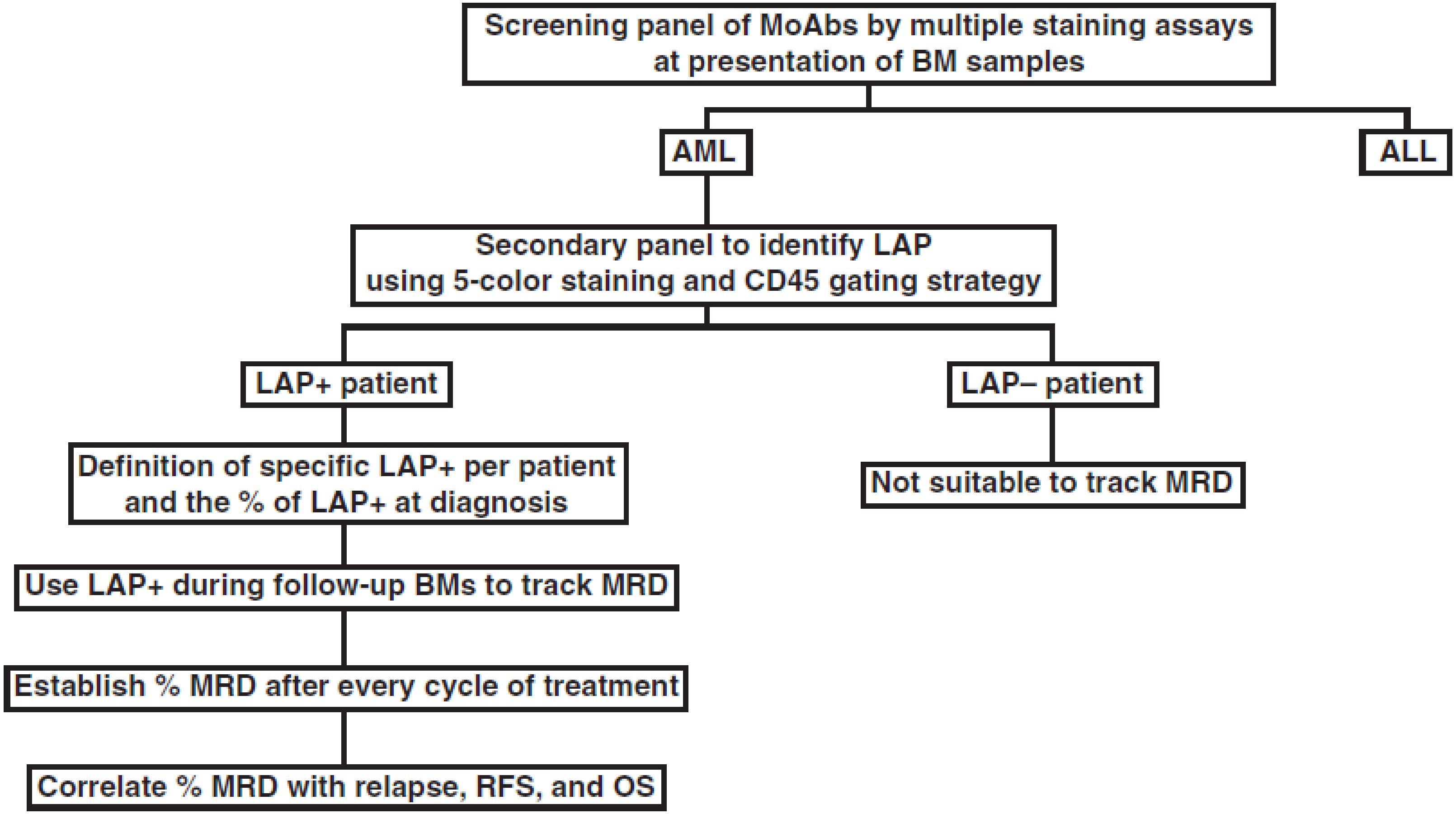


Technique	Advantages	Disadvantages	Sensitivity
Standard morphologic studies	—	Low sensitivity; not suitable for low level of leukemic cells	1%-5%
Cytogenetics	—	Labor-intensive; slow; requires metaphase chromosome preparations	5%
FISH	Dividing cells not required; large number of cells can be analyzed in a short time; interphase FISH precludes need for high-quality metaphases (cf standard cytogenetics)	Labor-intensive; limited sensitivity	0.3%-5%
Gene rearrangements by Southern blotting	Low risk of contamination; patient-specific	Labor-intensive; slow; limited sensitivity	1%-5%
PCR analysis	Can be identified with limited set of primers; high stability of DNA; relatively easy; rapid (1-3 d); no (or very low $<10^{-6}$) background in normal cells; sensitive; patient specific	False-positive results; applied in <50% of AML cases; relatively expensive	10^{-4} to 10^{-5}
Flow cytometric immunophenotyping	Applicable for most cases (>80%); quantification simple; single cell analysis; cell viability can be determined; information on normal cells; relatively easy; cheap; rapid (1-2 d); relatively patient-specific	Not as specific as PCR; presence of subpopulation in AML; immunophenotypic shifts can occur between diagnosis and relapse	10^{-4}

AML, acute myeloid leukemia; FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction.

Am J Clin Pathol 2009;131:16-26

Algorithm used for identifying leukemia-associated phenotype (LAP) in AML and for detection of MRD.



Screening panel of MoAbs by multiple staining assays at presentation of BM samples

AML

ALL

Secondary panel to identify LAP using 5-color staining and CD45 gating strategy

LAP+ patient

LAP– patient

Definition of specific LAP+ per patient and the % of LAP+ at diagnosis

Not suitable to track MRD

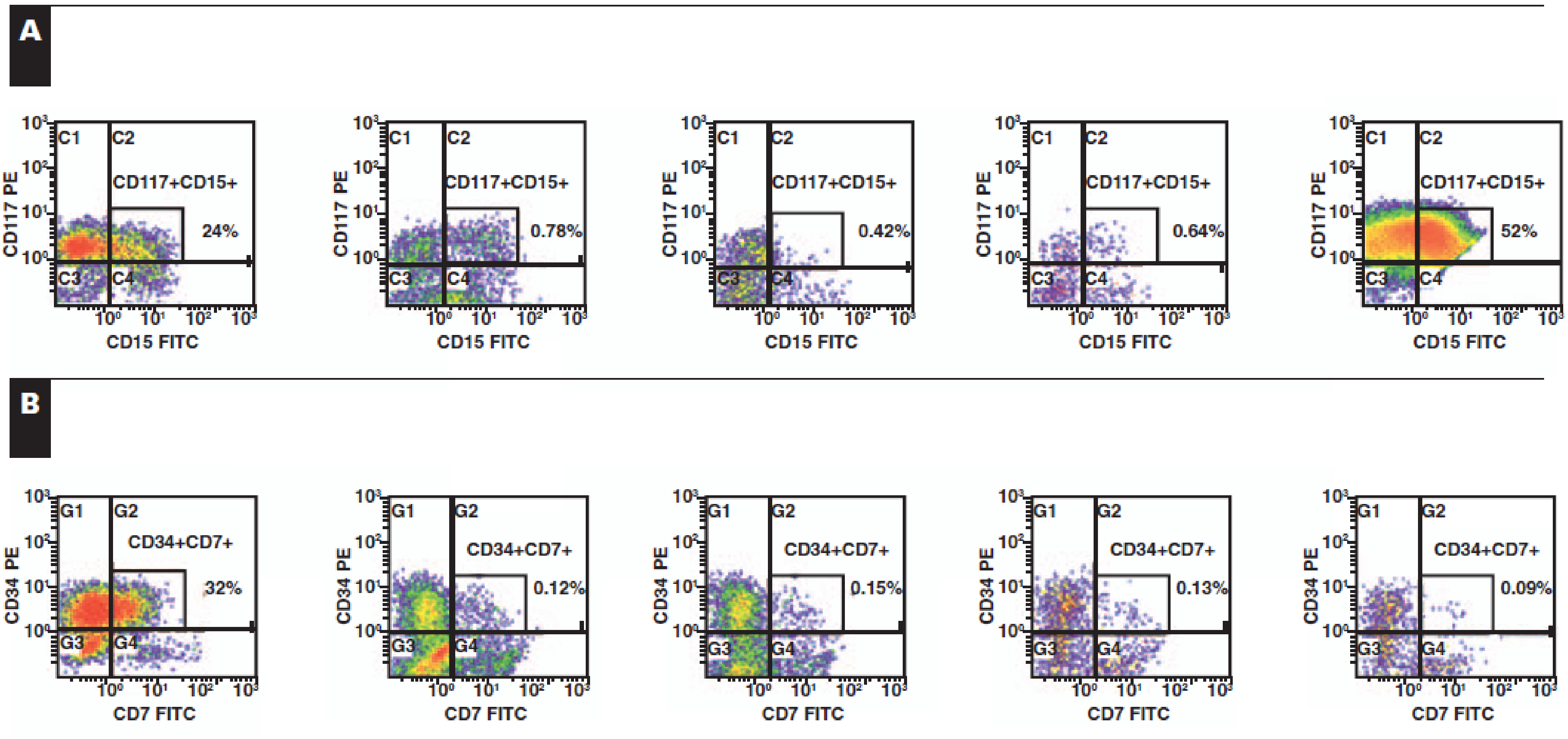
Use LAP+ during follow-up BMs to track MRD

Establish % MRD after every cycle of treatment

Correlate % MRD with relapse, RFS, and OS

Am J Clin Pathol 2009;131:16-26

Detection of MRD in consecutive BM samples from a patient with relapse (A) and a patient still in remission (B).



