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Identification of neoplastic cells in peripheral blood by flow cytometry in patients with B-cell non-Hodgkin's lymphoma

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Original article

ABSTRACT

Introduction: Non-Hodgkin lymphoma (NHL) is the most common hematological neoplasm in the world [(1)]. New techniques have been studied to allow for faster diagnosis of the disease, such as flow cytometry immunophenotyping (FCI). **Methods:** The possibility to identify the presence of circulating tumor cells (CTCs) in the peripheral blood of patients with NHL was attempted through FCI, and the findings were compared with the initial staging by bone marrow biopsy. Peripheral blood samples from nine patients with NHL were evaluated using a panel of monoclonal antibodies in order to detect B-cell chronic lymphoproliferative disorders. **Results:** Bone marrow biopsies and peripheral blood FCI were found to be discordant in three of the nine samples studied. Discordant cases were positive for neoplasia in peripheral blood immunophenotyping, and negative in immunohistochemical analysis of the bone marrow. In these three discordant cases, the tumor cells in the blood sample corresponded to less than 5% of the cellularity of the sample. **Conclusion:** Even though the use of peripheral blood remains poorly investigated regarding staging and monitoring of NHL, the findings of this study allowed us to define the importance of FCI in the early identification of neoplastic cells in patients with NHL.

Key words: diagnostic techniques and procedures, flow cytometry, non-Hodgkin's lymphoma, peripheral blood.

INTRODUCTION

The diagnosis of non-Hodgkin's lymphoma (NHL) is usually confirmed by conventional anatomopathological examination and immunohistochemical studies of the affected tissue. Since there are no antigenic markers specific for any lymphoid neoplasm, a combination of morphological features with an antibody panel is needed to achieve accurate diagnosis [(2)].

Fine-needle cytology can aid in the diagnosis of NHL, but as cytological features alone are not enough to diagnose and classify the disease, additional techniques are needed. Furthermore, diagnostic sensitivities and specificities differ widely [(3-6)]. The combination of fine-needle cytology and flow cytometry immunophenotyping (FCI) has played an important role in identifying useful antigens for prognostic evaluation [(7)], as well as antigens targeted for specific therapies. However, this methodology may be compromised because truly representative samples are not always obtained.

FCI has the advantage of allowing us to work with small samples of low cellularity, to analyze multiple parameters in a single cell, and to assess fluorescence intensities, which enable prompter differentiation between neoplastic and reactive populations [(8)]. Peripheral blood FCI is already widely used to diagnose some clinical entities such as chronic lymphocytic leukemia/monoclonal B lymphocytosis and autoimmune lymphoproliferative syndrome. Additionally, it is used as a screening tool for primary immunodeficiencies, acute leukemias with leukocytosis and paroxysmal nocturnal hemoglobinuria as well.

Currently, the use of FCI for diagnosing lymphomas is not well defined yet. However, it is known that circulating tumor cells (CTCs) are often present in some NHL subtypes, such as follicular and mantle cell lymphomas and, hence, these subtypes have been the target of studies, particularly for disease monitoring purposes. Therefore, that may also be useful for initial diagnosis [9-10]. Within this scenario, the present study aimed to identify CTCs in the peripheral blood of patients with NHL by FCI, and to compare the results with those of initial staging performed by bone marrow biopsy, subsequently following up on these patients during and at the end of treatment.

MATERIALS AND METHODS

Nine patients diagnosed with B-cell NHL treated at the Onco-Hematology Service at the Clinical Hospital (*Hospital de Clínicas*) of the Federal University of Triângulo Mineiro were evaluated from August 2019 to June 2021. Data regarding staging, type of treatment, and clinical outcomes are shown in Table I.

Table I. Clinical and diagnostic characteristics of the patients:

Identification	Age (years)	Pathological diagnosis (Ann Arbor staging)	Bone marrow trephine byopsy at the moment of the diagnosis	Treatment	Post-treatment status
Case 1	63	Orbital MALT Lymphoma (IIIA)	Negative for lymphoma	R-CHOP 21 (8 cycles) + Intrathecal CNS prophylaxis	Complete remission
Case 2	74	Follicular lymphoma for right breast (IAE)	Negative for lymphoma	R-miniCHOP 21 (6 cycles) + local RT	Complete remission
Case 3	75	Gastric DLBC lymphoma (IBE)	Not performed	R-miniCHOP 21 (6 cycles)	Complete remission
Case 4	63	Grade 3 follicular lymphoma (IVA)	Positive for lymphoma	R-CHOP 21 (7 cycles)	Complete remission
Case 5	85	DLBC lymphoma (IIBX)	Negative for lymphoma	R-miniCHOP 21 (8 cycles)	Partial remission

