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Study of Discriminating Markers for the Diagnosis of Chronic Lymphoid Leukemia and Evaluation of Matutes Scores

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ABSTRACT

The study of the expression level by lymphoma cells (MFI: mean fluorescence intensity) completed by ROC curve for diagnostic markers is a valuable aid for the daily interpretation of lymphocyte immunophenotyping as well as the differential diagnosis with other chronic lymphoproliferative B syndromes. The aim is to provide diagnostic guidance, increase the power of the diagnostic test based on the discriminating capacity of each marker and validate the value of the Matutes score adapted to our study population.

Materials and Methods: This is a technical validation study with retrospective recruitment of 91 patients diagnosed with B-CLL according to clinical, cytological and biological results. Statistical analysis is performed by the SPSS software.

Results: The ROC curve shows the discriminative power of each diagnostic marker. The combination of discriminatory markers (CD23, CD5, CD43) either in double or triple marking did not increase the power of the test; The diagnosis of B-CLL according to our threshold values is changed to a score value greater than or equal to 3.

Conclusion: The analysis of each marker, using the ROC curve allows us to identify the most discriminating markers useful to orient the diagnosis by elaborating a diagnostic score.

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Introduction

The diagnosis of B cell-chronic lymphoproliferatif leukemia (B-CLL) is essentially based on lymphocyte immunophenotyping using the Matutes score, which confirms the diagnosis when the score is greater than or equal to four [1]. The study of the median of mean fluorescence intensities (median = best central measure where a limited aberrants number can considerably affect the mean) is a precious help for the biologist in the interpretation of immunophenotyping, complemented by the ROC curves (Receiver Operating Characteristics) to raise the discriminating markers necessary to optimize the diagnosis. First, we combined the most discriminating markers to increase the power of our B-CLL diagnostic test. Secondly, the validation of the Matutes score is a pre-requisite since the expression (+/-) of every marker is technically dependent on each laboratory.

Materials and Methods

This is a diagnostic validity study conducted at the hematology laboratory at the Hassan II University Hospital Center in Fez, about 91 patients diagnosed with B-CLL according to the conclusion of immunophenotyping, clinical data, and cytology, between January 17, 2021 and april 1, 2022. Lymphocyte immunophenotyping is performed on a Beckman Coulter FC500 flow cytometer.

Methodology of Analysis

Technical Validation

Sampling (peripheral blood) collected on an anticoagulant: EDTA and lithium heparinate are recommended for immunophenotyping. It is important to check the quality of the sample before any technique, in particular the absence of clot. It should be analyzed within 24 hours for optimal results.

Sample Preparation for Immunophenotypic Analysis

It is usual to wash the samples, in particular to concentrate the cells, eliminate cellular debris and soluble Immunoglobulins that will consume the anti-isotype antibodies, neutralizing their fixation

on the B-lymphocytes, which will then appear devoid of membrane Immunoglobulin. The marking of lymphocyte subpopulations was performed with the following panel of monoclonal antibodies: Pan-leukocyte marker: CD45; T-cell markers: CD3, CD4, CD8; NK-cell markers: CD16 and CD56; B-cell marker: CD19.

B lymphocyte marking was performed with the following panel of monoclonal antibodies: Systematically: CD5, CD19, CD23, CD79b, FMC7, KAPPA and LAMBDA. In case of Matutes Score ≤ 3: CD10, CD20, CD22, CD43, CD38 and CD25.

The marking consist to incubate the pre-treated cells (50 µL) with the monoclonal antibody (10 µL) for 15 to 30 minutes at ambient temperature and in the dark. Erythrocyte lysis: eliminate interference with red blood cells in very high numbers.

Data Acquisition and Processing

The cells are generally passed with an FSC threshold eliminating dead cells, platelets and red blood cell stroma's, but it can be set to any other parameter (for example: CD45 marker save only leukocytes). The cytometry information is processed and analyzed by the CXP software. The orientation tube allows highlighting the presence of a B lymphocyte clone via the distribution of the Kappa and Lambda light chains and the presence of a positive CD5 clone.

