Immunophenotypic Characterization by Flow Cytometry of Chronic Lymphoid Leukemia

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Abstract

Introduction: Chronic lymphoid leukemia (CLL) is a malignant proliferative disorder of incompetent mature-looking lymphocytes that accumulate in peripheral blood (PB), bone marrow (BM) and lymphoid tissues. Currently, the flow cytometry (FC) technique has demonstrated its great utility in the immunophenotypic classification of this entity. **Objective:** To characterize the CLL immunophenotype. **Methods:** A descriptive, cross-sectional study was carried out to determine the cellular immunophenotype of samples from BM and PB from 64 patients with CLL using the FC technique at the Institute of Hematology and Immunology, during the period 2018-2020. Monoclonal antibodies directed against B and T lymphoid differentiation antigens were used. The reading was performed on a GALLIOS, Beckman Coulter cytometer and the data obtained were analyzed using the Kaluza computer program. **Results:** Out of the total patients studied, 28 were male and 36 were female. The most frequently expressed antigens were: CD45 (100%), CD19 (98.4%), CD5 (95.3%) and CD23 (79.7%). Women showed high expression of CD5 and CD5/CD19 (42.2%) in relation to men. The CD19$^+$/CD20$^+$ combination was statistically significant. There was low expression of CD38 (12.5%) and the prognostic marker CD49d predominated. **Conclusions:** Cell immunophenotyping proved to be a useful procedure to confirm the clinical and morphological diagnosis of CLL, which has a heterogeneous antigenic expression pattern.

Keywords: Immunophenotype, Chronic lymphoid leukemia, Flow cytometry

Introduction

Chronic lymphoid leukemia (CLL) is a malignant hematological disease characterized by the accumulation of mature lymphocytes with a defect in the induction of apoptosis that massively accumulate in peripheral blood (PB), bone marrow (BM) and lymphoid tissues. It is the most common lymphoproliferative disorder and adult leukemia in Western countries, it occurs more frequently in men than in women, and the average age at diagnosis is 72 years [1]. It is part of the chronic lymphoproliferative syndromes (CLPS) and in 95% of patients it involves B lymphocytes, in which a monoclonal proliferation occurs [2].

CLL patients can present with a wide range of symptoms and signs at the time of diagnosis. However, 70% of them are diagnosed incidentally during a routine blood count [3,4]. Diagnosis requires the presence of absolute lymphocytosis $>5 \times 10^9$ clonal B lymphocytes/L (5000/μL) in SP, morphological and immunophenotypic criteria. [5]

In Cuba, the diagnosis of CLL was made initially by leukemic cell morphology. Later, with the introduction of cellular immunophenotyping techniques, it was performed by indirect immunofluorescence and later, by the immunocytochemical ultramicromethod. Currently, the flow cytometry (FC) technique is used, the method of choice for the identification and immunophenotypic characterization of leukemic cells. It is characterized by the use of fluorochrome-conjugated monoclonal antibodies (mAb) that are detected and visualized by means of a computer system [6-8]. It has multiple advantages over the use of the fluorescence microscope and immunocytochemical techniques, among which are: analyzing a high number of particles in suspension, in a short period of time (5000 particles/sec), offering simultaneous information on several parameters, such as cell size and complexity, in an objective and precise way, in a large number of cells [9], which makes it a powerful tool for the immunological classification of patients with CLL.

This work aims to characterize the immunophenotype by flow cytometry of adult patients with CLL evaluated at the Institute of Hematology and Immunology (IHI) in Havana, Cuba.

Methods

Design, subjects and obtaining the sample

A descriptive, cross-sectional study was conducted from January 2018 to March 2020 in adult patients diagnosed with CLL, whose samples from BM and PB were processed in the Immunology laboratory of the IHI.

Inclusion criteria: Patients over 18 years of age with a clinical and morphological diagnosis of CLL.

Exclusion criteria: Patients who have started antileukemic treatment and received whole blood transfusion in the last month.

Samples from PB and BM were obtained by venipuncture extraction and through the medullogram respectively, 3 mL in each case, which were deposited in tubes with ethyleneaminetetraaceticacid.

Laboratory procedures and flow cytometry

50 μL of the samples from BM and PB were dispensed into each 15 ml tube, which were incubated with 5 μL of the corresponding combinations of mAb for 30 minutes, in the dark and at room temperature (RT). The red blood cells were lysed with ammonium chloride for 10 min at RT. Subsequently, the cells were washed twice with 0.9% sodium chloride and centrifuged for 10 min at 1500 rpm. Finally, they were read on a GALLIOS cytometer, Beckman Coulter, located in the Immunology department of the IHI.

