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Clonality Assessment of B Non-Hodgkin's Lymphoma by Multiparameter Flow Cytometry of Bone Marrow Biopsy: An Additional Diagnostic Tool

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ABSTRACT

Background: Bone marrow (BM) biopsies are generally included in the initial staging evaluation of non-Hodgkin's lymphoma (NHL) and BM involvement has unique prognostic implication in different histological subgroups of the disease. Flow cytometeric data should always be correlated with BM biopsy findings; immunophenotyping of core biopsy allows parallel morphologic examination and is capable of generating multivariate, quantitative, immunophenotypic data useful in diagnosis of NHL.

Objectives: To evaluate the role of flow cytometric immunophenotyping (FCI) of bone marrow biopsy samples as a complementary tool to immunohistochemistry and histopathology for more accurate diagnosis of BM infiltration of BM in NHL patients.

Subjects and Methods: The study included 60 B-NHL patients stage IV, diagnosed after histopathological examination of lymph node biopsy or fine needle aspiration (FNA) of other primary site and staged according to Ann Arbor system. Clonality assessment was established using Flow cytometric (FCI) immunophenotypic analysis of BMA and biopsy after obtaining single cell suspensions by mechanical disaggregation, with a restricted panel of monoclonal antibodies (CD45, CD20, CD3, CD19, anti-Kappa and anti-Lambda) using (BD FACSscan 4 color flowcytomtery). In addition to Histopathology of paraffin-embedded BM trephine biopsy with immunohistochemical (IHC) staining for morphological BM evaluation and clonality assessment.

Results: FCI analysis of BMB samples showed 24/60 cases (40%) positive for infiltration by B-monoclonal lymphocytes with light chain restriction, while BMA was positive in only 16.7%. FCI of core biopsy versus histopathological examination and light chain restriction detection by IHC revealed concordance rates of 63.6% and 85%. Clonality assessment and light chain expression detection revealed a kappa value of 0.708 for IHC versus FCM with concordance of 85%.

Conclusion: Our results showed that immunophenotypic analysis of BMB was rapid, specific, simple, having a definite role in detection of monoclonality of NHL making it an essential method to determine BM involvement when morphology is inconclusive, it is superior to IHC and flow cytometry on BMA. Better assessment of hematolymphoid neoplasms requires an integrated approach using multimodality technologies to identify the strengths, weaknesses and limitations to be an efficient and cost-effective method.

Key Words: *FCMI of BMB – NHL.*

INTRODUCTION

Non Hodgkin lymphoma (NHL) is a heterogeneous group of neoplasms with a wide spectrum of clinical behavior. Bone marrow biopsies (BMB) are generally included in the initial staging evaluation of patients with NHL and BM involvement may have unique prognostic implication in different histological subgroups of the disease **[1]**.

Morphologic examination of trephine BM biopsy, in search of involvement by disease, is a standard practice in staging patients with NHL and is useful for assessing response to therapy and evaluating relapse after treatment **[2]**. In general, the core biopsy sections are more useful than the aspirate smears in evaluating BM involvement in NHL **[3]**.

Flow cytometry of BM aspirates is thought to increase the sensitivity of BM involvement by NHL over morphological evaluation alone. Flow cytometric immunophenotyping offers the ability to fully characterize the initial diagnostic immunophenotype, determine cell surface monoclonaity, and detect intracytoplasmic antigens

and enzymes **[4]**. Flow cytometric data should always be correlated with BM biopsy findings.

Immunophenotyping of tissue samples may be performed by immunohistochemistry (IHC). Because of the limitations of IHC, a method to evaluate the core biopsy specimens by FCM is needed. Imunophenotypic analysis of BM cells could be done by mechanical disaggregation of core biopsies into cell suspensions. The isolated cells are stained with the appropriate antibodies and analyzed by FCI. This method is simple and reproducible. It allows for parallel morphologic examination and is capable of generating multivariate, quantitative, immunophenotypic data useful in diagnosis of BM involvement in NHL **[5]**.

The present study aimed to evaluate the role of flow cytometric immunophenotyping (FCI) of bone marrow biopsy samples as a complementary tool to immunohistochemistry and histopathology for better assessment of BM involvement in NHL patients.

MATERIAL AND METHODS

Patients:

The present study was carried out at the clinical pathology department, National Cancer Institute (NCI), Cairo University during the period between September 2012 and June 2013. It included 60 B-NHL patients, recruited from Medical Oncology outpatient clinic. The study was approved by the Institutional Review Board (IRB) and a written informed consent was obtained from all cases before participation in the study.

The patients were diagnosed as B-NHL after histopathological examination of lymph node biopsy or fine needle aspiration (FNA) of other primary site and clinically staged as stage IV NHL according to Ann Arbor system **[6]**. They were referred to the hematology laboratory for BM evaluation and initial staging. Their ages ranged from 3 to 88 with a median of 43 years. Thirty nine of them were males and 21 were females.

