Int J Clin Exp Pathol 2022;15(9):345-353
www.ijcep.com /ISSN:1936-2625/IJCEP0142087

Original Article

Diffuse large B-cell lymphoma: morphologic and immunohistochemical analysis of bone marrow for staging, with emphasis on lymphoid aggregates

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Received January 28, 2022; Accepted July 12, 2022; Epub September 15, 2022; Published September 30, 2022

Abstract: Diffuse large B-Cell lymphoma (DLBCL) may infiltrate bone marrow (BM) and evaluation of BM plays an important role in DLBCL staging. This study used BM samples from DLBCL patients for staging and analyzed the use of immunohistochemistry in the diagnostic management of these cases by the pathologist. Patients with DLBCL submitted to BM biopsy/aspiration for staging were studied according to clinical aspects, morphologic aspects, and expression of CD20 and CD3. The characteristics of lymphoid aggregates in the bone marrow and the power of histopathological diagnosis were studied, with immunohistochemistry as the gold standard for the decision of a neoplastic infiltration definition. An isolated morphological analysis showed low sensitivity (42.9%) for lymphoma detection in BM, which is disadvantageous. The median of three lymphoid aggregates in the BM (p-value = 0.02) and the presence of increased reticulin fibers (grade 2) in the lymphoid aggregate (p-value = 0.01) had significant associations with neoplastic infiltration. A morphological analysis must be accompanied by an immunohistochemical analysis in all cases, or when this is not possible, in cases with two or more lymphoid aggregates or an increase of reticulin within them.

Keywords: Lymphoma, bone marrow, immunohistochemistry, diffuse large B cell lymphoma, non-Hodgkin lymphoma

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma (NHL), in general, with an aggressive clinical behavior [1, 2]. Bone marrow (BM) involvement in these cases is estimated to occur in 12% to 35% of patients [3-5], determining more advanced stages and relevant prognostic implications [6].

Bone marrow biopsies are the classic method for staging and pathologic analysis may be done under morphological criteria, with the possibility of adding immunophenotypic aspects, by special immunohistochemistry (IHC) with lineage B and lineage T markers (CD20 and CD3, for example, respectively) [7].

DLBCL may infiltrate bone marrow tissue in a diffuse pattern, similar to the morphology observed in its main nodal or extranodal involvement, or in a discordant pattern, with a low-grade lymphoma infiltration interpreted, for example, in the context of the phenomenon of transformation [7]. For these situations, both the detection of lymphoid aggregates and the morphologic interpretation added to the reticulin and IHC stains, combine for more precise definitions as to the difference between benignity and malignancy. Talaulikar et al. (2007 [8] and 2008 [9]) and Baiyee et al. (2009) [10] show divergent results regarding the frequent use of IHC in the analysis of BM, suggesting the need for further investigations [8-10].

This research studied the BM staging of DLBCL patients in two situations: exclusively morpho-

Table 1. Signs and symptoms presented by the DLBCL patients

Signs and symptoms	N	%
Sweating	16/87	18.4
Fever	14/87	16.1
Itching	1/87	1.0
Weight loss	37/87	42.5
Extranodal mass	38/88	43.2
Adenopathies	50/88	56.8
Splenomegaly	3/101	3.0
Anemia	40/88	45.5
Thrombocytopenia	10/88	11.4
Leukopenia	9/88	10.2
Leukocytosis	10/88	11.4
Infiltration of CNS	5/79	6.3

CNS: central nervous system.

Table 2. Association between morphologic variables of lymphoid aggregates and the presence of infiltration in BM, defined only by IHC

Histopathologic analyses	Patients with infiltration in the BM (n = 21)		
	N	%	*p*-value*
Presence of lymphoid aggregates	21	100	<0.001
General increase of reticulin	2	9.5	0.597
Location of aggregates:			0.018
Peri-trabecular	13	61.9	
Non-peri-trabecular	5	23.8	
Clots	3	14.3	
Aggregates edges jagged	15	71.4	0.010
Increase of reticulin in aggregate	18	85.7	0.004
Blood vessels in aggregates	8	38.1	<0.001
Infiltration detected by H&E	9	42.9	<0.001

*Chi-squared or exact Fisher test, P<0,05. BM: Bone Marrow; HE: Hematoxylin-eosin stain.

logic analysis of BM, and morphologic analysis aided by IHC with CD20 and CD3 markers.