The identification and quantification of the antigens expressed in CLL was developed by the FC technique, with the use of mAbs directed against B and T lymphoid differentiation antigens (Ags). In all cases, mAbs were previously titrated and conjugated directly from the manufacturer to the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and phycoerythrin cyanine 5 (PC5). The mAb panel that was used was: AntiCD4FICT/CD8RPE/CD3PC5 *; Anti-CD20FICT/CD5RPE/CD19APC *; Anti-CD23RPE *; Anti-CD25 RPE **; Anti-CD38 FICT **; Anti-CD45 PC5/APC *; Anti-CD49dFICT *; of the firms Beckman Coulter (*) and Milteny (**).
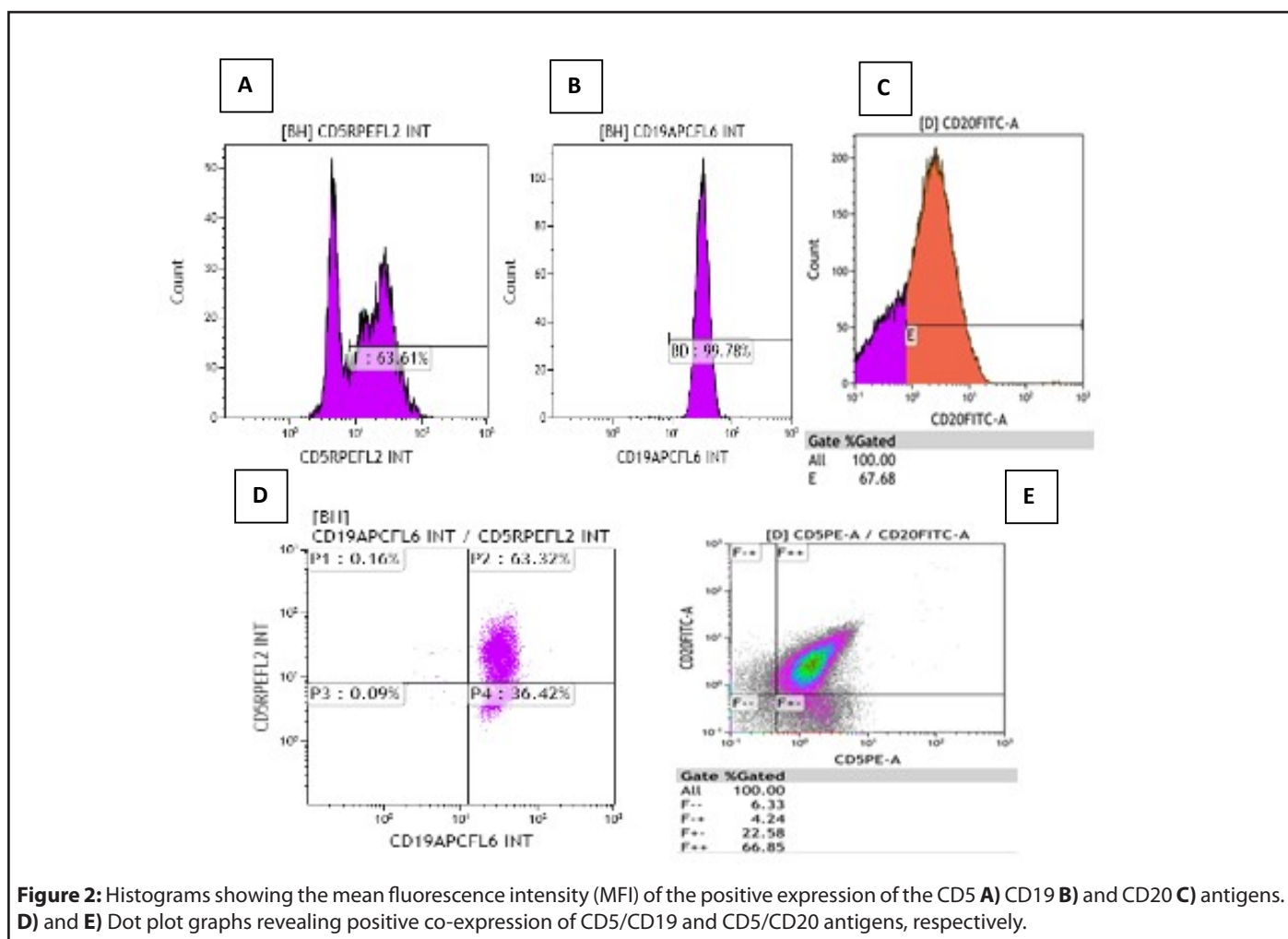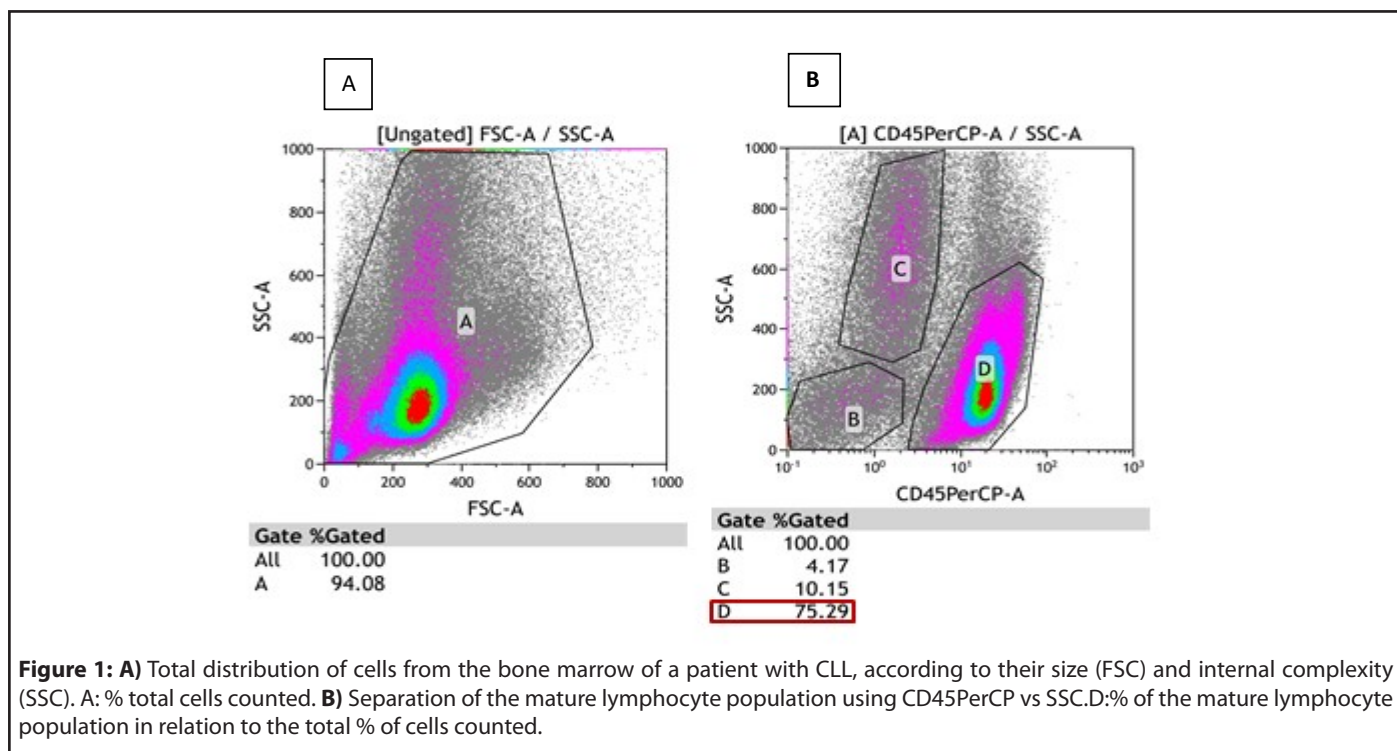