Case 6	64	DLBC lymphoma (IVA)	Positive for lymphoma	R-CHOP 21 (8 cycles) + Intrathecal CNS prophylaxis	Complete remission
Case 7	27	HIV-associated Burkitt lymphoma (IVB)	Positive for lymphoma	CODOX-M/ IVAC (4 cycles) + Intrathecal CNS prophylaxis	Complete remission
Case 8	42	DLBC lymphoma (IVBE)	Negative for lymphoma	CHOP 21 (3 cycles)	Death before completing therapy
Case 9	29	PMLBCL (IVA)	Negative for lymphoma	DA-EPOCH-R (1 cycle)	Change of follow-up location

MALT: Mucosa-associated lymphoma tissue; DLBC: diffuse large B cell; HIV: human immunodeficiency virus; PMLBCL: Primary mediastinal large B-cell lymphoma; CNS: central nervous system; RT: radiotherapy. R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone. R-miniCHOP: reduced-dose regimen of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone. CODOX-M/ IVAC: cyclophosphamide, vincristine, doxorubicin, and methotrexate (CODOX-M) alternating with ifosfamide, etoposide, and cytarabine (IVAC). DA-EPOCH-R: dose adjusted of rituximab, etoposide, prednisolone, vincristine, cyclophosphamide and doxorubicin.

Peripheral blood (10 mL) was collected from each patient by venipuncture using ethylenediaminetetraacetic acid (EDTA). The samples were collected on three occasions: before the onset of chemotherapy treatment, from six to nine weeks after the onset of treatment, and at the end of the treatment.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the density gradient separation method with Ficoll-Paque PLUS (GE Healthcare). PBMCs were added to a 10:1 solution of fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) for preservation at -80°C until analysis. For evaluation of immunophenotypic markers, the cells were thawed and then labeled with anti-human monoclonal antibodies (Table II). Antibody clones are described in the supplementary material.

Table II. The panel of monoclonal antibodies used in the study:

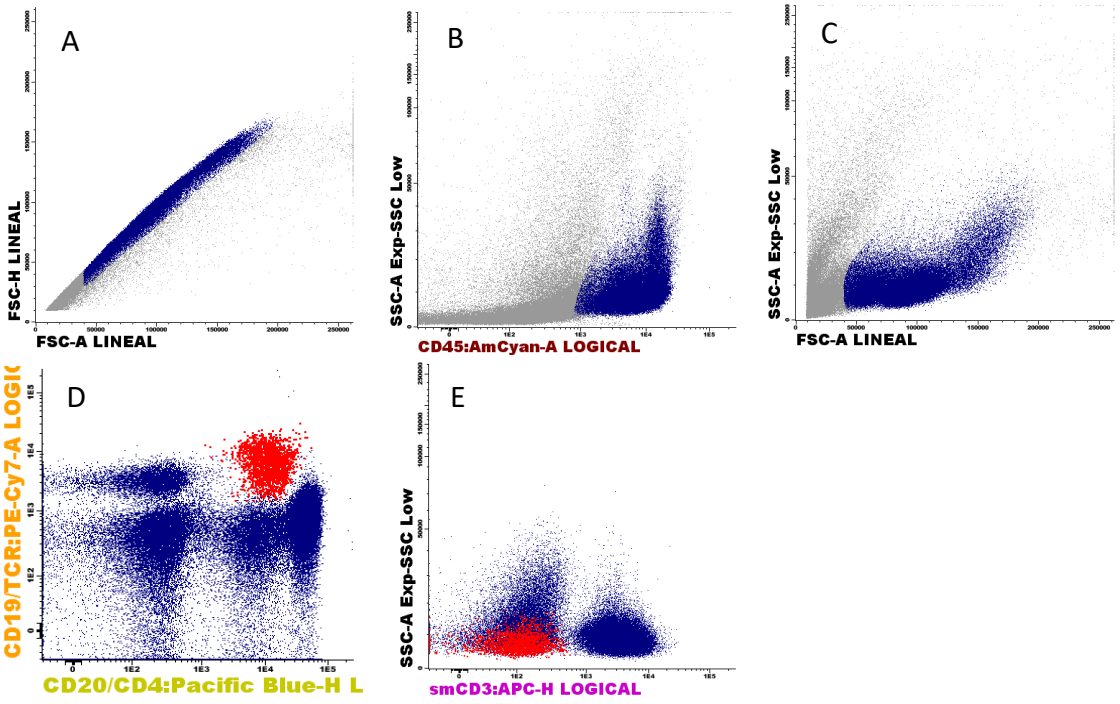
Fluorochrome	**Pacific Blue**	**Pacific Orange**	**FITC**	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**APC**	**APC-H7**
Tube 01	CD20/CD4	CD45	CD8/kappa	CD56/lambda	CD5	CD19/TCRγδ	CD3	CD38
Tube 02	CD20	CD45	CD23	CD10	CD79b	CD19	CD200	CD43