BM: AML at Diagnosis

After Induction

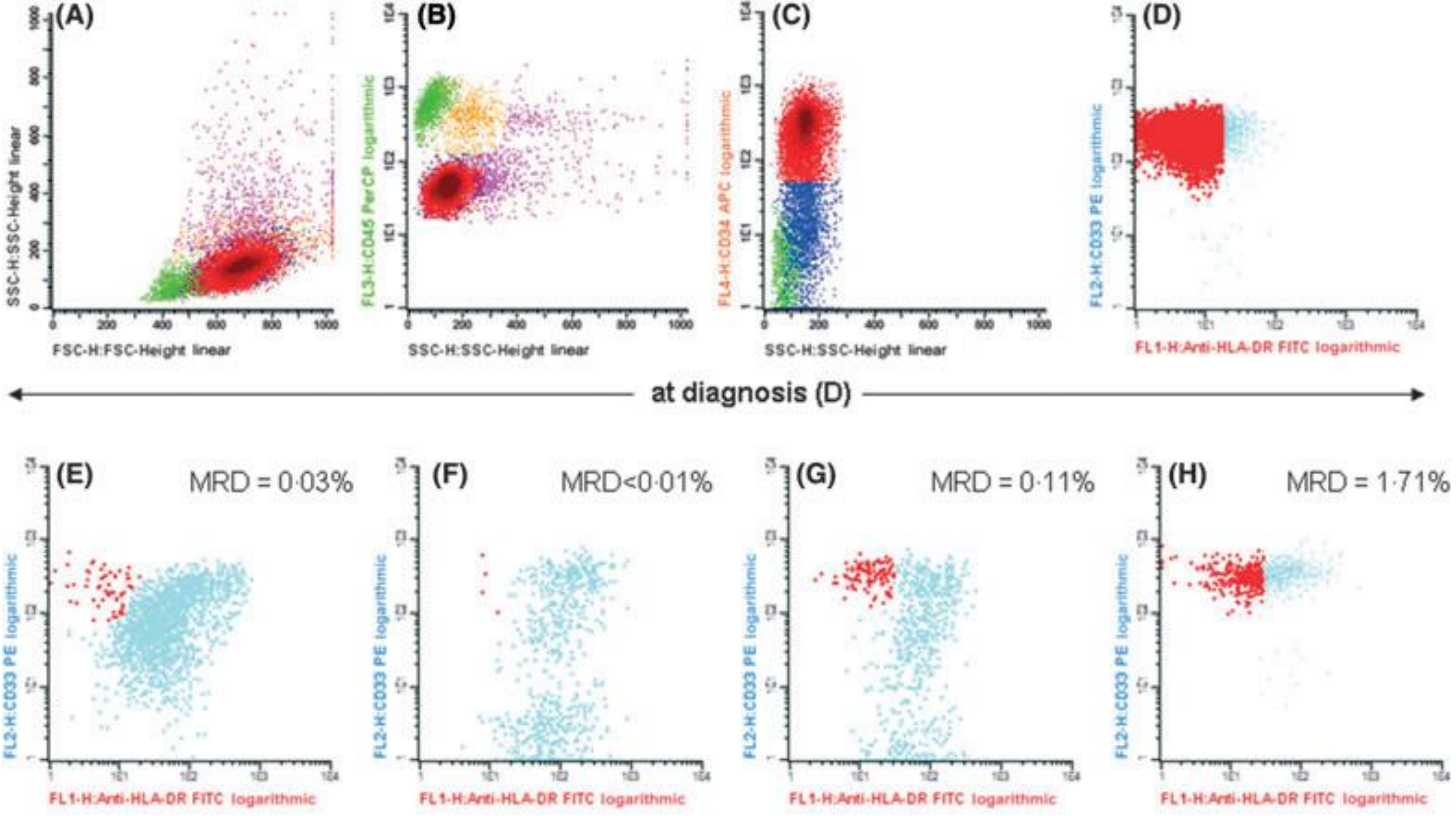
After Consolidation I

After Consolidation II

Follow-up

Am J Clin Pathol 2009;131:16-26

Absence of HLA-DR expression at diagnosis and tracing residual aberrant cells at follow up.



After 1st cycle

After D: 1 month

After 2nd cycle

5 months

After consolidation

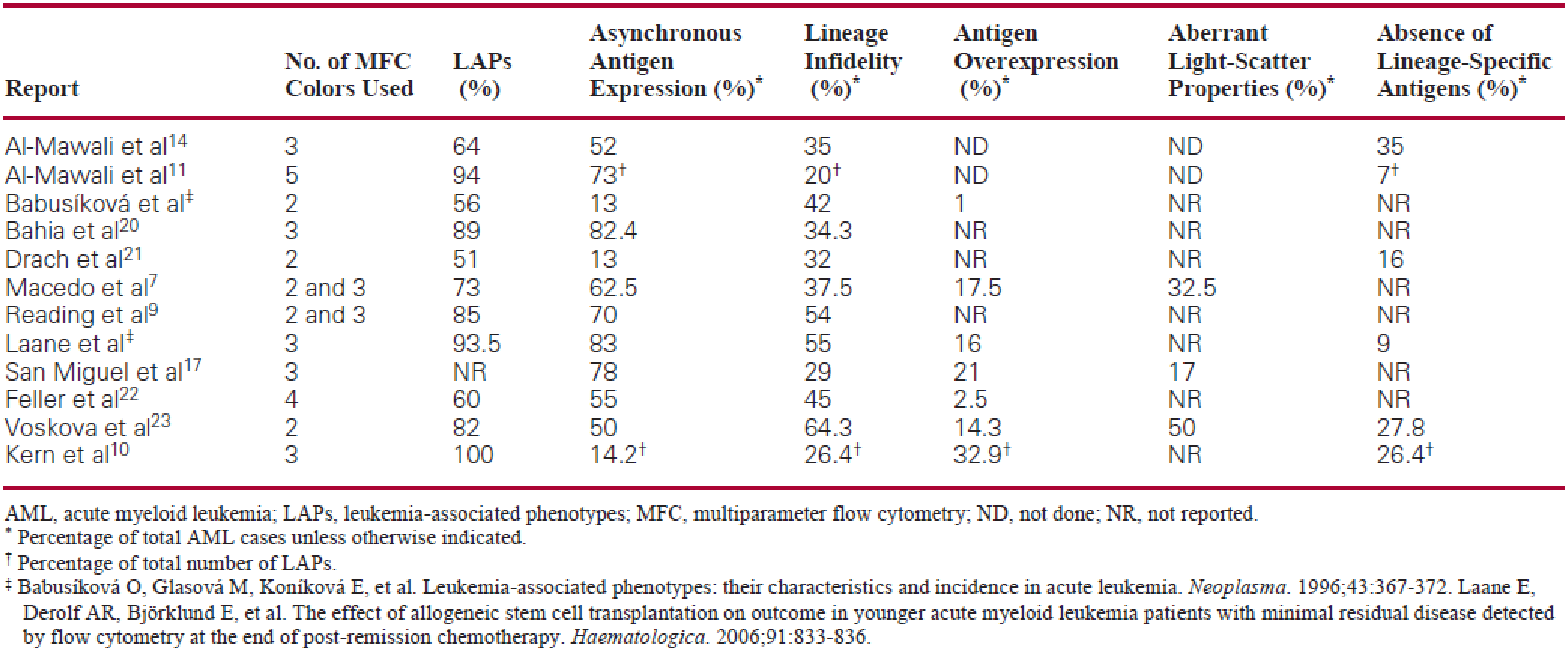
12 months

Relapse

17 months

British Journal of Haematology 2011;153:421–436

Various LAPs and Their Frequencies



Report	No. of MFC Colors Used	LAPs (%)	Asynchronous Antigen Expression (%)[*]	Lineage Infidelity (%)[*]	Antigen Overexpression (%)[*]	Aberrant Light-Scatter Properties (%)[*]	Absence of Lineage-Specific Antigens (%)[*]
Al-Mawali et al[14]	3	64	52	35	ND	ND	35
Al-Mawali et al[11]	5	94	73[†]	20[†]	ND	ND	7[†]
Babusíková et al[‡]	2	56	13	42	1	NR	NR
Bahia et al[20]	3	89	82.4	34.3	NR	NR	NR
Drach et al[21]	2	51	13	32	NR	NR	16
Macedo et al[7]	2 and 3	73	62.5	37.5	17.5	32.5	NR
Reading et al[9]	2 and 3	85	70	54	NR	NR	NR
Laane et al[‡]	3	93.5	83	55	16	NR	9
San Miguel et al[17]	3	NR	78	29	21	17	NR
Feller et al[22]	4	60	55	45	2.5	NR	NR
Voskova et al[23]	2	82	50	64.3	14.3	50	27.8
Kern et al[10]	3	100	14.2[†]	26.4[†]	32.9[†]	NR	26.4[†]

