CITOMETRIA A FLUSSO

APPLICAZIONI NELLA DIAGNOSTICA ONCO-EMATOLOGICA

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Flow cytometry
ANALYSIS OF SINGLE CELL SUSPENSIONS

Peripheral blood

Bone marrow aspirates

All type of body fluids

Fresh tissue biopsies

Core biopsies

Fine-needle aspirates

Flow cytometry
UNIQUE PROPERTIES

- Analysis of a broad array of antigens
- Quantification of population frequencies and antigen expression level in individual cells
- Definition of the antigen profile of specific cells by multicolor (8 and more) analysis
- Gating of discrete subpopulations based on specific parameters

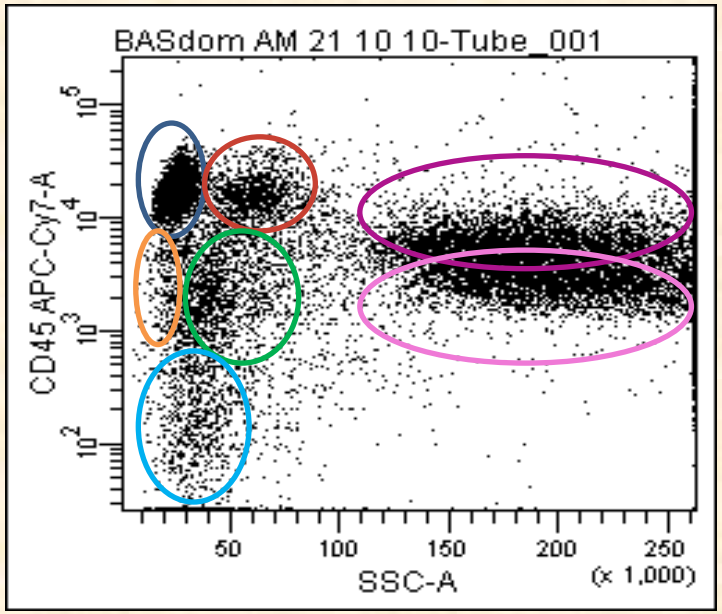
Flow cytometry
DIAGNOSTIC TOOLS

- Lineage assignment
- Maturational characterization of malignant cells
- Detection of clonality
- Heterogeneity and aberrant features of the malignant cell populations

$\Rightarrow$ Detection of minimal residual disease

The immunological gate CD45/SSC

A



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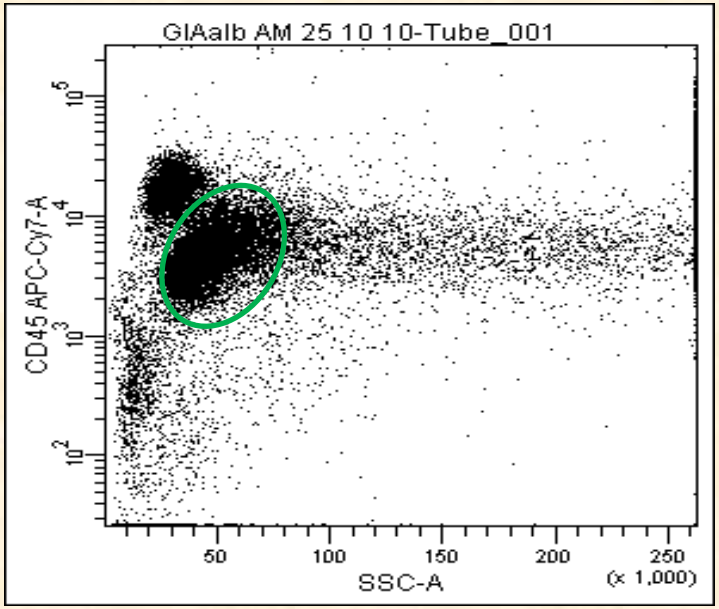


Normal bone marrow

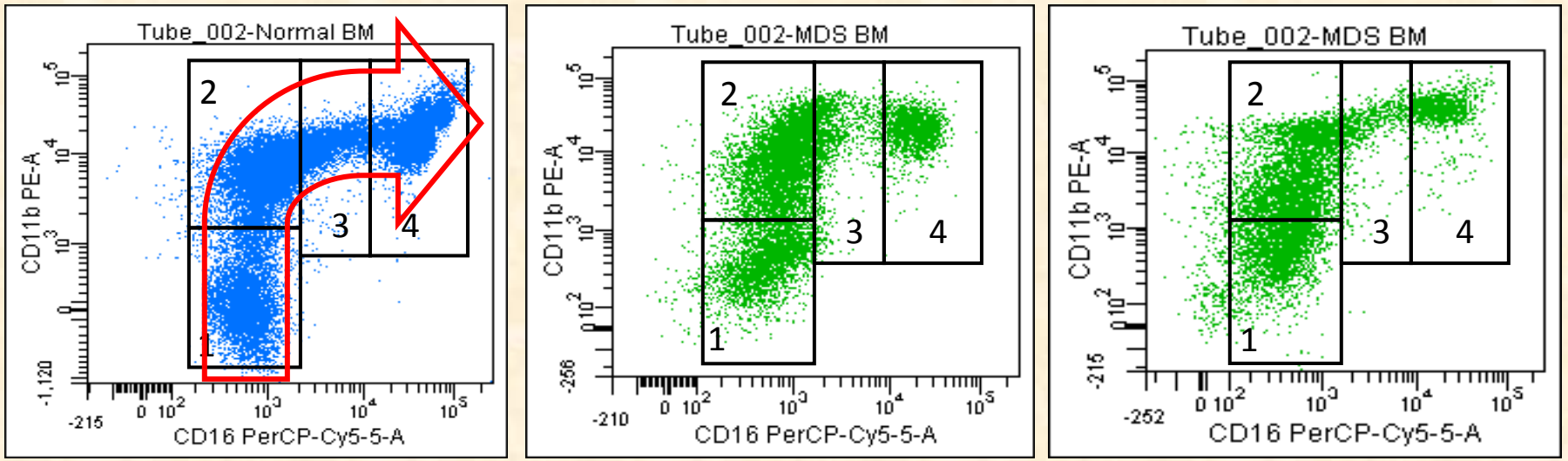
Lymphocytes
Lymphoblasts
Monocytes
Myeloblasts
Erythroblasts
Intermediate myeloid
Mature myeloid

Acute myeloid leukemia

Increase of myeloblasts

Decrease of granulocytes and monocytes

Granulocyte maturation by CD11b/CD16 combination



Normal BM **Myelodysplastic BM**

1 Promyelocytes
2 Myelocytes
3 Metamyelocytes and band forms
4 Mature granulocytes

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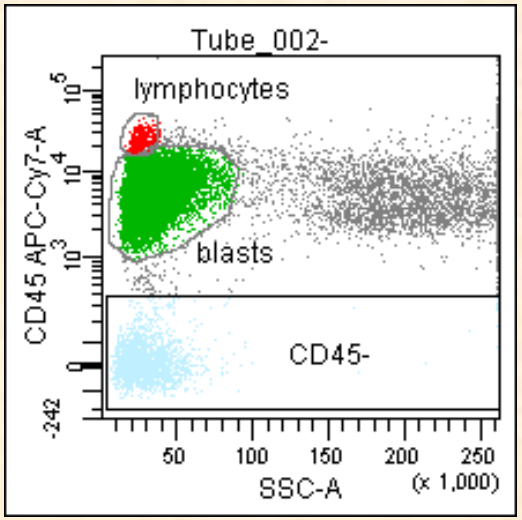
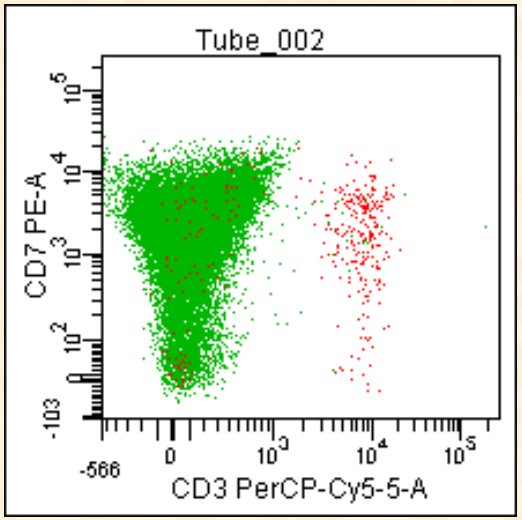
Leukemia-associated Aberrant Immunophenotypes (LAIP)