Tube 03	CD20	CD45	CD31	LAIR1	CD11c	CD19	IgM	CD81
Tube 04	CD20	CD45	CD103	CD95	CD22	CD19	- -	- -
Tube 05	CD20	CD45	CD62L	CD39	HLA-DR	CD19	CD27	- -

FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy5.5: peridinin-cyanine chlorophyll protein 5.5; PE-Cy7: phycoerythrin-cyanin 7; APC: allophycocyanin; APC-H7: allophycocyanin-H7.

Cell quantification after thawing ranged from 1.1×10^6 to 19.4×10^6 cells. All cells from each sample were acquired in a FACSCanto II flow cytometer (Becton Dickinson, California, USA). The number of events analyzed per case ranged from 81,021 to 4,947,146, excluding debris and doublets. The results were analyzed using Infinicyt™ version 2.0 software (Cytognos, Salamanca, Spain) according to the strategy described in Figure 01.

Figure 01. Gating strategy for analysis of B lymphocytes. Selection of events suitable for analysis (in blue) in the singlet region (A), CD45 positive (B), and exclusion of debris by the region of negative side scatter (SSC) and forward scatter (FSC) (C). Next, the B lymphocytes (in red) of each sample were selected by CD19 and CD20 positivity (D). In the first tube, in addition to the expression of CD19 and CD20, positive CD3 events were excluded (F).



Immunohistochemical staining was performed on histological sections of bone marrow fixed in formaldehyde and embedded in paraffin. The sections were then deparaffinized and rehydrated. After that, specific antibodies were incubated in a steam cooker at 121°C and 18 psi. A secondary antibody was added to the sections, and polymer reaction amplification and diaminobenzidine staining were performed. Positive controls were used for these markers. Immunohistochemical staining was performed for cell-type identification with antibodies specific for CD3 (Epsilon chain) and CD20 (L26) in all cases. In some cases, monoclonal antibodies to the following antigens were also used: BCL-6 (PG-B6P), CD10 (56C6), or a proliferation-related protein (Ki-67).

RESULTS

All the samples collected during and at the end of treatment were negative for neoplasia identification (Table III). Complete depletion of B lymphocytes (normal and neoplastic) was observed in all of these samples. The immunophenotypic analysis of PBMCs in the pre-treatment of the evaluated cases showed the following findings:

Table III. Results of the search for circulating mature B lymphoid tumor cells by FCI in the cases described:

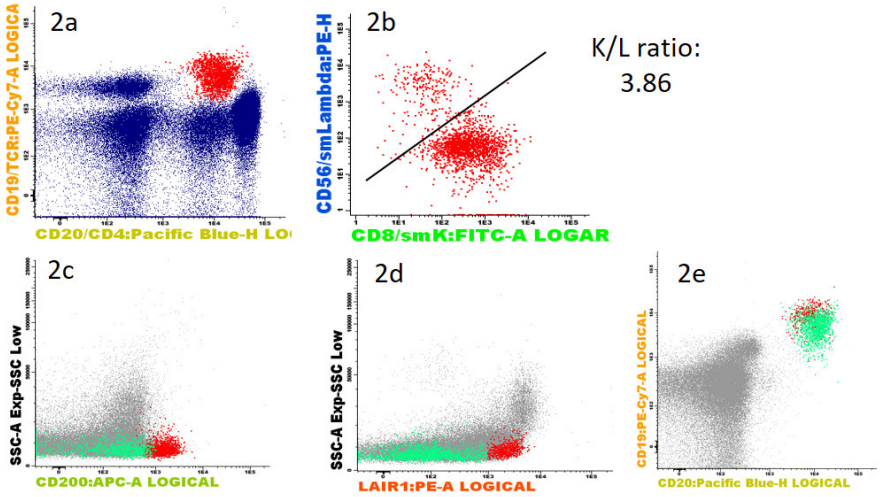
	Case 1	**Case 2**	**Case 3**	**Case 4**	**Case 5**	**Case 6**	**Case 7**	**Case 8**	**Case 9**
Pre-treatment	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Negative	Positive
During treatment	Negative	Negative	Negative	Negative	NP	Negative	Negative	NP	NP
End of treatment	Negative	Negative	Negative	NP	Negative	Negative	Negative	NP	NP

NP: Not performed. Case 4: it has not been possible to collect the third sample yet. Case 5: it was not possible to collect the second sample. Case 8: death, it was not possible to collect the second and third samples. Case 9: patient discontinued treatment in this institution, so it was not possible to collect the second and third samples.