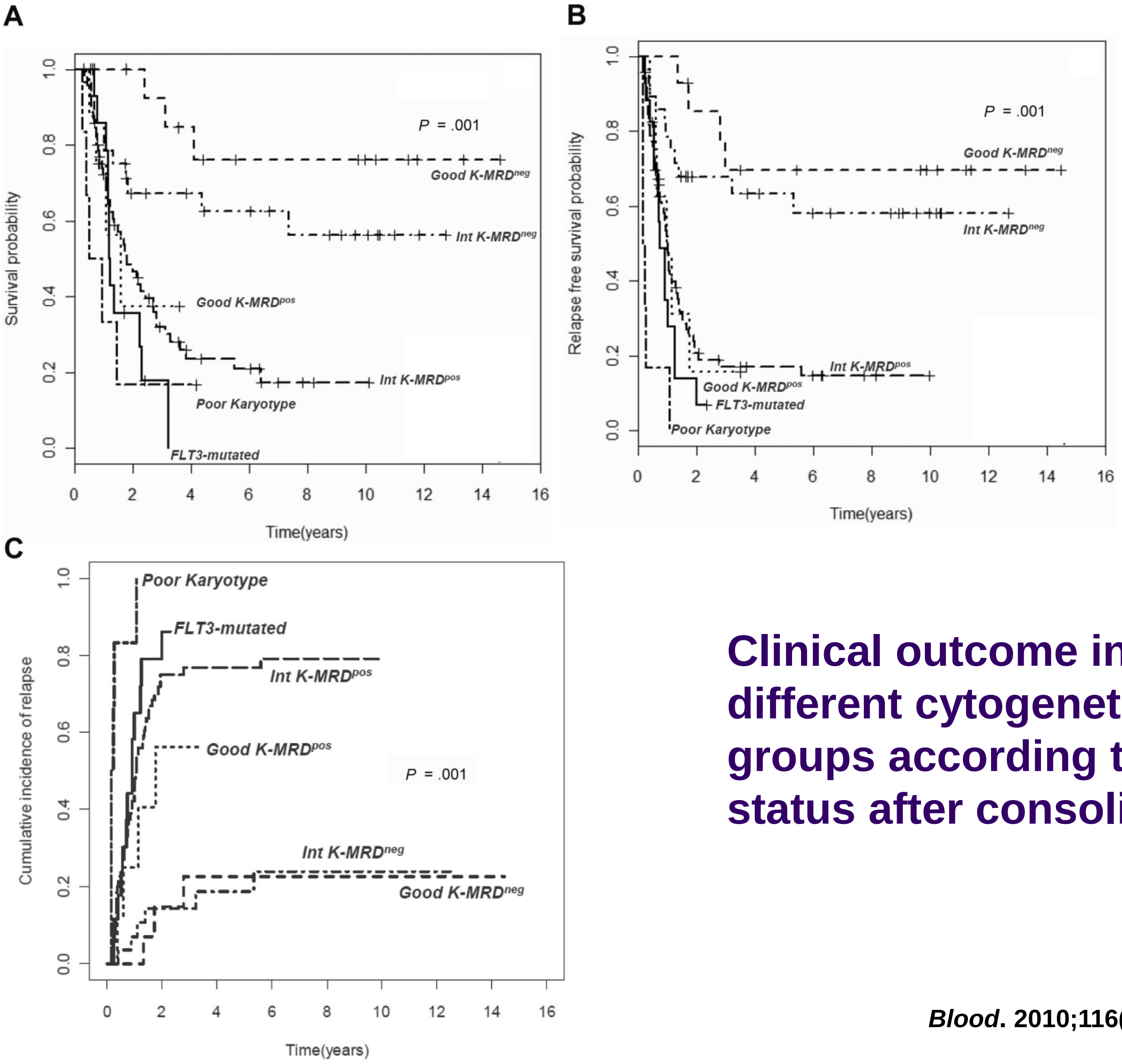
AML, acute myeloid leukemia; LAPs, leukemia-associated phenotypes; MFC, multiparameter flow cytometry; ND, not done; NR, not reported.

[*] Percentage of total AML cases unless otherwise indicated.

[†] Percentage of total number of LAPs.

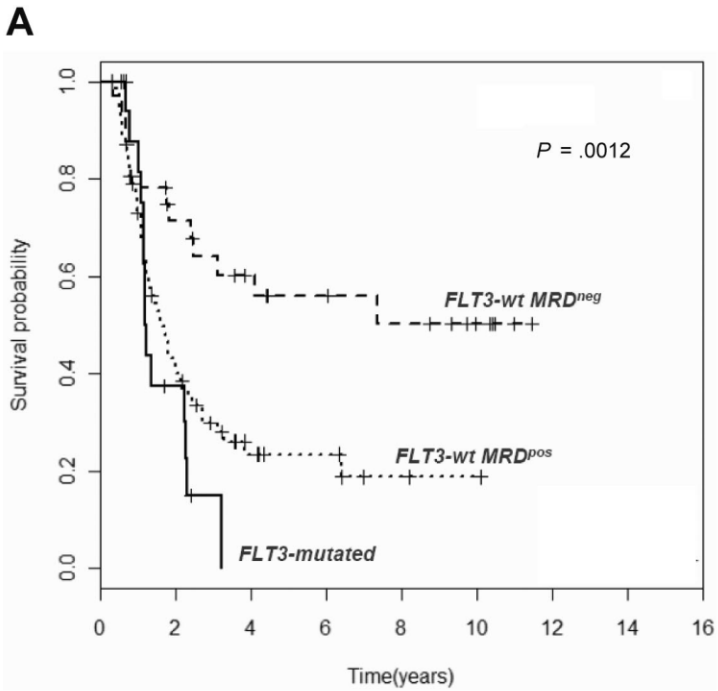
[‡] Babusíková O, Glasová M, Koníková E, et al. Leukemia-associated phenotypes: their characteristics and incidence in acute leukemia. *Neoplasma*. 1996;43:367-372. Laane E, Derolf AR, Björklund E, et al. The effect of allogeneic stem cell transplantation on outcome in younger acute myeloid leukemia patients with minimal residual disease detected by flow cytometry at the end of post-remission chemotherapy. *Haematologica*. 2006;91:833-836.

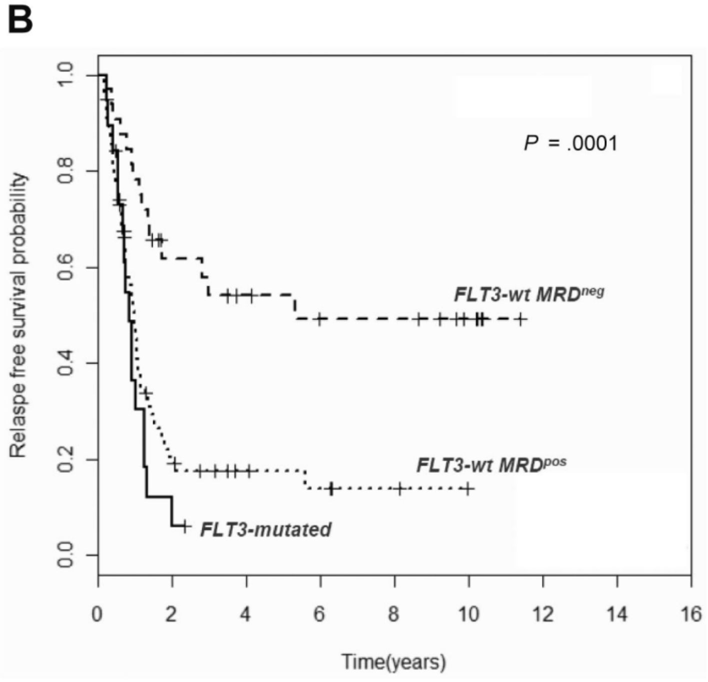
Am J Clin Pathol 2009;131:16-26

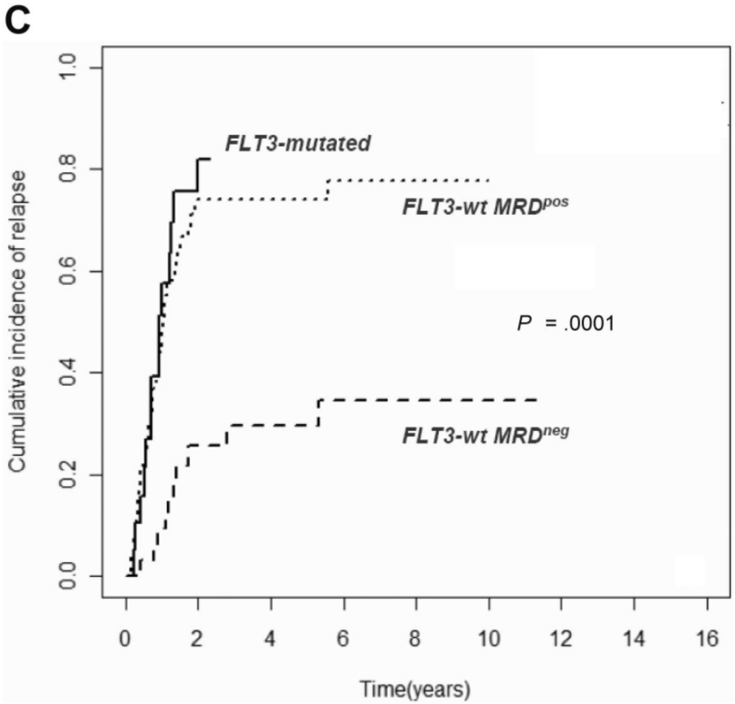


Clinical outcome in different cytogenetic groups according to MRD status after consolidation.

***Blood*. 2010;116(13):2295-2303**







Assessment of MRD at the end of consolidation splits FLT3 wild-type in 2 categories with different prognoses.

***Blood*. 2010;116(13):2295-2303**

Role of flow cytometric immunophenotyping in the detection of minimal residual CLL/SLL

- Traditionally, the goals of treatment in CLL/SLL have been palliation, delayed progression, and decreased disease burden.
- More recently, combination chemoimmunotherapy have been developed that can achieve MRD remission, and initial studies have shown longer duration of response or longer survival for patients without detectable MRD.
- Minimal residual CLL/SLL can be detected by PCR for clonal immunoglobulin gene rearrangement and flow cytometric immunophenotyping.
- A standardized multiparameter flow cytometric procedure has been developed for CLL/SLLMRD that can detect disease at a level similar to conventional PCR(ie, 1 10E4 or 0.01%).
- Although quantitative allele-specific oligonucleotide PCR is more sensitive, it requires the generation of patient specific primers, is not widely available, and for disease down to a level of 0.01% is closely correlated with the results of flow cytometric immunophenotyping.
- Therefore, standardized multiparameter flow cytometric immunophenotyping has been proposed to be the preferred method for the detection of CLL/SLLMRD10.