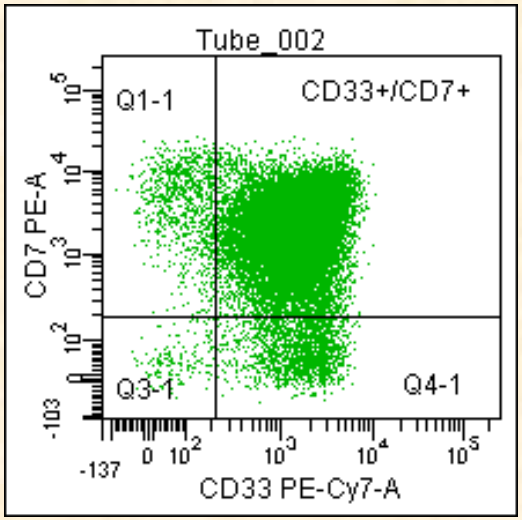
LAIP class	*Examples*
- Cross-lineage expression of lymphoid antigens	CD33+ CD2+ CD34+ CD34+ CD13+ CD19+
- Overexpression	HLA-DR++ CD33++ CD34++ CD64++ CD4++ CD45++
- Lack of expression of antigen	HLA-DR- CD33+ CD34+
- Asynchronous expression of antigens	CD15+ CD33+ CD34+ CD65+ CD33+ CD34+

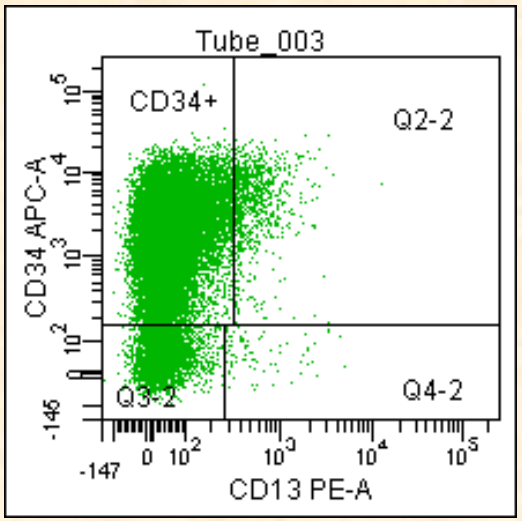
Kern W, Cancer 2008

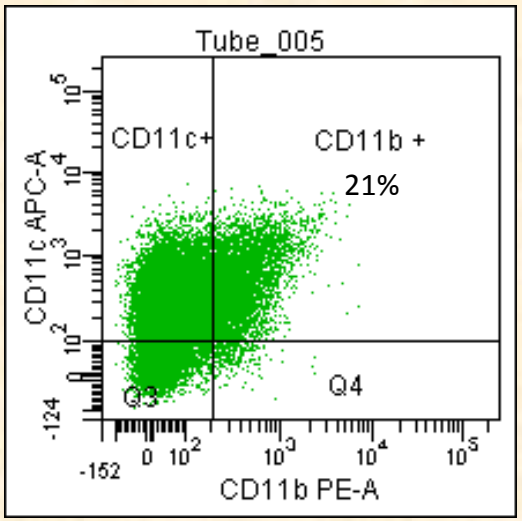
BM: lymphoproliferative disease?











AML

LAIP

Cross-lineage

CD33+/CD34+/CD7+

Asynchronous

CD34+/CD11b+

WHO 2008 - The mature B-cell neoplasms

Chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Splenic lymphoma/leukemia, unclassifiable
    Splenic diffuse red pulp small B-cell lymphoma
    Hairy cell leukemia-variant
Lymphoplasmacytic lymphoma
    Waldenström macroglobulinemia
Heavy chain diseases
    Alpha heavy chain disease
    Gamma heavy chain disease
    Mu heavy chain disease
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Extranodal marginal zone
B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone B-cell lymphoma (MZL)
    Pediatric type nodal MZL
Follicular lymphoma
    Pediatric type follicular lymphoma
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma (DLBCL), not otherwise specified
    T cell/histiocyte rich large B-cell lymphoma
    DLBCL associated with chronic inflammation
    Epstein-Barr virus (EBV)+ DLBCL of the elderly
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary cutaneous DLBCL, leg type
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma

Flow cytometric approach to the diagnosis and classification of B-cell lymphoid neoplasms

Immunophenotype		*Histotype*
CD5+	CD10-	**CLL, MCL**... PLL, MZL, DLBCL, LPL
CD5-	CD10+	**FL, DLBCL, BL**… HCL
CD5+	CD10+	FL, DLBCL, BL, MCL
CD5-	CD10-	**MZL, HCL**… FL, DLBCL, MCL

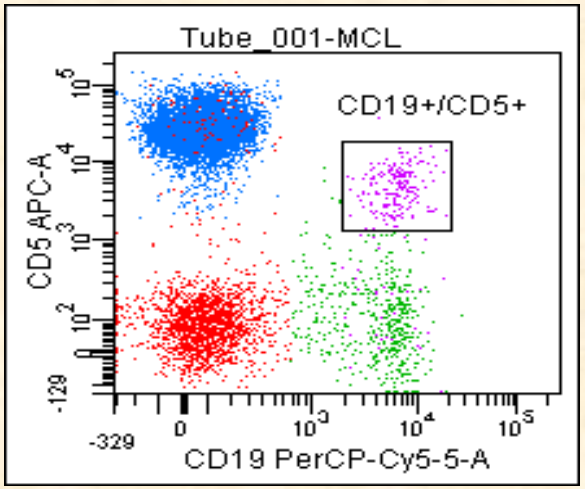
Craig F, Foon KA. Blood 2008

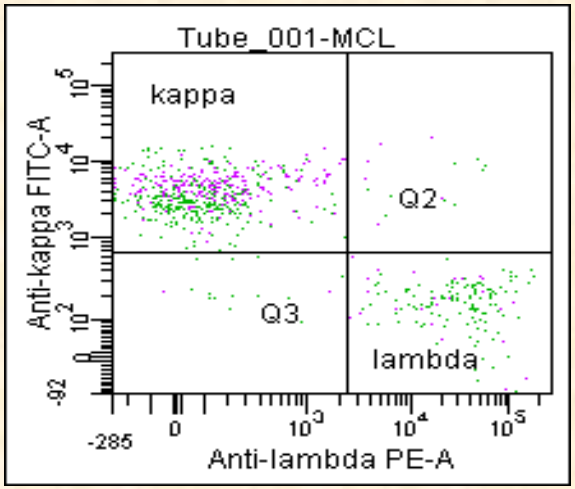
Additional immunological markers to differentiate B-cell lymphoma subtypes by FC

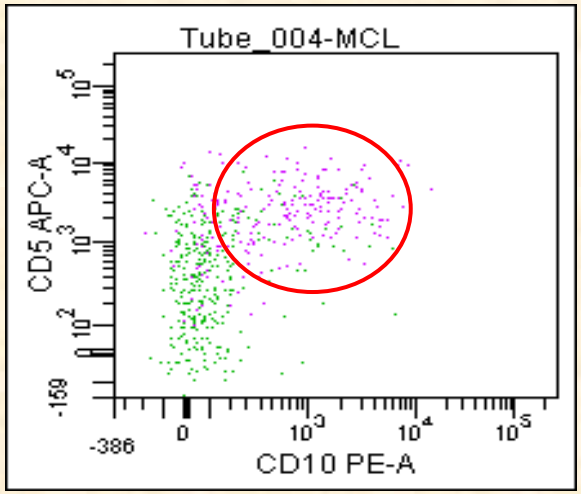
CD23	CLL+ MCL-
FMC7	CLL- MCL+
CD43	FL- CLL+ MCL+/- BL+ DLBCL+/-
BCL2	FL++ DLBCL+/- BL-
CD22	CLL- HCL++
CD11c, CD103	HCL++ MZL+/-

and more…..