Methods:

All Patients were subjected to the following:

1- Complete history taking and thorough clinical examination, chest X-ray, abdominal ultrasound and/or CT scan, histopathological evaluation of lymph node biopsy or FNA for primary diagnosis and to define the histopathological type of NHL.

2- *Laboratory Investigations:*

- Complete blood count (CBC) using an automated blood cell counter, Sysmex XE-2100 with examination of peripheral blood stained smears for differential leukocytic count and detection of circulating malignant lymphoid cells.
- Erythrocyte sedimentation rate (ESR) using Westergren method and serum LDH enzyme levels assay.
- Examination of BM aspirate stained smears for assessment of cellularity and morphological BM infiltration.
- Flow cytometric (FCI) immunophenotypic analysis of BMA was performed using restricted panel of monoclonal antibodies (CD45, CD20, CD3, CD19, anti-Kappa and anti-Lambda) using BD FACS Calibur 4 color flowcytomtery.
- FCI analysis of BM trephine Biopsy (BMB) after obtaining single cell suspension by mechanical disaggregation.
- Histopathological evaluation of paraffin- embedded BM trephine biopsy for assessment of morphological BM infiltration (gold standard diagnostic tool).
- IHC staining of paraffin- embedded BM trephine biopsy using CD20, CD3, anti-kappa and anti-lambda light chain for detection of infiltration and clonality assessment.

Flow cytometric studies:

Fresh EDTA-anticoagulated BM samples were kept at room temperature and processed for immunophenotyping within 6 hours of collection. Samples were diluted with phosphate buffered saline (PBS), pH 7.4 (Sigma Chemicals, St. Louis); the final cell count suspension was adjusted to $3\text{-}10x10^4$ cells/ml. For each sample, a set of tubes was prepared for a panel of fluorescein isothiocynate (FITC)/ phycoerythrin (PE) conjugated Mo-Abs of a restricted panel (CD45, CD20, CD3, CD19, anti- Kappa and anti-Lambda, from Dako, Denmark, using BD FACSscan 4 color flowcytomtery. Double and triple marker labeling was performed, including proper isotype controls. For detection of surface markers by direct staining technique,

10ul labeled Mo Ab was added to 100ul BM, incubated in the dark for 20 minutes, hemolyzed by lysing solutions (Becton and Dickinson) and washed twice by PBS then analyzed by BD FACS Calibur 4 color flowcytomtery **[7]**.

Preparation of single cell suspension from bone marrow core biopsy:

The core was placed in glass Petri dish and minced to a fine consistency with small scissors and blade in the presence of (RPMI) tissue culture medium, all tissue piece was loosened from the plate by using a Pasteur pipette to dispense media or saline over the plate then transferred to a 50ml plastic centrifuge tube. With the use of a syringe the minced tissue/ media gently and repeatedly moved in and out of the syringe until the liquid in the tube appears cloudy, and then the liquid was filtered twice through a piece of specter mesh-(polypropylene and nylon) into a clean 50ml centrifuge tube and centrifuged at 500g for 10min. The supernatant was removed and re-suspended in PRMI tissue culture medium and cell viability was assessed using Trypan Blue to confirm the presence of enough viable cells (>2 x10^4 cells/ml) **[8]**.

B-Cell Monoclonality detection:

By flow cytometry, B cell monoclonality was defined as κ/λ ratio <0.05 or >2.5 on CD19+ve gated cells; absence of both κ & λ expression also indicates malignancy **[9,10]**.

Interpretation of Immunohistochemistry:

Staining for all markers was defined as follows: Positive, moderate to intense staining of at least 5% of lesional cells, or negative, faint staining of lower than 5% of lesional cells to no staining of cells; positive and negative control samples should react appropriately with all antibodies **[11]**. Monoclonality was defined as κ/λ ratios <0.5 or >2.5.

All slides were reported by two pathologists blinded to the previous assessment on histology; IHC results were reviewed again after being un-blinded to routine histology results.

The presence of clusters of B-cells was classified as abnormal or malignant when there were large numbers of clusters, the clusters were large sized, or they contained disproportionate number of larger cells; the interstitially scattered malignant cells which could not be confirmed quite easily on routine histology were more easily discernible on IHC analysis. Such cases were classified as positive when the number of B cells was increased compared to controls. These were cases with morphologically normal marrows that were used to create a visual impression of normal amounts of background T and B cells. Overall, a fairly conservative approach was adopted to avoid false positives. Antibody was reported as positive/negative, normal/abnormal, individual scattered cells/ clusters, small/large cells, and percent of biopsy involved (1%, 1-5%, or.5%).

RESULTS

The present study was carried out on 60 stage IV B-NHL patient; they included 39 (65%) males and 21 (35%) females with an age range from 3 to 88 with a median of 43 years.

On BMB histopathological examination, 33/60 (55%) cases had BM infiltration, 24/60 (40%) were free and 3/60 (5%) showed undetermined infiltration. Because of the small number of the last group, it was included with the positive infiltration group in most descriptive statistical analysis and excluded from statistical analysis

The demoghraphic, clinical and hematological data of the patients at diagnosis and its relation to BM infiltration are presented in Tables (1,2). No statistically significant difference was found between the two patient's groups except for significantly higher degree of fibrosis and LDH level associated with BM infiltration (p=<0.001).