MRD in CLL

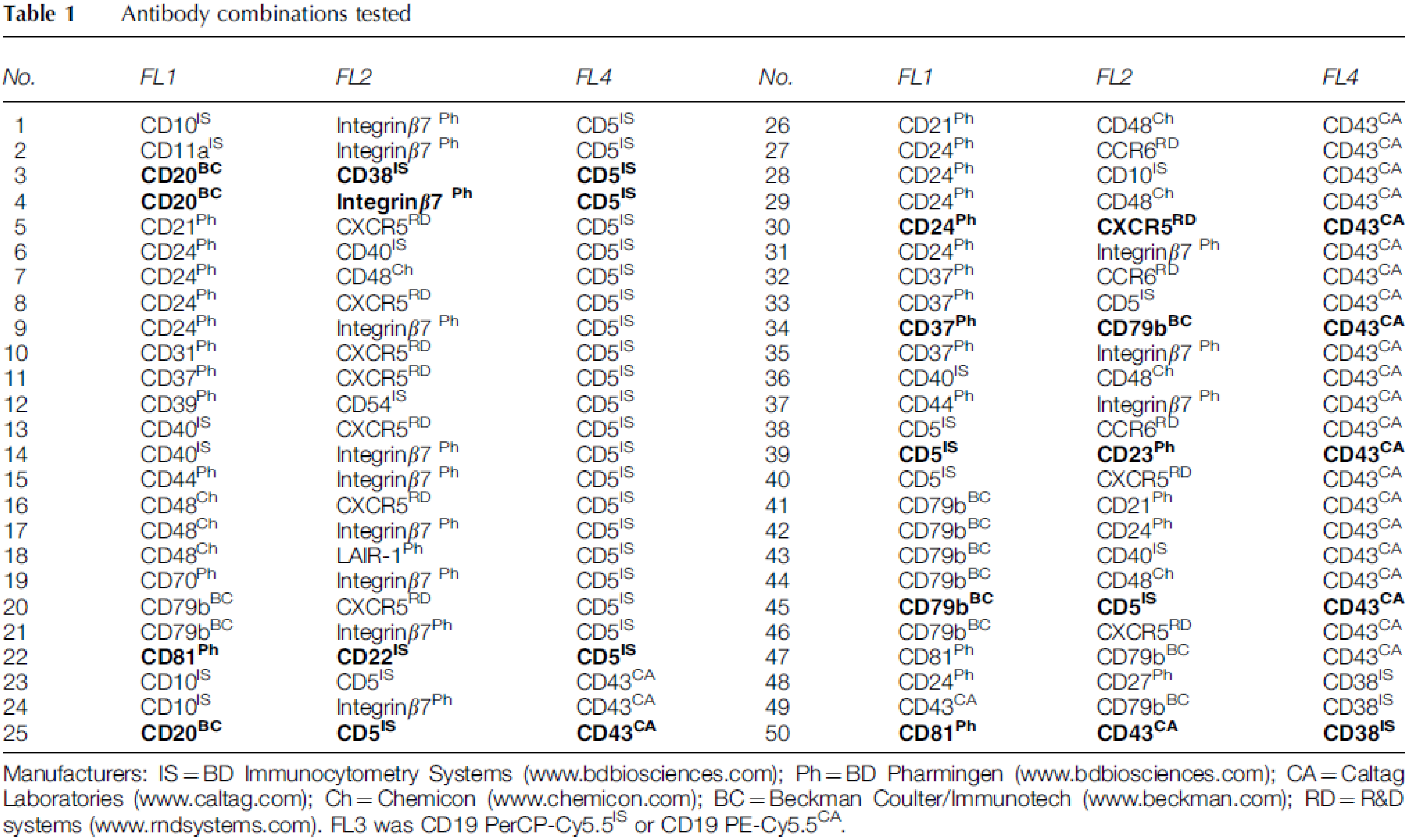


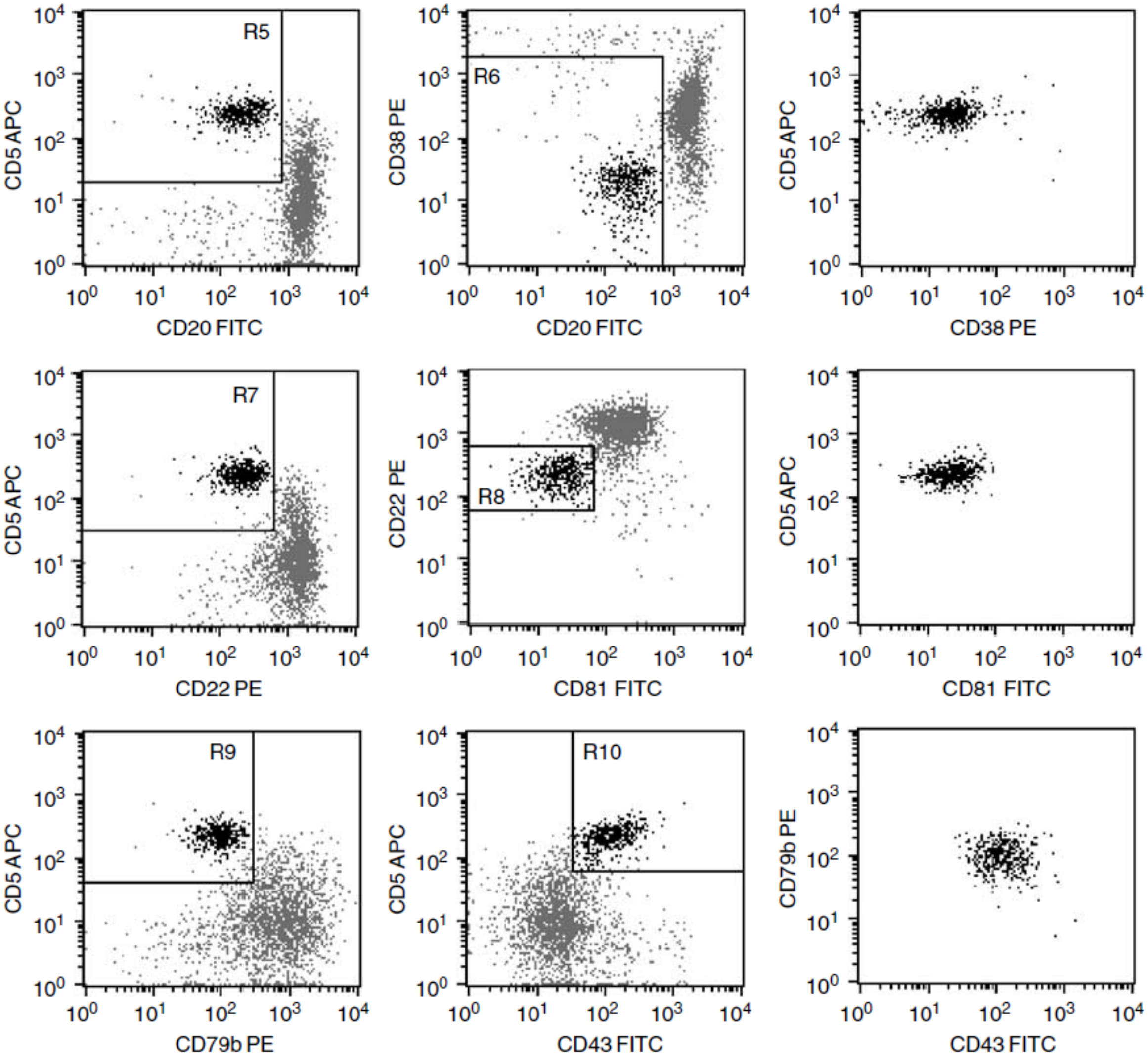
Table 1 Antibody combinations tested

No.	*FL1*	*FL2*	*FL4*	*No.*	*FL1*	*FL2*	*FL4*
1	CD10^{IS}	Integrinβ7 ^{Ph}	CD5^{IS}	26	CD21^{Ph}	CD48^{Ch}	CD43^{CA}
2	CD11a^{IS}	Integrinβ7 ^{Ph}	CD5^{IS}	27	CD24^{Ph}	CCR6RD	CD43^{CA}
3	**CD20**$^{\mathbf{BC}}$	**CD38**$^{\mathbf{IS}}$	**CD5**$^{\mathbf{IS}}$	28	CD24^{Ph}	CD10^{IS}	CD43^{CA}
4	**CD20**$^{\mathbf{BC}}$	**Integrin**$\boldsymbol{\beta}$**7** $^{\mathbf{Ph}}$	**CD5**$^{\mathbf{IS}}$	29	CD24^{Ph}	CD48^{Ch}	CD43^{CA}
5	CD21^{Ph}	CXCR5RD	CD5^{IS}	30	**CD24**$^{\mathbf{Ph}}$	**CXCR5**$^{\mathbf{RD}}$	**CD43**$^{\mathbf{CA}}$
6	CD24^{Ph}	CD40^{IS}	CD5^{IS}	31	CD24^{Ph}	Integrinβ7 ^{Ph}	CD43^{CA}
7	CD24^{Ph}	CD48^{Ch}	CD5^{IS}	32	CD37^{Ph}	CCR6RD	CD43^{CA}
8	CD24^{Ph}	CXCR5RD	CD5^{IS}	33	CD37^{Ph}	CD5^{IS}	CD43^{CA}
9	CD24^{Ph}	Integrinβ7 ^{Ph}	CD5^{IS}	34	**CD37**$^{\mathbf{Ph}}$	**CD79b**$^{\mathbf{BC}}$	**CD43**$^{\mathbf{CA}}$
10	CD31^{Ph}	CXCR5RD	CD5^{IS}	35	CD37^{Ph}	Integrinβ7 ^{Ph}	CD43^{CA}
11	CD37^{Ph}	CXCR5RD	CD5^{IS}	36	CD40^{IS}	CD48^{Ch}	CD43^{CA}
12	CD39^{Ph}	CD54^{IS}	CD5^{IS}	37	CD44^{Ph}	Integrinβ7 ^{Ph}	CD43^{CA}
13	CD40^{IS}	CXCR5RD	CD5^{IS}	38	CD5^{IS}	CCR6RD	CD43^{CA}
14	CD40^{IS}	Integrinβ7 ^{Ph}	CD5^{IS}	39	**CD5**$^{\mathbf{IS}}$	**CD23**$^{\mathbf{Ph}}$	**CD43**$^{\mathbf{CA}}$
15	CD44^{Ph}	Integrinβ7 ^{Ph}	CD5^{IS}	40	CD5^{IS}	CXCR5RD	CD43^{CA}
16	CD48^{Ch}	CXCR5RD	CD5^{IS}	41	CD79b^{BC}	CD21^{Ph}	CD43^{CA}
17	CD48^{Ch}	Integrinβ7 ^{Ph}	CD5^{IS}	42	CD79b^{BC}	CD24^{Ph}	CD43^{CA}
18	CD48^{Ch}	LAIR-1^{Ph}	CD5^{IS}	43	CD79b^{BC}	CD40^{IS}	CD43^{CA}
19	CD70^{Ph}	Integrinβ7 ^{Ph}	CD5^{IS}	44	CD79b^{BC}	CD48^{Ch}	CD43^{CA}
20	CD79b^{BC}	CXCR5RD	CD5^{IS}	45	**CD79b**$^{\mathbf{BC}}$	**CD5**$^{\mathbf{IS}}$	**CD43**$^{\mathbf{CA}}$
21	CD79b^{BC}	Integrinβ7^{Ph}	CD5^{IS}	46	CD79b^{BC}	CXCR5RD	CD43^{CA}
22	**CD81**$^{\mathbf{Ph}}$	**CD22**$^{\mathbf{IS}}$	**CD5**$^{\mathbf{IS}}$	47	CD81^{Ph}	CD79b^{BC}	CD43^{CA}
23	CD10^{IS}	CD5^{IS}	CD43^{CA}	48	CD24^{Ph}	CD27^{Ph}	CD38^{IS}
24	CD10^{IS}	Integrinβ7^{Ph}	CD43^{CA}	49	CD43^{CA}	CD79b^{BC}	CD38^{IS}
25	**CD20**$^{\mathbf{BC}}$	**CD5**$^{\mathbf{IS}}$	**CD43**$^{\mathbf{CA}}$	50	**CD81**$^{\mathbf{Ph}}$	**CD43**$^{\mathbf{CA}}$	**CD38**$^{\mathbf{IS}}$