At the time of acquiring the cells in the cytometer (10^4 cells), a previous calibration was carried out with the sample that contained only whole blood (negative control). When the diffracted light hit the same axis as the incident light, the cell size was evaluated through the "forward scatter" (FSC). On the other hand, when the diffracted and reflected light was detected at 90° from the laser direction, the internal cellular complexity or granularity was evaluated through the "sidescatter" (SSC) (Figure 1A). The histograms corresponding to the control tube were analyzed, through which the limits of the fluorescence emission of the fluorochromes used were established.
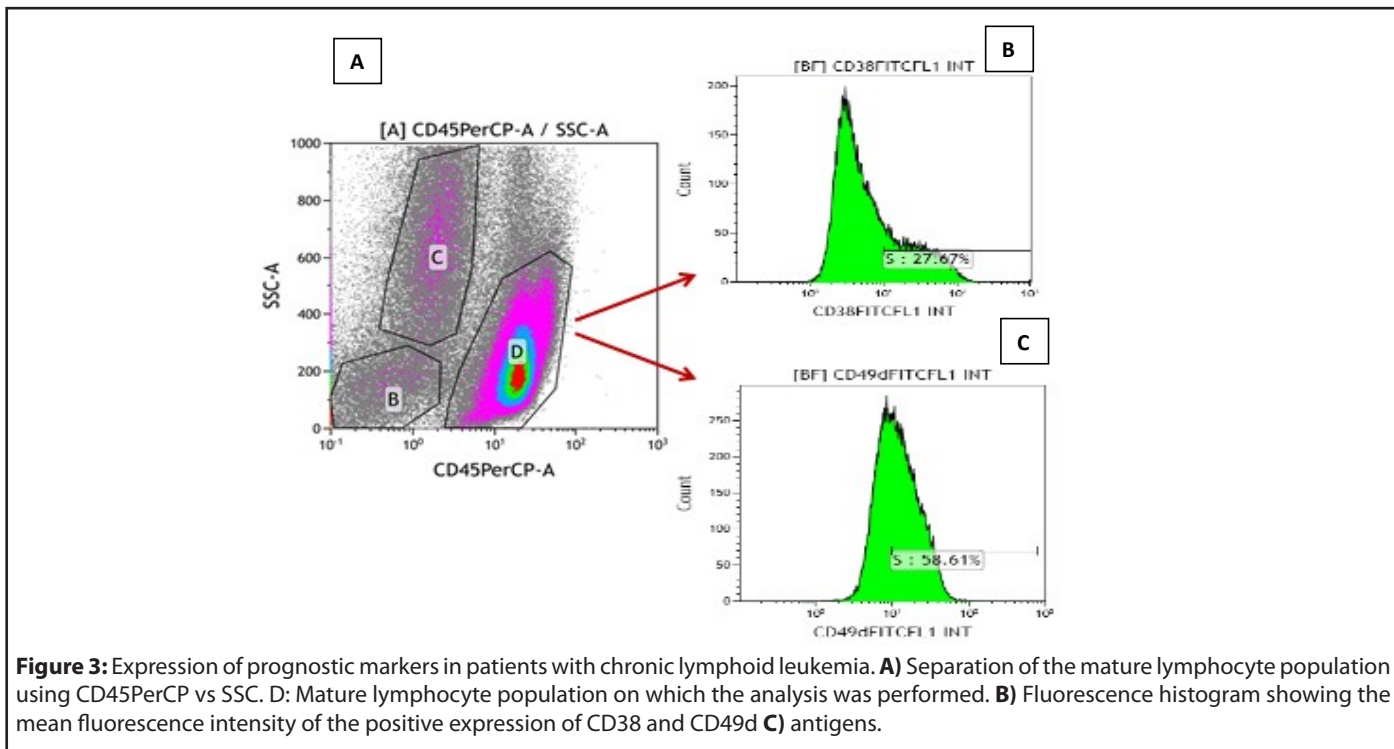
The plot of points CD45PerCP vs SSC was used to separate the lymphocyte population from the remaining cell populations and from the cellular debris, and the analysis was performed on this (Figure 1B).