BMA examination revealed 4/60 (6.7%) cases with dry tap (they were excluded from the analytical statistical studies), 45/60 (75%) negative for infiltration, 5/60 (8.3%) positive (by either high lymphocyte count or significant number of immature lymphocytes) and 6/60 cases (10%) equivocal due to infiltration by low number of immature lymphocytes (<5%). On the other hand, BMB revealed 33/60(55%) positive for infiltration, 24/60 (40%) negative and 3/60 (5%) cases equivocal (confirmed to be infiltrated by immunophenotyping).

On comparing BMA with biopsy as regards the morphological infiltration (after exclusion of 6 cases of undetermined infiltration by aspirate, 3 cases of undetermined infiltration by biopsy and 4 cases with dry tap), for the positive infiltration cases, concordance was 21.7%, discordance was 78.3% between both (aspirate and biopsy) and Kappa value for measurement of agreement between both was 0.197 indicating slight agreement (Table 3) making biopsy superior to aspirate in assessing morphological infiltration.

Table (1): Demographic and clinical data of 60 non-Hodgkin's lymphoma patients at diagnosis in relation to bone marrow (BM) infiltration.

Parameter	BM infiltration No: 36		No BM infiltration No: 24		Total		*p*
	No.	%	No.	%	No.	%	
Gender:							
Male	25	69.5	14	58.3	39	65	0.337
Female	11	30.5	10	41.7	21	35	
Age: years:							
≤40	17	47.2.	10	41.6	27	45	0.672
>40	19	52.8	14	58.4	33	55	
B-Symptoms:							
(+)	7	19.4	1	4	8	13	0.128
(–)	29	80.6	23	96	52	87	
Pathological types:							
DLBCL	11	30.6	12	50	23	38	
SLL	13	36.1	4	16.7	17	28	0.082
FL	8	22.2	2	8.3	10	17	
Others	4	11.1	6	25	10	17	
Degree of Fibrosis:							
0-1	3	8.3	24	100	27	45	<0.001
L l	13	36.1	0	0	13	21.7	
L ll	20	55.6	0	0	20	33.3	

Table (2): Hematological laboratory data of 60 non-Hodgkin's lymphoma patients at diagnosis in relation to bone marrow (BM) infiltration.

Parameter	BM infiltration	No BM infiltration	*p*
TLC $x10^9$/ L	9.10±4.23* 8.50 (0.27-19.0)	7.97±2.98 7.37 (3.2-13.6)	0.301
PLT $x10^9$/L	281.1±145.5 239.5 (9.0-650)	270.9±144.5 304.5 (82-754)	0.728
Hb: gm/dl	10.4±1.4 10.1 (6.0-13.9)	10.9±1.5 11.0 (8.8-15.2)	0.188
Absolute lymphocyte count $x10^9$/L	4.3±2.2 3.7 (0.1-10.7)	3.5±1.0 3.5 (1.5-5.7)	0.194
LDH: U/L	877.4±155.8 893.5 (567-1101)	325.9±144.4 268 (123-670)	<0.001*
ESR:mm/hour	71.4±17.6 77.5 (34-100)	66.7±16.8 62 (38-98)	0.210

TLC : Total leukocyte count.
PLT : Platelet count.
Hb : Hemoglobin level.
LDH : Lactic dehydrogenase.
ESR : Erythrocyte sedimentation rate.
* Mean ± SD. Median (range).

Table (3): Comparison of non-Hodgkin's lymphoma bone marrow (BM) biopsy morphological infiltration with BM aspirate, BM biopsy immunohistochemistry and flow cytometry of both BM aspirate and biopsy

Parameter	BMB Morphological infiltration						Kappa value
	Positive		Negative		Total		
	Count	%	Count	%	Count	%	
Bone marrow aspirate morphological infiltration							
Negative infiltration	18	42.9	24	57.1	42	89	
Positive infiltration	5	100	0	0	5	11	0.197
Total	23	49	24	51	47	100	
Immunohistochemistry of bone marrow biopsy:							
Negative infiltration	6	20	24	80	30	44	
Positive infiltration	24	100	0	0	24	56	0.462
Total	30	55.6	24	44.4	54	100	
Flow cytometry of bone marrow aspirate:							
Negative infiltration	19	44.2	24	55.8	43	81	
Positive infiltration	10	100	0	0	10	19	0.201
Total	29	54.7	24	45.3	53	100	
Flow cytometry of bone marrow biopsy:							
Negative infiltration	12	33.3	24	66.7	36	63.2	
Positive infiltration	21	100	0	0	21	36.8	0.352
Total	33	42.1	24	57.9	57	100	

Comparing immunophenotypic infiltration by IHC with morphological infiltration of BMB (after exclusion of 4 cases of undetermined infiltration by IHC, 3 cases of undetermined infiltration by histopathological examination of BMB and an overlapping in one case, was undetermined by both), it showed 80% sensitivity, 100% specificity, 80% NPV, 100% PPV, 88.8% accuracy and kappa value of 0.462 indicating moderate agreement between the two methods. The concordance between both was 100% for negative morphological infiltration while for positive infiltration, concordance was 80% and discordance was 20%.