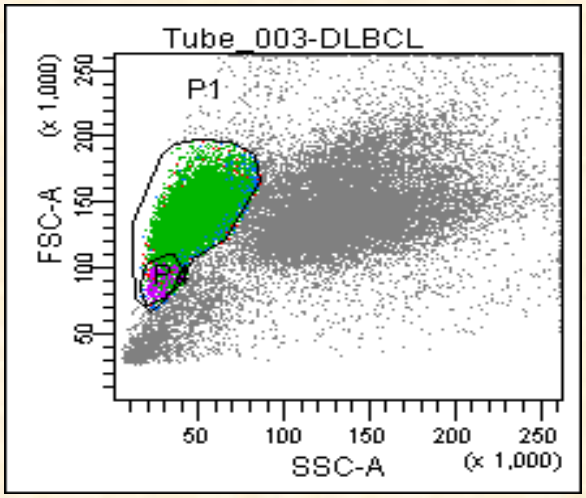
1. A case of CD5+ CD10+ mantle cell lymphoma

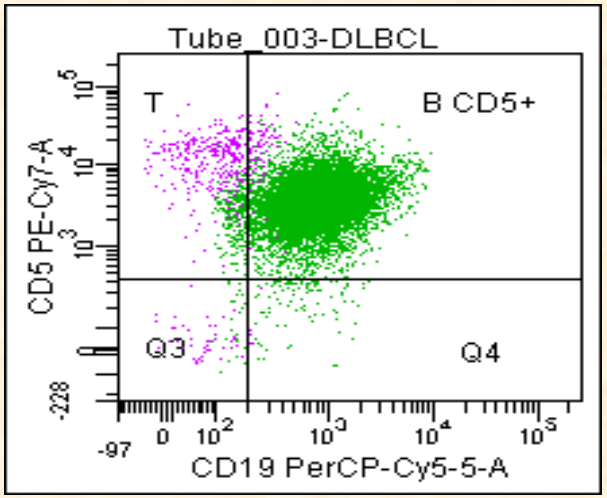


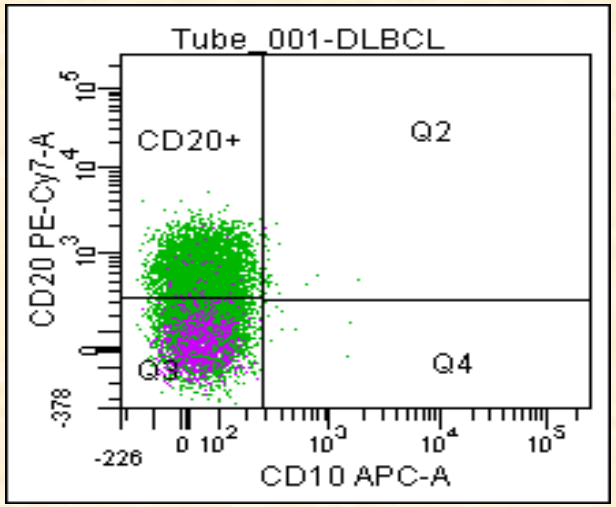




2. A case of CD5+ CD10- diffuse large B-cell lymphoma







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Diffuse large B-cell lymphoma, Burkitt's lymphoma, and the provisional intermediate category DLBCL/BL

	DLBCL	BL	DLBCL/BL
Volume	large	medium	medium, BL-like
Nucleoli	prominent	multiple	prominent
Mitotic rate	low	high	high
Apoptosis and starry-sky pattern	uncommon	yes	yes
BCL2	+	-	+
Ki-67	<90%	>95%	<95%
MYC R	Ig or non-Ig	Ig	non-Ig
BCL2 R	yes	no	yes (*BCL2 or BCL6* double-hit)
Karyotype	complex	simple	complex

Carbone A et al, Hum Pathol 2010

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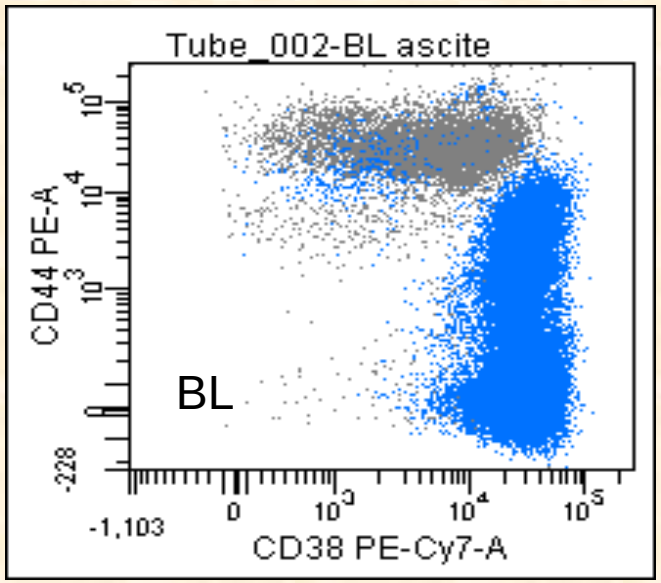
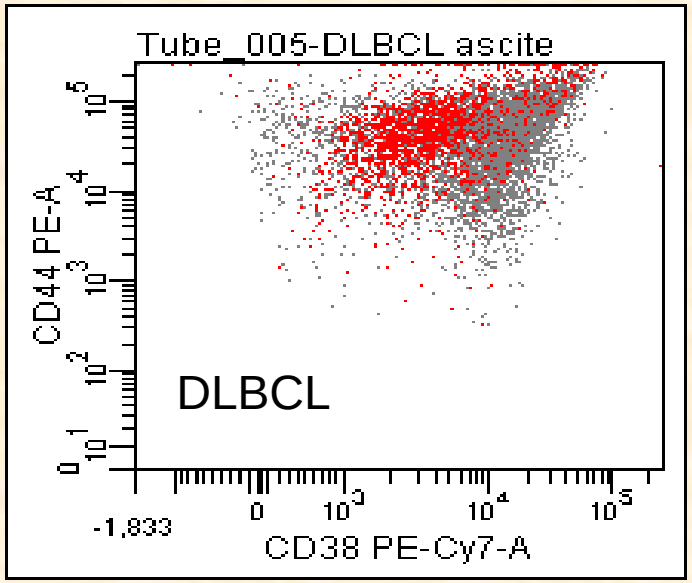
Flow cytometric antibody panel for distinguishing Burkitt lymphoma from CD10+ diffuse large B-cell lymphoma

Expression of CD44 and CD54 was detected at a significantly lower level in BL compared with CD10+ DLBCL (P = .001 and P = .01, respectively). There was not a significant difference in expression of CD18 and CD43.

