CAT
Critically Appraised Topic

Title:

Flow cytometric analysis of ZAP-70

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CLINICAL BOTTOM LINE

ZAP-70 expression is associated with a less favorable prognosis in CLL. Expression of ZAP-70 can be assessed by a variety of methods, including immunoblotting, quantitative reverse transcriptase polymerase chain reaction, immunohistochemical analysis, and flow cytometry. Although flow cytometry is generally considered the method of choice for ZAP-70 evaluation, several methodological and technical aspects hamper reliable determination of ZAP-70 status.

The staining intensity of ZAP-70 using commercially available fluorochrome conjugated antibodies is relatively weak, making it difficult to distinguish positive and negative cells. In addition, ZAP-70 is an intracellular target and detection requires cell permeabilization techniques that can lead to decreased staining intensity. Moreover, the permeabilization procedure and the antibody specificity can also be a source of nonspecific staining.

ZAP-70 expression in vitro appears to be labile over time and sensitive to different anticoagulants. EDTA anticoagulation and rapid delivery to the laboratory, preferably within 24 hours of specimen collection has been recommended.

Consensus has not yet been reached on the optimal method for determining which cells should be considered positive for ZAP-70, although a trend towards methods using the mean or median ZAP-70 fluorescence intensity of the CLL cells versus T and NK cells, and/or normal B cells can be expected.

Given the multiple variables involved in the flow cytometric analysis of ZAP-70 and since more robust and well defined alternative prognostic tests (i.e. IgVh mutation status, karyotyping and FISH analysis for detection of genetic abnormalities) are available at UZ Leuven, introduction of this test in the routine diagnostic work-up of CLL patients is questionable.

CLINICAL/DIAGNOSTIC SCENARIO

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in Western countries with an incidence of 4.2:100000 per year. CLL is extremely rare below the age of 30 years but the incidence increases to 30:100000 per year above the age of 75. The male/female incidence ratio is approximately 2:1.[1,2] In the WHO classification of hematopoietic malignancies CLL is always a disease of neoplastic B cells.[3] It is a chronic lymfoproliferative malignancy related to the accumulation in peripheral blood, bone marrow and lymphoid organs, of monoclonal B cells with the morphology of small lymphocytes with a narrow border of cytoplasm and a dense nucleus with partially aggregated chromatin and without recognizable nucleoli. Gumprecht nuclear shadows, or smudge cells, found as cell debris, are characteristic. Typically, CLL cells co-express the T-cell antigen CD5 and B-cell surface antigens CD19, CD20 and CD23. The levels of surface immunoglobulin, CD20 and CD79b are characteristically low compared with those found on normal B cells. Each clone of leukemic cells is restricted to expression of either κ or λ light chains. A scoring system for the diagnosis of CLL is shown in Table 1[4].

Despite these distinct characteristics, the clinical course of patients with CLL is heterogeneous. Some patients have aggressive disease requiring early therapy whereas others have indolent, asymptomatic disease and will not be in need of treatment for years.[5]

Table 1. Scoring system for the diagnosis of chronic lymphocytic leukemia. Scores in CLL are usually > 3.

marker	score point = 1	score point = 0
slg	weak	strong
CD5	positive	negative
CD23	positive	negative
FMC7	negative	positive
CD79b or CD22	weak	strong

The median survival of a patient with CLL from the time of diagnosis varies between 2 and more than 10 years. The staging classifications of Rai et al.[6] and Binet et al.[7,8], introduced over 30 years ago, are used to estimate the prognosis based on the extent of lymphadenopathy, splenomegaly, and hepatomegaly measured by palpations and anemia and thrombocytopenia measured by blood cell counts. Both systems reflect the overall tumor burden and allow assessing prognosis at the time of diagnosis (see table 2).

Table 2. Rai[6] and Binet[7,8] staging systems (adapted from Van Bockstaele et al.[62]).

	risk group	criteria	median survival (months)
Rai stage			
0	low	lymphocytosis[a]	> 150
I	intermediate	lymphocytosis + lymphadenopathy	101
II	intermediate	lymphocytosis	71
III	high	lymphocytosis + anemia[b]	19
IV	high	lymphocytosis + thrombocytopenia[c]	19
Binet stage			
A	low	< 3 nodal sites[d] involved	not reached
B	intermediate	≥ 3 nodal sites involved	84
C	high	anemia[e] and/or thrombocytopenia[c]	24

[a] > 5000/mm³
[b] Hb < 11 g/dl, with or without enlargement of lymph nodes, spleen or liver
[c] platelets < 100x10e9/L, with or without enlargement of lymph nodes, spleen or liver
[d] five possible nodal sites: axillary, cervical, inguinal, spleen and liver
[e] Hb < 10 g/dl, with or without enlargement of lymph nodes, spleen or liver

Because of their simplicity and reproducibility, these staging systems have been widely adopted, and their prognostic value has been validated in many studies. However, with 70-80% of patients nowadays being diagnosed as an incidental finding on a routine full blood count, it has been recognized that the Rai or Binet clinical staging systems alone are not sufficient to estimate individual prognosis. Moreover, a significant proportion of patients are managed by "watchful waiting" because meta-analysis has shown that patients at early stages of the disease do not benefit from treatment with chlorambucil.[9] However, in approximately half of such patients, the CLL will eventually progress and require treatment. For these patients, early treatment with newer agents such as fludarabine and rituximab, with the potential to induce complete remission, may be beneficial.[10]
A number of markers have been proposed as useful in predicting disease course in CLL, including serum LDH[11], serum β2-microglobulin[12], serum levels of sCD23[13], sCD27[14], sCD44[15] and circulating CD20[16] in plasma, lymphocyte count doubling time[17] and serum thymidine kinase[12]. With the development of fluorescence in situ hybridization (FISH), a more sensitive detection of chromosomal abnormalities became possible. Today, more than 80% of CLL patients show one or more cytogenetic defect. The most common are listed in table 3[18,21].

Table 3: Most common cytogenetic markers in CLL[18,21].

genetic abnormality	frequency (%)	median survival (months)	Clinical correlation
deletion 17p13	6-7	32-47	- atypical morphology - resistance to chemotherapy - advanced disease
deletion 11q22-23	18-19	79-117	- bulky lymphadenopathy - early relapse post autograft - progressive disease
trisomy 12	16-30	114-122	- atypical morphology - progressive disease
deletion 13q14	55-62	133-292	- prognosis > normal karyotype - stable disease

Approximately a decade ago, Damle et al.[19,] and Hamblin et al.[20] independently demonstrated and mutually corroborated that the mutational status of immunoglobulin heavy-chain variable region (IgVh) genes in CLL cells is strongly associated with clinical outcome (see figure 1). Approximately 50% to 70% of patients with CLL show evidence of somatic hypermutation in IgVh genes, indicating the cells giving rise to CLL have undergone germinal center maturation. Patients with CLL with unmutated IgVh genes (pre–germinal center origin) usually have an advanced stage of CLL and unfavourable cytogenetic features, require therapy and have significantly shorter survival than patients with mutated IgVh genes (post–germinal center origin).[19,20,21] However, sequencing of IgVh genes is difficult, time-consuming, costly and inaccessible to most clinical diagnostic laboratories. Therefore, a convenient surrogate marker for evaluation of IgVh status in individual patients in a clinical laboratory setting would be beneficial.