Excluding the 4 cases with dry tap, Flow cytometry of BMA showed no evidence of infiltration in 46/56 (82.14%); the other 10 cases (17.86%) showed evidence of infiltration with κ chain restriction in 7, λ chain restriction in 2 and lack of both κ and λ in one case.

While FCM analysis of BMB revealed; 24/60 cases (40%) were positive for infiltration by B- monoclonal lymphocytes with light chain restriction 19 (31.7%) cases with kappa, 2 (3.3%) showing lambda restriction and lack of expression of both light chains was detected in 3 (5%) cases), while 36/60 cases (60%) were negative for infiltration.

On comparing FCM of BMA with BMB morphological infiltration, FCI showed 34.5% sensitivity, 100% specificity, 55.8% NPV, 100% PPV, 64.2% accuracy and kappa value of 0.201 indicating fair agreement between the two methods. Concordance was 34.4% and 65.6% discordance for positive morphological infiltration cases, while concordance was 100% between both (FCM of BMA and histopathology of biopsy) for negative infiltration cases. Although FCI of BMA is 100% specific yet it has lower sensitivity in detection of infiltration when compared with BMB histopathology.

On the other side, comparing FCI and morphological infiltration of BMB, FCI showed 64% sensitivity, 100% specificity, 66.7% NPV,

100% PPV, 79% accuracy and kappa value of 0.352 indicating good agreement between the two methods. Concordance was 63.6% and discordance was 36.4% for positive morphological infiltration, while concordance was 100% for negative cases, and although the sensitivity of FCI of BMB is better than that of aspirate but still less than histopathology indicating that both methods are complementary to each other.

Finally using FCM analysis of light chain expression and restriction, 36/60 (60%) cases were negative for infiltration by B-lymphocytes, 19/60 (31.7%) showed monoclonal kappa restriction and 2/60 (3.3%) showed monoclonal lambda restriction, while 3/60 (5%) cases lacked expression of both light chains.

On the other hand IHC analysis of light chain expression and restriction, 30/60 (50%) cases were negative for infiltration, 19/60 (31.7%) showed monoclonal kappa restriction, 4/60 (6.7%) had monoclonal lambda restriction, 3/60 (5%) lacked expression of both light chains and clonality could not be detected by analysis of light chain expression in 4/60 (6.7%) cases and follow-up was recommended (Table 4), (Fig. 1).

Comparing light chain expression and clonality assessment by both FCM and IHC, it showed 0.708 kappa value, concordance 85%, and discordance 15% meaning complementarity of both methods (Table 4).

Table (4): Comparison of light chain monoclonality by Flowcytometry (FCM) and immunohistochemistry (IHC) of BMB.

IHC of BMB	FCM of BMB						Kappa value
	Infiltrated		Not infiltrated		Total		
	Count	%	Count	%	Count	%	
Negative infiltration	1	3.3	29	96	30	64	0.708
Positive infiltration	19	73	7	27	26	36	
Total	20	36	36	64	56	100	

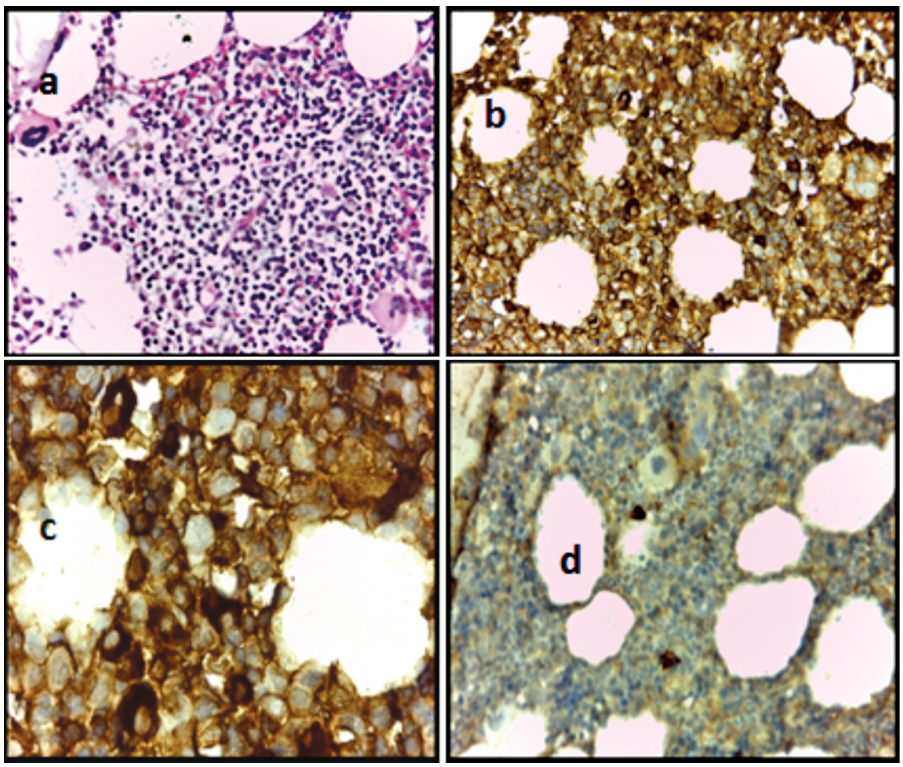

Fig. (1): (A) Nodular lymphoid infiltration of BMB, (B) Kappa light chain +ve (40x) (C) Kappa light chain +ve cells (100x), (D) Lambda light chain –ve cells (100x).

