Reproducible diagnosis of CLL by flow cytometry: an ERIC & ESCCA harmonisation project

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On behalf of the European Research Initiated on CLL (ERIC) and the European Society for Clinical Cell Analysis (ESCCA)

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Current criteria: flexibility in marker expression

- **WHO criteria:**
- CLL cells usually co-express CD5 and CD23
- Using flow cytometry, the tumour cells express dim surface IgM/IgD, CD20, CD22, CD5, CD19, CD79a, CD23, CD43 and CD11c (weak). CD10 is negative and FMC& and CD79b are usually negative or weakly expressed in typical CLL.
- **Some cases may have an atypical immunophenotype (e.g. CD5- or CD23-, FMC7+ or CD11c+, strong sIg, or CD79b+).**

- **IWCLL guidelines:**
- CLL cells co-express the T-cell antigen CD5 and B-cell surface antigens CD19, CD20, and CD23.
- The levels of surface Ig, CD20, & CD79b are characteristically low.
- Each clone is restricted to expression of either kappa or lambda.
- **Variations of the intensity of expression of these markers may exist and do not prevent inclusion of a patient in clinical trials for CLL.**

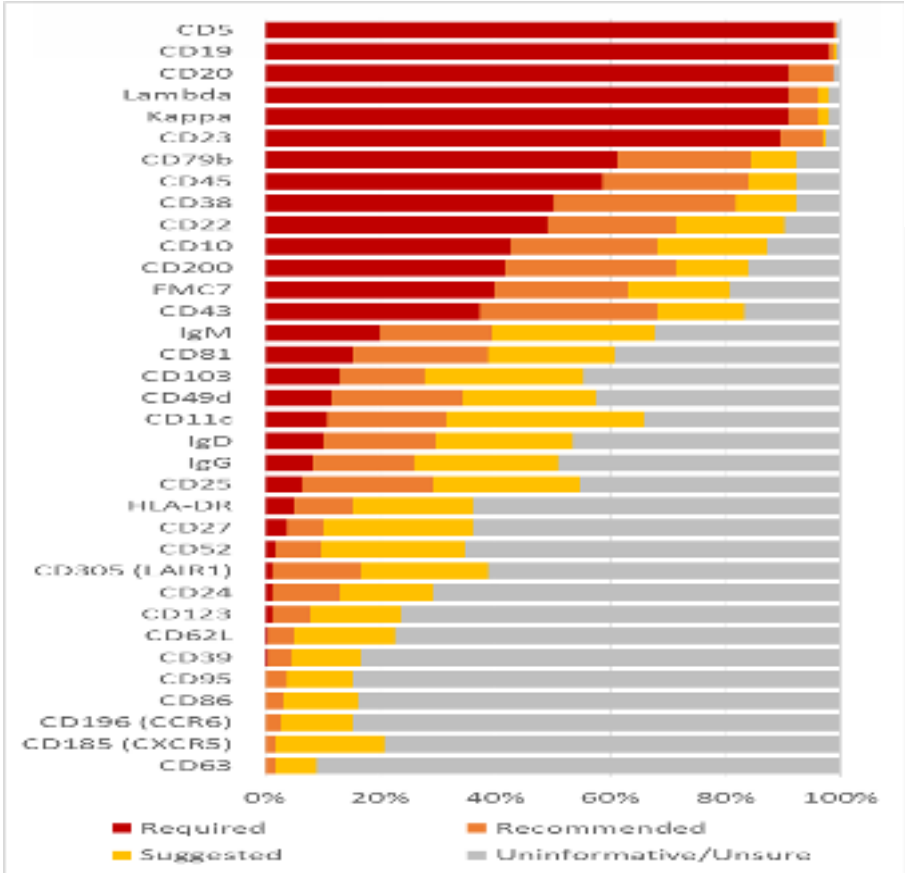
Trial cases referred to a central lab: ~2-5% not CLL & ~2-5% sub-optimal for MRD monitoring but this may vary according to trial treatment options

- ADMIRE/ARCTIC trial: FCR-based treatment (n=421)
  - 97% typical phenotype (2% with no CD200 or CD43 expression)
  - 3% $CD23^{neg}$, usually with additional aberrant markers but no t(11;14).
- IcICLLe trial: 40 patients, focussed on biological response to Ibrutinib to guide subsequent trials
  - 3 of first 20 patients not CLL: CD5+CD23-, rest of phenotype not consistent with CLL, not suitable for disease monitoring
  - Issued guidance that patients must have a fully typical phenotype before screening for trial.
- FLAIR trial: FCR vs. IR until MRD<0.01%, 754 patients. Of the first 103 patients
  - 93/103 typical phenotype suitable for disease monitoring
  - 5/103 CD5+CD23+ but not suitable for disease monitoring
  - 5/103 CD5±CD23- and excluded from the trial: 2 Mantle cell, 3 phenotype otherwise consistent with MZL/WM, no translocation or mutation identified, no tissue biopsy