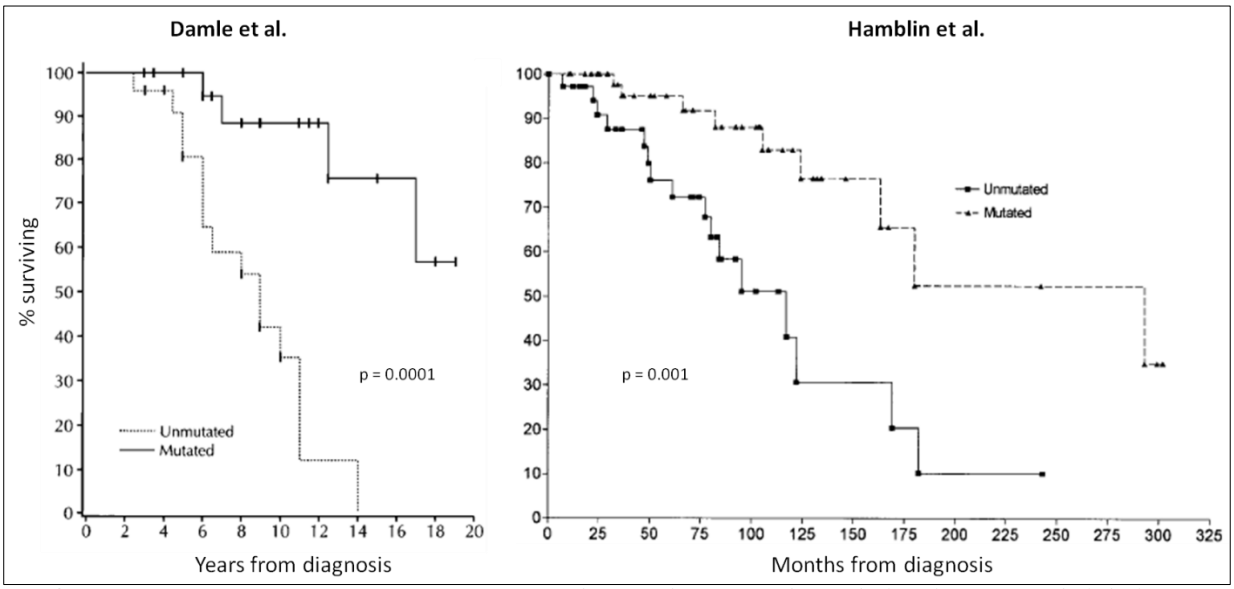

Figure 1: Kaplan-Meier plots comparing survival based on the absence (unmutated) or presence (mutated) of significant numbers (≥ 2%) of IgVh gene mutations. Damle et al.[19] found a median survival for the unmutated group of 9 years, whereas median survival of the mutated group was not reached. Hamblin et al.[20] described a median survival of 117 months (9.75 years) for the unmutated group versus a median survival of 293 months (24.4 years).

In this regard, CD38 expression on leukemic lymphocytes was the first marker that was found to correlate, although not absolutely, with IgVh mutations[19]. However, CD38 is an activation marker whose expression on CLL cells may vary over time.[22] In a pioneering gene expression profiling study, Rosenwald et al.[23] identified a panel of genes that could predict the IgVh mutational status of the CLL cells with high accuracy. One of the most differentially expressed genes was the gene encoding the ζ-associated protein of 70 kDa (ZAP-70), being more highly expressed in case of unmutated IgVh genes, whereas it was less expressed in case of somatically mutated IgVh genes, and thus supporting the possible role of ZAP-70 as a surrogate marker. ZAP-70 is an intracellular tyrosine kinase, normally expressed in T lymphocytes and natural killer (NK) cells and not in normal B cells. In addition to CLL, ZAP-70 expression has been reported in various B-cell malignancies as well as in human tonsillar cells.[24,25] CLL cells expressing ZAP-70 have been shown to be more responsive to cell receptor stimulation, in vitro, than CLL cells without ZAP-70 expression, indicating that ZAP-70 could enhance B cell receptor signalling for survival and proliferation and thereby contribute to disease progression.[26] DNA microarray studies also revealed that ZAP-70 mRNA expression in the leukemic clone across a patient population is a continuum ranging from absent to high. In some cases, ZAP-70 and IgVh mutation status yield discordant results.[27]

The expression of ZAP-70 can be assessed by different methods, such as Western blotting, quantitative RT-PCR, immunohistochemistry and flow cytometry[28,29,30].
In practice however, flow cytometry turned out to be the preferred technique since this is a fast and precise technique extensively used in routine diagnosis of hematological malignancies, it avoids the need to purify CLL cells and to run the appropriate controls to check that purity and it allows the simultaneous evaluation of ZAP-70 protein expression in CLL cells and in normal lymphocyte subsets. Crespo et al.[29] were the first to describe such a flow cytometric method, and they confirmed the value of ZAP-70 as a surrogate marker for the IgVh mutation status (see figure 2). In contrast with CD38, ZAP-70 levels seem to remain relatively stable over time irrespective of clinical treatment.[29,31]

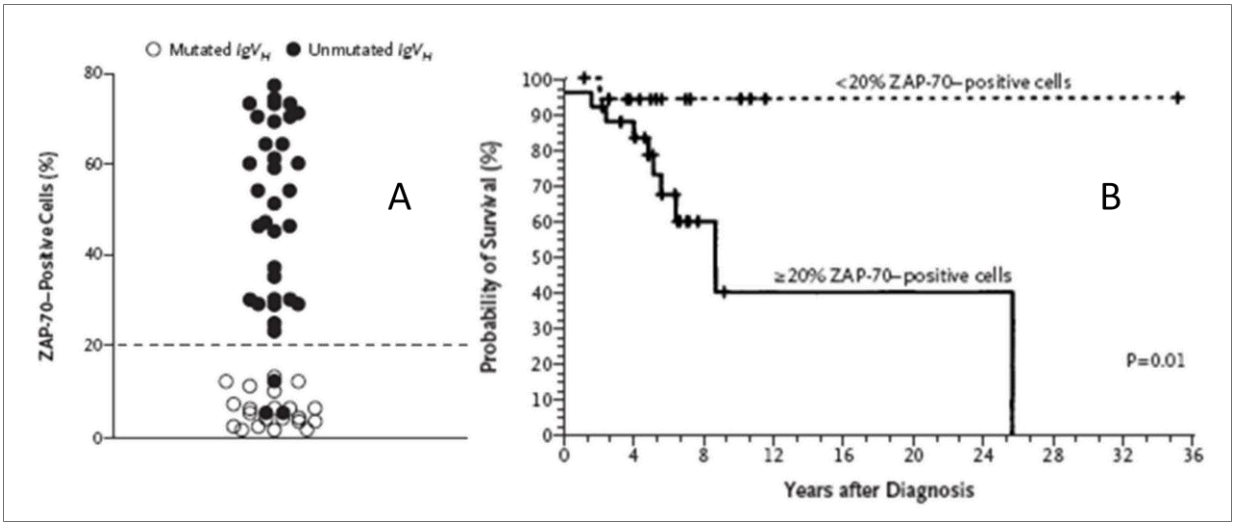

Figure 2: (A) A scatter plot representing the levels of ZAP-70 expression measured by flow cytometric analysis and the IgVh mutational status. When the threshold for categorizing ZAP-70 was established at 20%, two subgroups were clearly delineated: patients with unmutated IgVh and a high level of ZAP-70 expression and patients with mutant IgVh and low levels of ZAP-70 expression. Only three patients without IgVh mutations had low levels of ZAP-70 expression. (B) Kaplan-Meier plot comparing probability of survival based on the level of expression of ZAP-70. The median survival was 90 months among patients with a high level of ZAP-70 expression, whereas it was not reached among patients with a low level of ZAP-70 expression[29].