Fluorescence histogram graphs were used to evaluate the expression of the CD3, CD4, CD8, CD5, CD19, and CD20 antigens through the mean fluorescence intensity (MFI) and dot graphs to evaluate the co-expression of the CD3+/CD4+ antigens and CD3+/CD8+; CD5+/CD20+; CD5+/CD19+; CD19+/CD20+ (Figure 2). The data obtained were analyzed using the Kaluza software, version 1.2. It was considered positive if the expression of the antigen was ≥ 20% and >30% in the case of the markers CD49d and CD38, respectively (Figure 3). In relation to the density of antigenic expression, it was considered low, if the percentage was less than 20%, moderate, between 20 and 50%, and high, greater than 50% of the leukemic lymphocytes that expressed the antigen.

Statistic analysis

A Microsoft Excel database was created with the information obtained. The data were processed through the statistical program MedCalc using the percentage values, the absolute frequency and the mean for the sample description. The 95%

Figure 1: A) Total distribution of cells from the bone marrow of a patient with CLL, according to their size (FSC) and internal complexity (SSC). A: % total cells counted. **B)** Separation of the mature lymphocyte population using CD45PerCP vs SSC.D:% of the mature lymphocyte population in relation to the total % of cells counted.

Figure 2: Histograms showing the mean fluorescence intensity (MFI) of the positive expression of the CD5 **A)** CD19 **B)** and CD20 **C)** antigens. **D)** and **E)** Dot plot graphs revealing positive co-expression of CD5/CD19 and CD5/CD20 antigens, respectively.

Figure 3: Expression of prognostic markers in patients with chronic lymphoid leukemia. **A)** Separation of the mature lymphocyte population using CD45PerCP vs SSC. D: Mature lymphocyte population on which the analysis was performed. **B)** Fluorescence histogram showing the mean fluorescence intensity of the positive expression of CD38 and CD49d **C)** antigens.

confidence interval was calculated. The variables analyzed were age, sex, and expression of immunological markers, whose associations were analyzed with the Chi-square statistic. It was considered statistically significant if $p<0.05$.

Ethics

This study was conducted in accordance with the Declaration of Helsinki [10] and was approved by the IHI Research Ethics Committee.

Results

64 patients were studied, 36 of them female, representing 56, 25%, and 28 male, without statistical significance ($p=0.57$). The predominant age group was 70 to 79 years, which accounts

for 31.5% of all patients, with a mean of 75.1 and 72.5 years for women and men respectively, as shown in Table 1.

The most frequently expressed Ags were: CD45 in100% of the patients, CD19 (98.4%), CD5 (95.3%), CD5/CD19 co-expression (90.6%), CD23 (79.7%) and CD20 (53.1%). In only 3 patients, CD5 Ag was not expressed. There was low expression of the Ags CD3/CD4 and CD3/CD8, since only 11 and 16 patients expressed it respectively, with a predominance of the female sex in the expression of all markers. No antigenic expression showed statistical significance. It is necessary to take into account that the same patient expressed several antigens, which is why regardless of the numerical amount and percentage of antigenic expression of each patient studied, the total (64) remained the same (Table 2).