Reproducible diagnosis of CLL: AIMS

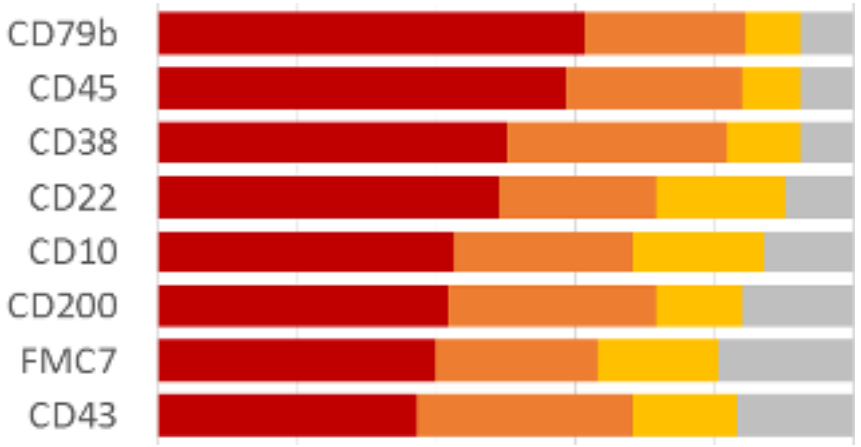
- Identify consensus minimum (required) and recommended markers for diagnosis in CLL
- Assess impact of using a reproducible strategy vs. a scoring system
- Develop an approach to ensure appropriate quality for the required markers
- Identify potential value of additional (recommended) markers in diagnosis
- Assess consensus prospectively

Ranking of 35 markers for CLL diagnosis by 154 ERIC/ESCCA members



>75% of respondents ➔ marker is **required** for CLL diagnosis:

>50% of respondents ➔ marker is **recommended** for CLL diagnosis:



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Proposal: required (minimum) and recommended panel for diagnosis

- Marker panel required for diagnosis:
  - **CD19 / CD5 / CD23 / CD20 / Kappa / Lambda**
- Additional markers recommended for diagnosis
  - **CD43 / CD79b / CD81* / CD22 / CD10 / CD200* / [ROR1]***
- Frequently recommended but not essential for diagnosis and monitoring:
  - **CD45 / CD38 / FMC7**
- Present in current diagnostic criteria but infrequently recommended:
  - **IgM/D and CD11c:**

Immunophenotypic scoring systems

Flow cytometric analysis of peripheral blood or bone marrow is performed for expression of the cell surface markers listed in the table below. The scores for each marker are summed.

A score ≥ 4 is indicative of CLL. A score of ≤ 3 should prompt consideration of an alternative diagnosis.

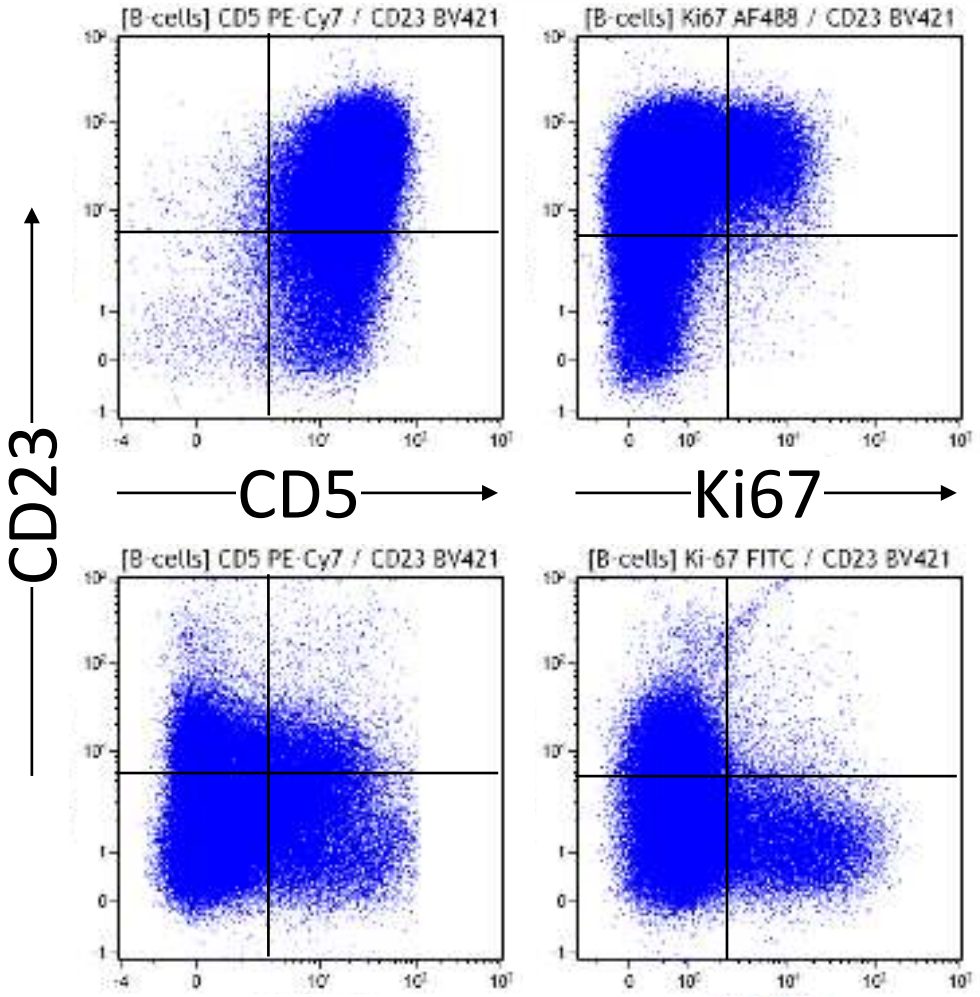
Cell surface marker	0 points	1 point
CD79b (or CD22)	Strong	Weak
CD23	Negative	Positive
CD5	Negative	Positive
FMC7	Positive	Negative
SmIg	Strong	Weak

Adapted from Matutes et al, 1994[1] and Moreau et al, 1997.[2]