Material and methods

Patients

This was a retrospective and cross-sectional study with 101 bone marrow samples performed to stage patients diagnosed with DLBCL, by World Health Organization (WHO) criteria of the 2017 [1] edition. The casuistic consisted of patients from the Hematology Service of the Clinical Hospital of the Botucatu School of Medicine from São Paulo State University (HC-FMB-UNESP), between 1998 and 2017. The project was submitted to and approved by the local research ethics committee (CAAE: 66028417.3.0000.5411).

Clinical review

The medical records were reviewed regarding sex, age at the time of diagnosis, clinical manifestations ("B Symptoms", nodal and/or extranodal site, systemic involvement), time of clinical follow-up, outcome (cure, maintenance of the disease or death), treatment, and staging. In our study, unfavorable outcome was classified as patients who died, relapsed or maintained the disease.

Morphological review

Two types of BM samples were available: bone marrow biopsy (BMB) or bone marrow clot (BMC). Each of the samples were initially evaluated for their representativeness. For BMB, at least seven intertrabecular spaces with viable hematopoietic tissue were required, and at least five spicules of viable hematopoietic tissue for BMC.

The main object of histologic evaluation was the lymphoid aggregates, which were described regarding number, location, borders, vascularization and reticulin fibers, according to the WHO-2017 [1]. Cases in which neoplastic involvement was detected were classified according to the infiltration pattern. These analyses occurred in the same way with the biopsies and the clots. During the research, BMCs were evaluated for agreement with the findings of BMB.

Immunohistochemical study

Immunohistochemical reactions were performed at the Department of Pathology at FMB/UNESP in paraffinized samples. The histologic sections were subjected to immunohistochemical examination in an automated system, with antigenic recovery in TPLink (Dako) and incubation, development, and counter-staining in AutoStainer Link48 (Dako), using highly sensitive polymer and ready-to-use FLEX antibodies. The markers used were CD20 (Dako IR604,

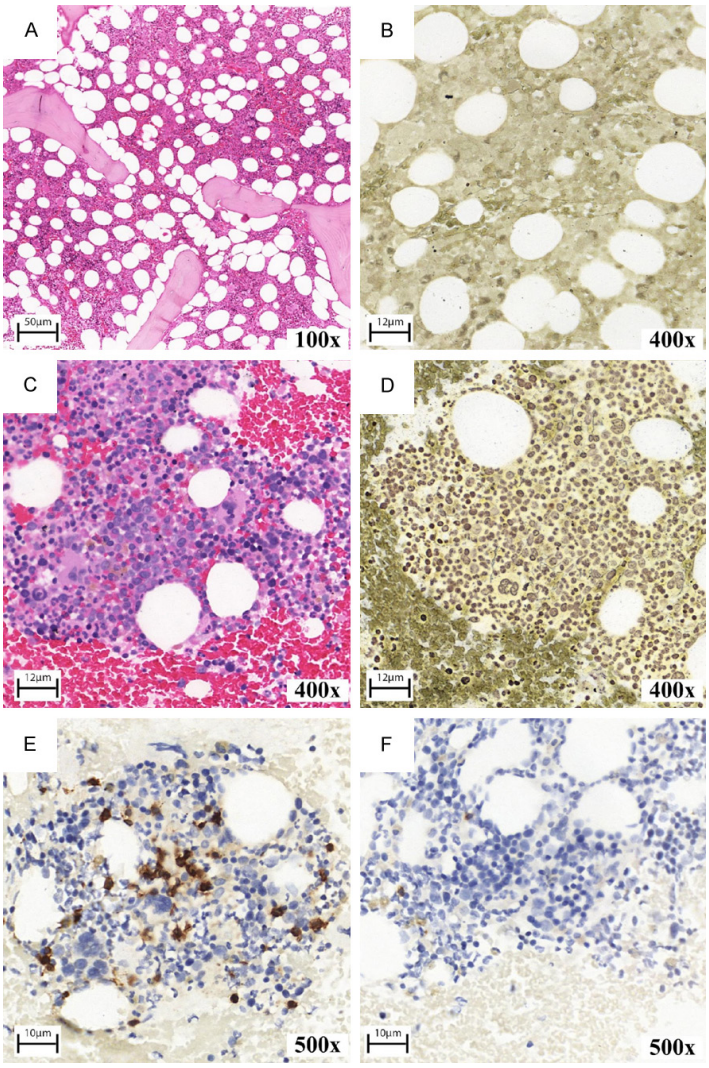

Figure 1. Photomicrograph of "pattern 1" lymphoid aggregate. Total predominance of T cells. This finding is considered a reactive aggregate. A. Hematoxylin-eosin stain, magnification 100×; B. Reticulin stain, magnification 400×; C. Hematoxylin-eosin stain, magnification 400×; D. Reticulin stain, magnification 400×; E. CD3 antibody, magnification 500×; F. CD20 antibody, magnification 500×.