However, ZAP-70 is not an easy target because in CLL cells, ZAP-70 is a relatively low copy number target and among CLL patients as a group, ZAP-70 expression is a continuum. Furthermore, ZAP-70 flow cytometric evaluation is currently a matter of debate because of the lack of standardization[32]. The main concerns are the choice of the anti-ZAP-70 clone and the fluorochrome used, the permeabilization/fixation procedure, the type of internal control, recording of mean fluorescent intensity (MFI) versus percentage of cells staining above isotype control and the cut-offs used to distinguish positive and negative samples.

Current routine diagnostic work-up at UZ Leuven.

Prior to treatment initiation, a number of laboratory tests are performed in our institution. For the clinical laboratory (Dienst Laboratoriumgeneeskunde, LAG) these include:

- complete and differential blood count (diagnosis and staging),
- morphologic examination of blood film (diagnosis),
- serum chemistry including LDH and bilirubin (diagnosis and staging),
- serum immunoglobulin (evaluation of hypogammaglobulinemia) (diagnosis),
- serum protein electrophoresis (evaluation of paraproteinemia) (diagnosis),
- direct antiglobulin test (evaluation of immune dysregulation) (diagnosis),
- serum β2-microglobulin (prognosis),
- flow cytometric analysis (CD19, CD5, CD23, FMC7, sIg (dim), CD79b) (diagnosis),
- flow cytometric analysis of CD38 (prognosis).

At the level of the Center for Human Genetics (Centrum Menselijke Erfelijkheid, CME) cytogenetic abnormalities can be detected using conventional karyotyping and FISH (fluorescence in situ hybridization) analysis and IgVh mutational status can be assessed, all for prognostic purposes.

The purpose of this *Critically Appraised Topic* (CAT) was to verify whether flow cytometric analysis of ZAP-70 is analytically robust enough to be introduced in the routine work-up of newly diagnosed CLL-patients. Does this analysis provide an added value over present tests when evaluating prognosis and what are the practical and financial implications for the laboratory?

QUESTION(S)

1) Which analytical challenges can be expected when performing flow cytometric analysis of ZAP-70?
2) What are the practical and financial implications when introducing this test?

APPRAISAL

1. Which analytical challenges can be expected when performing flow cytometric analysis of ZAP-70?

The lack of a standardized consensus method for the determination of ZAP-70 is the main bottleneck for the implementation of this parameter in clinical routine. From a biological and functional point of view, protein levels are more significant than mRNA levels; therefore a protein detection method is preferred over molecular techniques. An additional drawback of the latter approach – and of protein detection methods based on cell lysates, such as western blotting – is the need for highly purified CLL cells, since T and NK cells are characterized by high ZAP-70 expression levels. Flow cytometry overcomes this difficulty and allows the simultaneous evaluation of ZAP-70 protein expression in CLL cells and in normal lymphocyte subsets. Although Crespo and coworkers[29] reported high concordance (97%) between ZAP-70 expression and the presence of unmutated IgVh gene in CLL cells, subsequent studies in larger numbers of patients failed to demonstrate this high correlation, for example, Rassenti et al. reported about 23% discordance between ZAP-70 expression and IgVh mutational status[31]. The intra- and interlaboratory discrepancies can be explained by several methodological aspects requiring standardization, such as the nature of the sample, the selection of the ZAP-70 antibody (clone and labeling, direct or indirect), the choice of fluorochrome, the use of different fixation and permeabilization reagents and last but not least, the method of analyzing and expressing the results. A literature review was initiated to address these issues and the results are summarized in table 3.
Aside from the issues included in this overview, minimal impact can be expected from differences in staining procedures, differences in equipment and in software packages.

1.1 Sample type and stability

Different types of samples can be used for flow cytometric detection of ZAP-70: peripheral blood mononuclear cells, whole blood, bone marrow, lymph node tissue (see table 4). Although more laborious than whole blood samples, isolated peripheral blood mononuclear cells are frequently used when long term storage of samples is indicated. Passam and coworkers found that the use of whole blood displayed equivalent staining to peripheral blood mononuclear cells[33]. Additionally, for staining of fresh versus cryopreserved samples, no difference was noted in the percentage of ZAP-70 positivity for the samples in which both fresh and cryopreserved cells were available. This finding was also confirmed by Zucchetto et al[34] although a different cut-off value for predicting clinical behavior in fresh and frozen samples has been suggested[35].
Several studies have shown that blood sample storage can result in lower ZAP-70 levels in CLL cells detected with flow cytometry and analysis should take place as soon as possible post-venipuncture or in any case within 24 to maximum 48 h after sample collection[36,37,38,39].
One study (n= 29) investigated the influence of the anticoagulant used and found that heparin-preserved samples depicted a more pronounced decrease in ZAP-70 expression after 48 h, compared to samples collected into EDTA, however less variation in ZAP-70 expression was observed over the course of the first 24 h in samples collected into heparin rather than EDTA[40].

Table 3: Literature review of ZAP-70 flow cytometric analysis. In case of comparative studies, the optimal item is indicated in bold (when applicable).