In case 1, the sample comprised of 2.82% B cells, with a Kappa/Lambda ratio of 3.86 (expected from 0.3 to 3) [(11)]. Approximately 35% of the B cells showed a lower

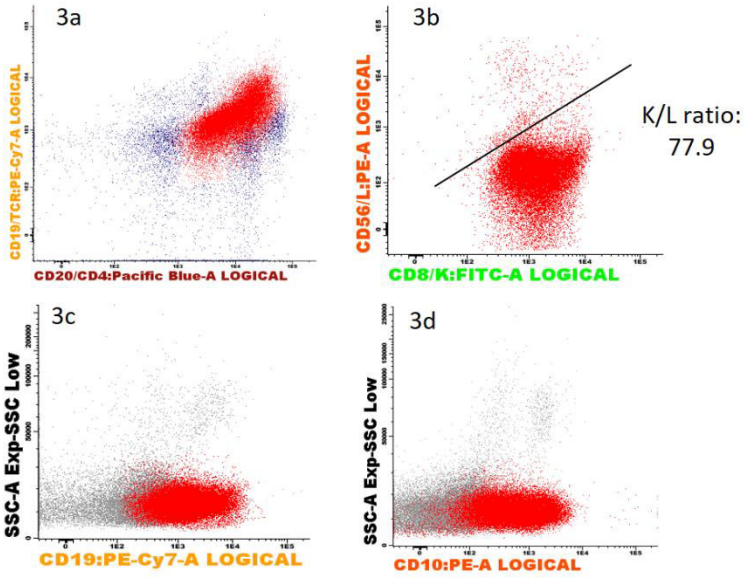
expression of CD200 and LAIR1, which may represent circulating marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) (Figure 02).

Figure 02. B lymphocytes in red (A). Kappa/Lambda ratio changed (B). In green, part of the B lymphocytes with loss of expression of CD200 (C) and LAIR-1 (D).



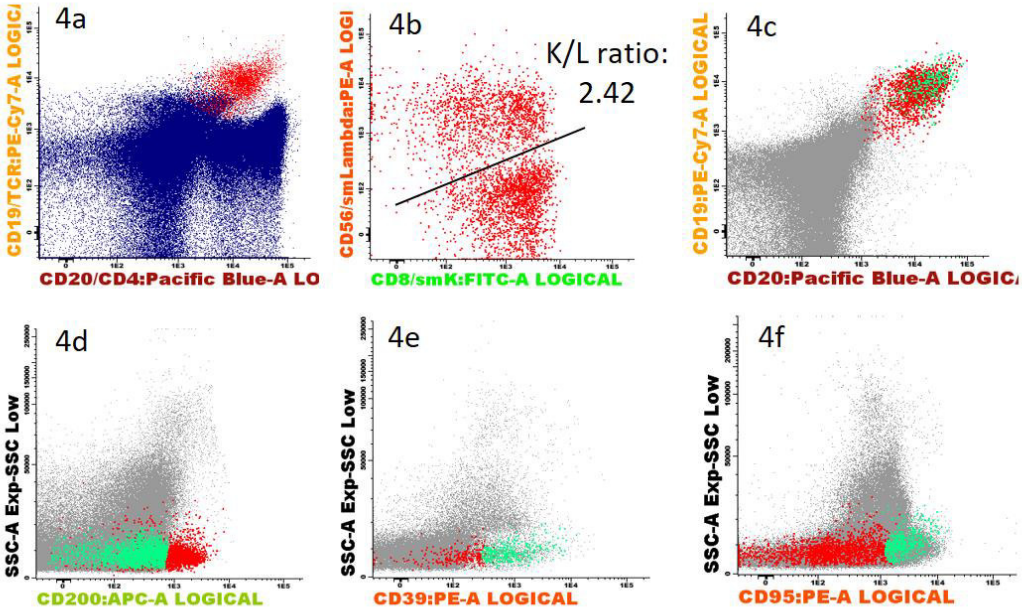
In case 2, 4.9% of the cells in the sample were B cells, and had a Kappa/Lambda ratio of 2.2. Events that could represent circulating follicular lymphoma cells were not detected. In case 3, 19.28% of B cells were observed in the sample, with a Kappa/Lambda ratio of 1.24. Some B cells expressed more CD23 and LAIR1, but there was no clear separation between possibly neoplastic and normal populations. The sample was considered negative for neoplasia. In case 4, the sample consisted of 84% of B cells with a Kappa/Lambda ratio of 77.9. The cells had a weaker expression of CD19 and CD10 than usual, which was compatible with infiltration by follicular lymphoma (Figure 03).