Issues for UK trials with new agents:

ADMIRE/ARCTIC (FCR-based) trials	97% typical CLL, 3% $CD23^{neg}$ and sub-optimal for MRD monitoring
IcICLLe (Ibrutinib monotherapy)	3 of first 20 screened were excluded: $CD23^{neg}$ and highly atypical
FLAIR (FCR vs. IR until MRD<0.01%)	90% typical CLL, 5% sub-optimal for MRD monitoring, 5% not CLL

CD23 expression is closely associated with the proliferation fraction in “typical” CLL



Typical CLL phenotype:
CD19+ CD5+CD23+ with weak CD20 & monoclonal sIg

Ki67+ fraction is always restricted to the cells with the strongest CD23 expression

Cases often classified as CLL:
CD19+ with weak CD5 &/or CD23 & strong CD20 / monoclonal sIg

Ki67+ fraction is not necessarily associated with CD23 expression

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Proposed minimum criteria for diagnosis

Antigen	Typical Expression (% pos vs. control)	Control Population in normal peripheral blood		Minimum Relative fluorescence intensity (preferred)
		Positive	Negative	
CD19	Positive (>95%)	B-cells	T-cells	>10 (>20)
CD5	Positive (>20%)*	T-cells	NK-cells	>14 (>18) †
CD23	Positive (>20%)*	CD23+ B-cells	CD19- Lymphocytes	>3 (>10)
CD20	Weak	CD19+ B-cells	CD3+ T-cells	>13 (>20) †
Igκ	Weak & restricted to either Igκ or Igλ	Igκ+Igλ- B-cells	Igκ-Igλ+ B-cells	>5
Igλ		Igκ-Igλ+ B-cells	Igκ+Igλ- B-cells	>5

Definition of weak: median fluorescence intensity at least 20%* lower than normal peripheral blood B-cells, range to be determined within each laboratory

* ICSH/ISLH/CLIA guidelines for stability require <20% variation, therefore reduction in fluorescence intensity less than 20% may reflect antigen/sample stability

† specifically validated (ERIC CLL MRD project) otherwise consensus

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Retrospective assessment of the minimum criteria

	Total CD5+ B-LPD diagnoses	Meet the proposed criteria and diagnosed with CLL	Not typical for CLL	
			Other diagnosis, e.g. Mantle Cell Lymphoma	Requires MDT or trial-specific decision
Primary referral	7286	5553 (76.2%)	989 (13.6%)	744 (10.2%)
Trial	2427	2267 (93.4%)	54 (2.2%)	106 (4.4%)

Retrospective assessment of the minimum criteria

Similar to current practice in Leeds –

- ~30% of cases require treatment & are diagnosed (usually CLL) after multi-disciplinary review
- ~70% of cases do not get a final diagnosis
  - low level disease not requiring treatment
  - co-morbidities
  - death

			t typical for CLL	
			e.g. ell ma	Requires MDT or trial-specific decision
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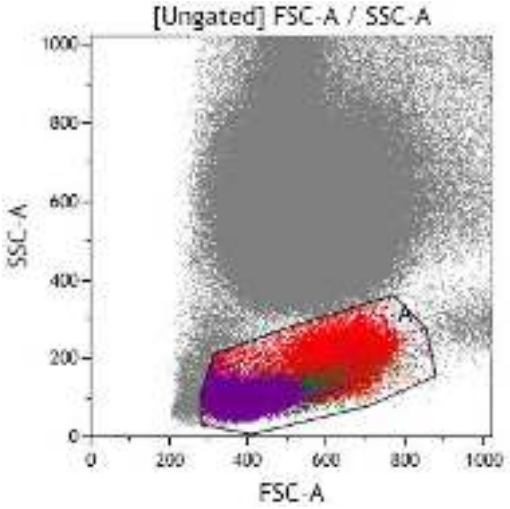
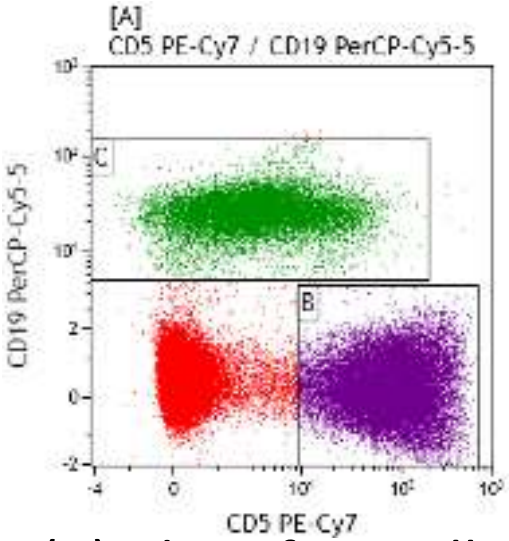
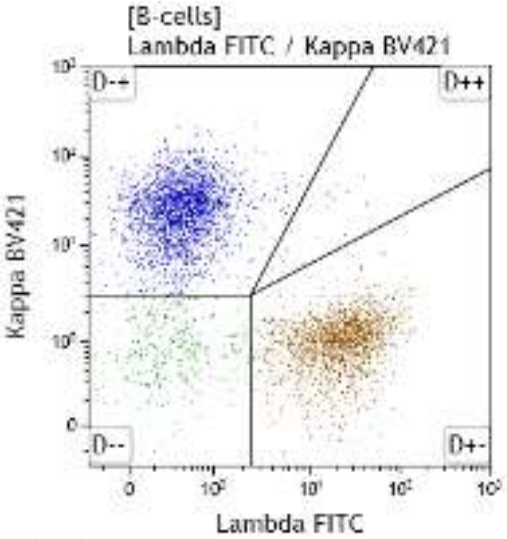