Schniederjan et al. Am J Clin Pathol 2010





Tube Name: Tube_002 Record Date: Oct 14, 2010 4:51:25 PM		
Population	CD44 PE-A Mean	CD38 PE-C... Mean
CD19+	1,711	30,208

Tube Name: Tube_005 Record Date: May 12, 2010 5:47:12 PM		
Population	CD44 PE-A Mean	CD38 PE-C... Mean
CD19+	62,573	6,531

BL **I.N.T.** BCL6+ BCL2- MUM1- MIB1 100%

DLBCL **I.N.T.** BCL6+ BCL2+ MUM1+ MIB1 >80%

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“Double-Hit” mature B-cell lymphomas show a common immunophenotype by flow cytometry

CD20 ↓ or - *

CD10 +

CD45 ↓ variable

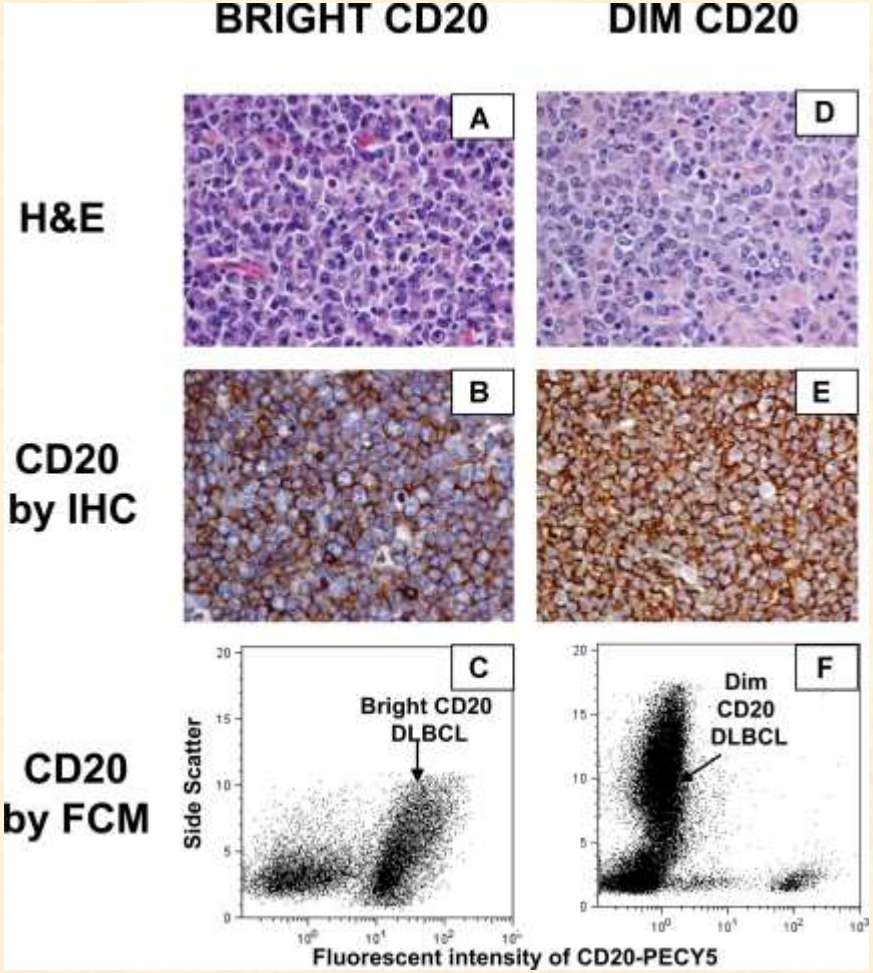
SIg ↓ or -

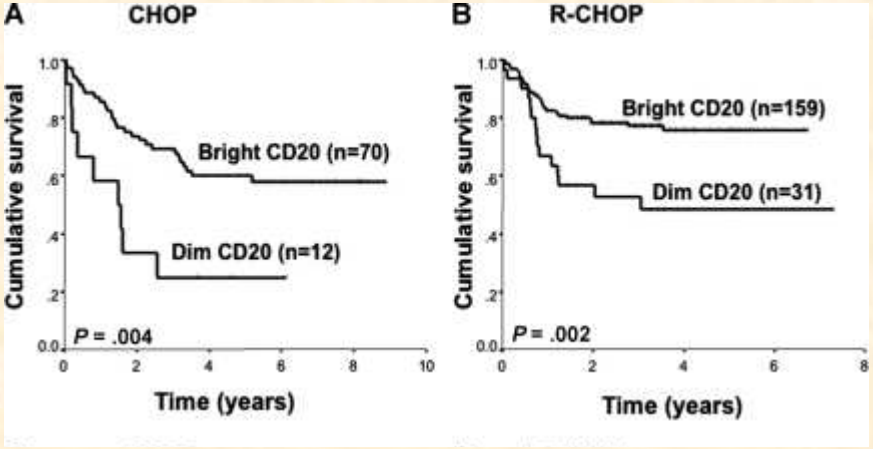
CD38 ↑ variable

FSC&SSC ↑

* in agreement with poor prognosis in $CD20^{low}$ DLBCL

Wu D. Am J Clin Pathol 2010





CD20^{dim} DLBCL are also highly associated with positivity for CD5 and BCL2

Johnson N, Blood 2009

CD5+ B-NHL

- **CLL** (CD23+ FMC7- $CD20^{dim}$, sIg^{dim})
- **MCL** (CD23- FMC7+ $CD20^{bright}$ sIg^{bright})

But also….

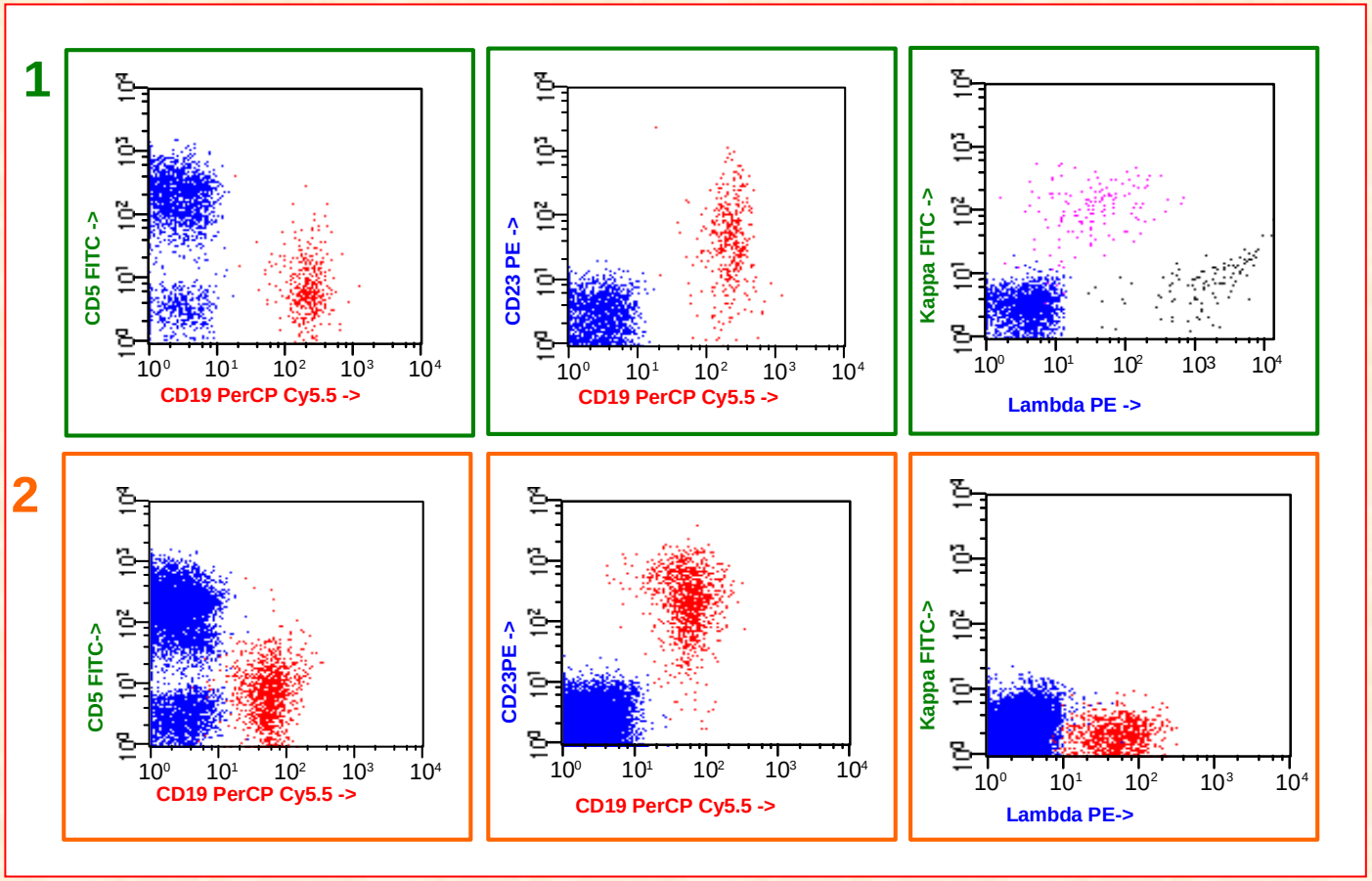
- MZL (splenic and nodular)
- LPL
- DLBCL
- MALT