reference (n = # of samples)	sample type	anti-ZAP-70 clone	fixation-permeabilization	analysis and interpretation		
				quadrant marker threshold	positive result	method
Durig et al. 2003[44]	PBMNC	unconjugated 2F3.2 (Upstate) + FITC	Fix & Perm kit (Caltag)	UR = CD3+/CD56+/ZAP-70+ (subjective)	>20%	%/T-Ly
(n = 67)						
Crespo et al. 2004[29]	PBMNC	unconjugated 2F3.2 (Upstate) + FITC	Fix & Perm kit (Caltag)	UR = CD3+/CD56+/ZAP-70+ (subjective)	>20%	%/T-Ly
(n = 65)	WB					
Orchard et al. 2004[45]	PBMNC	unconjugated 2F3.2 (Upstate) + FITC	paraformaldehyde/ethanol (in-house)	LL = isotype control (subjective)	>10%	%/ISO
(n = 167)						
Rassenti et al. 2004[31]	PBMNC	1E7.2-Alexa-488 (BD)	paraformaldehyde/saponin (in-house)	UR = 0,1% CD19+/ZAP-70+	>20%	%/B-Ly (HC)
(n = 307)						
Gibbs et al. 2005[46]	WB	unconjugated 2F3.2 (Upstate) + FITC	**Fix & Perm kit (Caltag)**	L = isotype controle (subjective)	>20%	%/ISO
(n = 33)		**1E7.2-Alexa-488 (Caltag)**	paraformaldehyde/methanol (in-house)			
Schroers et al. 2005[41]	PBMNC	unconjugated 2F3.2 (Upstate) + FITC	Fix & Perm kit (Caltag)	NS	>20%	%/T-Ly
(n = 252)						
Del Giudice et al. 2005[51]	PBMNC	unconjugated 2F3.2 (Upstate) + FITC	Fix & Perm kit (Caltag)	UR = 97% CD3+ZAP-70+	>20%	%/T-Ly
(n = 251)						
Hus et al. 2006[58]	PBMNC	unconjugated 2F3.2 (Biomol) + Alexa-488	paraformaldehyde/ethanol (in-house)	NS	>20%	%/ISO
(n = 156)						
Kröber et al. 2006[42]	PBMNC	unconjugated 2F3.2 (Upstate) + FITC	Intraprep (paraformaldehyde/saponin, BC)	NS	>20%	%/T-Ly
(n = 148)	WB					
Del Principe et al. 2006[59]	PBMNC	1E7.2-Alexa-488 (Caltag)	Fix & Perm kit (Caltag)	NS	>20%	%/ISO
(n = 289)						
Kay et al. 2006[63]	WB	unconjugated 2F3.2 (Upstate) + FITC	Fix & Perm kit (Caltag)	UR = 95% CD3+/CD56+/ZAP-70+	>20%	%/T-Ly
(n = 55)				NA	**>47000**	**MESF CLL**
Bakke et al. 2006[37]	PBMNC	unconjugated clone 29 (BD) + FITC	Fix & Perm kit (Caltag)	UR = CD3+/CD56+/ZAP-70+ (subjective)	>20%	%/T-Ly
(n = 37)		**unconjugated 2F3.2 (Upstate) + FITC**		NA	**upper tertile**	**CLL/T-Ly MFI ratio**
		clone 136F12-Alexa-488 (CST)				
		1E7.2-Alexa-488 (Caltag)				
Best et al. 2006[40]	WB	unconjugated 2F3.2 (Upstate) + FITC	paraformaldehyde/ethanol (in-house)	LR = <1% isotype control	>10% (Upstate), >15% (Caltag)	%/ISO
(n = 72)	PBMNC	**1E7.2-Alexa-488 (Caltag)**	**Fix & Perm kit (Caltag)**	UL = <1% CD2+/ZAP-70-	>10% (Upstate), >15% (Caltag)	%/T-Ly
				NA	**>1,3 (Upstate), >1,5 (Caltag)**	**CLL/ISO MFI ratio**
				NA	>0,18 (Upstate), >0,2 (Caltag)	CLL/T-Ly MFI ratio
Wilhelm et al. 2006[54]	WB	1E7.2-Alexa-488 (Caltag)	Fix & Perm kit (Caltag)	UR = CD3+/CD56/ZAP-70+ (subjective)	>20%	%/T-Ly
(n = 101)	BM			UR = no CD19+/ZAP-70+ (subjective)	>20%	%/B-Ly (HC)
				LL = isotype control (subjective)	>10%	%/ISO

Table 3: Literature review of ZAP-70 flow cytometric analysis. In case of comparative studies, the optimal item is indicated in bold (when applicable - continued).

reference (n = # of samples)	sample type	anti-ZAP-70 clone	fixation-permeabilization	analysis and interpretation		
				quadrant marker threshold	positive result	method
Shankey et al. 2006[36]	WB	unconjugated 2F3.2 (Upstate) + FITC	Intraprep (paraformaldehyde/saponin, BC)	NA	NS	CLL Z index
(n = NS)		clone 136F12-Alexa-647 (BC)	Fix & Perm kit (Caltag)	NA	NS	CLL/T-LyMFI ratio
		1E7.2-PE (Caltag)	Phosflow lyse/Fix (paraformaldehyde/methanol, BD)	UL = 99% CD19+/ZAP-70-	NS	%/B-Ly (no HC)
		unconjugated clone 29 (BD) + FITC	**formaldehyde/triton x-100 (in-house)**			
		SBZAP-PE (BC)				
Sheikholeslami et al. 2006[35]	WB (fresh)	1E7.2-PE (Caltag)	Fix & Perm kit (Caltag)	isotype control	>10% (fresh samples)	%/ISO
(n = NS)	PBMNC (frozen)				>15% (frozen samples)	
Shults et al. 2006[55]	WB	**unconjugated clone 136F12 (CST) + Alexa-647**	NS	55th percentile: negative (CLSI rank order)	>2100	MESF CLL
(n = 395)	cell line	1E7.2-PE (Caltag)		UR = 0,1% CD19+/ZAP-70+	>20%	%/B-Ly (HC)
	BM					
Zuccheto et al. 2006[34]	WB	1E7.2-Alexa-488 (Caltag)	Fix & Perm kit (Caltag)	**UR = <0,5% isotype control**	**>20% or >26,4%**	**%/ISO**
(n = 145)	PBMNC			UR = 98-99% CD3+CD56+/ZAP-70+	>20%	%/T-Ly
Van Bockstaele et al. 2006[52]	WB	unconjugated 2F3.2 (Upstate) + PE	Fix & Perm kit (Caltag)	UR = 95% CD3+/ZAP-70+	>11%	%/T-Ly
(n = 53)				Kolmogorov Smirnov statistics (D-value)	D<0,84	KS
Letestu et al. 2006[32]	stabilized blood	1E7.2-Alexa-488 (Caltag)	Fix & Perm kit (Caltag)	LL: isotype control	>10%	%/ISO
(n = 3)		**unconjugated 2F3.2 (Upstate) + FITC**		LL: CD3/CD16/CD56-CD5-	>20%	%/B-Ly (HC)
				NA	NS	**T-Ly/CLL MFI ratio**
				UR: CD3/CD16/CD56+CD5+	>20%	%/T-Ly
Passam et al. 2006[33]	WB	1E7.2-PE (Caltag)	Fix & Perm kit (Caltag)	UR: 1-2% CD19/isotype control	>20%	%/ISO
(n = 105)	PBMNC	1E7.2-Alexa-488 (Caltag)	Intraprep (paraformaldehyde/saponin, BC)			
	fresh vs frozen		Leukoperm (Serotec)			
Munoz et al. 2007[50]	BM	2F3.2-FITC (Upstate)	Fix & Perm kit (Dako)	LL: 5% of T cells	>20%	%/T-Ly
(n = 53)				LL: 5% of NK cells	>20%	%/NK
D'Arena et al. 2007[43]	WB	1E7.2-PE (Caltag)	Fix & Perm kit (Caltag)	UR: CD3+/CD56+/ZAP-70+ (subjective)	>20%	%/T-Ly
(n = 157)						
Slack et al. 2007[60]	WB	clone 136F12-Alexa-647 (BC)	Intraprep (paraformaldehyde/saponin, BC)	**LL: isotype control**	**>25%**	**%/ISO**
(n = 45)				fixed threshold	NS	fixed region
				UR: T/NK-cells (subjective)	NS	%/T-Ly
Le Garff et al. 2007[56]	PBMNC	2F3.2-FITC (Upstate)	Fix & Perm kit (Caltag)	LL: isotype control	> 20%	%/ISO
(n = 237)		1E7.2-PE (sBioscience)		UR: T/NK-cells	>20%	%/T-Ly
		1E7.2-PE (Caltag)		NA	**<4**	**T-Ly/CLL MFI ratio**
		1E7.2-Alexa-488 (Caltag)				

Table 3: Literature review of ZAP-70 flow cytometric analysis. In case of comparative studies, the optimal item is indicated in bold (when applicable - continued 2).