Statistical Analysis

Statistical Exploitation of the MFI (Mean Fluorescence Intensity) of the Different Diagnostic Markers of B-CLL

The ROC (Receiver Operating Characteristics) curve is a graphical representation of all sensitivity/specificity pairs corresponding to all possible thresholds, with the sensitivity of the test (the proportion of true positives) on the ordinate axis and the specificity (1-Sp) of the test (the proportion of false positives) on the abscissa axis. Thus, the point on the curve closest to the upper left corner is the threshold value for which the proportion of correct results (true positive and true negative) is the highest [2]. The different measures of fluorescence intensity of the markers tested were listed on an Excel table using an appropriate coding, and will be analyzed by the SPSS (Statistical Package for the Social Sciences) software in the form of curves to better identify the discriminating markers. In addition, the calculation of the area under the curve (= AUC for Area under the Curve) of ROC allows us to quantify the diagnostic performance of a marker of interest [3].

Study of the Discriminating Power and Evaluation of the Matutes Score

a) Determining threshold values with better specificity for B-CLL

To support a hypothesis diagnosis, the clinician frequently uses clinical symptoms and signs as well as additional tests. These tests should ideally give an unequivocal answer to the diagnostic question: the disease is present if the test is positive, absent if it is negative. Unfortunately, in practice, there are few clinical signs or tests that can be unequivocally interpreted. Some patients present sign or a positive test, without suffering from the disease sought; others, on the contrary, even if they have the disease sought, they do not present this clinical sign or present a negative test. Thus, the clinician must have the characteristics of a test enabling him to interpret the results and apply them to his patient: these are the qualifiers of sensitivity, specificity and predictive values. In the literature, the authors try to define the situations in which sensitivity or specificity should be preferred [4].

The sensitivity of a test is preferred when

- • The aim of the diagnostic process is to eliminate the disease
- The disease is more curable if treated early
- A false positive result has any serious consequences
- The disease is rare and we are in a screening situation.

Specificity is Preferred When

- The aim of the diagnostic approach is to affirm or confirm the diagnosis.
- The risk of a false positive result is serious and not acceptable.
- The disease is incurable.

The reality is more complex in choosing to privilege sensitivity or specificity. Increasing sensitivity cannot be done without taking into consideration the cost of decreasing specificity at the same time. The choice to favour one or the other must take into account the consequences of this choice [5, 6].

In our case and in combination with several tests with complementary properties in a B-CLL diagnostic strategy, we will focus on specificity in order to minimize the risk of false positive results.

b) Raised the B-CLL discriminant markers from our study series and coupled them to increase the power of the assay by improving the sensitivity/specificity parameters.

c) Calculate the Negative Predictive Value NPV of the test which is the probability that disease is absent when the test result is negative and the Positive Predictive Value PPV which is the probability that disease is present when the test result is positive: to better identify our study population.

d) Evaluation of the Matutes score according to our threshold values

Results

The MFI values studied for each of the markers correspond to B lymphocyte population CD19+ (Table I).

Table I: Different measures of mean fluorescence intensity of the tested markers given by the CXP cytometer software

Markers	Mean Fluorescence Intensity (MFI)			
	Minimum	Maximum	mean	Median
CD5	502	8234	3813,6923	3056
CD23	512	11234	4470,16484	4001
CD79b	302	2101	976,21978	873
FMC7	42	450	156,197802	127
KAPPA	331	3077	1676,18681	1677
LAMBDA	211	2976	1444,01099	1304
CD43	567	5007	2399,17582	2111
CD25	39	694	244,934066	151
CD11c	43	288	103,087912	89
CD103	27	210	92,7362637	89
CD10	23	311	117,384615	95
CD22	350	3221	1486,95604	1320
CD38	1002	7409	4197,49451	4507

Based on the ROC curves, the most discriminating markers were removed: CD5, CD23, CD43 with an air under the curve higher than 50% and we fixed threshold values according to a better specificity.

To increase the power of our diagnostic test, we held the most discriminating markers together and complemented with the NPV and PPV calculation (Figure 1) (Table II).