Figure 03. B lymphocytes in red (A). Kappa/Lambda ratio changed (B). B lymphocytes with hypoexpression of CD19 (C) and positivity for CD10 (D).



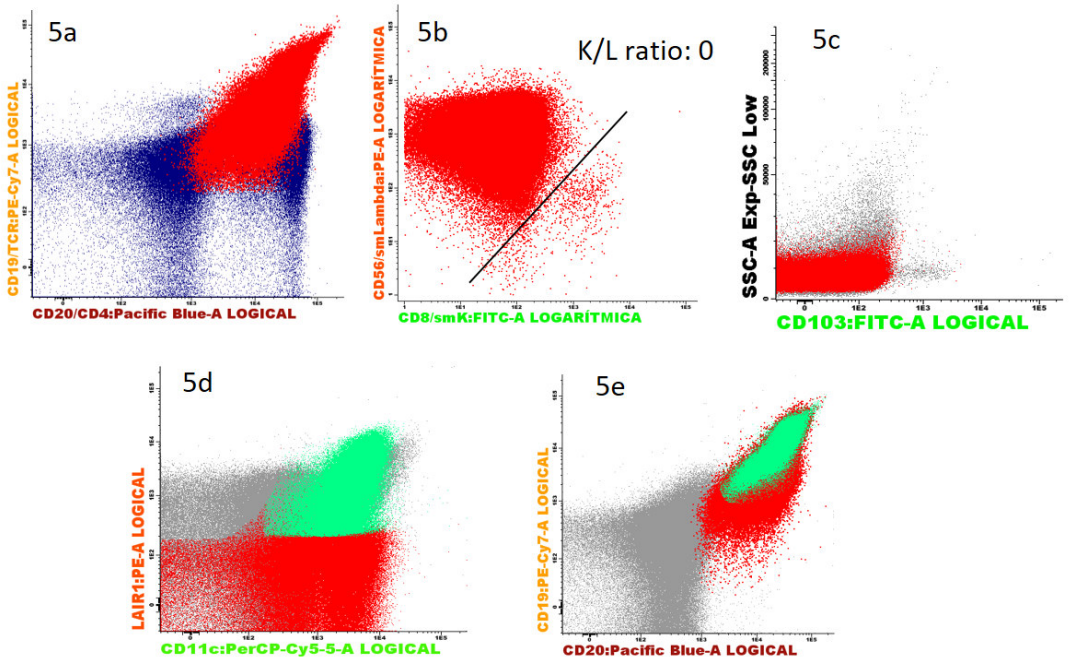
In case 5, 3.16% of the cells in the sample were B cells with a Kappa/Lambda ratio of 2.42. Despite the normal Kappa/Lambda ratio, a population of approximately 39% of B cells expressing CD95 and CD39 was observed, which may represent diffuse large B-cell (DLBC) CD10 negative cells (Figure 04).

Figure 04. B lymphocytes in red (A). Normal Kappa/Lambda ratio (B) but, in green, part of the B lymphocytes with loss of expression of CD200 (D) and overexpression of CD39 (E) and CD95 (F).