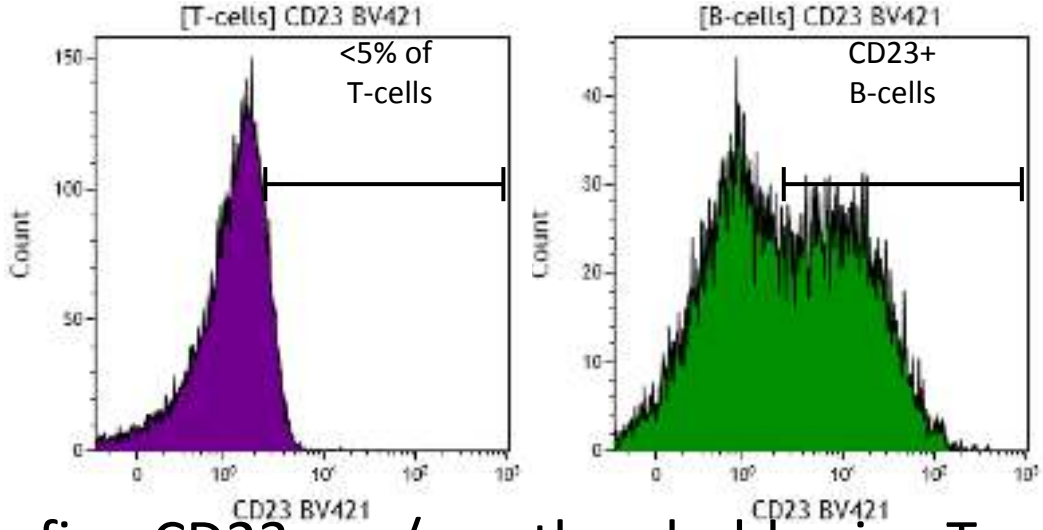
Mononuclear cell (MNC) gate



(2) Identify T-cell and B-cell gates



(3) Identify κ+λ- & κ-λ+ B-cell gates



Define CD23 pos/neg threshold using T-cell expression and identify CD23+ B-cells

Quality assessment on diagnostic panel

Antigen	CD19	CD20	CD5	Kappa	Lambda	CD23
Proposed relative signal	>10	>10	>14	>5	>5	>3
Centre 1	5462 (4291-6393)	64.8 (36.6-103)	41.1 (17.7-57.2)	17.1 (4.9-37.6)	2.9 (2.1-4.9)	4 (3.1-6.8)
Centre 2	17.9 (5.6-23.5)	175 (102-306)	237 (52.8-368)	35.6 (12.6-60)	430 (148-612)	49 (2.5-223)
Centre 3	106 (89.9-175)	53.6 (41.2-67.4)	26.2 (17.9-39)	22.1 (6.9-45.1)	149 (72.2-287)	16.9 (8.6-35)
Centre 4	55.2 (50-62.7)	32.8 (25.9-56.5)	54 (44.4-75.3)	7 (3-23.2)	10.3 (4.2-37.4)	4 (2.4-5.4)
Centre 5	12126 (85.1-14264)	5.4 (2.5-7.1)	44.2 (2.8-102)	20.2 (7.1-55.5)	35.8 (8.4-116)	43.2 (0.8-1670)
Centre 6	16.5 (11.2-18.8)	24.6 (16.7-30.2)	42.9 (15-56.7)	22.6 (10.3-65.1)	17.5 (10.3-24.2)	18.7 (8.6-31.7)
Centre 7	12 (3.9-41.4)	29 (16.3-41.4)	5.4 (0.2-8.5)	11.7 (4.7-29.4)	7 (2.9-33.2)	2.6 (1.2-10.8)

Control cases meeting target signal:noise

100% 70-90% <50%

Retrospective assessment of the minimum criteria

	Total CD5+ B-LPD diagnoses	Meet the proposed criteria and diagnosed with CLL	[illegible]	
Primary referral	7286	5553 (76.2%)	989 (13.6%)	744 (10.2%)
Trial	2427	2267 (93.4%)	54 (2.2%)	106 (4.4%)

? Accuracy of diagnosis

? Frequency of alternative abnormalities, e.g. MYD88

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Retrospective assessment of the minimum criteria

? possible to improve or refine the diagnosis

	Total CD5+ B-LPD diagnoses	Meet the proposed criteria and diagnosed with CLL	Not typical for CLL	
			Other diagnosis, e.g. Mantle Cell Lymphoma	Requires MDT or trial-specific decision
Primary referral	7286	5553 (76.2%)	989 (13.6%)	744 (10.2%)
Trial	2427	2267 (93.4%)	54 (2.2%)	106 (4.4%)

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CD20	Weak	CD19+ B-cells	CD3+ T-cells	>5 (>20)
Igκ Igλ	Weak & restricted to either Igκ or Igλ	Igκ+ or Igλ+ B-cells	Igλ- or Igκ+ B-cells	>5
CD43	Positive (>20%)	CD3+ T-cells	CD20+ B-cells	>7 (>50) †
CD79b	Weak	CD20+ B-cells	CD3+ T-cells	>11 (>30) †
CD81	Weak	CD3+ T-cells	Granulocytes	>5 (>8) †
CD22	Weak	CD20+ B-cells	CD3+ T-cells	>5 (>20)
CD10	Negative (<20%)	Granulocytes	Memory B-cells	>3 (>10)
CD200	Positive (>20%)	CD20+ B-cells	CD3+ T-cells	>3 (>10)
ROR1	Positive (>20%)	B-progenitors	CD20+ B-cells	>3 (>10)