reference (n = # of samples)	sample type	anti-ZAP-70 clone	fixation-permeabilization	analysis and interpretation		
				quadrant marker threshold	positive result	method
Chen et al. 2007[26]	WB	1E7.2-PE (Caltag)	Intraprep (paraformaldehyde/saponin, BC)	UR: 97% CD5+CD19-ZAP-70+	>20%	%/T-Ly
(n = 154)	BM			UL: 97% CD5-CD19+ZAP-70-	>10%	%/B-Ly (no HC)
	lymph node			NA	**>1,33**	**MESF CLL/B-Ly (no HC)**
				NA	>0,11	MESF CLL/NK
				NA	**>0,25**	**MESF CLL/T-Ly**
				UR: CD5+CD19-ZAP-70+ (subjective)	**>21%**	**%/T-Ly**
Rassenti et al. 2008[57]	PBMNC	NS	NS	isotype control	>20%	%/ISO
(n = 1012)						
Bekkema et al. 2008[38]	WB	1E7.2-PE (Caltag)	Fix & Perm kit (Caltag)	UR: 98% CD19-CD5+CD3+	>20%	%/T-Ly
(n = 65)			**FACS Lysing Solution (BD)**	NA	>0,10	CLL/T-Ly MFI ratio
Preobazhensky et al. 2008[39]	PBMNC	2F3.2-FITC (Upstate)	Fix & Perm kit (Caltag)	LR: <1% CD19+CD5-ZAP-70+	>20%	%/B-Ly (HC)
(n = NS)		1E7.2-FITC (sBioscience)	Intraprep (paraformaldehyde/saponin, BC)			
		1E7.2-Alexa-488 (Caltag)	**paraformaldehyde/saponin (in-house)**			
			paraformaldehyde/triton X-100 (in-house)			
Gachard et al. 2008[61]	PBMNC	unconjugated 2F3.2 (Upstate) + ?	paraformaldehyde/triton X-100 (in-house)	NA	**>1,4**	**CLL/B-Ly MFI ratio (HC)**
(n = 153)		1E7.2-Alexa-488 (Caltag)				T-Ly/CLL MFI ratio*
		SB70-FITC (Dako)				%/B-Ly*
		SB70-PE (Dako)				%/T-Ly*
		SBZAP-PE (BC)				
Kern et al. 2009[49]	WB	unconjugated SBZAP (BC) + PE	paraformaldehyde/triton X-100 (in-house)	UR: 97% CD3+ZAP-70+	>20%	%/T-Ly
(n = 1229)	BM	SBZAP-CDS (BC)		NA	NS	MFI B-Ly
				NA	**<4,5**	**T-Ly/B-Ly MFI ratio**
Rossi et al. 2010[48]	PBMNC	1E7.2-Alexa-488 (Caltag)	Fix & Perm kit (Caltag)	UR: <1% isotype control	>11%	%/ISO
(n = 514)				UR: 98% T/NK-cells	>20%	%/T-Ly
				NA	**<3**	**T-Ly/B-Ly MFI ratio**
Hassanein et al. 2010[53]	WB	1E7.2-PE (Caltag)	BDLyse (BD)/paraformaldehyde	NA	>2	CLL/B-Ly MFI ratio (HC)
(n = 111)		**1E7.2-Alexa-488 (Caltag)**		UR: <1% CD19+CD5- (HC)	>40%	%/B-Ly

NS: not specified
NA: not applicable
BC: Beckman Coulter
BD: Becton Dickinson
Caltag: Caltag Laboratories
Dako: DakoCytomation
Upstate: UpstateBiotechnology Inc.
**: data not shown*

PBMNC: peripheral blood mononuclear cells
WB: whole blood
BM: bone marrow
T-Ly: T lymphocytes
B-Ly: B lymphocytes
MFI: mean fluorescence intensity
MESF: molecules of equivalent soluble fluorochrome
subjective: no accurate (%) specification
HC: healthy control

For multicentric harmonization purposes, analysis on stabilized samples was evaluated. However, stabilization using a preservative medium (BCT, BD), results in decreased expression of CD19. This makes gating more difficult and therefore this approach is not recommended[32]. Both the problematic analysis of stabilized samples as well as the prerequisite to perform ZAP-70 analysis within 24h to 48 h since venipuncture, poses a problem for the analysis of external samples.

Table 4. Different types of samples used for flow cytometric analysis of ZAP-70 (based on table 3).

sample type	number of references
peripheral blood mononuclear cells	19
whole blood	18
bone marrow	5
lymph node tissue	1
stabilized blood	1

1.2 Fixation and permeabilization

Due to the intracellular localization of ZAP-70, the staining procedure requires a fixation and permeabilization step. Fixation reagents are used to stabilize delicate cell structures (formation of methylene bridging between protein aldehyde functions) and are usually formaldehyde-based solutions. Permeabilization agents, on the other hand can consist of a mild detergent (saponin, triton-x, tween,…) or of methanol/ethanol based solutions. Although some authors use "in-house" fixation and permeabilization protocols for ZAP-70 flow cytometric analysis, several fixation and permeabilization kits are commercially available and are usually more reliable and easier to use. For example, some "in house" methods appear to disrupt the Alexa Fluor 488 fluorochrome in an unpredictable way[46].

Table 5. Range of in-house and commercial fixation and permeabilization kits cited in literature as sole or as optimal method (based on table 3)

kit	number of references
in-house	
paraformaldehyde/ethanol	2
paraformaldehyde/saponin	2
paraformaldehyde/triton X-100	3
commercial	
Fix & Perm kit (Caltag Laboratories)	18
Intraprep (Beckman Coulter)	4
FACS Lysing Solution (Becton Dickinson)	1
BDLyse/paraformaldehyde (Becton Dickinson)	1
Fix & Perm kit (DakoCytomation)	1
Leukoperm (AbD Serotec)	1

Literature review, as summarized in table 5, demonstrates the wide use of commercial fixation and permeabilization kits, with the *Fix and Perm kit* by Caltag Laboratories (Burlingame, CA, USA) as the preferred kit in many studies, however, as can be deducted from the comparative studies, no general consensus can be made and the ideal fixation and permeabilization kit must be evaluated for each method within every laboratory (see table 6).

Finally, the effects of varying both time and concentration of these two key steps, to obtain higher signal-to-noise ratios, can also have an impact on the ZAP-70 expression and needs to be optimized as well for each method.