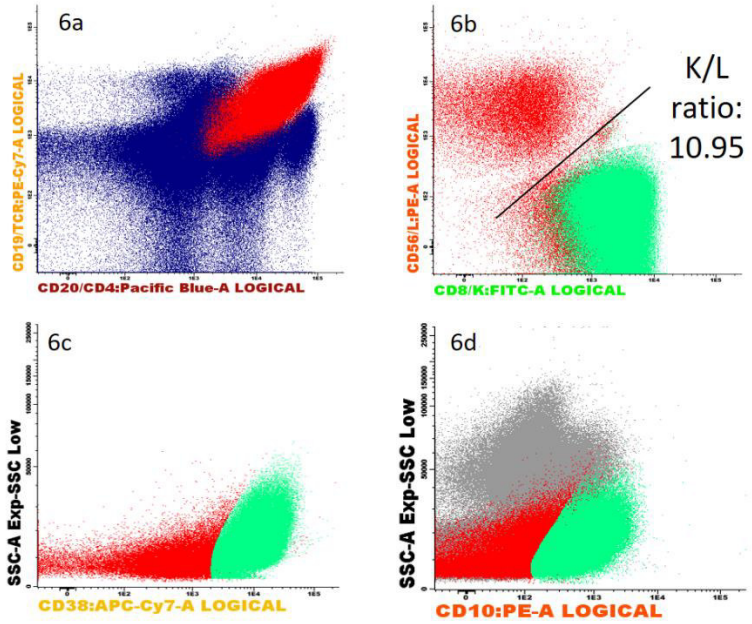
In case 6, 60.2% of B cells in the sample had a Kappa/Lambda ratio of 0.0 (99.8% of the B cells were Lambda). Of these, 45% expressed CD11c, LAIR 1, and CD23. In this case, the CD103 marker was negative. The patient had massive splenomegaly and bone lesions. Pathological examination of the right acetabular lesion showed DLBC lymphoma, but the clonal lymphocyte population was divided into two groups based on the peripheral blood immunophenotype. A part of it coexpressed CD11c and LAIR1, leading us to question whether it could be a compound lymphoma (Figure 05).

Figure 05. B lymphocytes in red (A). Change in the Kappa/Lambda ratio (B). B lymphocytes were negative for CD103 (C), but part of them coexpressed CD11c and LAIR1 (D).



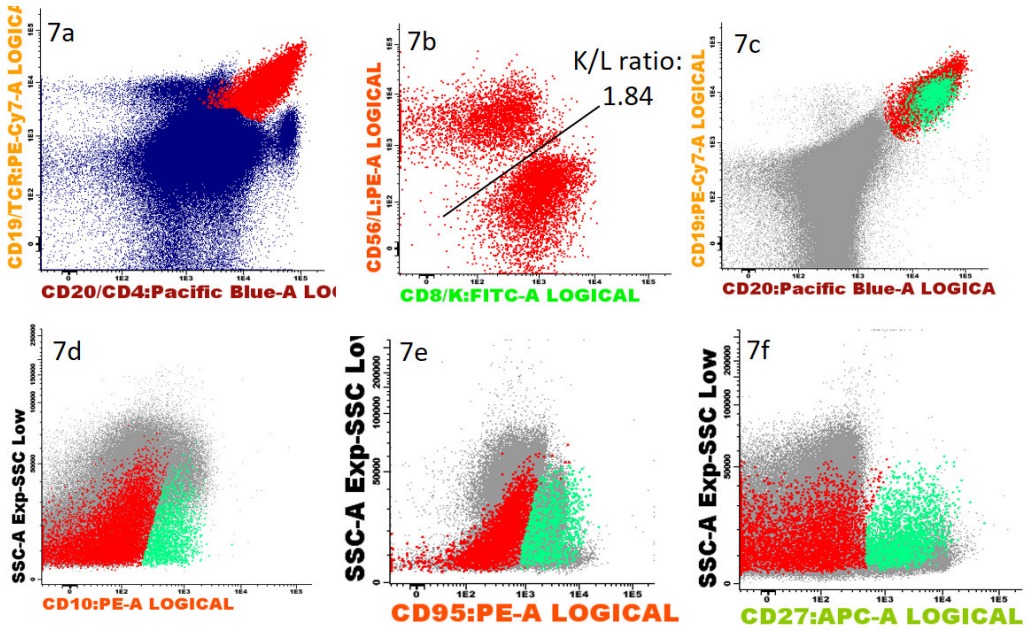
In case 7, 53% of B cells were found in the sample, with a Kappa/Lambda ratio of 10.95. Kappa cells overexpress CD38. Positivity for CD10, sIgM, and CD81 (Figure 06) was also observed and was compatible with infiltration by Burkitt's lymphoma.

Figure 06. B lymphocytes in red (A). Change in the Kappa/Lambda ratio (B). Part of B lymphocytes (in green) expressing CD38 (C) and CD10 (D).



In case 8, 3.54% of the sample were B cells, with a Kappa/Lambda ratio of 1.84. We considered this sample negative for neoplasia. In case 9, 14.93% of B cells were found in the sample, with a Kappa/Lambda ratio of 1.52/1. Despite the normal Kappa/Lambda ratio, a population of approximately 14% of B cells expressed CD10, 24.5% expressed CD95, and 25% expressed CD27, which probably represents DLBC circulating cells (Figure 07).

Figure 07. B lymphocytes in red (A). Normal Kappa/Lambda ratio (B). Part of B lymphocytes (in green) express CD10 (D), CD95 (E) and CD27 (F).