Jevremovic D et al. Leukemia Res 2010

Dronca RS et al. Cytometry 2010

Baseggio L et al. Haematologica 2010

Pitfalls in using the traditional CD5/CD23/CD19 antibody combination to detect B-CLL cells



PBL from a B-CLL patient (follow-up, negative)

PBL from a B-CLL positive patient

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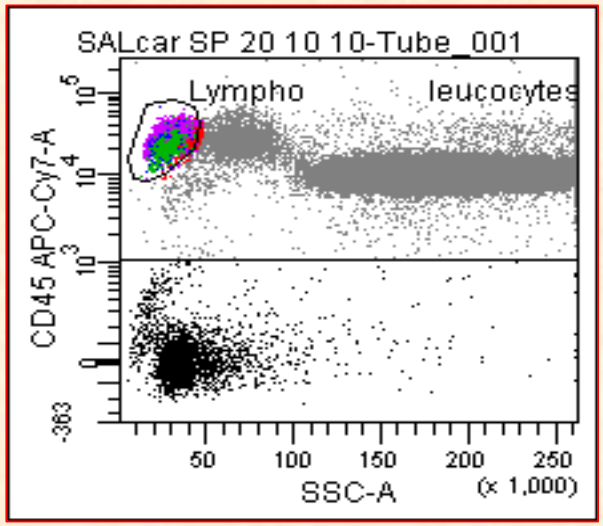
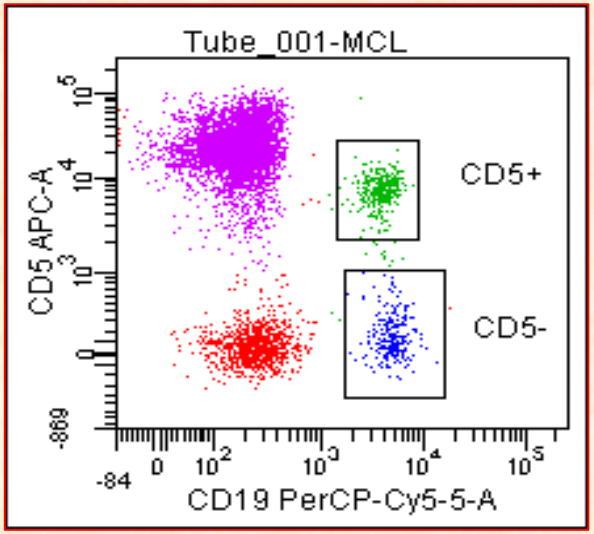
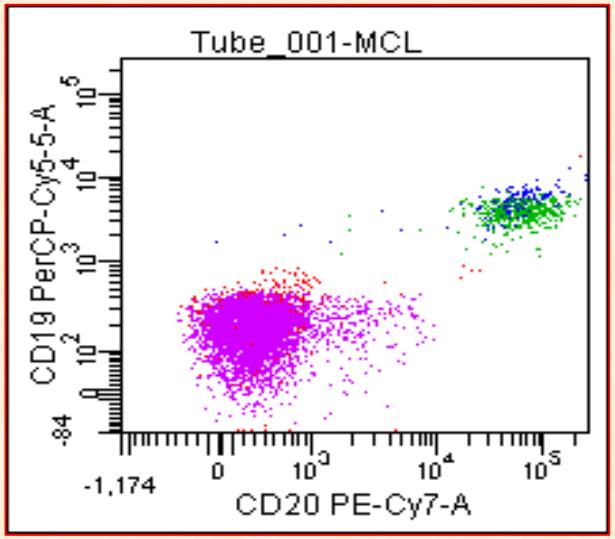
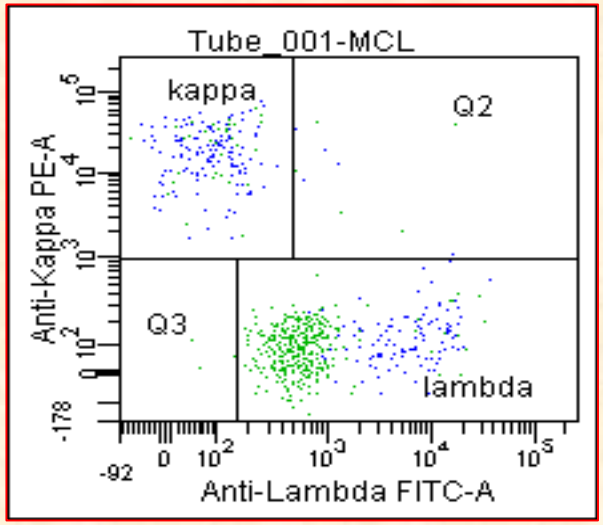
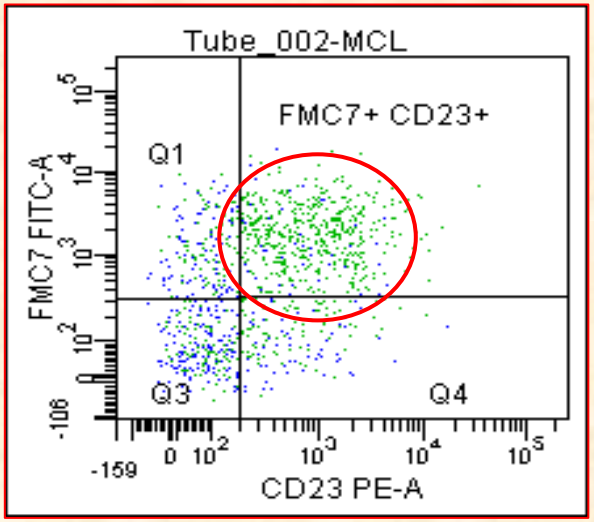
CD23+ Ig^{low} MCL or FMC7+ CD20++ CLL?











Tube Name: Tube_001			
Record Date: Oct 20, 2010 5:04:55 PM			
			CD20 PE-...
Population	Parent Name	%Parent	Mean
CD5-	CD19+	39.9	65,876
CD5+	CD19+	57.3	71,915

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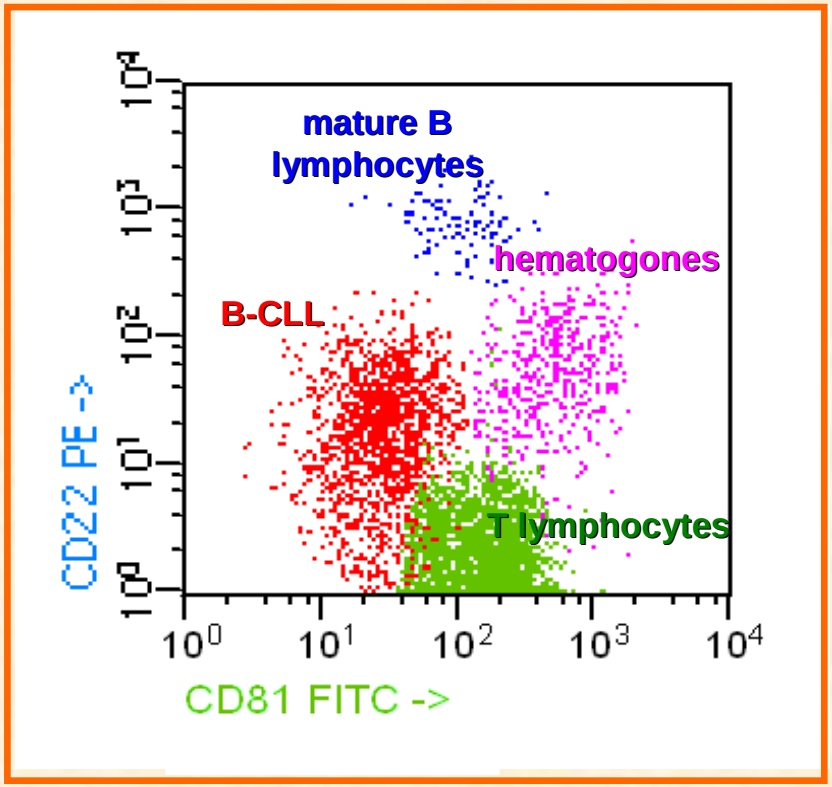
Tube1 staining: **L** FITC / **K** PE / **CD19** PerCPCy5.5 / **CD20** PE-Cy7 / **CD5** APC / **CD45** APC-H7