DISCUSSION

In the present study BM biopsy of 60 stage IV NHL patients were reviewed. Their histopathological findings were compared with the corresponding FCM findings of both BMA and biopsy and with that of IHC. The results were reviewed independently to determine diagnostic value of each in the assessment of BM involvement by NHL and detection of monoclonality.

The morphological infiltration of BMA was negative in 45 cases (75%), positive in 5 (8.3%) and undetermined in 6 cases (10%) due to the presence of low number of immature lymphocytes not sufficient to confirm infiltration and 4 cases (6.7%) had dry tap with failed morphological examination. While, the histopathological evaluation of BM trephine biopsy was positive in 55% of cases, negative in 40% and undetermined in 5% of cases due to the presence of low number of interstitially infiltrating cells that could not be confirmed whether they are reactive or neoplastic. On assessment of the agreement level of both BMA and BMB considering that histo-pathological examination of biopsy as the gold standard diagnostic tool **[12]**, our results are different from what was found by Sah and coworkers **[2]**; they reported that the sensitivity for the detection of BM involvement in different malignancies ranged from 69 to 82% (21.7% in our study), the specificity ranged from 80 to 90%, the PPV ranged from 61 to 76%, and the NPV ranged from 82 to 87%.

When considering only the indolent NHL samples, the sensitivity of BMA was 82% and the PPV was 82%, whereas sensitivity and PPV were 40% and 29%, respectively, in the aggressive NHL specimens. The differences in BMA sensitivity observed may be attributable, in part, to the small size of lymphoid infiltration in the aggressive NHL group **[2]**. The present study is consistent with others who concluded that BMA was 100% specific in most disorders including B-NHL but sensitivity and accuracy depended upon the disease being evaluated **[13,14]**.

They stated that in B-NHL, only two thirds of NHL positive marrow was picked upon aspirate and 23.9% were missed on aspiration and concluded that BM biopsy renders information which cannot be determined from aspiration, such as spatial distribution, extent of infiltrate, overall cellularity and fibrosis. They also implied that trephine biopsy may be more useful in post chemotherapy patients to assess the residual tumor cell burden and degree of chemotherapy response **[13,14]**.

On the contrary, our findings are inconsistent with that encountered by Sovani and his colleagues **[15]**, who stated that with the exception of cases associated with stromal fibrosis e.g. DLBCL, aspirate involvement by lymphoma closely parallels that of trephines. They concluded also that detection of lymphoma cells in peripheral blood is approximately half as frequent as in aspirates and has been correlated with extensive disease. This debate may be due to differences in subtypes studied, as they were mainly indolent lymphoma which has a trend for diffuse infiltration with low stroma that can be easily aspirated, thus rising the sensitivity of BMA for detection of infiltration by NHL **[15]**.

In conclusion, morphological examination of BMA and BMB are not entirely complementary in positive cases. Because the utility of obtaining an aspirate is supported by its earlier and easier availability for BM examination, and the larger amount of marrow that can be examined by combining the two procedures, thus, BMA may be considered an additional procedure to core biopsy, but it can't substitute it. Recently, in most hematological centers it is established that the detection of occult BM involvement by lymphoma depends on the adequacy of both routine histopathological examination and IHC.

It was previously believed that bilateral trephine biopsies increased the yield from (for) staging BMs in NHL **[16]**. However, it was later established that bilateral sampling is not required if adequate tissue (including adequate trephine length and number of levels examined) is available for diagnosis **[17]**. Other authors recognized that in most cases of NHL, the biopsy specimens needed should be at least 16mm in length and that several levels required examination for optimal results. The use of IHC in staging bone marrow in NHL is largely limited to ambiguous cases particularly those with lymphoid aggregates. Its role in routine clinical practice remains un-established **[18,19]**.

Most of their studies have focused on the use of IHC on BM trephines in ambiguous cases

to differentiate between benign lymphoid aggregates and malignant infiltration. They aimed to determine whether the routine use of IHC in NHL would improve the detection of lymphomatous involvement in the bone marrow, using the same panel as in the present study (CD20, CD3, anti kappa and anti lambda light chains). In their study, IHC detected lymphomatous involvement in an additional 11% of cases compared with histopathological examination alone. They concluded that IHC performed routinely on bone marrow trephines has the ability to improve detection of occult lymphoma in experienced hands **[18,19]**. In the present study, on comparing IHC with histopathological examination of BMB, exclusion of 3 cases were undetermined by morphological examination, and 4 by IHC (only one undetermined by both), the Kappa value was 0.462 indicating moderate agreement between the two methods, with 100% concordance in negative infiltration cases, while for positive cases, the concordance was 80% and discordance 20%. This discordance was due to the presence of 6 cases showing positive morphological infiltration and negative by IHC (2 normal reactive T lymphocytes and 4 normal reactive hyperplasia of B-lymphocytes with normal k/L ratio).