In the present study, the results of bone marrow biopsy and FCI analysis of peripheral blood were found to be in discordance in three of the nine samples studied. Discordant cases showed positivity for neoplasia in peripheral blood immunophenotyping, and negativity in the immunohistochemical study of the bone marrow (Table IV). In these three discordant cases, the tumor cells in the blood sample corresponded to less than 5% of the cellularity of the sample.

Table IV. Agreement between immunophenotypic examination of peripheral blood and bone marrow biopsy at the time of diagnosis concerning neoplastic involvement

	Case 1	**Case 2**	**Case 3**	**Case 4**	**Case 5**	**Case 6**	**Case 7**	**Case 8**	**Case 9**
FCI analysis of peripheral blood	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.
Bone marrow biopsy	Neg.	Neg.	NP	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.

Pos.: positive. Neg.: negative. NP: not performed.

DISCUSSION

Complete and accurate staging of NHL is of paramount importance to determine the extent of the disease and, therefore, therapeutic decision making and prognostic evaluation too. Bone marrow involvement in lymphoma is assessed using bone marrow biopsy as a standard technique [12]. However, with advanced diagnostic techniques, several researchers have studied the role of FCI in the diagnosis and staging of patients with NHL. Most of these studies evaluated bone marrow aspirate in search of tumor cells; however, data and standardization of protocols supporting this process in clinical practice are still limited. Some studies have also investigated the role of peripheral blood analysis in the diagnosis and follow-up of NHL [13].

The concordance rates between NHL staging by flow cytometry and histopathological examination vary between studies. Borahm Kim *et al.* [12] compared 248 samples of various subtypes of NHL and found a concordance rate of 91.5% between these techniques. FCI has not yet replaced bone marrow biopsy in the staging process; however, multiparametric FCI may increase the sensitivity of this assessment, especially when the biopsy is inconclusive. Furthermore, FCI also has the advantage of enabling study of small populations. Despite the role of minimal bone marrow involvement in the diagnosis of NHL, it is not well elucidated whether it is deemed as a prognostic factor. Comparison of flow cytometry and histopathological results between the three discordant cases showed less than 5% of tumor cells in the blood samples. We can infer a minimal infiltration in the bone marrow and, thus, hindered identification by anatomopathological examination.

The immunophenotypic patterns obtained by multiparametric flow cytometry are also used for accurate diagnosis of specific lymphoma subtypes, and they help discover peculiar clinical presentations, such as composite lymphomas [14]. Composite

lymphomas are defined as the occurrence of two types of lymphomas in a single patient. That may be the occurrence of two distinct types of NHL or a Hodgkin and non-Hodgkin lymphoma simultaneously, which is an extremely rare situation [14]. Coincidentally, in this study, a case classified as diffuse large B-cell non-Hodgkin's lymphoma (DLBCNHL) was detected by performing anatomopathological examination of an acetabular lesion with spinal cord involvement; however, in the immunophenotypic examination of peripheral blood, a portion of neoplastic B cells (clonal for Lambda) was found to express CD11c and LAIR1, with negative CD103. This was believed to be a splenic marginal zone lymphoma or hairy cell leukemia concomitant with DLBCNHL. Clinically, the patient presented with massive splenomegaly.

FCI can also be performed in tissues affected by neoplasms by using a manual tissue disaggregation method. There was a good correlation between anatomopathological and immunophenotypic diagnoses in tissues [15]. In the context of oncological urgency, as is the case of tumors with a high proliferation rate, for instance, the results of immunophenotypic tissue analysis may direct early therapy, whereas immunohistochemical examination determines the definitive diagnosis because it is faster.

In addition to aiding in diagnosis and staging, FCI plays an important role in monitoring NHL. Measurable residual disease (MRD) in NHL is a matter of interest and debate, as novel treatments offer higher rates of complete responses than in the past, thus having a positive impact on long-term survival [16]. The definition of MRD includes all methods capable of measuring a disease more accurately than conventional tools. Technologies used to assess MRD can be divided into three main categories: (1) those derived from molecular biology, (2) flow cytometry, and (3) imaging.

In NHL, the role of FCI in MRD research is not well established. Some studies have reported that the correlation between flow cytometry and molecular results agrees in 80-85% of cases, with 10% of samples defined as MRD positive by polymerase chain reaction (PCR), but negative by flow cytometry. With respect to conventional microscopy, half of the cases were found to be concordant (negative in the microscope, but positive in the FCI). The differences in the sensitivity of the methods (1×10^{-5} for PCR, 1×10^{-4} /10^{-5} for FCI, and 1×10^{-2} for microscopy) may explain the discordances observed between the methods [17-19]. Despite having lower sensitivity compared to molecular techniques, flow cytometry can be performed in a shorter timeframe and at relatively low costs [20]. However, even reputable guidelines such as those edited by the European Society of Medical Oncology, despite recognizing the role of MRD in NHL, have not yet included it as a necessary routine test on patient management, which show the need for definitive standardization of the tools used [21].