Table 1: Distribution of patients with chronic lymphoid leukemia according to age and sex.									
Age (years)	**Female (n=36)**				**Male (n=28)**				
	Absolute frequency	**Mean**	**CI**	**% of total**	**Absolute frequency**	**Mean**	**CI**	**% of total**	**Total No. (%)**
<50	-	-	-	-	2	44.0	-	3.1	2 (3.1)
50-59	11	53.5	51.7 - 55.3	17.2	8	55.6	52.9 - 58.3	12.5	19 (29.7)
60-69	10	64.7	62.7 - 66.6	15.7	7	66.0	63.8 - 68.1	10.9	17 (26.6)
70-79	11	75.1	73.3 - 76.9	17.2	9	72.5	70.2 - 74.8	14.1	20 (31.3)
80-90	4	86.7	80.3 - 93.1	6.25	2	86.5	67.4 -105.5	3.1	6 (9.3)
Total	36	66.9	63.04- 70.7	56.2	28	65.04	60.8 - 69.2	43.8	64 (100)
p=0.57; n: number of patients; CI: Confidence Interval									

Table 2: Distribution of patients with chronic lymphoid leukemia according to sex and antigen expression.

Antigens	Female (n=36)		Male (n=28)		Total No. (%)	p
	Absolute frequency	% of total	Absolute frequency	% of total		
CD3/ CD4	7	19.4	4	14.3	11 (17.1)	0.84
CD3/ CD8	9	25	7	25	16 (25)	0.77
CD5	34	94.4	27	96.4	61 (95.3)	0.82
CD19	35	97.2	28	100	63 (98.4)	0.89
CD20	22	61.1	12	48.9	34 (53.1)	0.23
CD5/ CD19	34	94.4	24	85.7	58 (90.6)	0.45
CD5/ CD20	16	44.4	11	39.3	27 (42.1)	0.88
CD19/ CD20	13	36.1	13	46.4	26 (40.6)	0.56
CD23	27	75	24	85.7	51 (79.7)	0.46
CD45	36	100	28	100	64 (100)	-
n: number of patients						

Considering the origin of the analysis sample, 43 were obtained from BM (15 men and 28 women) and 21 from SP (13 men and 8 women) (Results not shown in the Table 2).

Taking into account the density of antigenic expression in relation to sex (Table 3), CD45 predominated with high expression in both women and men (53.1% and 42.2%, respectively), followed in women by CD5 and the co-expression of CD5/CD19, both with 42.2%. CD19/CD20 co-expression was statistically significant (p=0.04). In both female and male sex, with moderate expression, CD20 and CD5/CD20 co-expression predominated in 17.2% and 9.4%, respectively. The CD3/CD4 combination was the one with the lowest expression (female 46.8% and male 34.4%).

In relation to prognostic markers, CD49d expression predominated in 24 female patients in relation to men (18), for a total of 42 patients, which constituted 65.6% of the total of those patients who expressed prognostic Ags (50), much higher than CD38, which represented only 12.5% of the total, expressed in 8 patients, 5 of them were female and 3 were male. The expression of both markers did not show statistical significance (CD49d, p=0.94 and CD38, p=1, respectively). Taking into account the combination of these antigens, the expression of high CD49d/high CD38 6, 25%, low CD49d/low CD38 26.6% was obtained. They showed a discordant phenotype CD49d high/CD38 low 60, 9% and CD49d low/CD38 high 6, 25% of the patients studied, respectively (Results not shown in tables).

Table 3: Antigenic expression density in patients with chronic lymphoid leukemia, according to sex.

Antigens	Low Expression Fem (n=36) Male (n=28) No. (%) No. (%)	p	Moderate Expression Fem (n=36) Male (n=28) No. (%) No. (%)	P	High Expression Fem (n=36) Male (n= 28) No. (%) No. (%)	p
CD3/ CD4	30 (46.8) 22 (34.4)	0.87	6 (9.4) 5 (7.8)	0.84	1 (1.6) 0 (0)	0.89
CD3/ CD8	26 (40.6) 22 (34.4)	0.77	7 (10.9) 5 (7.8)	0.87	1 (1.6) 3 (4.7)	0.43
CD5	2 (3.1) 1 (1.6)	0.82	8 (12.5) 6 (9.4)	0.82	27 (42.2) 20 (31.2)	0.97
CD19	1 (1.6) 1 (1.6)	0.58	8 (12.5) 3 (4.7)	0.38	26 (40.6) 25 (39.1)	0.17
CD20	14 (21.9) 17 (26.6)	0.14	11 (17.2) 6 (9.4)	0.59	6 (9.4) 10 (15.7)	0.15
CD5/ CD19	1 (1.6) 5 (7.8)	0.10	9 (14.1) 3 (4.7)	0.26	27 (42.2) 19 (29.7)	0.73
CD5/ CD20	20 (31.3) 16 (25)	0.89	11 (17.2) 6 (9.4)	0.59	5 (7.8) 6 (9.4)	0.65
CD19/ CD20	23 (35.9) 15 (23.4)	0.56	8 (12.5) 2 (3.1)	0.19	5 (7.8) 11 (17.2)	0.04*
CD23	9 (14.1) 3 (4.7)	0.26	4 (6.3) 6 (9.4)	0.43	23 (35.9) 19 (29.7)	0.94
CD45	0 (0) 0 (0)	-	2 (3.1) 1 (1.6)	0.82	34 (53.1) 27 (42.2)	0.82
n: number of patients; Fem: Female; *: statistically significant						