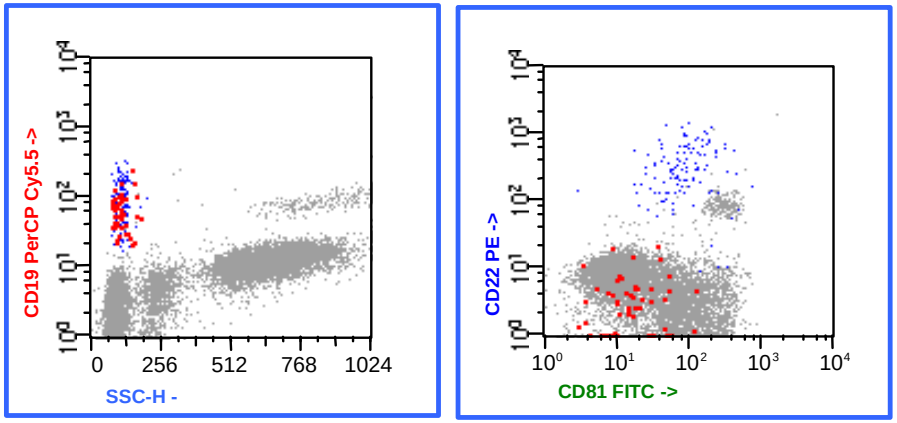
CD79b, CD22, CD81 and CD200 in B-CLL and B-NHL

CD79b **CD22** **CD81**	✓A.C. Rawstron et al. *Leukemia* (2006); **20**:2102 ✓A.C.Rawstron et al. *Leukemia*. (2007), **21**:956 ✓H. Sayala et al. *Best Pract & Res Clin Haematol.* (2007); **20**:499 ✓G A Jasper et al. *Cytometry Part B* (2010); Sep 24 ✓R F Luo et al. *Hum Pathol* (2010); **41**:271 (IHC)
CD200	✓ Palumbo GA et al. *Leukemia Res*. (2009); **33**: 1212 ✓D M Dorfman et al. *Am J Clin Pathol* (2010); **135**:726 (IHC) ✓ Brunetti L et al. *Br J Haematol. (*2009); **145**:665

CD81/CD22 expression in B-CLL and MRD detection

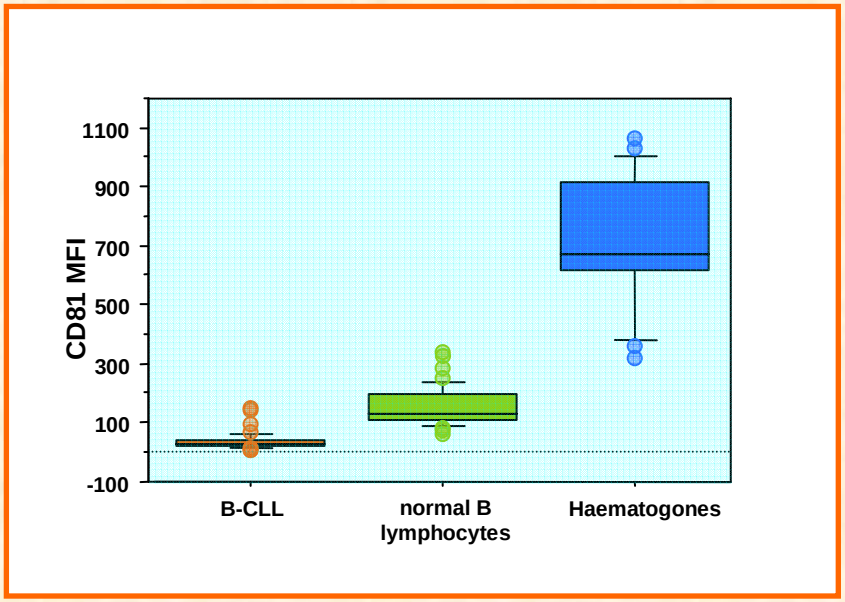


MRD detection



Residual B-CLL population: 4.5×10^{-3} (red dots)

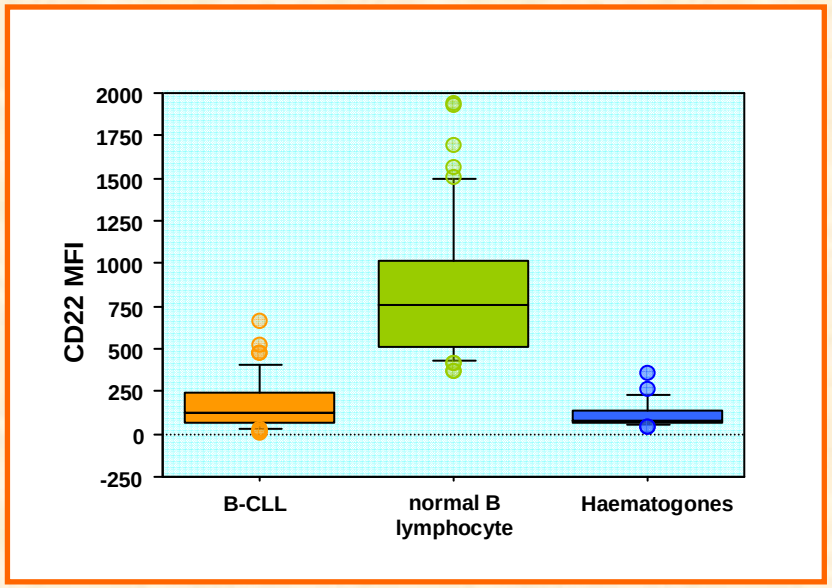
CD81 in B-CLL



CD81	n° samples	Mean	Std. Dev.	Median	Minimum	Maximun
B-CLL	47 *	**37,8**	29,6	**29,0**	11	150
normal B lymphocytes	42	**150,3**	65,9	**127,5**	60	343
hematogones	19	**710,2**	226,2	**673,0**	320	1064

* MFI calculated on 45 B-CLL positive samples, 1 sample was CD81- and 1 not evaluable

CD22 in B-CLL



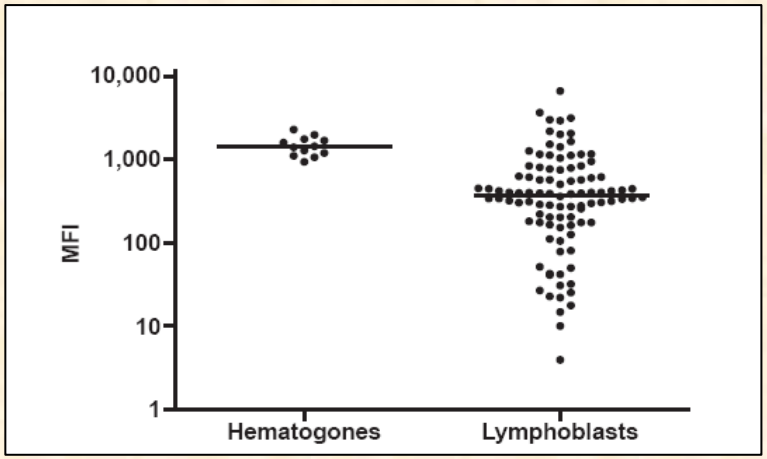
CD22	n° samples	Mean	Std. Dev.	Median	Minimum	Maximun
B-CLL	47 *	177,0	153,2	122,0	13	660
normal B lymphocytes	48	832,7	413,7	752,5	370	1938
hematogones	19	110.5	83,3	75,0	38	362