Table 6: Summary of studies comparing different fixation and permeabilization kits (in-house and commercial, based on table 3).

optimal fixation/permeabilization kit	comparative study	comparator kit(s)
Fix & Perm kit (Caltag)	Gibbs et al. 2005[46]	paraformaldehyde/methanol (in-house)
	Best et al. 2006[40]	paraformaldehyde/ethanol (in-house)
FACS Lysing Solution (BD)	Bekkema et al. 2008[38]	Fix & Perm kit (Caltag)
paraformaldehyde/saponin (in-house)	Preobrazhensky et al. 2008[39]	paraformaldehyde/triton X-100 (in-house)
		Fix & Perm kit (Caltag)
		Intraprep (BC)
paraformaldehyde/triton X-100 (in-house)	Shankey et al. 2006[36]	Phosflow lyse/Fix (BD)
		Fix & Perm kit (Caltag)
		Intraprep (BC)

1.3 ZAP-70 antibody and fluorochrome

The early studies used the anti-ZAP-70 clone 2F3.2 developed by Upstate Biotechnology Inc. (Lake Placid, NY, USA), which is unconjugated[29,44,45]. Downside of this approach is that conjugation of the antibody with an adequate fluorochrome, i.e. fluorescein isothiocyanate (FITC) or phycoerythrin (PE), requires an additional step. Moreover, with the addition of other antibodies, such as CD19, the problem of non-specific binding of this fluorochrome between antibodies necessitates an additional blocking step. The 2F3.2 clone in its unconjugated form is unstable at 4°C. Finally, clone 2F3.2 was developed for immunoblotting and immunoprecipitation rather than flow cytometry[46]. Rassenti and coworkers were the first to describe a flow cytometric analysis of ZAP-70 using the anti-ZAP-70 clone 1E7.2 directly conjugated with Alexa-488 as a fluorochrome[31]. The 2F3.2 clone is raised against the two SH2 domains of human ZAP-70 and the 1E7.2 clone targets an epitope within the tyrosine kinase domain. Both clones are frequently used (see table 3), with a slight advantage for the Alexa-488 directly conjugated 1E7.2 clone, because the fluorochrome conjugation step can be omitted and because of a higher stability. Nevertheless, subsequent comparative studies (see table 7) produced discrepant results with regard to any superiority of one of the formerly mentioned clones[32,37,40,46].

Several other directly or indirectly conjugated anti-ZAP-70 clones were evaluated over the years, some more successfully than others: 2 comparative studies referred to SBZAP as an optimal clone, two other publications showed that clone 136F12 was superior to clones 1E7.2, 2F3.2 and 29 (see table 7).

Although no consensus can be derived from literature, there is a trend towards the use of conjugated antibodies for flow cytometric analysis of ZAP-70, because they are easier to use in a daily routine and because they are at least as good as the originally introduced unconjugated 2F3.2 clone.

Table 3 shows that the following fluorochromes are primarily used for direct or indirect conjugation with ant-ZAP-70 antibody: FITC, Alexa-488 and PE. No consensus was found for the use of either of them.

Table 7: Summary of studies comparing different ZAP-70 antibody clones (based on table 3).

optimal ZAP-70 antibody clone	comparative study	comparator clone(s)
1E7.2-Alexa-488	Gibbs et al. 2005[46]	unconjugated 2F3.2 + FITC
	Best et al. 2006[40]	unconjugated 2F3.2 + FITC
	Preobrazhensky et al. 2008[39]	2F3.2-FITC
		1E7.2-FITC
	Hassanein et al. 2010[53]	1E7.2-PE
2F3.2 + FITC	Bakke et al. 2006[37]	1E7.2-Alexa-488
		unconjugated clone 29 + FITC
		136F12-Alexa-488*
	Letestu et al. 2006[32]	1E7.2-Alexa-488
SBZAP-PE	Shankey et al. 2006[36]	unconjugated 2F3.2 + FITC
		136F12-Alexa-647
		unconjugated clone 29 + FITC
		1E7.2-PE*
	Gachard et al. 2008[61]	unconjugated 2F3.2 + ?
		1E7.2-Alexa-488
		SB70-FITC
		SB70-PE
136F12-Alexa-488	Bakke et al. 2006[37]	unconjugated clone 29 + FITC
		1E7.2-Alexa-488
		unconjugated 2F3.2 + FITC*
136F12 + Alexa-647	Shults et al. 2006[55]	1E7.2-PE
1E7.2-PE	Shankey et al. 2006[36]	unconjugated clone 29 + FITC
		unconjugated 2F3.2 + FITC
		136F12-Alexa-647
		SBZAP-PE*
** = comparator clone performed equally well as optimal ZAP-70 antibody clone*		

1.4 Method of analysis and interpretation of the results

After gating the lymphocyte population using a forward-scatter versus sideward-scatter plot, the following membrane markers are used to identify the different lymphocyte subsets in potential CLL samples: CD19, CD3, CD16/56, CD5. For cost effectiveness, some authors replace CD3, CD16/56 by CD2 alone[40]. The use of anti-CD20 as a fifth color might allow better discrimination of the normal B lymphocyte population, however this would also increase the cost (Letestu et al.; data not published).

A number of different approaches to index or measure ZAP-70 levels in the CLL population have been published over the years (see table 3 and 8). Crespo et al. considered the expression of ZAP-70 in patients' T and NK cells, constitutively expressing the protein, as an internal positive control population (= *%/T-Ly method*)[29]. A CLL sample is defined as ZAP-70 positive when at least 20% of CLL cells express ZAP-70 at levels comparable to those found in the residual T cell component. However, this study did not specify the exact cursor position used to mark the boundary for the internal ZAP-70 positive population. Since CLL cells stain uniformly for ZAP-70, two studies have subsequently demonstrated that relatively small changes in the cursor settings used to define ZAP-70 positive levels can have a profound impact on the assay results[37,47], a problem exacerbated for ZAP-70 assays having poor separation between positive and negative populations[37].

Table 8: Overview of different approaches used in literature to index or measure ZAP-70 levels in a CLL population (based on table 3).

method	synonym
% ZAP-70 positive CLL cells compared to T cells	%/T-Ly
% ZAP-70 positive CLL cells compared to an isotype control	%/ISO
% ZAP-70 positive CLL cells compared to normal B cells	%/B-Ly
% ZAP-70 positive CLL cells compared to NK cells	%/NK
Kolmogorov Smirnov statistical method	KS
ratio of MFI in CLL (or B) and T cells*	CLL/T-Ly MFI ratio
ratio of MFI in CLL (or B) and isotype control	CLL/ISO MFI ratio
ratio of MFI in CLL (or B) and normal B cells	CLL/B-Ly MFI ratio
MFI in B (or CLL) cells	MFI B-Ly
(CLL MFI- B cell MFI)/(T cell MFI – B cell MFI)	CLL Z index
ratio of MESF in CLL (or B) and T cells	MESF CLL/T-Ly
ratio of MESF in CLL (or B) and normal B cells	MESF CLL/B-Ly
ratio of MESF in CLL (or B) and NK cells	MESF CLL/T-Ly
MESF in CLL cells	MESF CLL
Use of a constant baseline threshold	Fixed region
**= T-Ly/CLL MFI ratio or T-Ly/B-Ly MFI ratio*	

Figure 3 shows how a single patient can be transformed from positive to negative by minor changes in the cursor positioning[37]. In an attempt to reduce this variability, later studies used a cursor position with a standard percentage, ranging from 95% to 99%, of T and NK cells falling into the ZAP-70 positive region[34,38,40,47,63,48,49,50,51,52]. However, variability can also be introduced due to a difference in staining intensity between T and NK cells. T cells stain less intensely and since different patients have different levels of T and NK cells. Furthermore, few normal T and NK cells may be left in some CLL patients and, as shown in one study the level of ZAP-70 on normal patient T cells may be significantly higher in ZAP-70 positive cases[53]. These additional variables can also lead to inconsistent cursor positioning.

