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Scientific Research

# Association of Morphology and Immunophenotype in Diffuse Large B-Cell Lymphomas with Bone Marrow Infiltration in a Sample Mexican Population*

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## ABSTRACT

**Introduction:** Diffuse large B-cell lymphoma (DLBCL), not otherwise specified, is a large B-cell lymphoma with a diffuse growth pattern and aggressive clinical course. It is divided in subgroups according to its morphology, immunophenotype, and primary site. Dissemination to bone marrow occurs in 11% to 35% of cases and can be of concordant or discordant morphology. **Objective:** To examine the association, the type of bone marrow involvement in relation to the primary site, morphology, immunohistochemistry of DLBCLs and to determine the cases of Epstein-Barr virus positive DLBCLs. **Materials and Methods:** We reviewed lymph node and extranodal biopsies as well as the respective bone marrow biopsies in all cases of DLBCL diagnosed in the Hospital General de México during the period from 2002 to 2010. We used immunohystochemistry for immunophenotype identification (Hans's algorithm) and an *in-situ* hybridization technique to detect presence of Epstein Barr encoded RNA (EBER). **Results:** We included 108 patients with a mean age of 51.9 years, 59 (55%) were men. DLBCL involved lymph nodes in 60% of cases and palatine tonsils in 13%. The centroblastic variant predominated (80%) and 58% originated from activated B-cells. Infiltration of bone marrow was present in 30% of cases and was discordant in 55% of these cases. Correlation between morphology and bone marrow infiltration was statistically significant ($P = 0.0003$). Presence of Epstein-Barr virus was demonstrated in 15% of patients older than 50 years. **Conclusions:** Dissemination to bone marrow occurred in 30% of cases and discordant involvement was most common. DLBCL originating from activated B-lymphocytes predominated and the most common extranodal sites were palatine tonsils, suggesting that our population has a clinical behavior similar to Asiatic populations.

**Keywords:** Dissemination to Bone Marrow; Diffuse Large B-Cell Lymphoma; Immunophenotype

## 1. Introduction

Diffuse large B-cell lymphoma (DLBCL), not otherwise specified, is a type of non-Hodgkin lymphoma, and comprises a group of clinically aggressive heterogeneous entities. It accounts for 25% to 30% of lymphomas in adults in the USA and western Europe [1]. It arises in lymph nodes in 60% of cases and extranodal sites in 40%. The most common extranodal site is the gastrointestinal tract (35% - 37%) [1,2]. Biological, clinical, and morphological studies have classify the DLBCL into morphological, immunophenotypical, and molecular subtypes [3-7]. Dissemination to bone marrow (BM) occurs in advanced clinical stages and varies in different series from 11% to 35% [1,2]. Minimal infiltration can be detected if auxiliary studies are conducted together with the morphological evaluation, using immunohistochemistry markers or polymerase chain reaction (PCR) in the bone marrow (BM) specimen biopsy [8]. The prevalence of discordant infiltration varies in different reports and ranges from rare to 70% [9,10]. Dissemination to bone marrow at the time of diagnosis plays a crucial role in determining treatment and predicting clinical follow-up of patients [11]. There are controversial data concerning the prognostic significance of BM involvement in DLBCL, when relying only on routine histology, percentage of medullar involvement, and type of infiltration [12,13].

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Hence, some authors recommend determining BM involvement by other auxiliary methods such as flow cytometry and immunohistochemistry [14].

In a previous study at our institution, we documented that DLBCL accounted for 59% of all lymphoid neoplasms, and the mean age at diagnosis was 50 years with a predominance for the male sex [3]. In some populations, such as Western Europe and in the United States the mean age at diagnosis is between 60 and 70 years of age but it is also encountered in children and young adults [1-3]. The objective of the present study was to association of morphology, immunophenotype, clinical site of the DLCBL and the presence of Epstein Barr virus with the type of BM infiltration to determine possible prognostic factors at the time of clinical staging.

## 2. Materials and Methods

### Case Selection

We reviewed the biopsy of the primary site of DLCBL and the immunohistochemistry reports filed during the period from 2002 to 2010 at the surgical pathology archives of the Pathology Unit of the General Hospital of Mexico, and selected all cases diagnosed as DLCBL arising in lymph nodes and extranodal sites, such as palatine tonsils, oropharynx, gastrointestinal tract, cervix, and mammary glands, and that had a bone marrow biopsy. We reviewed the histologic sections stained with hematoxylin-eosin, periodic acid-Schiff (PAS), Giemsa, and reticulin. We included BM biopsies that sampled at least 8 - 10 medullary spaces or at least 1 cm long and determined discordant versus concordant infiltration. We employed the streptavidin-biotin peroxidase technique for immunohistochemistry studies of tissue embedded in paraffin from the primary tumor and BMs with suspected discordant morphology. Antigenic recovery was performed in a pressure boiler, with citrate buffer at a pH of 8 and pressure of 1 bar (20 PSI) for 30 seconds and subsequently incubated for 40 min at room temperature. To perform the immunohistochemistry tests, the tissue was cut into 4-µm thick sections and deparaffinized. Endogenous peroxidase activity was blocked using methanol and hydrogen peroxide. Subsequently it was washed, placed in phosphate buffered saline, and then incubated with normal sheep serum. The sample was decanted and the primary antibody was incubated for 18 h at 4˚C, washed, and placed in phosphate buffered saline. The secondary biotin-marked antibody was added for 60 min and washed with PBS. Streptavidin-biotin was added and the antigen-antibody binding visualized with diaminobenzidine. It was washed under running tap water and then counterstained with Hill hematoxylin, washed, rehydrated, cleared in xylol, and mounted on the corresponding slides. The monoclonal antibodies used were CD 20 (L-26 clone Dako Cytomation), CD 5 (clone, CD5/54/B4), CD 2 (rabbit monoclonal antibodies; Dako Cytomation), CD 10 (clone, 56C; Novocastra Laboratories), MUM-1 protein (clone, MUM 1p; Dako Cytomation), bcl2 (clone 124; Dako Cytomation) and bcl6 (clone G-B6p; Dako Cytomation).