Discussion

CLL is more common in men than women, and it usually occurs later in life. However, 10% of the patients reported in a variety of clinical trials are under 50 years of age. This differs from what was found in this study, where there was a female prevalence of 56, 25%, which we attributed to the peculiar demographic characteristics of our country, although it was diagnosed in only 2 patients under 50 years of age (3.1%), a result that is in agreement with that reported by other researchers [11].

The immune system is inherently different between men and women, and the genetic basis translates into disparities in, among other things, lymphocyte groups, cytokine and chemokine production, and response intensities to antibiotics. In particular, hormones shape the development and functioning of the immune system and influence the functional activity of immune cells and their responses, which also results in the sex-specific prevalence of infectious and autoimmune diseases, respectively. As neoplasms originating from immune cells, it has been hypothesized that chronic infectious and inflammatory conditions cause the formation and growth of preneoplastic cells that can develop into lymphoid neoplasms. For CLL, chronic antigenic stimulation has been strongly implicated for leukemogenesis, based on the high frequency of nearly identical B-cell receptors in unrelated patients. The hormonal profiles of male and female patients are significantly altered compared to healthy individuals of the same sex [12,13].

As another cause of the sexual disparities observed in cancer incidence and survival, the sex chromosomes have been implicated. A study by Dunford and colleagues [14], conducted in mainly solid but also lymphoid tumors, found sex-specific loss-of-function mutations and consequently differential expression of several genes on the sex chromosomes. Furthermore, differential DNA methylation by sex has been reported [12]. In CLL patients, a strong change in DNA methylation pattern is reported, suggesting that this may play a role in sex-related differences [14,15].

The disease is seen primarily in the elderly, with the incidence increasing rapidly with age. The highest incidence occurs in the age group 75 to 84 years, with a mean age of diagnosis of 72 years [16]. In the present study, the peak incidence was in the age group 70 to 79 years, with a mean 75.1 and 72.5 years for women and men, respectively.

Gogia and colleagues reported a median age of 59 years from a large serial study in India, which emphasized the basis for ethnic variations in epidemiology. In fact, early epidemiological studies have emphasized the importance of racial and genetic influences in the development of the disease [16]. For their part, Cmunt et al., in an immunophenotyping study of 100 patients with B-CLL, found that the mean age was 58.2 years [17], different from the data obtained in this investigation.

The characterization of CLPS by immunophenotyping of cell membrane proteins using the FC technique has become an important and widely used diagnostic method in hematology, taking on special importance in the case of CLL, since it allows the diagnosis to be made both in the PB as well as the BM [18].

Quantitative measurements of MFI by FC evaluate the number of mAb molecules bound to a cell. The fluorescence intensity of the staining correlates with the number of fluorochrome-conjugated mAb molecules bound to the cell and, consequently, with the number of receptors existing in the cell. Thus, the number of antigen receptors can be determined by estimating the exact number of bound fluorescent antibody molecules, provided that suitable fluorescence standards and controls are tested in parallel and reagents are used in saturating amounts [19]. Historically, routine testing often uses percentages and compares antigenic expression with the distribution of an immunoglobulin isotype. Typically, a limit of 20% above the isotype limit is used to designate a marker as positive or negative [20].

The leukemic cells of CLL-B have an immunophenotypic pattern defined by positive expression of the specific Ags of B cells: CD19, CD20, CD22; strong positivity of TCD5 Ag, although there is an absence of other T markers; they express only a light chain of immunoglobulins (κ or λ) and a low density of surface immunoglobulins. All these elements are necessary for an accurate diagnosis of CLL and its differentiation from other CLPS [21,22].

The antigenic frequency found in the patients with B-CLL in this investigation was similar to that reported by other authors, since there was an expression of the Ags CD19 in 98.4% and CD5 in 95.3%. Dwilewicz-Trojaczek, in his immunophenotypic analysis of 54 patients with B-CLL, found that 100% expressed CD5 Ag and 97% CD19. [23] However, although CD5 Ag expression by neoplastic cells is considered a diagnostic criterion for B-CLL, it is well known that 7-20% of patients do not express this Ag [23,24], a higher percentage than that obtained in this research, in which there was not expression of this marker in 4.7% of the cases studied.