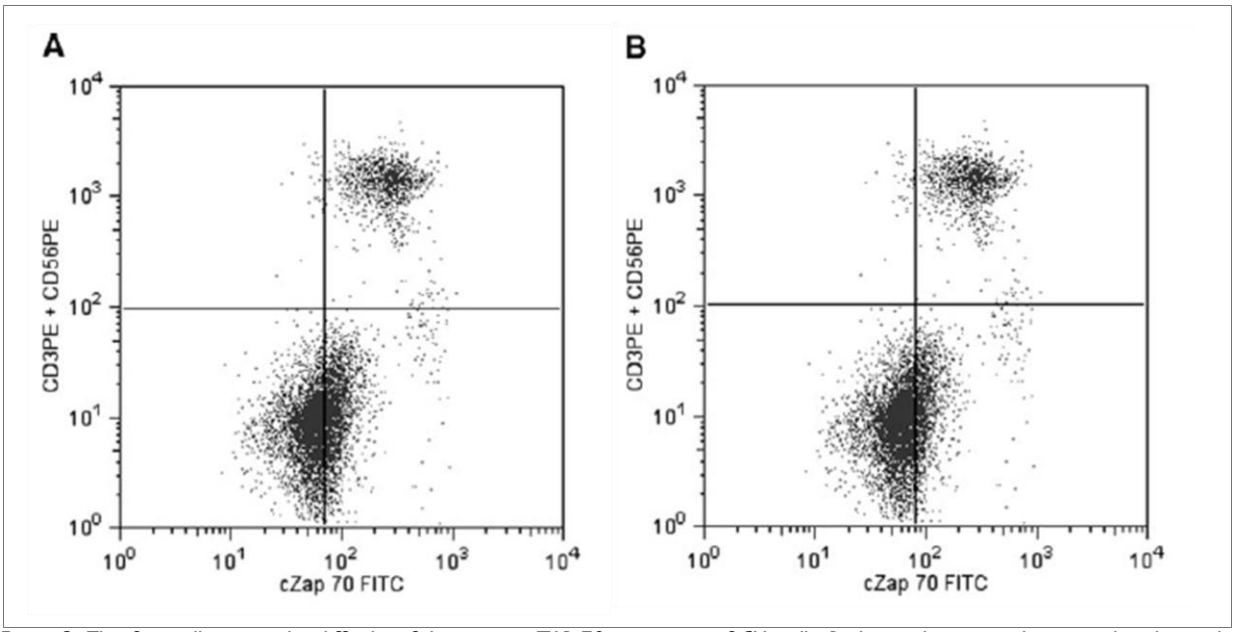

Figure 3: This figure illustrates the difficulty of determining ZAP-70 expression of CLL cells. Both panels contain the same data but with small differences in the placement of the vertical cursor. (A) T and NK cells are dual positive for ZAP-70 and CD3 + CD56 expression (upper right quadrant). With the vertical cursor set at the left of all T and NK cells, the patient's CLL cells are 29% positive for ZAP-70 expression (lower right quadrant), which would be considered positive for ZAP-70. (B) With the vertical cursor set a few channels to the right, but still left of the major T and NK cluster, the patient's CLL wells are now 12% positive for ZAP-70 and would be read negative for ZAP-70 expression[37].

Another approach is to set a ZAP-70 negative boundary using either residual or normal B-cells from healthy controls (= *%/B-Ly method*). By setting this boundary to make 99.9% of all normal B-cells negative for ZAP-70 expression, Rassenti and coworkers were able to set a dichotomous cut-off-point to separate progressors from

non-progressors, and more importantly, they were able to demonstrate that this approach provided a better predictor for disease course than IgVh mutational status in these same patients[31]. Again, most authors used a cut-off of 20% to define ZAP-70 positivity[31,32,39,54,55] and again, the percentage of positive cells can be influenced by the placement of the quadrant marker, especially since a healthy control sample is most often used[31,53]. The latter approach is necessary because in approximately 10% of CLL samples, insufficient normal, residual B cells can be detected[36].

Table 9: Overview of comparative studies and their optimal interpretation method for ZAP-70 positivity (based on table 3).

optimal interpretation method	comparative study	comparator method(s)
T-Ly/CLL MFI ratio	Bakke et al. 2006[37] (CLL/T-Ly MFI ratio)	%/T-Ly
	Letestu et al. 2006[32]	%/T-Ly
		%/B-Ly
		%/ISO
	Le Garff et al. 2007[56]	%/T-Ly
		%/ISO
	Kern et al. 2009[49] (T-Ly/B-Ly MFI ratio)	%/B-Ly
		MFI B-Ly
	Rossi et al. 2010[48] (T-Ly/B-Ly MFI ratio)	%/T-Ly
		%/ISO
CLL/ISO MFI ratio	Best et al. 2006[40]	%/ISO
		%/T-Ly
		CLL/T-Ly MFI ratio
CLL/B-Ly MFI ratio	Gachard et al. 2008[61]	T-Ly/CLL MFI ratio*
		%/B-Ly*
		%/T-Ly*
%/ISO	Zucchetto et al. 2006[34]	%/T-Ly
	Slack et al. 2007[60]	%/T-Ly
		fixed threshold
MESF CLL/T-Ly; MESF CLL/B-Ly	Chen et al. 2007[47]	%/T-Ly**
		%/B-Ly
		MESF CLL/NK
MESF CLL	Kay et al. 2006[63]	%/T-Ly
* = data not shown		
** = %/T-Ly (subjective cut-off) performed equally well as MESF CLL/T-Ly or MESF CLL/B-Ly		

An alternate method of evaluating ZAP-70 staining has employed comparison to an isotype control (= *%/ISO method*)[45]. Also in this interpretation methodology, many authors use a 20% cut-off for ZAP-70 positivity[34,46,56,57,58,59], although in the literature a range from 10% to 25% can be found[32,35,40,45,48,54,60]. Besides the problems with cursor placement, different commercial ZAP-70 antibodies cannot be consistently compared to isotype controls, because of lot-to-lot variability in the isotype staining. Therefore, comparison to isotypic controls is also not a universally applicable procedure[37].

Since all of the aforementioned methodologies, based on a cut-off percentage, suffer from high variability due to correct cursor positioning, an increasing number of reports indicated that discrimination between ZAP-70 positive and negative cases should not be based on a cut-off percentage, but on a population-derived parameter, such as the mean fluorescence intensity (MFI) ratio of two populations (MFI CLL/T-Ly, MFI CLL/B-Ly)[26,32,36,37,48,49,56,61] or the mean fluorescence intensity ratio of stained cells and an isotypic control (MFI CLL/ISO)[40]. These methods include the entire CLL population, as well as one or more internal or external control populations, such as normal B cells, T and NK cells or an isotypic control. Therefore, they offer a more robust measure for ZAP-70 expression[62] and have demonstrated to be more reproducible in multicenter comparison[32,56,61]. Cut-off values for positivity differ among different groups and range from >1.4 to >2 for the CLL/B-Ly MFI ratio method, from >0.1 to > 0.2 for the CLL/T-ly MFI ratio method and from <4.5 to <3 for the T-Ly/B-Ly (or T-Ly/CLL) MFI ratio method (see table 3). If the MFI T-Ly/B-Ly in normal blood is lower than 4,

the assay should be rejected as a technical failure[53]. Despite the fact that some authors express no preference[38] and others use both MFI and percentage methodologies[53], table 9 illustrates that, several comparative studies favor the use of an MFI ratio of CLL cells versus T and NK cells.
Other approaches have used added beads to calculate the molecules of equivalent soluble fluorochrome (MESF) of ZAP-70 in CLL cells in an attempt to provide clinicians with a quantitative result. The MESF concept designates that a sample labeled with a fluorochrome has the same fluorescence intensity as an equivalent number of molecules of the fluorochrome free in a solution under the same environmental conditions. This fluorescence unit provides a tool to calculate the molecular equivalents of a protein (i.e. ZAP-70) in the cell and compare quantitative flow cytometry data over time, across instruments and laboratories. Although useful, the main drawback of this approach is the added cost to each test[47,55,63].