Manufacturers: IS = BD Immunocytometry Systems (www.bdbiosciences.com); Ph = BD Pharmingen (www.bdbiosciences.com); CA = Caltag Laboratories (www.caltag.com); Ch = Chemicon (www.chemicon.com); BC = Beckman Coulter/Immunotech (www.beckman.com); RD = R&D systems (www.rndsystems.com). FL3 was CD19 PerCP-Cy5.5^{IS} or CD19 PE-Cy5.5^{CA}.

Leukemia (2007) 21, 956–964

The immunophenotypic profile of CLL cells and normal B cells using the optimized CLL MRD panel



CD20/CD38/CD19/CD5

The plots show B cells gated according to their CD19 expression and light scatter characteristics.

CD81/CD22/CD19/CD5

CD43/CD79b/CD19/CD5

Leukemia (2007) 21, 956–964

MRD in CLL

500.000 leukocytes in all tubes
Limit of detection 0.01%

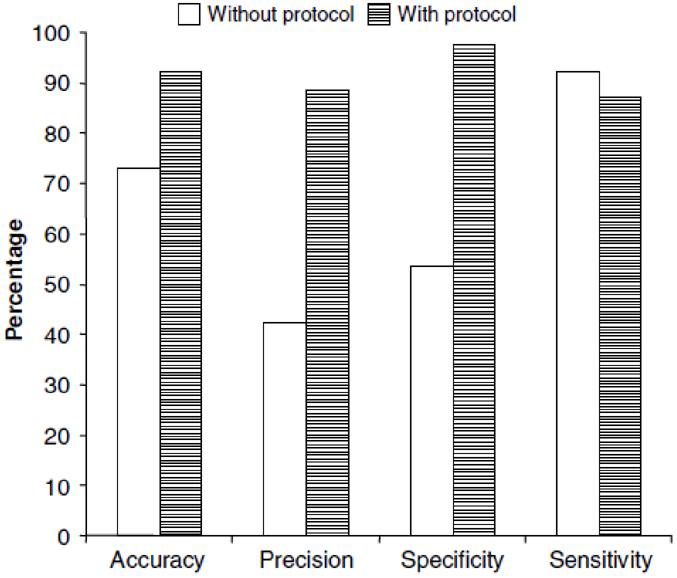


Figure 3 Use of a consensus protocol improves precision, consistency and specificity for detection of MRD by operators who are experienced in flow cytometric analysis of CLL.

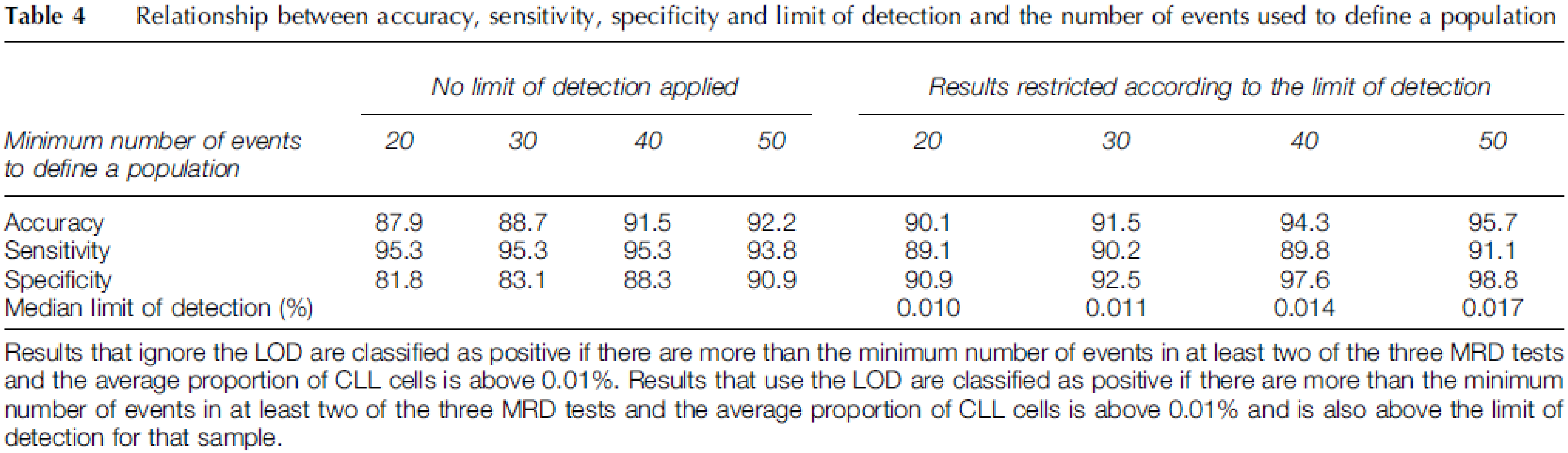


Table 4 Relationship between accuracy, sensitivity, specificity and limit of detection and the number of events used to define a population

	No limit of detection applied				*Results restricted according to the limit of detection*			
Minimum number of events to define a population	*20*	*30*	*40*	*50*	*20*	*30*	*40*	*50*
Accuracy	87.9	88.7	91.5	92.2	90.1	91.5	94.3	95.7
Sensitivity	95.3	95.3	95.3	93.8	89.1	90.2	89.8	91.1
Specificity	81.8	83.1	88.3	90.9	90.9	92.5	97.6	98.8
Median limit of detection (%)					0.010	0.011	0.014	0.017

Results that ignore the LOD are classified as positive if there are more than the minimum number of events in at least two of the three MRD tests and the average proportion of CLL cells is above 0.01%. Results that use the LOD are classified as positive if there are more than the minimum number of events in at least two of the three MRD tests and the average proportion of CLL cells is also above 0.01% and is also above the limit of detection for that sample.