The CD5 molecule is a type I membrane glycoprotein belonging to group B of the SF-SRCR-type cysteine-rich domains superfamily of receptors. They are expressed in mature T lymphocytes, medullary thymocytes, and in a subtype of B cells called B1a, which is involved in the production of polyreactive autoantibodies in autoimmune diseases and in lymphoproliferative processes such as B-CLL [13,16,25,26].

The positivity of the Ags CD5 and CD23 were significantly correlated with the diagnosis. For its part, the frequency of expression of CD23 represented 64% of all patients and was absent in 23 patients, a figure higher than that of a study carried out by Marcela et al., where the non-expression of CD23 was seen in 5 patients and they occurred in three that also presented additional adverse prognostic factors (high

β2 microglobulin, accelerated leukocyte doubling, among others) [27]. In a study by Shirin [28], CD23 positivity was observed in 81.25%, higher than that found in this study. The importance of CD23 expression in CLL is currently debated, because it has been found to be associated with a good prognosis, active disease, and prolymphocytoid morphology. It has been suggested that CD23- cases could be leukemic cases of centrocyticlymphoma, which may also be CD5+ [29].

CD45 Ag belongs to a complex family of high molecular weight glycoproteins, composed of five isoforms; it possesses tyrosine phosphatase activity and performs an important function in the regulation of cell differentiation. It is expressed on the cell surface of all hematopoietic cells; its density increases in the final stages of hematopoiesis; except, in erythroid cells and platelets where it loses its expression throughout differentiation and maturation [30]. In this study, the frequency of CD45 expression was 100%, an expected result, since it is a hemopathy that occurs in mature lymphocytes. It can be assumed that the CD45 isoforms expressed by various B cell malignancies reflect the stage of differentiation of those B cells that expand in the neoplastic process [31].

In this study, other Ags that showed low frequency of expression were CD3/CD4 (17.1%) and CD3/CD8 (25%), which is in agreement with the expected results in the context of patients with B-CLL. CD3 is an important component of the T cell receptor, where antigen recognition takes place during an immune response, while CD4 and CD8 act as coreceptors. Thus, in CLL, a low number of CD3 receptors can lead to a reduction in the initial activation signal on the surface of T cells, contributing to an altered immune response [19].

On the other hand, the Ags with a higher density of moderate expression were CD20 and CD5/CD20 coexpression in the female sex, both account for 17.2%. In this series of cases, the high density of CD20 expression was 25%, higher in males, represented by 10 patients (15.7%). In a study by Ayalew and colleagues, the intensity of the CD20 antigen was classified as weak in 58 patients (62%), moderate in 11 (12%), and strong in 24 (26%) [32]. Differential expression of CD20 has been reported in CLL, depending on the site of origin of the sample (BM, lymph node (LN) or PB), with the highest mean number of anti-CD20 antibodies bound per cell, in lymphocytes from SP; and the lowest average number of anti-CD20 antibodies bound per cell; found in BM and LN aspirate samples [33]. Heterogeneity in CD20 expression exists not only between different CLL patients but also between anatomical sites [34]. In this sense, in this study most of the samples from PB were made up of men with 13 patients, a result in accordance with that reported in the literature. The differences in the density of expression of the CD20 antigen by site in CLL have relevance with respect to the clinical response to anti-CD20 mAb therapy [35]. Of the total number of patients who expressed CD20, 61.1% was represented by the female sex, where a better prognosis is expected, regardless of age and CLL stage,

and a better response to treatment [36]. CD20 is expressed in B cells and in a small population of T cells in PB and BM, and its biological function is the proliferation and activation of B lymphocytes. [37] Quantification of this significant disease marker by development of standardized reference standards, controls, and protocols would increase their clinical utility. A possible therapeutic implication concerns the infusion toxicity of Rituxan, the anti-CD20 mAb, which may be related to higher levels of expression of the CD20 antigen [38].

The high density of expression was represented by the Ags CD45, CD5 and CD19/CD5 in both sexes, with significant CD19/CD20 co-expression. This last combination was found only in a few patients. Alternatively, the strong expression of CD19/CD20 in this disease could result from the expansion of a subset of cells with high expression of the CD19 and CD20 antigens, which is rarely represented in normal tissues. However, it is certain whether the cell-bound antibody values for CD19 and CD20 in leukemia reflect different stages of cell development or represent abnormal phenotypes, due to neoplastic transformation [39]. The percentage and intensity of CD20 in CLL probably reflects an earlier stage of lymphocyte maturation compared to normal blood B lymphocytes and other malignant B cells [40].