Antigen	Typical Expression (% pos vs. control) ‡	Control Population in normal peripheral blood		Minimum Relative fluorescence intensity (preferred)
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Igκ Igλ	Weak & restricted to either Igκ or Igλ	Igκ+ or Igλ+ B-cells	Igλ- or Igκ+ B-cells	>5
CD43	Positive (>20%			>7 (>50) †
CD79b	Weak			>11 (>30) †
CD81	Weak	CD3+ T-cells	Granulocytes	>5 (>8) †
CD22	Weak	CD20+ B-cells	CD3+ T-cells	>5 (>20)
CD10	Negative (<20%)	Granulocytes	Memory B-cells	>3 (>10)
CD200	Positive (>20%)	CD20+ B-cells	CD3+ T-cells	>3 (>10)
ROR1	Positive (>20%)	B-progenitors	CD20+ B-cells	>3 (>10)

Required for MRD detection – should be assessed in clinical trials

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CD20	Weak	CD19+ B-cells	CD3+ T-cells	>5 (>20)
Igκ Igλ	Weak & restricted to either Igκ or Igλ	Igκ+ or Igλ+ B-cells	Igλ- or Igκ+ B-cells	>5
CD43	Positive (>2			>7 (>50) †
CD79b	Weak	CD20+ B-cells	CD3+ T-cells	>11 (>30) †
CD81	Weak	CD3+ T-cells	Granulocytes	>5 (>8) †
CD22	Weak	CD20+ B-cells	CD3+ T-cells	>5 (>20)
CD10	Negative (<20%)	Granulocytes	Memory B-cells	>3 (>10)
CD200	Positive (>20%)	CD20+ B-cells	CD3+ T-cells	>3 (>10)
ROR1	Positive (>20%)	B-progenitors	CD20+ B-cells	>3 (>10)

Potential to refine the diagnosis

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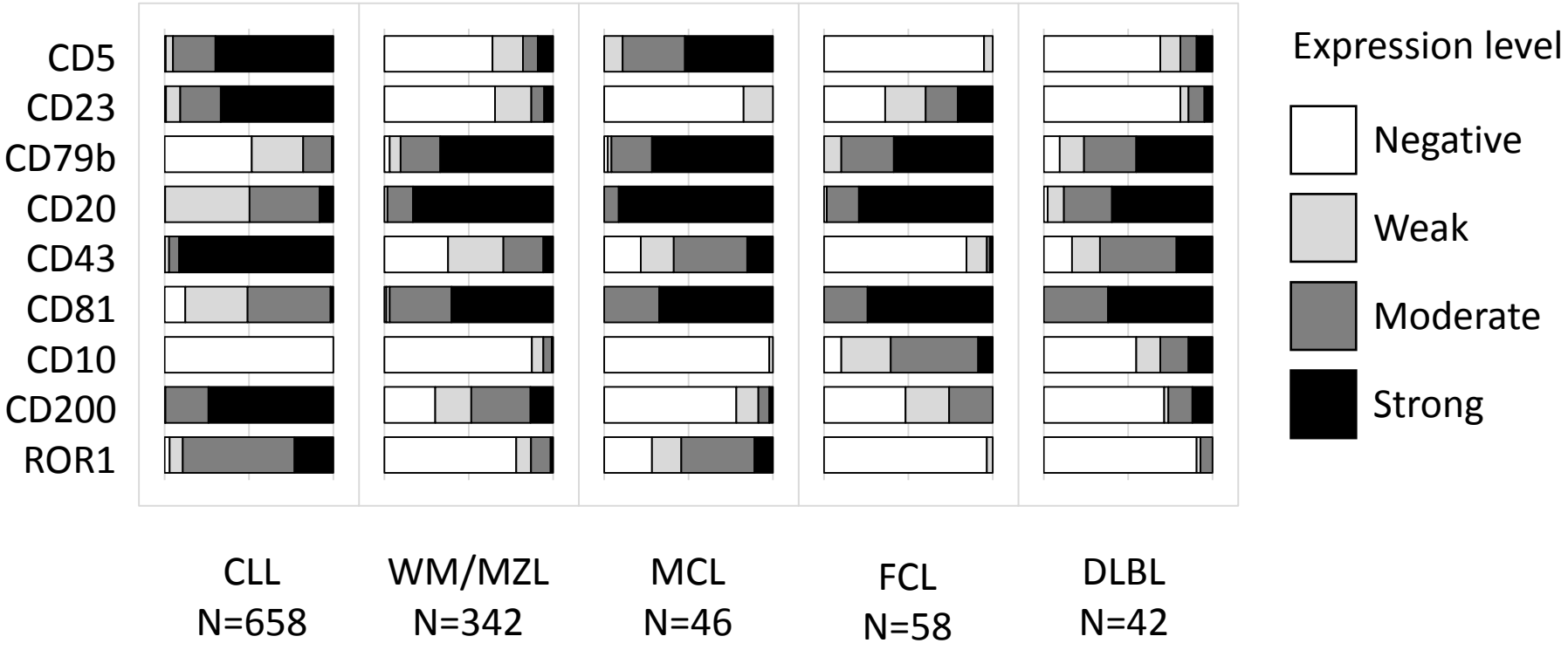
Evaluation of diagnostic markers in 3,000 cases