clone L26, ready-to-use kit, Mouse) and CD3 (Dako IR503, UCHT1 clone, ready-to-use kit, Rabbit). The results were interpreted regarding their positivity or negativity and their proportion of T lymphocytes and B lymphocytes, either globally or in lymphoid aggregates, when present.

The lymphoid aggregate patterns were classified from 1 to 5, where 'Pattern 1' consists of accumulation exclusively populated by T lymphocytes, 'Pattern 2' consists of accumulation predominantly populated by T lymphocytes but with a small number of B cells, 'Pattern 3' with an equal proportion of B and T cells, 'Pattern 4' with a predominance of B cells, and 'Pattern 5' has a total population of B cells. Only patterns 4 and 5 were considered positive for malignant infiltration.

Statistical analysis

A descriptive evaluation of morphological, immunohistochemical, and clinical data was performed. For diagnostic tests, sensitivity (S), specificity (E), positive predictive value (PPV), negative predictive value (NPV), and accuracy (A), when appropriate, were calculated, including their 95% confidence intervals. Based on the obtained sensitivity and specificity, *receiver operating characteristic* (ROC) curves were constructed for certain morphologic variables. The variables, as needed, were subjected to normality tests and then analyzed by parametric and non-parametric tests. The kappa agreement index was performed, and its result was interpreted according to Landis and Koch (1977): <0.0 insignificant; 0.0-0.20 weak; 0.21-0.40 reasonable; 0.41-0.60 moderate; 0.61-0.80 strong; 0.81 to 1.00 almost perfect [11]. Data were analyzed using SPSS for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA). A *p*-value less than 0.05 was considered significant.

Results

Clinical profile of the patients

Among 101 patients, the median age was 57 years old (7 to 89 years old). **Table 1** shows the frequency of clinical findings, considering patients with medical records and available information. None of the clinical characteristics studied showed significant differences when they were tested for associations with the presence of BM neoplastic infiltration.