Our finding is in accordance with Merli and coworkers, who confirmed in 2010 the importance of adding IHC to the routinely histological examination of BMB. In their study, 16 BMBs were plastic-embedded and evaluated by morphology alone, without IHC, the nature of the lymphoid component could not be definitively established. On the other hand, in the same series, paraffin embedded cases with similar morphological findings were evaluated for lymphoma localization by applying a wider IHC study to demonstrate the phenotypic composition of the lymphoid component and to ascertain specific phenotypic aberrancies. Moreover, they reported that paraffin-embedded BMBs could also be used for molecular studies in the limited number of cases in which morphology plus IHC could not discriminate between benign and neoplastic lymphoid component **[20]**.

Talaulikar and his coworkers concluded that the role of IHC in staging BM has been upstaged, to some extent, by FCM immunophenotyping of BMA. This might be due to the results obtained with FCM being quantitative and perceived to be less prone to the errors that may be associated with a more subjective diagnostic tool such as IHC, particularly in inexperienced hands **[18, 19]**.

The ready availability of flow cytometric analysis in most laboratories has also led to its widespread use. Indeed, there is evidence that FCM plays an important role in staging NHL and has an impact on outcome factors **[19]**. BM involvement in NHL is more often apparent on BMB rather than BMA **[3, 21, 22]**. It follows that flow cytometric assessment of aspirates can also yield false-negative results **[19,23,24]**, whereas if FCM assessment of trephine biopsy samples was performed, a greater positivity rate may be obtained. However, there is no published literature addressing the value of this issue. Also, using some of the biopsy specimen for FCM analysis could compromise availability of tissue for histological evaluation and obtaining additional biopsy tissue for FCM analysis may be inconvenient to the patients specifically, thus renders immunophenotyping on BM trephines using IHC therefore, a logical ancillary study in staging BM, although, FCM is superior to it **[3,17]**.

In the present study, an additional 1cm of trephine core bone marrow biopsy was taken for analysis by FCM and compared with that of BMA. As regards detection of BM infiltration of core biopsy by FCM, in the present study, the method used for tissue disaggregation and preparation of a single cell suspension was a manual mechanical tissue disaggregation using disruption with blade and scissors. The cell viability and yield in this method were considered good and the method was simple, rapid and reliable. The prepared BM core suspensions were allowed for a complete immunophenotypic profile by FCM in all cases. In other studies an automated mechanical tissue disaggregation using medi machine was used **[25]**. They reported that the potential shortcoming of this technique is the failure to obtain sufficient cells for immunophenotyping when the cell yield is low in cases of low infiltration. However, in other studies authors have processed samples for FCM containing as few as $1x10^4$ **[26]**. Other authors suggested that both automated and manual techniques for tissue disaggregation will not work optimal for the disaggregation of necrotic tumors. Also, loss of architectural

relationships in the course of processing specimens for FCM is a disadvantage when small foci of lymphoma or tumor cells exist together with large amounts of stroma or normal lymphocytes. Thus, the FCM data must always be correlated with histological sections of the BM biopsy **[27]**.

In their study, Boyd and his colleagues, in 2015, concluded that FNA was found to be significantly better than trephine bone marrow core FCM as regards to yield and viability of cells, but BMB resulted in higher determinate final diagnosis, which was expected due to the benefit of histopathological correlation and low yield of malignant cells obtained by FNA in cases of NHL, and this is in agreement with the current study **[28]**. This difference between BMA and BMB samples on FCM analysis in our study was explained by a poor yield of the aspirate compared with the BMB, which showed paratrabecular infiltration in some of these cases. It is possible that no disruption of these aggregates takes place during aspiration and an intact paratrabecular aggregate is removed only by block resection during biopsy. Also the patchy nature of many lymphomas involving the BM may produce aspirate free of the disease, although the biopsy is being involved. Furthermore, dilution of the aspirate samples with peripheral blood during the procedure may decrease the proportion of neoplastic cells below the threshold of detection by FCM, in addition to the main role of FCM of BMB in diagnosis of cases with failed BM aspiration "dry tap".