- Sequential cases from 01-July-2014 to 11-Dec-2015: n = 3082 (1041 diagnosis, 2042 follow-up pre-treatment or relapse)
- Split into learning and validation sets based on analysis date, then duplicate analyses excluded (selection for cases with definitive diagnosis > FISH/molecular data > trephine or tissue biopsy)

	BLS	XB1	XB2	XB3	XB4
BV421	κ	CD23	CD95	CD25	CCR6
BV510	CD45	CD43	CD31	CD11c	CXCR5
FITC	λ	CD81	CD49d	CD103	IgG
PE	CD305	CD79b	CD305	CD200	IgD
PerC5.5	CD19	CD19	CD19	CD19	CD19
PC7	CD5	CD5	CD38	CD39	CD27
APC	CD10	ROR1	CD10	CD22	IgM
APC-H7	CD20	CD20	CD20	CD20	CD20

Differential expression patterns of ROR1 & other markers commonly used in the diagnosis of CLL



Baskar et al, Clin Cancer Res 2008; 14(2) p396
Daneshmanesh et al, Int J Cancer 2008; 123(5) p123
Uhrmacher et al, Leuk Res 2011, 35(10) p1360

Markers ranked according to specificity for discrimination CLL vs. MCL and CLL vs. WM/MZL

Specificity for diagnosis of CLL vs. Mantle Cell Lymphoma	
CD20 weak	**91.3%**
CD23 pos	**82.6%**
CD200 pos	**78.3%**
sIg weak	71.7%
CD81 weak	67.4%
CD43 pos	**41.3%**
ROR1 pos	**28.3%**
CD5 pos	NA

Specificity for diagnosis of CLL vs. WM/MZL	
CD20 weak	**83.0%**
ROR1 pos	**78.1%**
CD43 pos	**70.5%**
CD79b weak	67.0%
CD23 pos	**65.5%**
CD5 pos	64.0%
CD81 weak	60.2%
CD200 pos	**30.1%**

Specificity = TN/TN+FP where TN is the absence of the CLL-associated profile in another disorder (e.g. CD5-negative WM/MZL) and FP is the presence of the CLL-associated profile in another diagnosis (e.g. CD23-positive MCL).
CLL n = 658, WM/MZL n = 342, MCL n = 46.

Using the minimum criteria to identify CLL then refining diagnosis according to other markers: validation set

	Total n (%)	CCND1/IGH fusion *	MYD88 L256P mutation *	CLL Score 4-5
CLL: CD5+CD23+ weak sIg & CD20	774	1.9% (1/54)	0.0% (0/27)	100
CLL CD5+CD23+ mod/strong sIg or CD20 ROR1+CD43+CD200+	68	0.0% (0/14)	22.2% (4/18)	7.3%
CD5+CD23+ other	138	13.2% (9/68)	34.0% (33/97)	1.5%
CD5+CD23-	181	39.1% (41/105)	26.9% (40/149)	6.1%
CD5-	308	0.0% (0/2)	50.0% (91/182)	1.6%

* Represents upper limit as testing selection based on aberrant phenotype with respect to other markers also, e.g. weak CD19 or CXCR5, bi-modal LAIR1, expression of CD95.

Using the minimum criteria to identify CLL then refining diagnosis according to other markers

	Total n (%)	CCND1/IGH fusion *	MYD88 L256P mutation *	CLL Score 4-5
CLL: CD5+CD23+ weak sIg & CD20	1524 (51.3%)	<1.3% (1/77)	<5.1% (7/138) 5.4% (6/138)§	100%
CLL CD5+CD23+ mod/strong sIg or CD20 ROR1+CD43+CD200+	133 (4.5%)	0.0% (0/26)	<15.8% (6/38) 2.6% (1/38)§	6.0%
CD5+CD23+ other	240 (8.1%)	16.4% (19/116)	29.3% (53/181) 0.6% (1/181)§	2.1%
CD5+CD23-	366 (12.3%)	40.2% (86/214)	24.0% (74/309)	8.2%
CD5-	706 (23.8%)	0.0% (0/4)	50.0% (227/454)	3.3%

* Represents upper limit as testing selection based on aberrant phenotype with respect to other markers also, e.g. weak CD19 or CXCR5, bi-modal LAIR1, expression of CD95.

§ Cases with an additional CD5neg MBL population

Using the full consensus required and recommended panel

	Total n (%)	CCND1/IGH fusion *	MYD88 L256P mutation *	CLL Score 4-5
CLL: CD5+CD23+ ROR1+CD43+CD200+ weak sIg & CD20 & CD81	1460 (49.2%)	0.0% (0/64)	<1.8% (2/111) 5.4% (6/111)§	100%
CLL: CD5+CD23+ ROR1+CD43+CD200+ mod sIg or CD20 or CD81	149 (5%)	0.0% (0/26)	<15.0% (6/40) 2.5% (1/40)§	16.1%
CD5+CD23+ other	288 (9.7%)	15.5% (20/129)	28.2% (58/206) 0.5% (1/206)§	18.4%
CD5+CD23-	366 (12.3%)	40.2% (86/214)	24.0% (74/309)	8.2%
CD5-	706 (23.8%)	0.0% (0/4)	50.0% (227/454)	3.3%