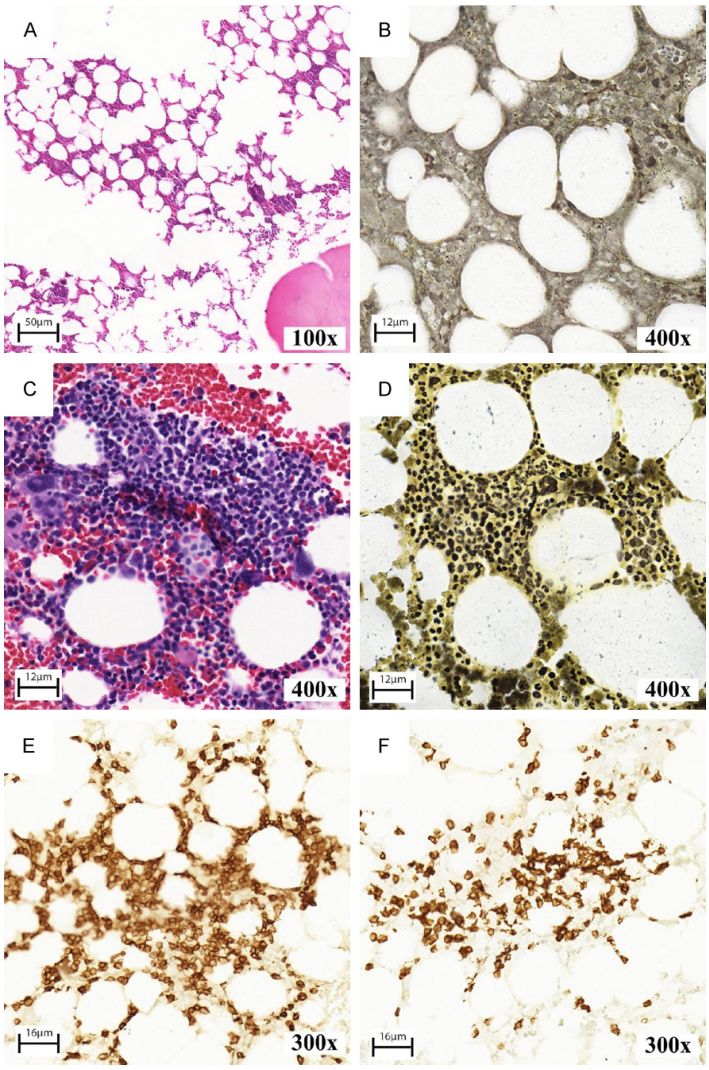

Figure 2. Photomicrograph of "pattern 2" lymphoid aggregate. High predominance of T cells. This finding is considered a reactive aggregate. A. Hematoxylin-eosin stain, magnification 100×; B. Reticulin stain, magnification 400×; C. Hematoxylin-eosin stain, magnification 400×; D. Reticulin stain, magnification 400×; E. CD3 antibody, magnification 300×; F. CD20 antibody, magnification 300×.

Of the patients who underwent chemotherapy treatment, 27/88 (30.7%) used the regimen with rituximab (R-CHOP). Radiotherapy was applied to 25/88 patients (28.4%). Unfavorable outcome was detected in patients who died (39.2% of cases), relapsed (35.2%), or maintained the disease (5.4%). Among the 87 individuals with available clinical-morphological information, 15 exhibited neoplastic infiltration in the BM, and, of these, nine (64.3%) died (P = 0.035).

The lactic dehydrogenase (LDH) median was 725 mg/dL (152-4,386; reference value: <242 mg/dL), which did not differ statistically between the patients with infiltrated BM and non-infiltrated BM by neoplasia (P = 0.227, Mann-Whitney).

Morphology and immunohistochemistry

Table 2 shows the histopathological analyses of the 101 patients. The presence of lymphoid aggregates was detected in 64 cases (63.4%). In 31 cases (31/64; 48.4%), lymphoid aggregates were highlighted only by IHC. Moreover, in 11 (11/64; 17.2%) an increase in number of accumulations were detected only by IHC.

Lymphoid aggregates were classified according to the number, varying between 1 and 9 accumulations, with a median of 2. Lymphoid aggregates were located in the peri-trabecular region in 24 cases (24/64; 37.5%), non-peri-trabecular in 27 (27/64; 42.2%) and in biopsied clots in 13 (13/64; 20.3%). The edges of the lymphoid aggregates were classified as regular in 33 cases (33/64; 51.6%) and an increase of reticulin in the lymphoid aggregates was found in 39 cases (39/64; 60.9%), with grade 2 in 37 (37/64; 57.8%) and grade 3 in 2 (2/64; 3.1%). Blood vessels were found in the lymphoid aggregates in 9 (9/64; 14.1%) patients. There was agreement between the diagnosis of BMO and the diagnosis of BMO in 62 patients (71.3%).

Considering only the histologic evaluation of samples from 101 patients, the diagnosis of neoplastic infiltration was detected in 16 (15.8%) patients. The use of immunohistochemistry increased this rate to 21 (20.8%) patients. Therefore, the use of immunohistochemistry increased by 31.25% the number of cases of bone marrow compromised by neoplasia.

The performance of IHC did not change the morphological diagnostic result in 83 of the 101 cases, representing 82.2%. Staging was elevated in 11 patients (10.9%) and reduced in