MRD in CLL

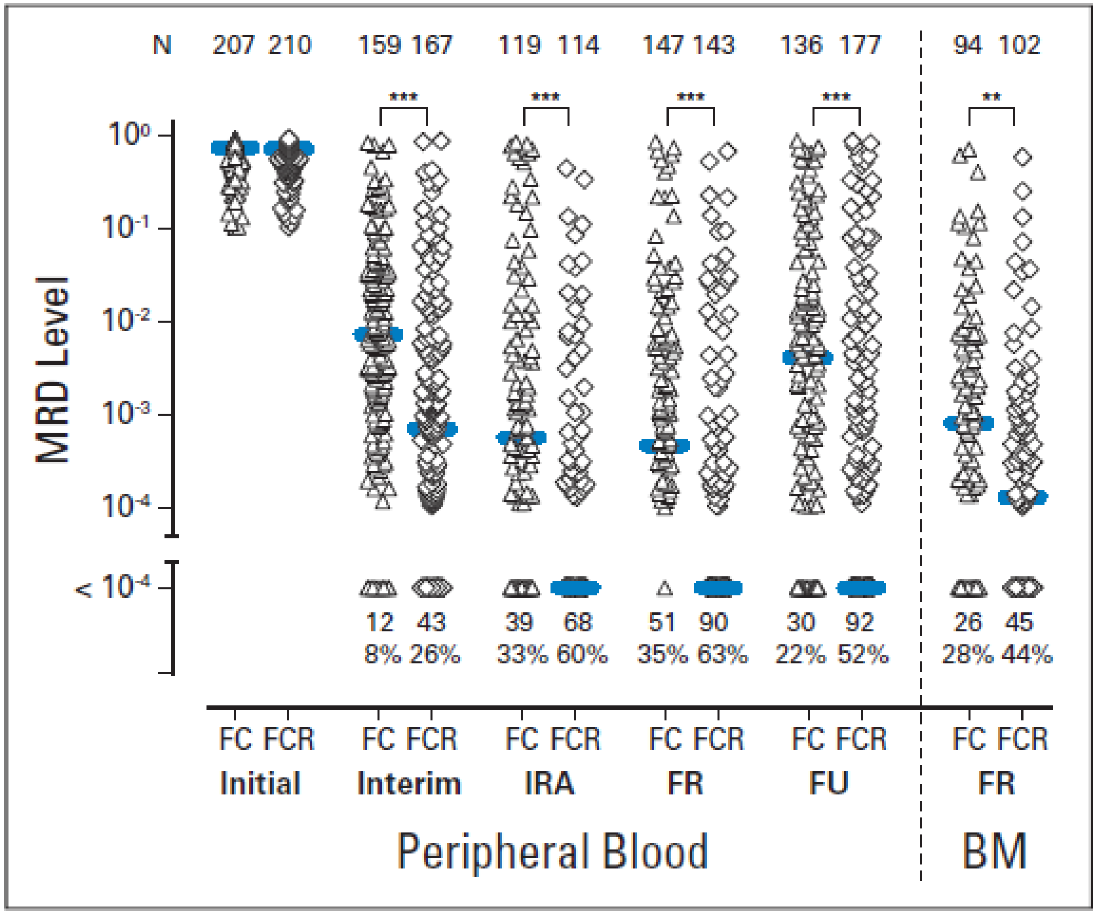


Fig 1. Minimal residual disease (MRD) levels at different time points in patients treated with fludarabine and cyclophosphamide (FC; triangles) and FC plus rituximab (FCR; diamonds). Individual triangles and diamonds symbolize patients; blue lines depict the medians. Top line numbers: total number of patients assessed; bottom numbers: number and percentage of patients with MRD levels $< 10^{-4}$. Comparison between treatment arms: *** $P < .0001$; ** $P = .0007$. Comparisons of consecutive time points within a treatment arm in peripheral blood: initial versus interim and interim versus initial response assessment (IRA): $P < .0001$ each in both FC and FCR arms. IRA versus final restaging (FR): not significant in both treatment arms. FR versus follow-up (FU): $P = .0001$ (FC) and $P = .027$ (FCR). BM, bone marrow.

J Clin Oncol 2012;30:980-988

MRD in CLL

J Clin Oncol 2012;30:980-988

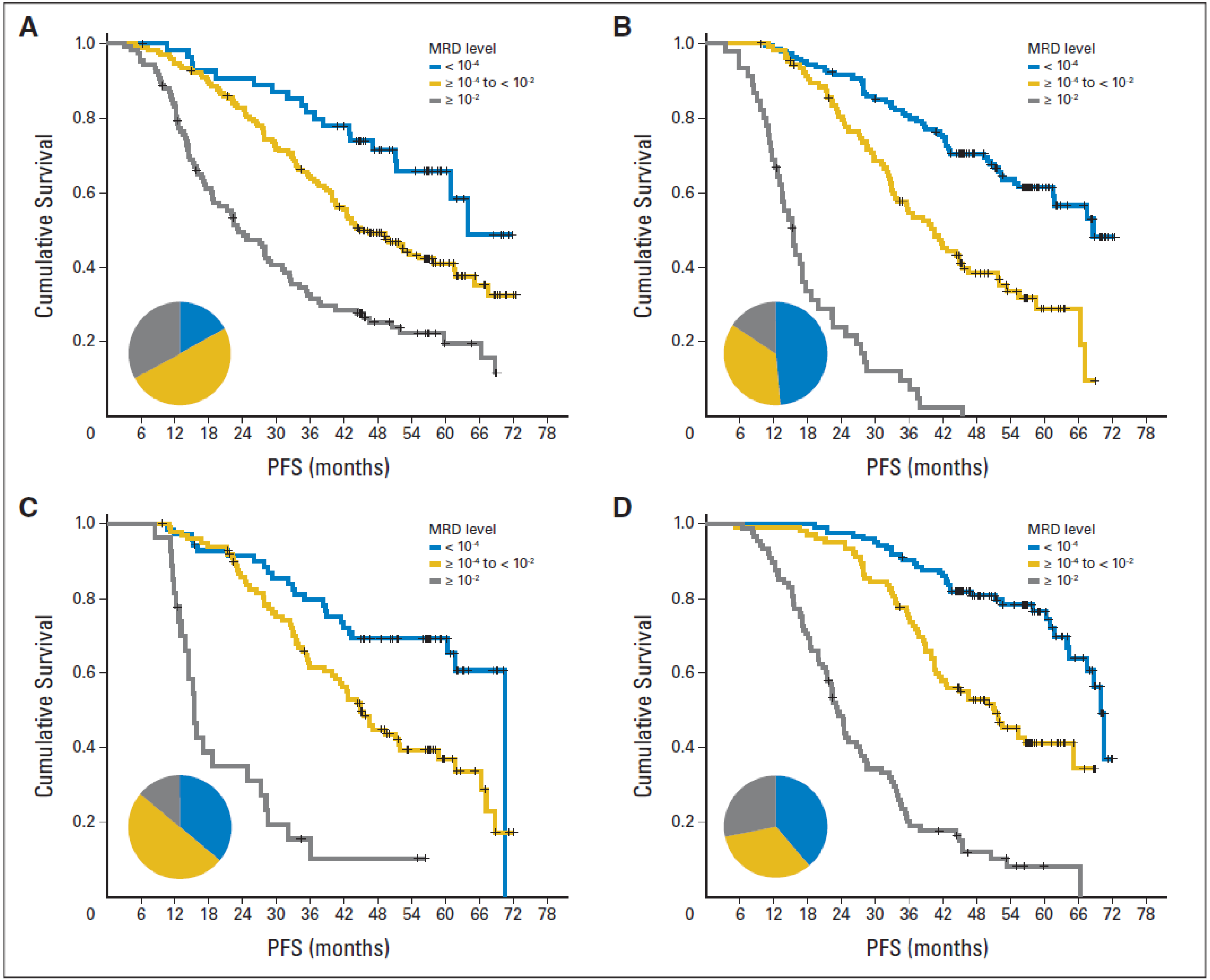
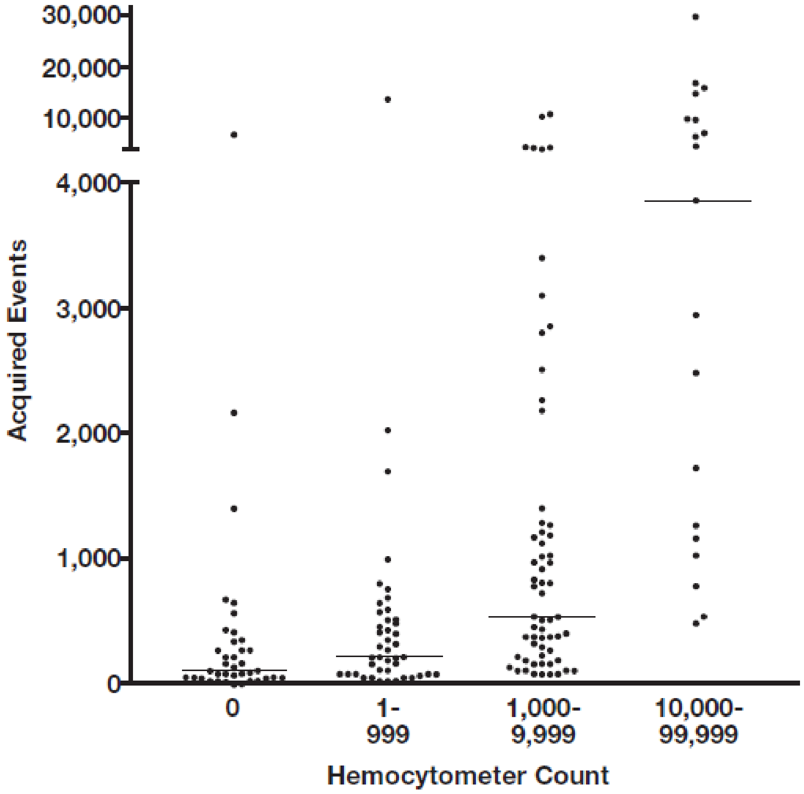


Fig 2. Progression-free survival (PFS) in patients with chronic lymphocytic leukemia grouped by minimal residual disease (MRD) levels assessed (A) in peripheral blood (PB) at interim staging, (B) in PB at final restaging, (C) in bone marrow at final restaging, and (D) in PB during follow-up. The inserted pie charts represent the frequency distributions. $P < .0001$ for all analyses by log-rank test.

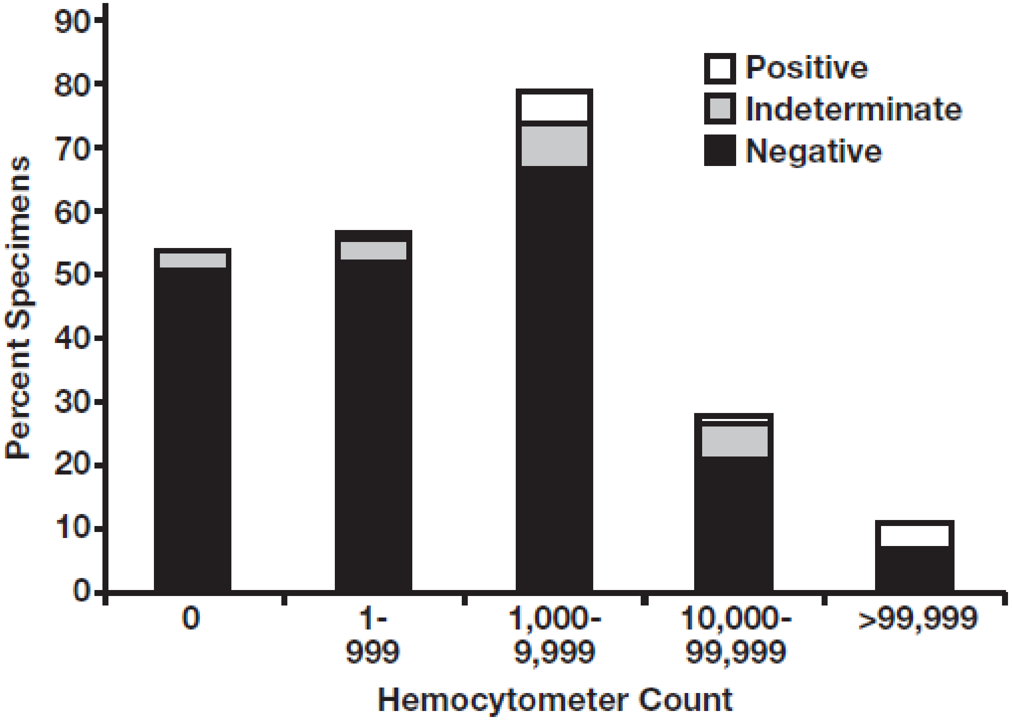
Flow Cytometric Immunophenotyping of Cerebrospinal Fluid Specimens



Flow cytometry laboratory hemocytometer count vs number of events acquired on the flow cytometer for lower cellularity specimens.