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Using a scoring system

CLL Score	Total n (%)	CCND1/IGH fusion *	MYD88 L256P mutation *	CLL by consensus panel
5	1524 (51.3%)	1.3% (1/77)	<5.1% (7/138) 5.4% (6/138)§	96.9%
4	66 (2.2%)	23.5% (4/17)	35.9% (14/39)	12.1%
3	261 (8.8%)	8.2% (5/61)	28.0% (35/125) 1.6% (2/125)§	36.4%
2	362 (12.2%)	23.3% (31/133)	29.9% (79/264)	8.3%
1	505 (17%)	44.2% (65/147)	34.8% (130/374)	0
0	251 (8.5%)	0.0% (0/2)	56.7% (102/180)	0

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Reproducible diagnosis of CLL

- Identify consensus minimum (required) and recommended markers for diagnosis in CLL
  - Required: CD19, CD5, CD23, CD20, Kappa, Lambda
  - Additional recommended markers: CD43, CD79b, CD81*, CD22, CD10, CD200*, ROR1*
- Assess impact of using a reproducible strategy vs. a scoring system – retrospective → prospective analysis
- Develop an approach to ensure appropriate quality for the required markers – complete
- Identify potential value of additional (recommended) markers in diagnosis – validate prospectively

Prospective project

- Obtain antibodies from companies and test to ensure that they meet the minimum specification
  - CD19, CD5, CD20, CD23, κ, λ — drop in CD22, CD10
  - CD19, CD5, CD20, CD43, CD79b, CD81 — drop in ROR1, CD200
- Identify centres that can assess this panel in >100 cases with
  - paired trephine or node biopsy
  - CCND1/IGH fusion FISH
    - Very few CCND2 translocations, limited success with IHC for CyclinD2/D3 and CyclinD1/2/3 RNA expression
  - Mutation analysis: MYD88, CXCR4, KLF2, NOTCH2 (in addition to CLL prognostic markers)

Acknowledgements

ERIC
european research initiative on CLL

Emili Montserrat
Paolo Ghia
David Oscier
Michael Hallek
Peter Hillmen

NCRI
National Cancer Research Institute

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Piers Patten
Chris Pepper
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Walter Gregory

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Laura Collett
David Stones
Sadia Aslam
James Baglin
Julia Brown

LEUKAEMIA & LYMPHOMA RESEARCH
Beating Blood Cancers

TAP
From Leukaemia & Lymphoma Research

The Leeds Teaching Hospitals NHS
NHS Trust

ESCCA
European Society for Clinical Cell Analysis

ERIC
european research initiative on CLL

Flow cytometry for the diagnosis of CLL: proposed categories for ERIC prospective evaluation (2)

- **10% of CD5+ B-LPD (128/1142) have a borderline phenotype: CD5+CD23+ but either CD43- &/or CD200- &/or ROR1-.**
- **flow report → phenotype is not typical for CLL**
  - ~20% (10/51) have CyclinD1 translocation
  - 40% (31/79) have MYD88 mutation (+ other MZL/WM features) **? exclude a CLL Dx**
  - not classifiable in 25/51 PB only, 17/25 (68%) have B-cell count <10 x 10^9/L
  - 17/78 BMA only, 12/17 (71%) have <30% infiltration
  - **Currently ~15% have final diagnosis of CLL ? → CLL variant Dx permitted ?**

- **15% of CD5+ B-LPD (181/1142) have no CD23 expression:**
- **flow report → phenotype is not consistent with CLL**
  - ~40% (41/105) have CyclinD1 translocation
  - ~30% (40/149) haveMYD88 mutation(+ other MZL/WM features) **? exclude CLL Dx**
  - not classifiable in 32/72 PB only, 24/32 (75%) have B-cell count <10 x 10^9/L
  - 27/93 BM only, 17/27 (63%) have <30% infiltrate.
  - **Currently ~5% have final diagnosis of CLL ? → CLL variant Dx permitted ?**

ERIC/ESCCA project to identify the minimum and recommended markers for analysis

- Reproducible criteria using marker specification that can be readily validated using normal peripheral blood and accessible across a range of resource setting
- Robust identification of new relevant markers and a foundation for assessing which molecular pathways are central to the pathogenesis of CLL
- Marker panel required for diagnosis:
  - **CD19 / CD5 / CD23 / CD20 / Kappa / Lambda**
- Additional markers recommended for diagnosis and required for clinical trials:
  - **CD43 / CD79b / CD81 / CD22 / CD10 / CD200 / ROR1**

Leeds validation set: 1450 B-LPD of which 1142/1450 (78%) have weak or moderate CD5 expression

758/1142 (66.4%) CLL-phenotype : CD5+ CD23+ sIg^{wk}CD20^{wk}

740/758 express both CD200 and ROR1

727/740 (98%) optimal for disease monitoring (CD43^{+}CD81^{wk})

2/21 MYD88 mutation, both also have CD5neg MBL.