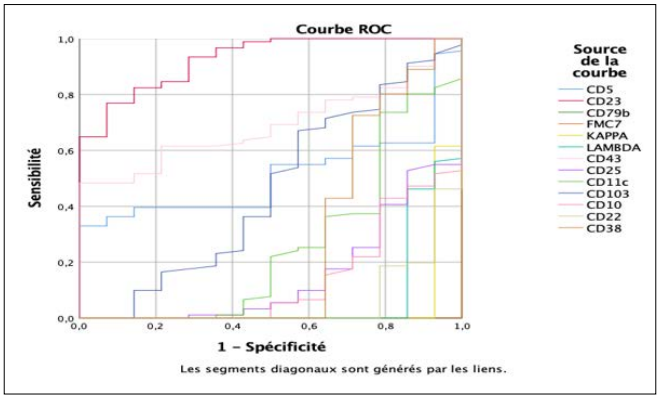

Figure 1: ROC Curve of MFI for Different Markers of Interest in B-CLL

Table II. Most discriminating test result variables CD5/CD23/CD43

Test result variable(s)	Area under the curve	asymptotic confidence interval 95%	
		Lower Borne	Upper Borne
CD5	0.693	539	779
CD23	0.918	882	989
CD43	0.792	699	885

CD5: When coupled with CD23 and/or CD43 the sensitivity decreased to 16% and 17% respectively, a triple marking CD5CD23CD43 has a sensitivity of 9% , while a simple marking CD5 has a sensitivity 32% better than the combined markings hence the interest of working with mono-marking (Table III).

Table III: PPV and NPV of discriminating markers

	Sensitivity	Specificity	PPV	NPV
CD5	32%	100%	100%	29%
CD23	60%	100%	100%	41%
CD43	48%	100%	100%	31%
CD5CD23	16%	100%	100%	25%
CD5CD43	17%	100%	100%	22%
CD23CD43	28%	100%	100%	25%
CD5CD23CD43	9%	100%	100%	21%

CD23: The combined CD23 /CD43 and CD23/CD5 labelling present sensibilities of 28% and 16% respectively, the tri-labelling CD23CD5CD43 has a sensitivity of 9%, while the unique CD23 labelling has a sensitivity of 60%, so the coupling of the three markers does not improve the sensitivity of the test (Table III).
CD43 : CD43 is a discriminating marker which has a sensitivity of 48% in single marking, whereas in combined marking with CD23 and CD5 there is a significant decrease in sensitivity, hence the need to work with a single marking (Table III).

For The Evaluation of the Matutes Score According to the New Threshold Values, the Following are Assigned
One point according to the Matutes score for all patients with a CD5 MFI value greater than or equal to 5224: specificity is preferred.

One point according to Matutes score for positive expression of CD23 (greater than 2838)
One point according to the Matutes score for weak or negative expression of CD79b (less than 56661).

One point according to the Matutes score for any weak or negative expression of surface immunoglobulin (less than 20311).

One point according to Matutes score for any negative expression of FMC7 (less than 2911).

For a Matutes Score Higher than Four According to our Threshold Values we have
TP (true positive = 69) represents the number of patients (CLL+) with a Matutes Score ≥ 4.
FP (false positive = 0) represents the number of healthy persons with a Matutes score ≥ 4.
FN (false negatives = 22) represents the number of patients with a Matutes score ≤ 4.
TN (true negatives = 26) represents the number of healthy persons with a Matutes score ≤ 4.

For a Matutes Score Greater than or Equal to Three According to our Threshold Values we have
TP (true positive = 90) represents the number of patients (CLL+) with a Matutes Score ≥ 3.
FP (false positive = 0) represents the number of healthy individuals with a score ≥ 3.
FN (false negatives = 1) represents the number of patients with a Matutes Score ≤ 3.
TN (true negatives = 26) represents the number of healthy individuals with a Matutes Score ≤ 3.

Kappa Coefficient
The principle of the Kappa coefficient is to correct the observed concordance by the concordance that could be linked to chance [7].