Craig FE et al Am J Clin Pathol 2011;135:22-34

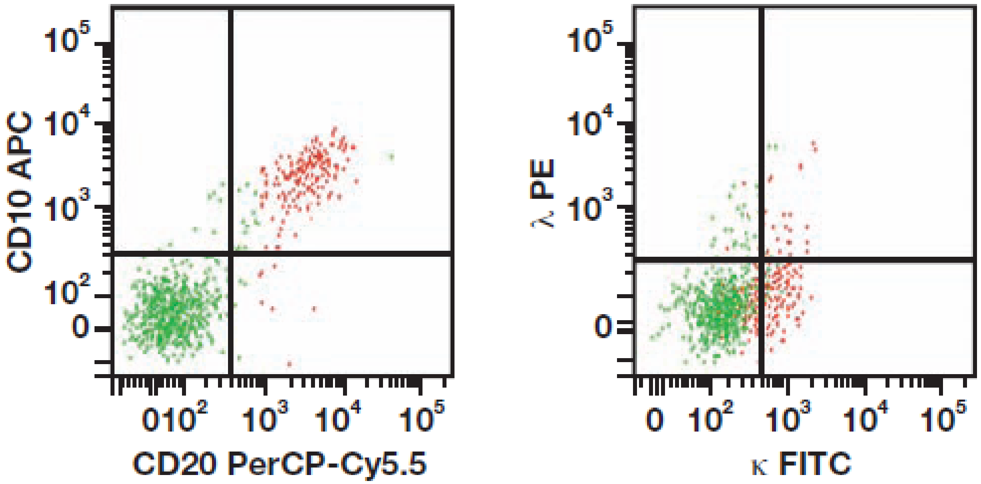
FC and LCR



Results of flow cytometric testing (positive, indeterminate, or negative) at different levels of cellularity as determined by hemocytometer counts performed in the flow cytometry laboratory.

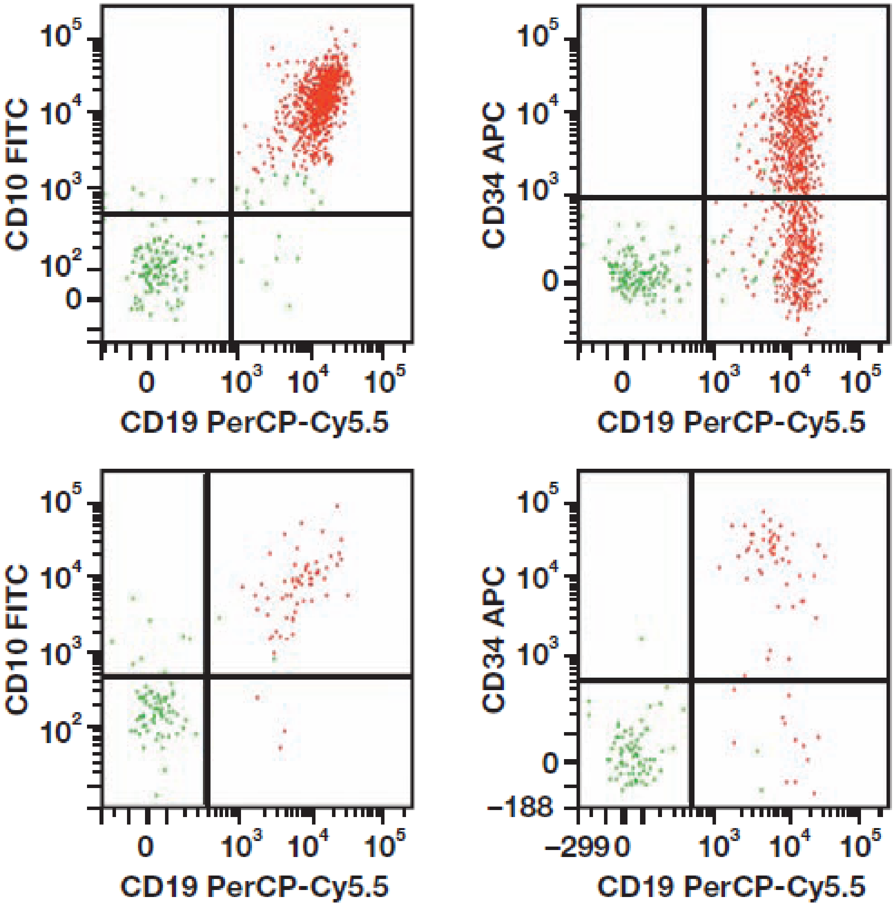
Craig FE et al Am J Clin Pathol 2011;135:22-34

Positive flow cytometric immunophenotyping: B-cell lymphoma



Craig FE et al Am J Clin Pathol 2011;135:22-34

Positive flow cytometric immunophenotyping: B-lymphoblastic leukemia

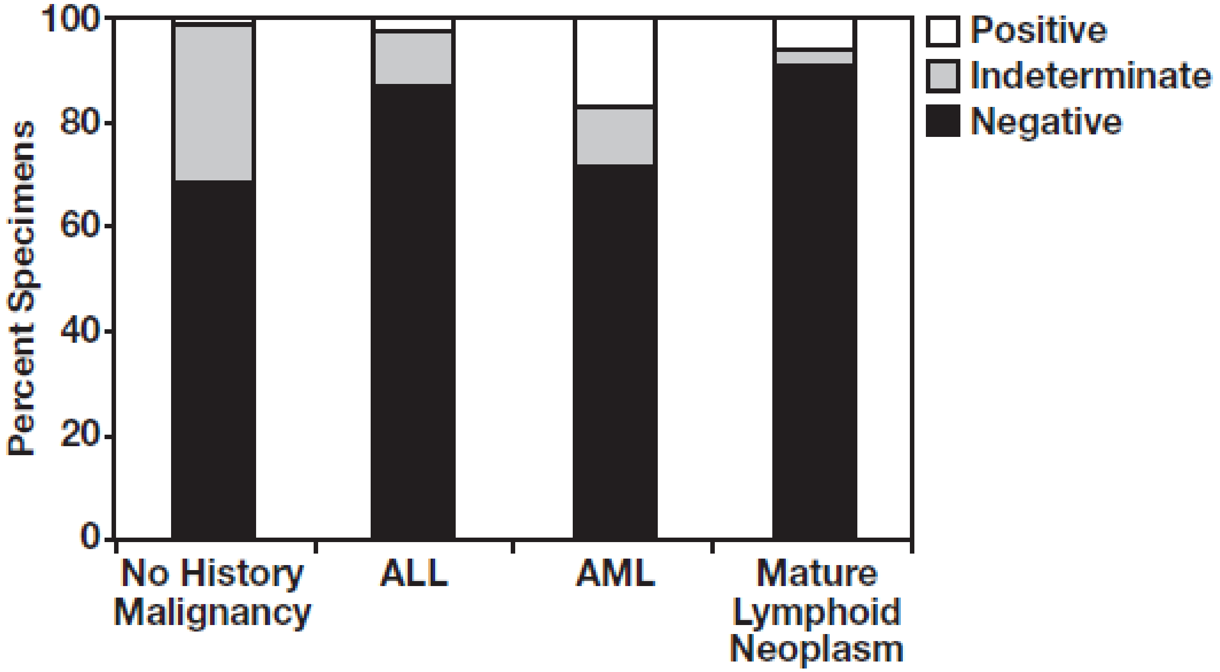


At diagnosis

after 21 days

Craig FE et al Am J Clin Pathol 2011;135:22-34

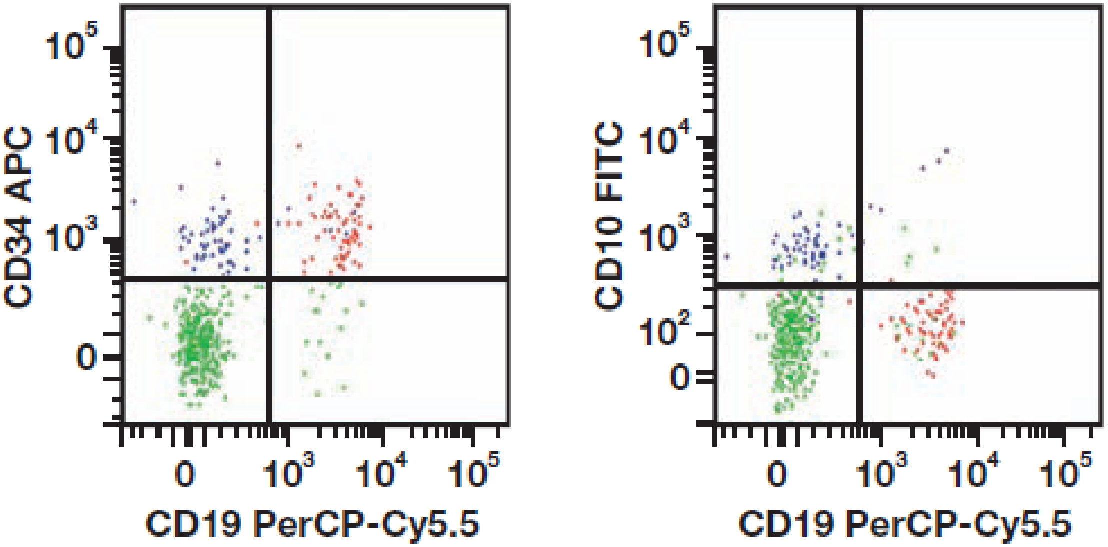
LCR in hematological neoplasia



Results of flow cytometric testing (positive, indeterminate, or negative) by clinical history. ALL, history of lymphoblastic leukemia, B or T cell; AML, history of acute myeloid leukemia.