Furthermore, in patients with solid tumors, epithelial cells can detach from the tumor mass and be found in the blood circulation [22]. These cells are commonly called circulating tumor cells (CTCs). Research on CTCs can be used as an early biomarker of cancer, as a parameter to assess the evolution of the disease or even response to treatment. [23]. Several techniques have currently been used to research CTCs, including: Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), Automated Digital Microscopy (ADM); Fiber-optic Array Scanning Technology (FAST), fluorescence microscopy and flow cytometry [24].

Lopresti *et al* [23] evaluated peripheral blood by flow cytometry from patients with refractory breast cancer and untreated colon cancer and compared it with healthy donors and found a significant difference ($p<0.0001$) in the quantity of circulating CTCs:

in healthy donors, less than 3 cells cells per millimeter of blood were found (average of 0.93+-0.75cells/mL) while the average CTCs in donors with colorectal cancer was 11.2 CTCs/mL and in donors with breast cancer it was 21.93 CTCs/mL. Mușină *et al* [25] also evaluated the peripheral blood of patients with colorectal cancer using flow cytometry three days after surgical treatment and identified that CTCs research can be used to identify patients at high risk of recurrence, which would justify the use of more aggressive therapies in this group.

In addition to CTCs, other particles can be released by tumors as cell-free nucleic acids, exosomes, or tumor-educated platelets (TEPs), and be found in any body fluid (i.e., blood, urine, cerebrospinal fluid, or others [26]. The search for these particles is known as liquid biopsy. In the context of hematologic malignancies, the analysis of circulating tumor DNA (ctDNA) from plasma represents the most used application of liquid biopsy, especially no for minimal residual disease (MRD) monitoring [27]. The limitation is that molecular methods, such next-generation sequencing (NGS) is not widely available in clinical routine. In this context, search for circulating tumor cells by flow cytometry may be more accessible. However, extensive studies should be carried out to demonstrate the benefit of its application.

There are few published papers on the immunophenotypic study of peripheral blood in the context of NHL staging and MRD. A study by Mancuso *et al.* [11] centered around the question: “if the NHL is in the bone marrow, will it also be in the peripheral blood?”. The researchers compared bone marrow and peripheral blood samples of 591 patients with NHL, including cases of follicular lymphoma, DLBCNHL, mantle cell lymphoma, marginal zone/ MALT lymphoma, lymphocytic lymphoma, CLL, and Waldenstrom’s macroglobulinemia. In their study, peripheral blood and bone marrow

immunophenotyping were found to be 95.1% concordant, with bone marrow examination being slightly more sensitive. Among the discordant cases, the most frequent diagnosis involved Waldenstrom's macroglobulinemia. Sixty-two percent of the discordant samples were collected after chemotherapy. The most frequent therapy associated with the discordant results was the use of rituximab as monotherapy or in combination with chemotherapy. Moreover, a concordant result between bone marrow immunophenotyping and bone marrow biopsy was reported to be 83% at diagnosis and 85% after treatment. Finally, the researchers concluded that, upon excluding Waldenstrom's macroglobulinemia, an overlap between peripheral blood and bone marrow FCI can be observed in NHL, hence suggesting the potentially important role of peripheral blood, even as an alternative to bone marrow biopsy, in the assessment of patients with NHL.

CONCLUSIONS

Peripheral blood immunophenotyping in patients with NHL provides relevant information, particularly at the time of diagnosis. In our study, although only a few cases were involved, 33% of them were discordant regarding anatomopathological staging. Furthermore, the discordant cases presented with low tumor burden, which can be explained by the higher sensitivity of the FCI of peripheral blood. In this study, peripheral blood FCI during and at the end of treatment was negative for NHL. Further investigation is needed to allow for a longer follow-up period of these patients, as well as for relapse prediction and for prognostic impact analysis of the examination. The use of peripheral blood samples for the staging and follow-up of patients with NHL has proved to be of paramount importance because of its benefits, namely the following: it is a sample that

is easy to obtain at a low cost and with little probability of adverse effects, and it is a relatively quick and sensitive laboratory technique. Clinical consensus must be created so that the technique is widely used in a validated and safe manner in the context of NHL.

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