0/36 CCND1/IGH fusion

2/740 Express ROR1 but no CD200

1 Mantle Cell, 1 DLBL with t(8/14)

→ CD200 expression should be required for CLL diagnosis

16/740 CD19+CD5^{+} CD23^{+} sIg^{wk}CD20^{wk} ROR1^{-}CD200^{+}

0/4 MYD88 mutation – needs further testing

93/1142 (8.1%) CD5+CD23+ strong CD20/sIg CD43+CD200+ROR1+

6/36 (17%) MYD88 mutation

0/29 (0%) CCND1/IGH

Validation set: 1450 B-LPD of which 1142/1450 (78%) have weak or moderate CD5 expression

110/1142 (9.6%) CD5+CD23+ no CD43 or no CD200 or no ROR1 expression

14/110 (14%) MDT diagnosis of CLL

31/79 (39%) MYD88 mutation (c.f. 52% of CD5neg B-LPD)

10/51 (20%) CCND1/IGH

181/1142 (15.8%) CD5+CD23-

6/181 (3.3%) MDT diagnosis of CLL (transport artefact)

40/149 (27%) MYD88 mutation (c.f. 52% of CD5neg B-LPD)

41/105 (39%) CCND1/IGH

101/1142 (8.9%) unclassifiable with no diagnostic molecular abnormality:

61 PB with no further samples, of which 39/61 had <10 x 10^9/L B-cells

18 BMA only with no further samples, of which 12/18 have <50% BM infiltrate

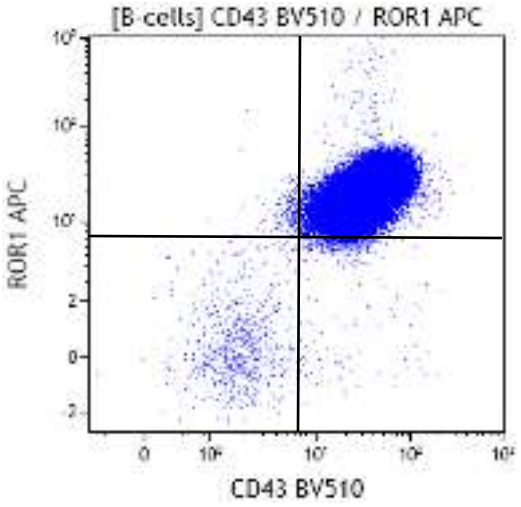
21 TBP/LU of which 14/21 have <50% BM infiltration

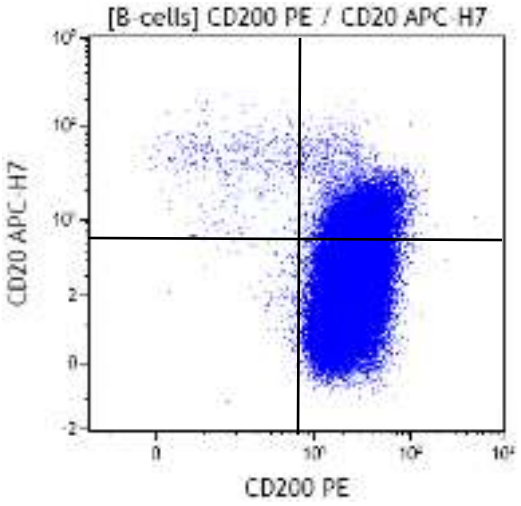
1 Tissue biopsy, proliferation centres but no CD23 confirmed in PB & BMA.

Flow cytometry for the diagnosis of CLL: proposed categories for ERIC prospective evaluation (1)

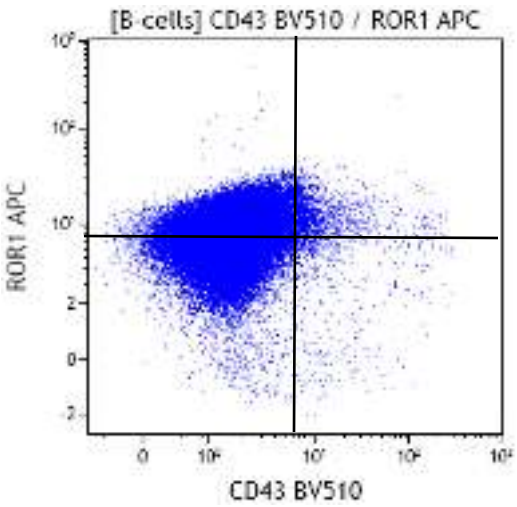
- **75% of CD5+ cases (833/1142) can be diagnosed as CLL based on flow cytometry**
- **CD19+ CD5+ CD23+ CD43+CD200+ROR1+ monoclonal B-cells**
  - **740 (89%) "typical": sIg^{wk}CD20^{wk}**
    - of which 730 optimal for disease monitoring (i.e. also CD81wk)
    - No MYD88 mutation unless CD5neg MBL also present.
  - **93 (11%) "variant": moderate to strong sIg or CD20**
    - ? may have MYD88 mutation (10-20%)
  - ? need to test for CyclinD1 translocation ?
  - Not detected so far, validate on 1,000 cases
  - **>95% of cases submitted for clinical trials meet the criteria**

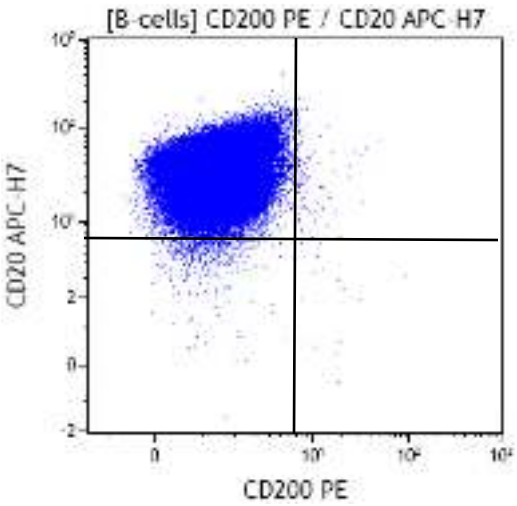
CLL profile:
ROR1+ CD43++
CD20wk CD200+





MCL profile:
ROR1± CD43±
CD20++ CD200-





WM/LPL/MZL profile:
ROR1- CD43-
CD20++ CD200±