In the present study on examination of BMB, among 33 cases positive for morphological infiltration by biopsy, only 21 were positive by FCM with biopsy, with agreement level of 64%. Among the 12 cases which were positive for morphological infiltration and negative by FCM, 9 were reported to have BM involvement by less than 10% with lymphoid elements and 3 were involved by less than 30% with lymphoid elements. Out of the 12 cases, 6 had paratrabecular pattern of infiltration, 2 had nodular pattern and 4 had interstitial pattern. The yield of cells in these cases were more or less unsatisfactory (less than $2x10^4$). No cases were detected to be positive by FCM and negative by morphological examination of BMB, there were 24 cases negative for both FCM and BMB with agreement level of 100%. According to BMB morphological examination and its comparison with FCM, the latter showed Specificity of 100%, NPV 66.7%, Sensitivity 63.6%, PPV 100% and accuracy of 78.9%. On comparing immunophenotyping infiltration by FCM with morphological infiltration of BMB, 3 cases of undetermined infiltration by morphological examination of BMB were excluded. It showed kappa value of 0.352, which means fair agreement between the two methods, with 79% concordance and 21% discordance, indicating that both methods are complementary to each other and FCM is confirmatory to morphological examination of BMB but couldn't substitute it especially in cases with low infiltration with low yield of cells.

Negative FCM and positive histopathological findings were also suggested previously by old studies **[29]**, but recently, different Authors that investigated the role of flow cytometery in detecting BM involvement with NHL didn't provide evidence of much benefit beyond morphological examination alone of the biopsy **[30]**. A low percentage of concordance between FCM and BMB was also estimated earlier by others, who reviewed 273 bone marrows from patients known to have NHL, the FCM was detected only in 60% of positive BMB cases **[31]**. The latter, did not support a significant benefit of FCM of BM beyond pathological examination of core biopsy alone and would not encourage the continued use of FCM in NHL staging, they stated that the low rate of detection by FCM may be partly related to the use of BMA and the application of less sensitive and specific single or dual color method. It might be too attributed to the patchy nature of many lymphomas, poor specimen viability, and the presence of a large reactive lymphoid population such as T-cell rich B-cell NHL **[31]**. Conversely, the present study did not go in line with a study **[32]** who found a good correlation (81% and 85% respectively) between morphology of BMB and FCM, this might be attributed to the inclusion of large number of CLL cases in their studies.

However, Crotty and colleagues in 1998 compared FCM, morphology and molecular gene rearrangement by PCR in the evaluation of BM involvement and concluded that FCM remains the method of choice for detection of clonality in B-cell neoplasms because of its higher sensitivity **[33]**. Although, the recent report of an international workshop to standard-

ize response criteria for NHL in 2016 recommended that patients whose BM is histologically normal but had a small clonal B-cell population detected by FCM should be considered as having normal BM until clinical studies demonstrated a different outcome for this group.

Hehn et al., used lymph node FNA samples for morphological examination and FCM, they concluded that FNA cytological examination was not helpful and may misguide treatment of patients with malignant lymphoma with sensitivity of 12%. This conclusion is in line with our study which showed similar results regarding the value of FNA cytological examination in spite of the difference in sample type **[34]**. In agreement too, recently **[35,36]** concluded that FCM combined with morphological examination of aspirate and biopsy has been demonstrated to be useful in the diagnosis and classification of NHL. FCM is mainly effective in discriminating benign reactive hyperplesia from small cell NHL, and in their classification. Conversely, they stated that traditional BM morphological examination alone may be helpful in the diagnosis of large cell NHL. The combination of the two techniques enhances their diagnostic possibilities.

Bangerter et al., **[37]** suggested that the combination of FNA cytological examination and FCM was very useful in the diagnosis of malignant lymphoma. They found that a definitive diagnosis was given in 115 (88%) out of 131 cases, resulting in a sensitivity of 85.6% and a specificity of 100%. They stressed in their study on the problem of the differentiation of malignant lymphoma from reactive lymphoid hyperplasia. So, concluding that correlating histopathological examination with FCM has shown to be absolutely mandatory to maximize the use of both methods and that combination of both allows further subclassification in most cases on the basis of the new WHO classification **[37]**.

As regards to assessment of monoclonality, mature B-lymphocytes express both immunoglobulin heavy and light chains (κ,λ) on the cell surface. The relative number of lymphocytes that express one immunoglobulin light chain versus the other generally was used to determine the clonality of the lymphoid proliferation in question which is the key for lymphoma diagnosis **[38]**. A lymphocyte population was considered unequivocally clonal when the κ/λ was more than 4:1 or less than 1:2 in at least 1,000 gated B-lymphocytes. A lymphoma was considered negative for immunoglobulin light chains when light chain expression was present in fewer than 5% of total gated B lymphocytes **[9]**.

FCM is one of the most convenient and powerful means to establish B-cell clonality. Whereas, benign B cells may also be present in a lymphoma specimen which may mask neoplastic cells leading to a partially normalized ratio κ/λ. In these cases, often other clues to the presence of neoplastic cells may be found for example, altered forward scatter or SSC, or the expression of immunophenotypic markers that distinguish them from benign cells. Multiparametric FCM, using multicolor antibody combinations, allows detailed information on the different cell subsets present in cellular suspensions to be obtained and permits the identification of a clonal population, even if masked in an apparently non tumoral background **[39]**. By using FCM for clonality detection, in almost all cases, an all-or-non phenomenon was observed; either most (>80%) or few (<5%) B lymphocytes expressed surface immunoglobulin light chains. An expression by fewer than 5% or more than 20% to 25% of the gated lymphocytes was used as a cutoff for the negative or positive expression respectively **[40]**. In IHC, morphology and antigen label can be visualized simultaneously by light microscopy which is not the case in FCM, enabling accurate identification of the cells or region of interest. It is considered more suitable in the detection of partial or focal involvement by neoplastic cells and it is also more beneficial in nuclear antigen assessment. Another privilege of IHC is the long-term preservation of the biological-specimen after processing and the ability to re-examine it at any time **[41]**.