* MFI calculated on 46 B-CLL positive samples, 1 was not evaluable

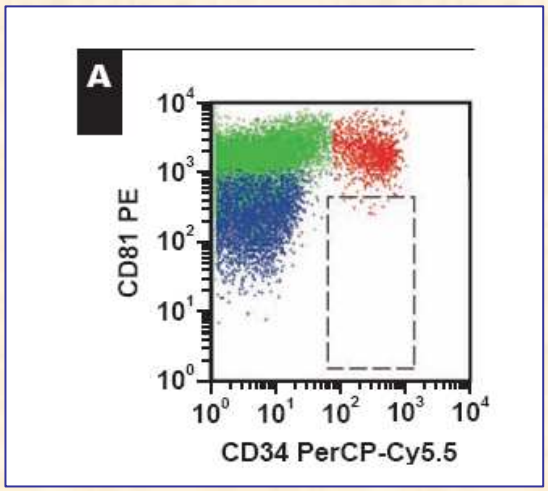
CD81 in B-CLL and B-NHL

	Disease	CD81 down-regulation	CD81 up-regulation	
Low grade	**CLL**	53/53 **(100%)**		
	MZL	3/7 **(43%)**		
	LPL	1/5 (20%)		
	HCL	3/3 **(100%)**		
	MCL	2/12 (17%)		
	FL	2/5 (40%)	2*/5 (40%)	*1 HG transformed
High grade	**BL**	0/8 (0%)	7/8 **(88%)**	
	DLBCL	1/5 (20%)	2/5 (40%)	

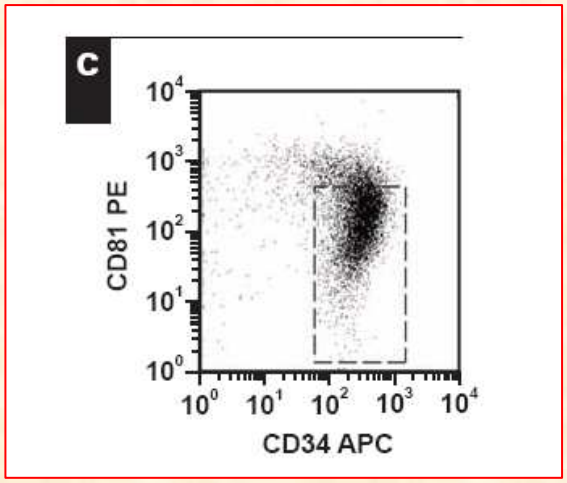
Aberrant underexpression of CD81 in precursors B-cell acute lymphoblastic leukemia



Normal	B-ALL
MFI 1460	MFI 647



Normal BM



B-ALL

Muzzafar T. Am J Clin Pathol 2009

CD200 in B-CLL and B-NHL

Disease	N° positive cases	
B-CLL	53/53* (100%)	bright
MZL	2/7 (28%)	
LPL	4/5 (80%)	
HCL	3/3 (100%)	
MCL	0/11° (0%)	
FL	3/5 ^ (60%)	dim
BL	0/8 (0%)	
DLBCL	2/6 (33%)	

***** 2 CD200-negative B-CLL cases were subsequently re-evaluated as FL and MZL

° 2 CD200-positive MCL cases were subsequently re-evaluated as B-CLL

^ The two CD200-negative FL expressed high levels of CD81 antigen; the clinical and histological features of one of them were suggestive of high grade transformation

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6-color staining for B-CLL and CD5+ B-NHL

FITC	PE	PerCP-Cy5	PE-Cy7	APC	APC-H7
CD81	**CD200**	**CD19**	**CD5**	**CD22**	**CD45**
CD43	CD23	CD19	CD5	CD79b	CD45
Kappa	**Lambda**	**CD19**	**CD20**	**CD5**	**CD45**

6-color staining for B-CLL and CD5+ B-NHL

	FITC	PE	PerCP-Cy5	PE-Cy7	APC	APC-H7
	CD81	CD200	CD19	CD5	CD22	CD45
CLL	dim	++	+	+	dim	+
MCL	+	-	+	+	+	+

B-NHL re-classification
(1) MCL → B-CLL

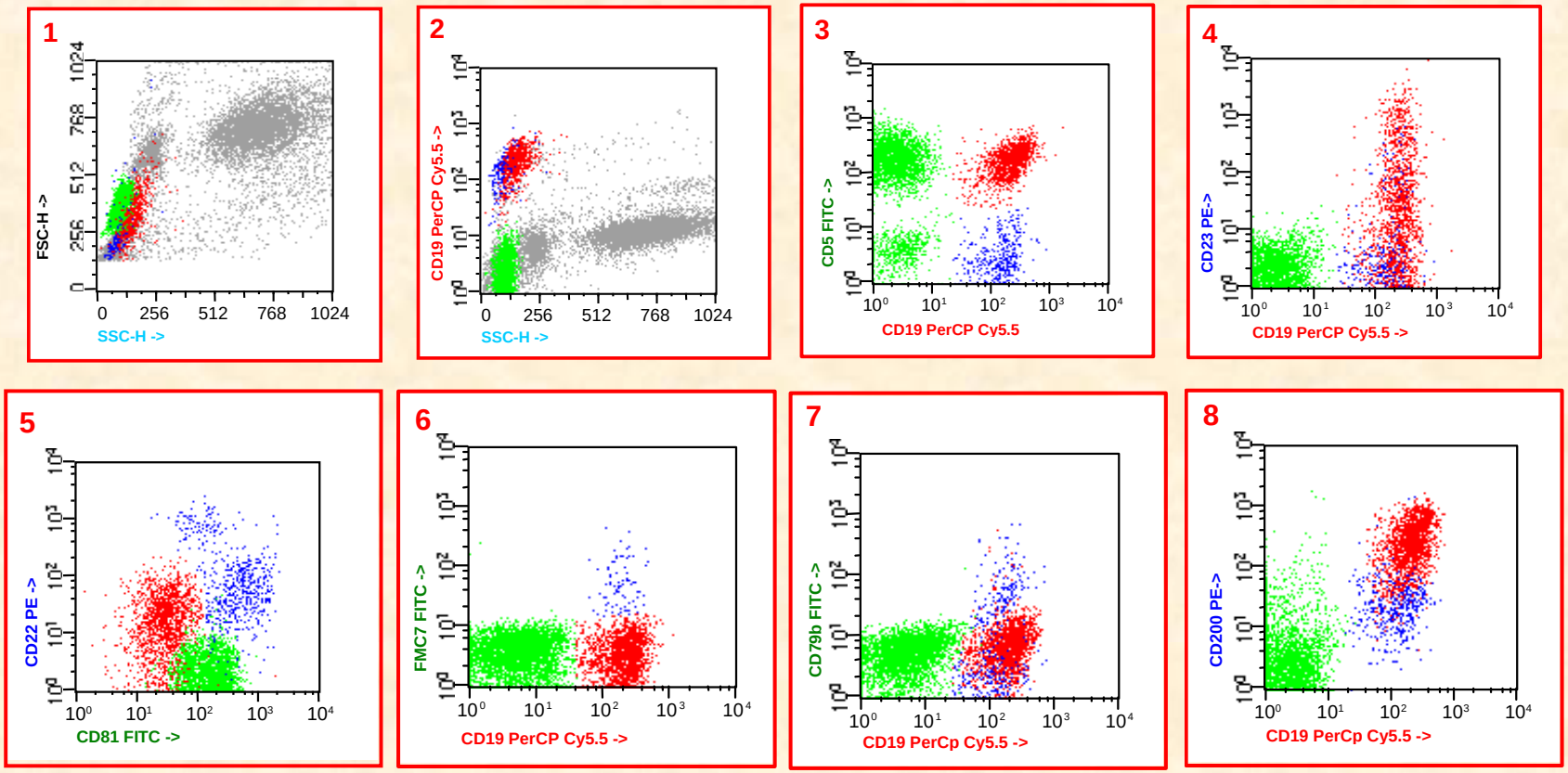
- Male age 38 Diagnosis: MCL stage IV
- IHC: CD20+, CD5+, CD43+, Bcl2+, CD23-, CD10-, Cyclin D1-
- PCR and FISH: BCL1 not rearranged
- CR by high dose chemotherapy and radiotherapy
- 4 years later: rare CD19+ CD5+ $CD23^{dim}$ B cells in BMA by flow cytometry
- 2 years later: relapse and complete re-stadiation