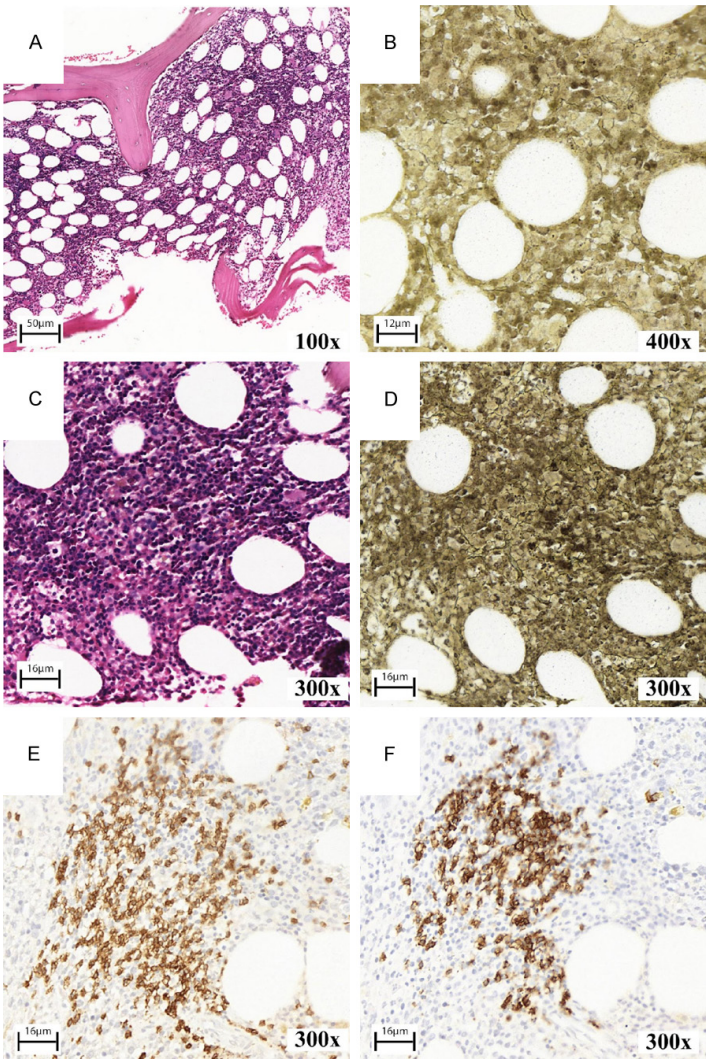

Figure 3. Photomicrograph of "pattern 3" lymphoid aggregate. Equal proportion of T and B cells. This finding is considered an inconclusive aggregate. A. Hematoxylin-eosin stain, magnification 100×; B. Reticulin stain, magnification 400×; C. Hematoxylin-eosin stain, magnification 300×; D. Reticulin stain, magnification 300×; E. CD3 antibody, magnification 300×; F. CD20 antibody, magnification 300×.

Morphologic analysis shows low sensitivity in relation to the immunohistochemical study

Morphologic analysis by H&E showed sensitivity of 42.9% (95% CI; 21.7-64.0) and specificity of 91.3% (95% CI %; 81.1-97.4), with IHC as the gold standard method (**Table 3**). Accuracy was 81.2% (95% CI; 73.6-88.8), with a positive predictive value (PPV) of 53.3% (95% CI; 31.9-80.6). The negative predictive value (NPV) was 85.9% (95% CI; 78.5-93.3), showing the percentage of truly negative cases among all reported as non-infiltrated. The Kappa index was 0.37, indicating a reasonable degree of agreement between the evaluation by isolated morphology versus combined morphology.

The overall median of reticulin grade in patients with infiltrated bone marrow was Grade 1 (0-2), the same as for non-infiltrated patients (P = 0.285, Mann-Whitney), showing that there was no association of this finding with BM infiltration. The median of reticulin grade in lymphoid aggregates in patients with infiltrated bone marrow was Grade 2 (1-3), greater than the median for non-infiltrated patients, who had a median Grade 1 (0-3), with P = 0.01 (Mann-Whitney), indicating an association with BM infiltration.

seven patients (6.9%). Three of the patients that had increased staging with IHC had an unfavorable outcome (P = 0.554), and two died (P = 0.562).

Lymphoid aggregates were present in peri-trabecular in 13 cases (61.9%; P = 0.018). The borders were jagged in 15 cases (71.4%; P = 0.010), in addition to an increase of reticulin in lymphoid aggregates of 85.7% (P = 0.004). Among the patients with infiltration, 17 cases had 'pattern 4' infiltration (80.9%), and 4 cases had 'pattern 5' (19.1%). Examples of each pattern are illustrated in **Figures 1-5**.

Finally, the median number of lymphoid aggregates among the infiltrated bone marrow samples was 3 accumulations (1-9), against a median of 1 accumulation (0-8) in neoplasia-free cases (P = 0.020; Mann-Whitney), also showing an association with BM infiltration.