For a Matutes score greater than or equal to 4 the kappa accord measure is 58%.

For a Matutes score greater than or equal to 3 the kappa accord measure is 97%.

Discussion
The study of the MFI medians of the different markers of the B-CLLL diagnostic panel at the Hassan II University Hospital of Fez allows to refine the diagnosis by the realization of phenotypic profiles specific to the pathology. In fact, few cases were reported in our study (91 cases), and few published data on the MFI because these measurements are very "technique dependent" and specific to each laboratory. This makes the measurement of MFI medians insufficient to interpret phenotyping for diagnostic purposes, hence the interest of further investigation in order to assert the diagnosis.

In Order to study with precision the discriminating power of each marker between a populations of interest, we have established ROC curves, for a test, which allows to highlight the discriminating capacity of each marker, useful for the realization of scores aimed at diagnosis with more certitude.

In B-CLL, the diagnostic value of a single marker is insufficient, so it is useful to combine the results of several diagnostic markers in order to propose a diagnostic hypothesis with greater certainty. Threshold values for the most discriminating markers in B-CLL

according to the ROC curve are chosen according to a better specificity because it is a chronic hemopathy of older subjects whose diagnostic approach consists in confirming the diagnosis by flow cytometry and a false negative result has no serious consequences to the patient because it is an incurable pathology even with an early diagnosis.

The association of the discriminatory markers either in double or triple labeling did not increase the power of the test; the specificity of 100% of the three discriminatory markers did not change either in single or combined labeling while the sensitivity of the test decreases by associating the markers and also we risk losing many real positives (patients); Note that the expression of CD5, CD23 and CD43 is specific for B-CLL according to our results, but the absence of one or more of these markers does not exclude the diagnosis of CLL, because there are cases of CLL negative CD5, for example, described in the literature, hence the interest in interpreting the results according to the expression of each marker, opting for other specific markers in order to improve the power of the test, especially for cases of atypical LLC such as CD200 and CD180 [8, 9].

The evaluation of the Matutes score according to our own threshold values implies the study of the different performance indicators of this diagnostic test. The study of the distribution of patients with a diagnosis of B-CLL according to the value of the Matutes score will allow us to validate the value of the score adapted to our threshold values determined according to our study population, our own marking techniques, reagents, adjustments, etc.

For a Matutes diagnostic test greater than or equal to 4, sensitivity and specificity give us little information because we do not know if the patient presents the diagnosis or not, but we know to the contrary the result of the test that must be interpreted [10].

This information is provided by another pair of indicators; the Positive Predictive Value which is 100%, corresponds to the probability that the diagnosis of CLL is actually positive with a diagnostic test greater than or equal to 4, while the Negative Predictive Value, which is the probability that the diagnosis of CLL is actually negative with a Matutes score less than 4, is 54%.

Results that must be completed by another diagnostic performance indicator the kappa coefficient to evaluate the concordance between the score value and the presence or absence of disease. In this situation, the kappa coefficient is only 58%. These results disagree with the literature because the diagnosis of B-CLL is straightforward with a Matutes score greater than or equal to 4 except for a few cases of atypical CLL with a score of 3. For a Matutes diagnostic test greater than or equal to 3 the PPV is 100% and the NPV is 96% the kappa coefficient is 97% so we can conclude that the diagnosis of B-CLL is changed to a score value greater than or equal to 3 instead of greater than or equal to 4.

These results and in addition to the patient's clinical and biological data may imply the diagnosis of B-CLL in front of a score greater than or equal to 3 in our structure, and we insist on enlarging the diagnostic panel of B-CLL through other more specific and more discriminating markers.

Conclusion

The validation of Matutes scores adapted to a Moroccan population suggests the development of recommendations for inter-laboratory standardization and Continue this work by enlarging our cohort with a larger number of prospective and comparing other markers to confirm these initial results in order to obtain statistically significant and usable results for optimal diagnosis and treatment.

Declaration of Interest

The authors declare that they have no conflict of interest in relation with this article.

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