Several immunophenotypic prognostic markers in CLL are described. The inclusion of mAb panels of those directed against Ags CD38, CD49d and ZAP70 is recommended. It is considered a poor prognosis when the patient has more than 30% of leukemic cells that express CD38, CD49d, or both, or more than 20% of ZAP70 expression. [2] The prognostic markers CD49d and CD38 are involved in various cellular functions relevant to the pathogenesis of CLL: localization of CLL cells in lymphoid organs, survival, and proliferation [41].

A low frequency of CD38 Ag expression (12, 5%) was found in the study patients. Other authors such as Domenech, [42] have found it expressed in 19% of the patients studied. In a study carried out by Massimo et al., an incidence of patients with CD38+ CLL of 14% was obtained, according to the results obtained in this study [43], well below those reported by Damle et al.; Del Poeta and colleagues, who ranged from 27% to 55% [44,45]. The low rate of patients expressing CD38+ lymphocytes in their lymphocytes can be explained by the strict immunophenotypic and morphological diagnostic criteria used, which allowed us to analyze only the patients with CLL and ruled out the inclusion of other CD5+ lymphoproliferative disorders with leukemic effusion.

Clinical studies indicate that B-CLL CD38+ is characterized by a more aggressive clinical course and with more complex cytogenetic alterations than CD38-B-CLL, data that reinforce the value of CD38 as a prognostic factor in this disease [1,24,42]. At the end of the 1990s, different investigations, including those carried out by Damle and Domenech, concluded that the CD38 surface antigen was an independent prognostic marker, whose expression it was associated with

shorter overall and treatment-free survival [42,44].

In addition, CD49d is the α4 chain of the integrin that pairs non-covalently with CD29 (β1 chain) to form the very late activation antigen 4 (VLA-4) and not only participates in cell adhesion to fibronectin and vascular cell adhesion molecule 1 (VCAM-1), but also in intercellular leukocyte interactions [8]. It is a critical regulator of CLL cell migration, allowing tumor cells to pass from the blood to the protective microenvironment of LN and BM. [46] The high level in patients with B-CLL is associated with an aggressive course of the disease and is considered one of the negative prognostic markers [47]. In this series of cases, it was found that 65.6% of the patients expressed CD49d, results that coincide with those of the study carried out by Al-Rubaie HA et al. [48] in Iraq that showed positivity for CD49d (60%). However, in a study by Haq and colleagues [49], CD49d was positive in 21 (41.2%) cases, lower than those obtained in the present study.

Zucchetto et al. were the first in 2006 to report a strong association between the expression of CD38 and CD49d in CLL cells using both parameters as categorical variables. When comparing overall survival rates, a combined low CD38/low CD49d phenotype was attributed to the better prognosis. Of the 115 samples 27 cases were investigated (23%). However, they showed a discordant phenotype CD38 low/CD49d high or CD38 high/CD49d low. Patients with this discordant phenotype showed better overall survival rates compared to the combined CD38 high/CD49d high phenotype. Recently, they found a comparable 21.5% rate of discordant cases when analyzing 144 samples [41,50]. In this study, the expression of CD49d high/CD38 low was obtained in 60.9%, CD49d low/CD38 high and CD49d high/CD38 high in 6.25%, CD49d low/CD38 low in 26.6%, respectively, results different from those found by other researchers.

The authors consider that they constitute limitations of the study not to have quantified the expression of the intracytoplasmic antigen ZAP-70, as well as the expression of the light chains of immunoglobulins κ and λ, evaluations that will be taken into account in future investigations.

Conclusions

Characterization by FC proved to be a useful procedure to confirm the clinical and morphological diagnosis of CLL, which constitutes one of the pillars on which the diagnosis and follow-up of patients with this entity is supported. CLL has a heterogeneous antigenic expression pattern; some are case specific and differ from normal polyclonal B lymphocytes. In this sense, the antigenic coexpression of the surface membrane provides a basis for the more extensive exploration of its role in the pathogenesis of the same, which should be investigated in a larger group of patients. This should provide a stable platform to evaluate the contribution of cellular markers to the diagnosis and prognosis of this entity. In turn, the more in-depth study of CLL, will allow the best understanding in the

clarification of its nature related to sex, in order to optimize the approaches of personalized medicine.

Competing Interests

No conflicts of interest are declared between the participating institutions and researchers.

Declarations of Interest

None.

CRediT Author Statement

Yenisey Triana Marrero: Conceptualization, Methodology, Investigation, Validation, Data Curation, Writing - Original Draft. **Vianed Marsán Suárez:** Conceptualization, Investigation, Writing – Review & Editing. **Yaneisy Duarte Pérez:** Investigation. **Gabriela Diaz Dominguez:** Investigation. **Imilla Casado Hernández:** Investigation. **Elizabeth Hernández Ramos:** Formal analysis, Investigation. **Rosa María Lam Díaz:** Formal analysis. **Consuelo Macías Abraham:** Review & Editing, Supervision.

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