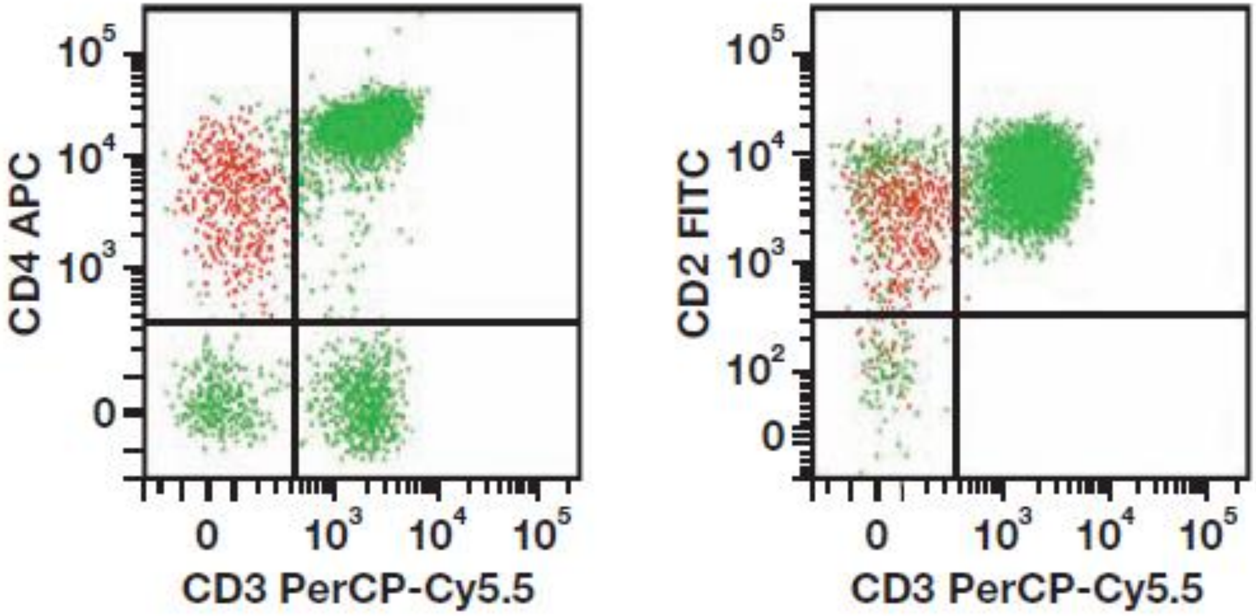
Craig FE et al Am J Clin Pathol 2011;135:22-34

Indeterminate flow cytometric immunophenotyping, limited primarily by possible artifact



Craig FE et al Am J Clin Pathol 2011;135:22-34

Indeterminate flow cytometric immunophenotyping, limited primarily by insufficient phenotypic findings



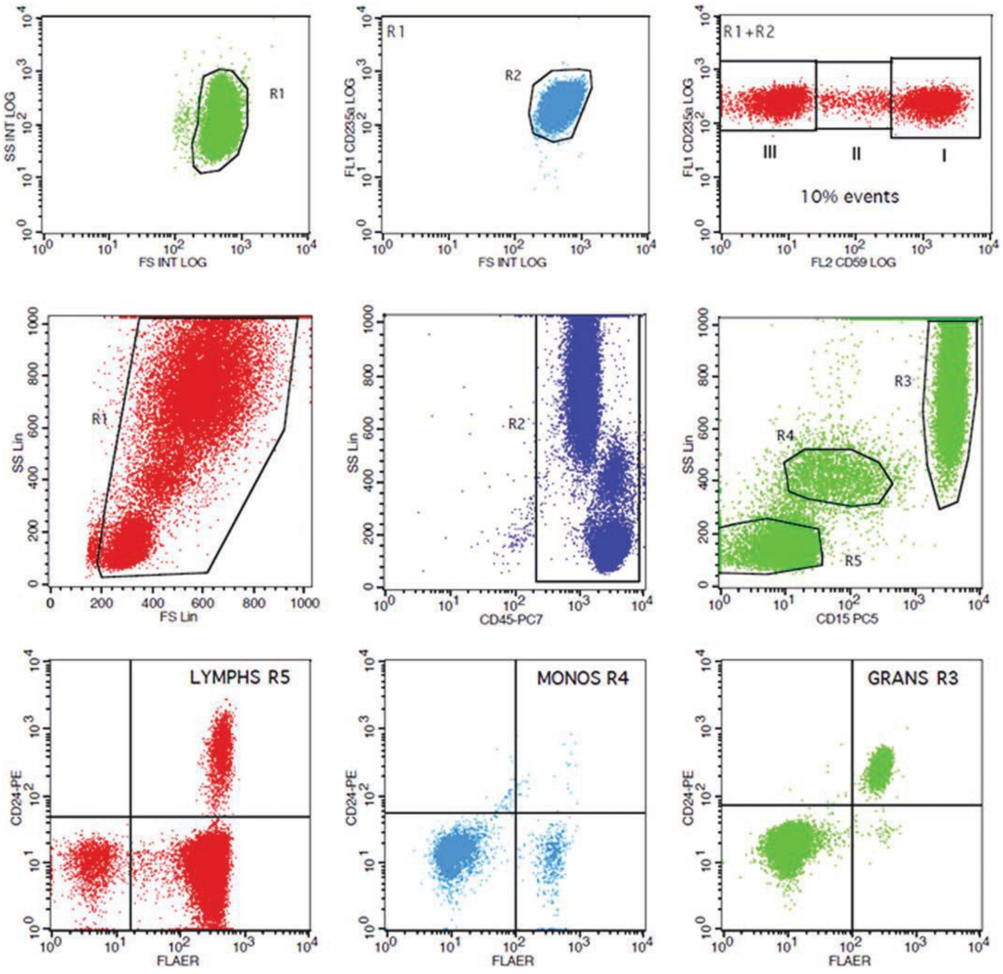
The patient was treated for suspected herpes encephalitis.

Craig FE et al Am J Clin Pathol 2011;135:22-34

Paroxysmal Nocturnal Hemoglobinuria

- FCM is a standard method for diagnosis of paroxysmal nocturnal hemoglobinuria (PNH).
- In PNH, the somatic mutation of the X-linked phosphatidylinositol glycan complementation Class A (PIGA) gene causes a partial or absolute inability to make GPIanchored proteins.
  - Antigens such as CD55, CD58, CD59, CD14, CD16, and CD24 are affected.
- The channel-forming toxin aerolysin and its preform pro-aerolysin bind selectively and with high affinity to GPI anchor.
- An inactive aerolysin variant conjugated with Alexa Fluor 488 (FLAER-A) is now widely used to detect GPI-anchor-deficient cell populations.
- Current guidelines include a combination of
  - CD235a-FITC and CD59-PE for detection of GPI-deficient RBC,
  - FLAER-A/CD24-PE/CD15-PECy5/CD45-PECy7 for detection of GPI-deficient ranulocytes,
  - FLAER-A/CD14–PE/CD64–PECy5/CD45-PECy7 for GPI-deficient monocytes
- High-resolution assays allow detection of GPIdeficient RBC at sensitivity level 10^{-5} and GPI-deficient WBC at 10^{-4}, which has been noted in patients with aplastic anemia and MDS.

Enumeration of blood for markers associated with paroxysmal nocturnal hemoglobinuria (PNH).



- Upper row: the red blood cell (RBC) assay using CD23a–FITC/CD59-PE staining. RBCs are gated on FS and SS (R1, Upper left plot) and displayed on FCS versus CD235a–FITC plot (upper middle). CD235a positive RBCs are gated (R2). RBCs from region R1 + R2 are analyzed for CD59 expression (right upper plot). Normal RBCs (CD59 bright) are in region I. RBCs with PNH-related phenotypes (i.e., with CD59dim expression or CD59 negative) are in regions II and III, respectively. Middle and lower row: white blood cell (WBC assay) using staining with FLAER, CD24PE, CD15PECy5, and CD45PECy7. Light scatter voltages were established so that all nucleated cells were visible above the forward scatter threshold (middle left) and debris was excluded with a combination of light scatter and CD45 gating (middle plot). CD45+ events were displayed on CD15 versus SS plot (middle right plot) and granulocytes (bright CD15, high SS), monocytes (dim CD15 and intermediate SS) and lymphocytes (CD15-negative, low SS) were gated. Each of these populations was displayed on a FLAER versus CD24 plot (bottom row). PNH granulocytes (FLAER-negative, CD24-negative) were enumerated in the bottom right plot (lower left quadrant). Normal granulocytes were enumerated in the upper right quadrant. Gated monocytes were similarly displayed (bottom row middle) and the PNH monocytes (FLAER-negative, CD24-negative) were enumerated in the lower left quadrant. Gated lymphocytes (bottom row left) were assessed for PNH phenotypes in the lower left quadrant. Normal T-lymphocytes (FLAER+, CD24-negative) are visible in the lower right quadrant and normal B-lymphocytes (FLAER+, CD24+) are visible in the upper right quadrant.

Other analysis

- Myeloproliferative Neoplasms
- Reticulocyte Enumeration
- Hemoglobin F (Fetal-Maternal Hemorrhage and Sickle Cell Anemia)
- Analysis of Platelets
- Human Leukocyte Antigen Antibody Detection
- Primary Immunodeficiency Diseases
- Flow Cytometry Detection of HLA-B27
- Human Immunodeficiency Virus Infection
- Analysis of Antigen-Specific T-cells
- Cellular Dna Content And Cell Cycle Analysis
- Functional Assays
  - Monitoring of Cytokine Profiles
  - Protein Phosphorylation
  - Apoptosis