ROC curves of morphological values in relation to the 'gold standard'

Figure 6 shows the ROC (*receiver operating characteristic*) curves drawn from the specificity and sensitivity of the morphologic values. It presents three important features: the general

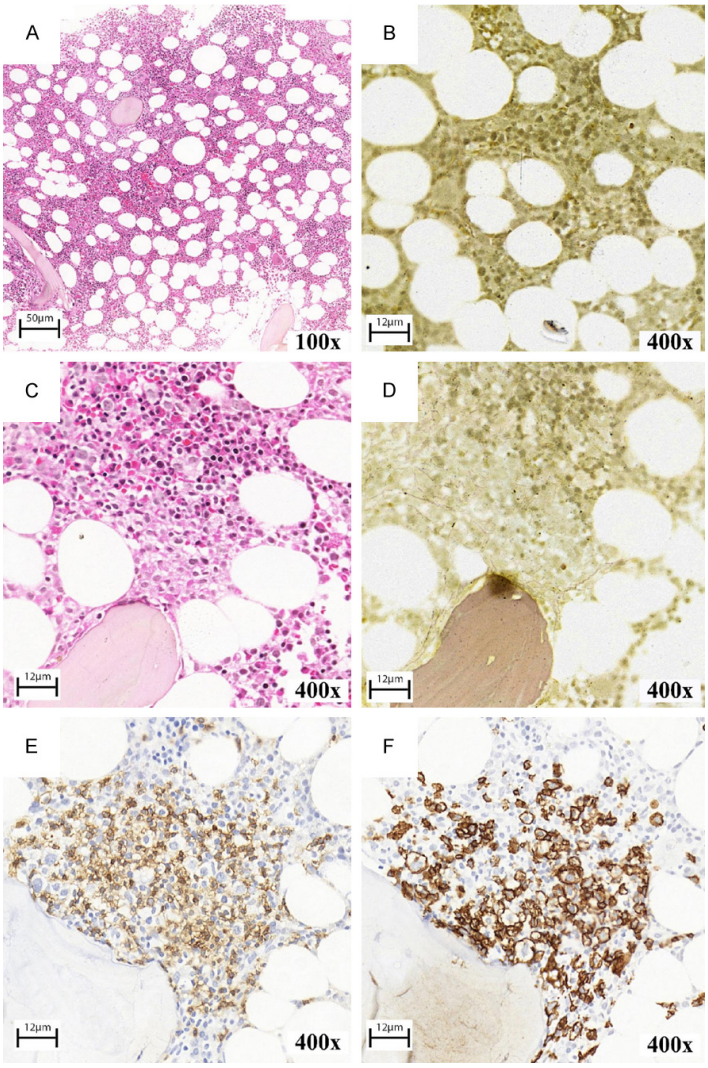

Figure 4. Photomicrograph of "pattern 4" lymphoid aggregate. High predominance of B cells. This finding is considered a reactive aggregate. A. Hematoxylin-eosin stain, magnification 100×; B. Reticulin stain, magnification 400×; C. Hematoxylin-eosin stain, magnification 400×; D. Reticulin stain, magnification 400×; E. CD3 antibody, magnification 400×; F. CD20 antibody, magnification 400×.

increase in reticulin in bone marrow sample, the number of lymphoid aggregates, and the increase of reticulin in lymphoid aggregates.

The general increase of reticulin (blue line) did not demonstrate a good relation between sensitivity and specificity for detecting infiltration (area under the curve of 0.570; 95% CI 0.438-0.701, P = 0.328). The sensitivity and specificity, for example, for reticulin grade II were 85.7% and 52.2%, respectively.

The number of lymphoid aggregates (green line) showed an area under the curve of 0.734 (95% CI 0.602-0.867, P = 0.002), indicating sensitivity of 57.1% and specificity of 76.7% for cutoff between 2 and 3 accumulations.

The increase in reticulin in lymphoid aggregates (yellow line) showed an area under the curve of 67.3 (95% CI; 0.541-0.804, P = 0.026), revealing a sensitivity of 85.7% and specificity of 52.2% for cutoff of at least grade 2.

Discussion

Lymphoid aggregates were found in 63% of BMs. Immunostaining (IHC) was important for identification and classification of the lymphoid aggregates, since 73% of the total were thereby identified, which was a 27% increase in number. In addition, the study observed good specificity (91.3%) but low sensitivity (43%) for bone marrow analyzed only by H&E, which means that this is a method with deficiencies in selecting negative cases. It is noteworthy that the infiltrative cases had lymphoid aggregates and the application of certain values, such as the number of accumulations and reticulin in these accumulations, combined for high specificity.