In the present study, on comparing IHC with FCM results regarding the determination of monoclonality and light chain restriction, the positivity percent of markers and staining intensity using IHC were compared to the positivity percent and the first mean fluorescence intensity of these markers using FCM. FCM analysis of trephine BMB for clonality assessment showed 31.7% kappa restriction, 3.3% of cases showed lambda restriction while both kappa and lambda chains were absent in 5% of cases infiltrated by B-lymphocytes. In 60% of cases there was no detectable malignant or

neoplastic lymphocytes and reported as negative for infiltration. As regards IHC, kappa light chain restriction was detected in 31.6% of cases, lambda chain restriction in 6.7% and lack of both light chain expression in 5% while in 6.7% of cases light chain could not be determined due to technically interfering background in spite of following restricted precautions. In general detection of full immunophenotype and determination of monoclonality by IHC was technically more difficult than detection by FCM.

In spite of the numerous commercially available antibodies advances in heat-induced epitope retrieval and the availability of a full panel of paraffin-reactive antibodies in the workup of B-cell lymphoid neoplasm, IHC is still not considered a satisfactory method for light chain detection. Fifty percent of cases were negative for infiltration by B-lymphocytes. On comparing the results of analysis of light chains expression by both FCM and IHC for detection of monoclonality (exclusion of 4 cases that could not be determined by IHC), kappa value was 0.708 meaning a substantial agreement between the two methods, concordance was 85%, discordance was 15%, indicating that both methods are complementary to each other to a great extent.

The present study is near to what was found by Abdel-Ghafar and his collagues, who studied immunophenotyping of chronic B-cell neoplasms by FCM versus IHC. They measured the sensitivity of IHC by comparing it with the results of FCM. In their study, IHC showed sensitivity of 90%, 40% and 25% for CD20, kappa and lambda respectively **[42]**. Earlier studies, compared IHC of fresh frozen tissues with FCM of NHL cases. They obtained lower concordant results as regards CD20 being 86%. The sensitivity of light chain restriction by IHC was 30% near to what we found **[43]**. Similar results also found a concordance between FCM and histopathology/IHC in 89% and 87.2% of B-lymphoma cases respectively **[44,45]**. On the same line, a study done by El-Sayed and his colleagues in Egypt, they measured the overall concordance between IHC and FCM without specifying each marker. The results of the study showed 88% concordance between FCM and histopathology/IHC in the diagnosis of lymphoma **[46]**.

A more recent study concluded that IHC can be a reliable alternative choice for FCM in work up of staging NHL **[47]**. In the present study lack of expression of both light chains was detected in 5% of cases in the presence of infiltration by B-lymphocytes. Lack of expression of immunoglobulin light chains of either kind on the surface of neoplastic lymphoid cells of B-NHL is a rare phenomenon first described by De Martini and coworkers in 1988 who reported 33 cases of surface immunoglobulin negative B-cell NHL from a total of 271 cases, although, clinicopathologic details were lacking **[48]**. Other rare cases have been also described by other studies, but didn't provide clinicopathologic details or documentation of these tumors **[49]**. The true incidence, tumor characteristics, and mechanisms responsible for this phenomenon are largely unknown. Our finding is in accordance with Kaleem and colleagues, who found 10 well documented cases of B cell NHL that did not express surface immunoglobulin light chains by dual color FCM **[50]**. Furthermore, Tomita et al., in 2009, found that FCM does not always detect surface light chain expression on lymphoma cells, a phenomenon most commonly reported in DLBCL, also described in other B-NHLs **[10]**. This apparent lack of surface immunoglobulin was postulated to be due to post-transcriptional de fects of the immunoglobulin molecule **[9]**.

Conclusion: B-NHL is not a diagnostic challenge when the material is adequate and appropriate ancillary studies are available. However, it can be challenging when the type or amount of specimen available for evaluation is limited.

Our results showed fair agreement level for IHC and FCM of BMB, yet FCM is faster, specific and has a more definite role in detection of monoclonality of NHL, so accurate assessment of hematolymphoid neoplasms requires an integrated multiparameter approach. Although morphologic histopathologic examination remains the mainstay of initial assessment, immunophenotypic analysis of core biopsy is essential to determine the pattern of differentiation and detect minimal disease when morphology is inconclusive. Finally, an integrated approach using multimodality technologies is a must with identifying the strengths, weaknesses, and limitations to be an efficient and cost-effective method for better assessment.

Conflict of interest:

None of the authors of this paper has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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