B-NHL re-classification

(1) MCL → B-CLL

BMA

6% pos cells



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B-NHL re-classification
(2) B-CLL ⟶ FL

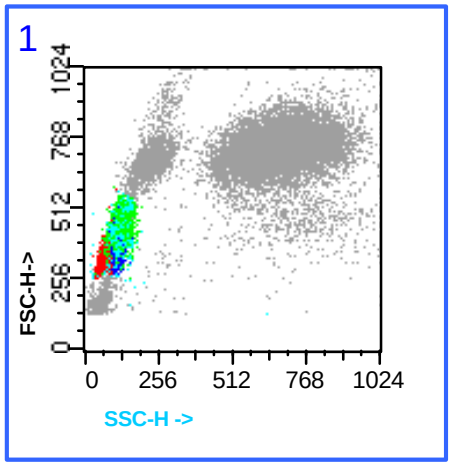
- Male Age 32
- Clinics: abdominal lymph adenopathies, spleen enlargement, focal liver lesions
- Diagnosis: MCL
- Second opinion (I.N.T.): histologic diagnosis of SLL/B-CLL
- CR after CHOP therapy for several years
- Six years after diagnosis, relapse and progression with deep and superficial lymph node enlargement and BM involvement. IHC on BM: CD20+ CD5- CD23- Cyclin D1-

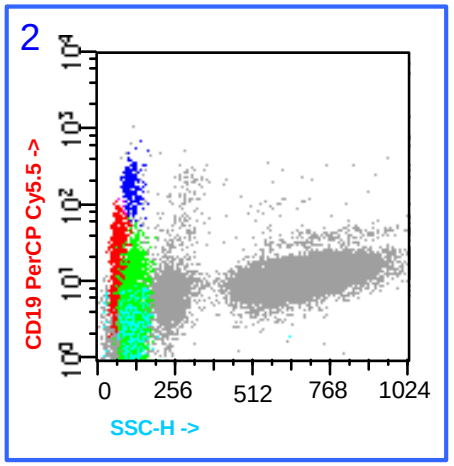
B-NHL re-classification

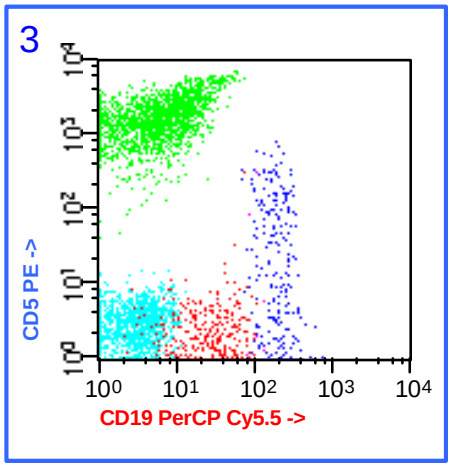
(2) B-CLL → FL

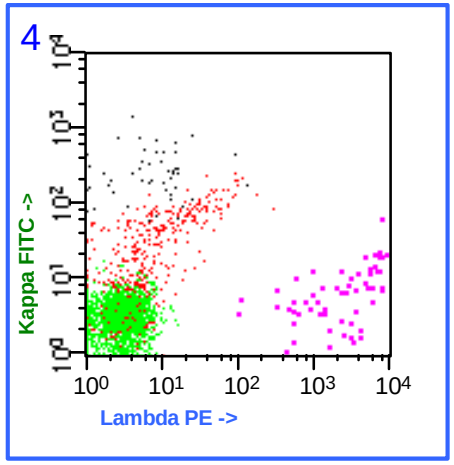
SLL/CLL: BMA

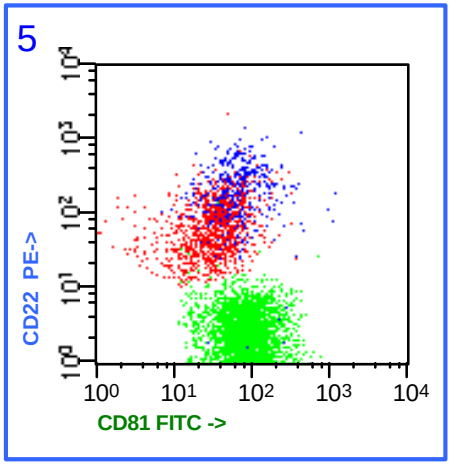
3.5% pos cells

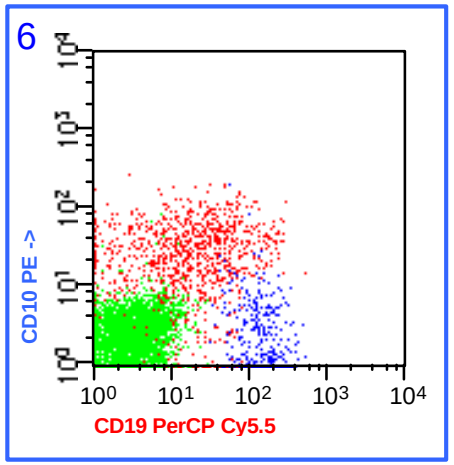


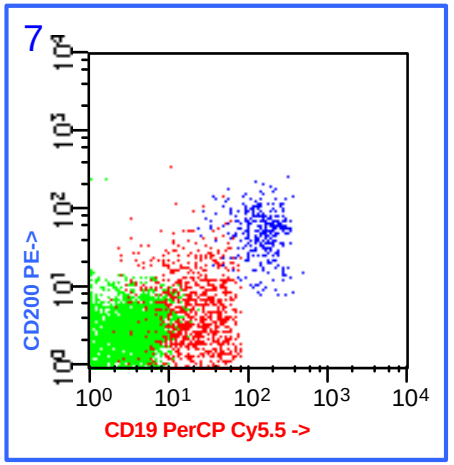


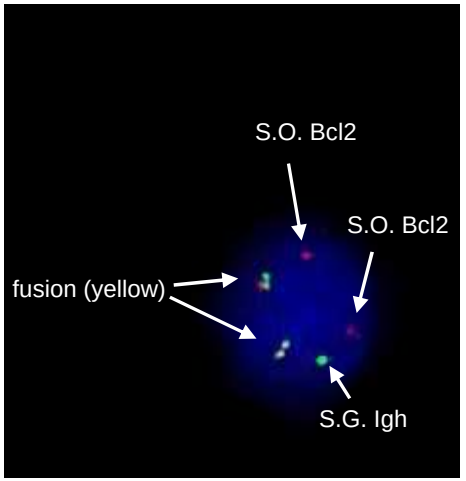












FONDAZIONE IRCCS ISTITUTO NAZIONALE TUMORI MILANO

Conclusions

Flow cytometry contribution to the diagnosis of hematological neoplasia

Multicolor Immunophenotyping

- *LAIP determination (MRD)*
- *Differential diagnosis (and MRD)*
- *Quantitative analysis of antigens for target therapies (CD20, CD22, CD200)*
- *New diagnostic markers (CD200, CD81)*
- *New prognostic markers (CD49d)*