The exclusively morphologic analysis had 24% false negative cases in relation to BM infiltration, and the patterns adopted identified 5 more cases of infiltration, totaling 21 with infiltrated BM, which corresponded to 20.8% of the total cases, similar to what was seen by Chung et al. (2007) [12] and Campbell et al. (2006) [13].

Despite the controversial opinions regarding the collection of bone marrow clot, the research showed a satisfactory sample in 86% of the total, including diagnoses in agreement with the biopsies. Cantadori et al. (2019) report that the use of a clot, in addition to increasing the accuracy of the study of the marrow as a whole, is still more suitable for performing IHC - and, when necessary, molecular evaluation (FISH) - since it does not need decalcification [14].

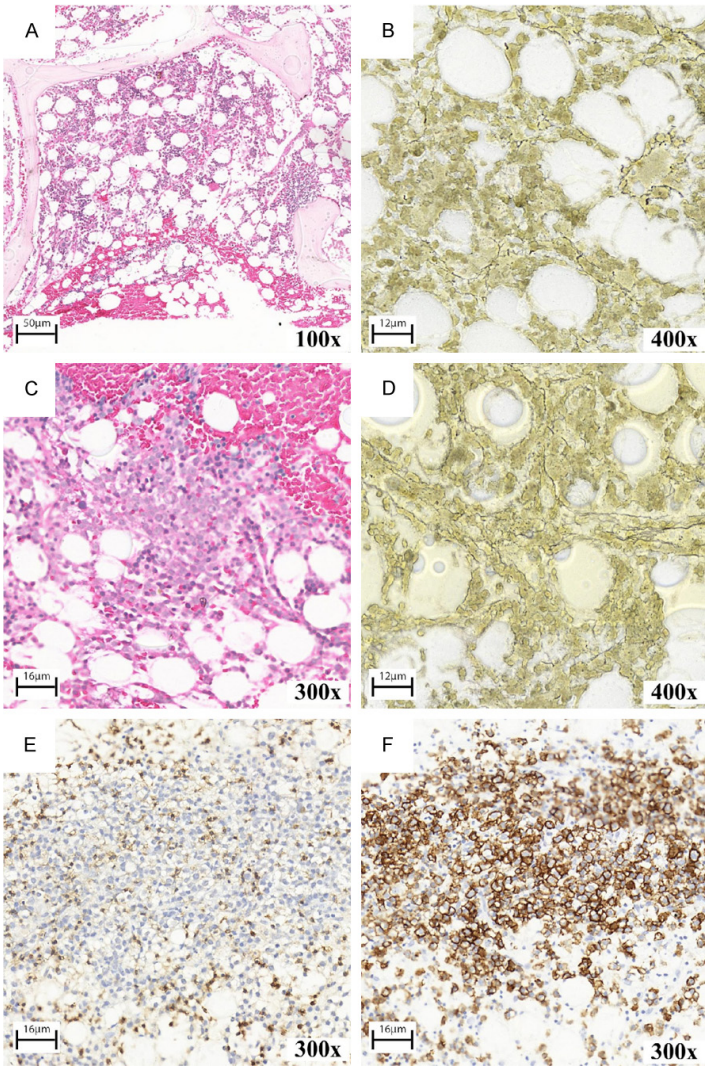

Figure 5. Photomicrograph of "pattern 5" lymphoid aggregate. Total predominance of B cells. This finding is considered a reactive aggregate. A. Hematoxylin-eosin stain, magnification 100×; B. Reticulin stain, magnification 400×; C. Hematoxylin-eosin stain, magnification 300×; D. Reticulin stain, magnification 400×; E. CD3 antibody, magnification 300×; F. CD20 antibody, magnification 300×.

Table 3. Evaluation of the diagnostic power of isolated morphologic analysis for the detection of bone marrow infiltration in patients with DLBCL, using immunohistochemistry as the gold standard for this detection

Isolated morphological analysis	Value	IC 95%
Sensitivity	42.9%	21.7-64.0
Specificity	91.3%	81.1-97.4
Accuracy	81.2%	73.6-88.8
Positive predictive value	56.3%	31.9-80.6
Negative predictive value	85.9%	78.5-93.3
Kappa concordance index	0.37	0.12-0.63

CI: Confidence value.