1.5 Benchmarking

Clinical laboratories of other major hematological centers in Belgium were contacted with regard to this analysis: OLV-Ziekenhuis Aalst, UZ Antwerpen, AZ Sint-Jan Brugge, UZ Brussel, Cliniques Universitaires Saint-Luc, Hôpital Erasme, UZ Gent, Jessa Ziekenhuis Hasselt and Ziekenhuis Oost-Limburg.
Of these 9 centers, only UZ Brussel and UZ Gent offer (similar) flow cytometric analysis of ZAP-70 albeit on an infrequent and request-only base:

-	UZ Brussel:	ZAP-70 antibody:	unconjugated 2F3.2
		fluorochome:	FITC-labelled goat-anti-mouse antibody
		interpretation:	percentage of ZAP-70 positive CLL cells
		positivity threshold:	95% of CD3 positive cells are positive for ZAP-70 (%/T-Ly)
		cut-off:	> 20% of CLL cells are positive for ZAP-70
-	UZ Gent:	ZAP-70 antibody:	unconjugated 2F3.2
		fluorochrome:	PE-labelled goat-anti-mouse antibody
		interpretation:	percentage of ZAP-70 positive CLL cells
		positivity threshold:	95% of CD3 positive cells are positive for ZAP-70 (%/T-Ly)
		cut-off:	> 20% of CLL cells are positive for ZAP-70

2. What are the practical and financial implications when introducing this test?

Routine flow cytometric work-up of CLL samples is comprised of the following combinations of markers (see attachment 1):

Screen NHL B and T:
- CD3-FITC/CD16.56-PE/CD45-PerCP/CD4-PE-CY7/CD19-APC/CD8-APC-H7
- Kappa-FITC/Lambda-PE/CD19-PerCP/CD10-PE-CY7/CD5-APC/CD20-APC-H7

Diagnosis lymphoma set 2 B-NHL:
- FMC7-FITC/CD23-PE/CD19-PerCP/CD38-PE-CY7/CD79b-APC/-

Flow cytometric analysis of ZAP-70 could be achieved by introducing a fourth panel:

- ZAP-70-FITC/CD16.56-PE/CD3-PerCP/CD5-APC/CD19-APC-H7/-

Using these markers, the CD19/CD5 double positive cell population can be isolated and subsequently, the ZAP-70 expression can be quantified in MFI values. Alexa fluor-488 conjugated ZAP-70 antibodies can be detected in the FITC channel.

Taking into consideration the previously (see chapter 1) mentioned trends or suggestions with regard to antibody, fixation and permeabilization kit, implementation of this parameter in the routine diagnostic work-up of samples from CLL patients would generate the following costs:

- *Cost of the ZAP-70 antibody clone 1E7.2 Alexa fluor-488 (Invitrogen):* *3.82 euro (excl. BTW)*
- *Cost of CD16.56-PE, CD3-PerCP, CD5-APC and CD19-APC-H7 antibodies (BD):* *16.25 euro (excl. BTW)*
- *Cost of the Fix & Perm kit (Imtec Diagnostics)* *1.60 euro (excl BTW)*
- *Cost of the lab technician (preparation, analysis and interpretation):* *7 euro*

This would result in an additional cost of 28.67 euro per sample. Considering a total of 94 newly diagnosed CLL cases yearly at UZ Leuven (60 cases intramuros, 34 cases extramuros), an estimated added cost of 2695 euro is to be expected.
From a laboratory point of view, no cost savings can be expected when introducing this test.

The exact implications of the flow cytometric detection of ZAP-70 and the impact on the prognosis in terms of patient care are difficult to determine and to translate in actual costs.

3. Conclusions

The IgVh mutational status can be considered a "gold standard" for assessing prognosis of CLL patients at presentation. Since determination of IgVh mutation status is complex, time consuming and costly, a hunt for surrogate markers was initiated and ZAP-70 expression has rapidly been identified as one of the most important candidates. Among the different possible techniques, flow cytometric analysis of ZAP-70 expression was thought to be the most convenient one. However, the necessary standardization of technical determination of flow cytometric evaluation of ZAP-70 expression has proven for more challenging than expected. Several factors can be identified for introducing variability: sample type, antibody clone, fluorochrome to be used, staining strategy and not in the least, the expression of the results.

Several authors have tried to address these issues, but up to now, large international multicenter studies encompassing all these variables are lacking. Nevertheless, some general considerations can be made: first of all, analysis on fresh whole blood samples within 24 h (or maximum within 48 h) is preferable since ZAP-70 expression changes on storage. Secondly, directly labeled antibodies and commercially available fixation and permeabilization kits can be used, simplifying the staining protocol. Finally, an increasing number of reports suggest that ZAP-70 expression should be interpreted using evaluation of MFI values, as measured in the context of both CLL cells and (normal) B and/or T cells, rather than computing the percentage of positive cells. It is clear, however, that ZAP-70 expression shows a continuous spectrum in CLL, and that no clear-cut distinction can be made between ZAP-70 positive and ZAP-70 negative patients.

Since no recommendations with regard to ZAP-70 expression are available in clinical practice guidelines and since more robust techniques are available at our institution, analysis of ZAP-70 is not a priority.

TO DO/ACTIONS

Although the concept of flow cytometric analysis of ZAP-70 remains promising, no consensus method is available to warrant a practical evaluation of this test. Moreover, more robust methods (i.e. IgVh mutation status and karyotyping and FISH analysis for detection of chromosomal aberrations) are available in our hospital for evaluation of prognosis. Therefore, no further action is warranted.

Attachment 1 : SOP 090 – Logblad 4: "diagnose NHL"

DIAGNOSE NHL (6 kleur)

Patient- en staal identificatie

Cytose (10e9/L):

Aantal MoAB:

		FITC	PE	PerCP	PE-CY7	APC	APC-H7
Screen NHL B en T							
TBNK	☐	CD3	CD16.56	CD45	CD4	CD19	CD8
sIgB	☐	Kappa	Lambda	CD19	CD10	CD5	CD20
Diagnose lymfoom set 2 B-NHL							
B-NHL 1	☐	FMC7	CD23	CD19	CD38	CD79b	x
HCL							
HCL 1	☐	CD103	CD22	CD20	CD25	CD11c	CD19
sIg zware ketens							
sIgA	☐	IgA	CD19	CD45	x	x	x
sIgD	☐	IgD	CD19	CD45	x	x	x
sIgM	☐	IgM	CD19	CD45	x	x	x
sIgG	☐	IgG	CD19	CD45	x	x	x
Diagnose lymfoom set 2 T-NHL							
T-NHL 1	☐	CD7	CD2	CD45	x	CD5	x
T-NHL 2	☐	CD57	CD16	CD45	x	CD8	x
T-NHL 3	☐	TCRab	TCRgd	CD45	x	CD56	x
TCR V beta mix							
	☐	TCR-Vbeta panel					

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term: "ZAP-70, chronic lymphocytic leukemia, prognosis, flow cytometry"*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)*
3) *UpToDate Online version 18.3*

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