Peri-trabecular localization, jagged borders, and increase in reticulin were present in more than 60% of infiltrated bone marrow samples, confirmed by IHC, agreeing with the findings of Johnston et al. (2014) [15], showing the use of these features in the evaluation of lymphoid aggregates. However, only 42.8% of infiltrated cases were detected by only morphologic analysis, which is also reflected in the false-positive index (43.7%), which added 7 cases of the 16 that the morphological analysis identified as infiltrated.

The study adopted morphological analysis combined with immunohistochemistry as the 'gold standard' for detecting BM infiltration, and the distribution pattern of lymphocytes in the accumulations considered positive were those classified as 'Pattern 4' or 'Pattern 5' When strict morphologic analysis was compared, the study obtained high specificity but low sensitivity, which is unfavorable for this type of analysis. Thus, screened cases would eventually have to be subjected to immunohistochemical analysis. Another worrisome factor was the low positive predictive value, implying a considerable amount of possible false positives, exposing patients to unnecessary chemotherapy treatment that may be more aggressive. This fact, however, is not exclusive to the morphologic analysis, as seen by Caimi et al. (2016) [16], who also detected a low positive predictive value (PPV) in the analysis of BM by PET/CT. The accuracy of the latter, however, proved to be high, with about 81% rate of correct reports, superior to histologic analysis. Bone marrow is a dynamic organ, with different series in distinct topologies, and relatively sensitive to systemic variation. That is, changes in global and specific cellularity may occur in these patients which are not always infiltrations by lymphoma. The mor-

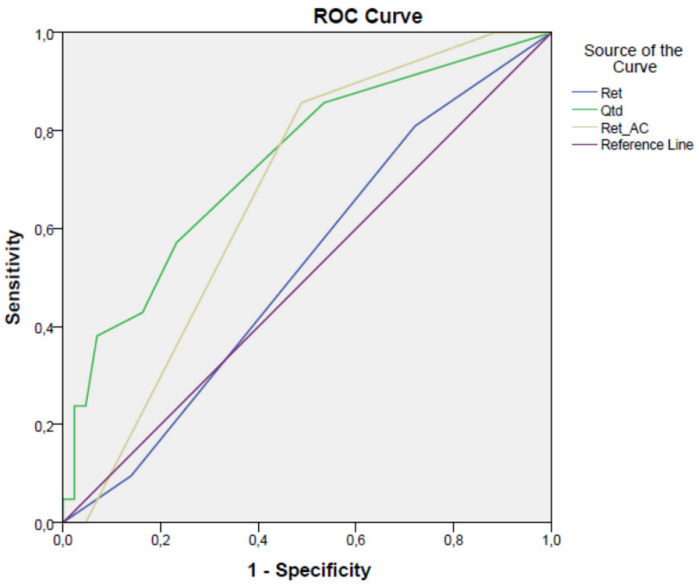

Figure 6. ROC curve of sensitivity and specificity of the morphologic findings evaluated. The blue line represents the situation of an increase of reticulin in the BM as a whole. The green line represents the number of lymphoid aggregates. The yellow line represents the reticulin inside the lymphoid aggregate. The purple line is the reference line.

phology and the study by PET/CT may fail in this sense, and the use of IHC is one more tool to obtain accuracy of the result.

Of the three morphologic aspects tested to help identify impairment of BM, an increase of reticulin in BM as a whole did not show an ability to identify positive cases or filter negative ones. The number of lymphoid aggregates in the sample (from two accumulations) and the increase in reticulin (grades 2 and 3) specifically in the lymphoid aggregates, showed to be of value for a pure morphological evaluation and can be useful in daily practice or in suboptimal circumstances, which is in line with the results and conclusions found in the literature.

Finally, the results indicate that the morphological analysis must be followed by an immunohistochemical study, or there is a risk of underdiagnosis of cases with worse prognosis, depriving these patients of adjusted therapy or a risk of overdiagnosis of non-infiltrated cases. These results can guide pathologists who need to make diagnostic decisions but do not have access to immunohistochemistry, when choosing which morphological parameters to value in reading the BM. Therefore, it is possible to consider that finding 2 or more lymphoid aggregates, and an increase of reticulin within them, may mean an infiltration of the bone marrow.

Disclosure of conflict of interest

None.

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