The *in-situ* hybridization technique was carried out using fluorescein-labeled oligonucleotide probes to detect nuclear presence of encoded Epstein Barr RNA (EBER). We added conjugated alkaline phosphatase, rabbit antibody and antifluorescein isothiocyanate, followed by 4-nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche Diagnostics, Indianapolis, IN, USA). Hill's hematoxylin was used to counter-stain and the procedure was continued as described in the immunohistochemistry technique above. As a positive control we used a nasal T/NK cell lymphoma sample.

The following independent variables were analyzed: age, gender, primary site, and morphological subtype (centroblastic: monomorphic or polymorphic, immunoblastic and anaplastic). The immunohistochemistry markers were reported as positive when at least 30% of the neoplastic cells showed positivity for CD 10, bcl6, bcl2, and MUM-1. For the *in-situ* hybridization test, EBER was reported as either negative or positive. The dependent variable, bone marrow infiltration, was judged by the size of lymphoid cells as either discordant (small lymphocyte) or concordant (large lymphocyte with blastic resemblance). Statistical analysis was performed using the SPSS software (version 11) and the level of statistical significance for comparisons was set at $P \leq 0.05$ using $x^2$.

## 3. Results

We evaluated 108 patients who had a suitable bone marrow biopsy. Fifty-nine (55%) were male and 49 (45%) were female. The primary sites were localized as follows: lymph nodes (60.1%), palatine tonsils (13%), ocular adnexa (6.5%), digestive tract (3.7%), the rest of the primary DLBCL sites included soft tissues, mammary glands and cervix. Skin, mediastinum, and central nervous system was not included (**Table 1**). The mean age of diagnosis in the patients presenting DLBCL affecting lymph nodes and tonsils was 52 years. For DLCBL affecting ocular adnexa and other extranodal sites, the mean age at diagnosis was 47.8 and 47.9 years, respecttively, while for those primarily affecting the digestive tube, the mean age at diagnosis was 65 years. Concerning morphology, the centroblastic (CB) variant was predominant with 87 cases (80.5%). The CB variant was further subdivided, according to Kiel's classification criteria into polymorphic and monomorphic subtypes, considering the

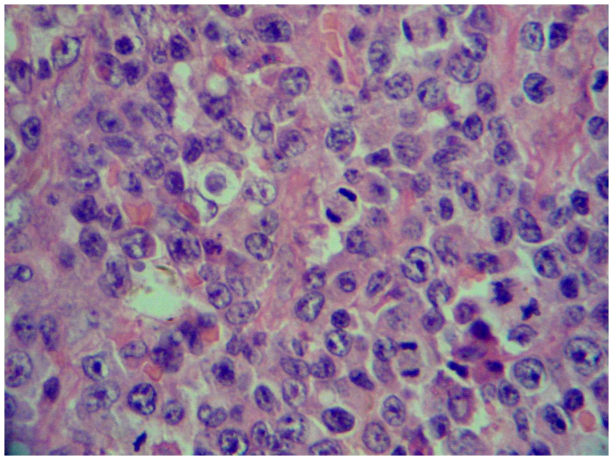
**Table 1. Association between gender, the morphologic type of DLBCL and the primary site.**

| DLBCLtype | Gender | Primary site of DLBCL | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Lymph nodes | | Palatine tonsils | | Ocular adnexa | | Digestive tract | | Other extranodal sites | |
| | | Count | Column N % | Count | Column N % | Count | Column N % | Count | Column N % | Count | Column N % |
| Polymorphic | Female | 15 | 40.5 | 5 | 41.7 | 3 | 50.0 | 1 | 100.0 | 5 | 71.4 |
| | Male | 22 | 59.5 | 7 | 58.3 | 3 | 50.0 | 0 | 0.0 | 2 | 28.6 |
| Monomorphic | Female | 6 | 42.9 | 1 | 100.0 | 1 | 100.0 | 0 | 0.0 | 3 | 60.0 |
| | Male | 8 | 57.1 | 0 | 0.0 | 0 | 0.0 | 2 | 100.0 | 2 | 40.0 |
| Immunoblastic | Female | 3 | 42.9 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 1 | 50.0 |
| | Male | 4 | 57.1 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 1 | 50.0 |
| Anaplastic | Female | 2 | 40.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 3 | 100.0 |
| | Male | 3 | 60.0 | 1 | 100.0 | 0 | 0.0 | 1 | 100.0 | 0 | 0.0 |

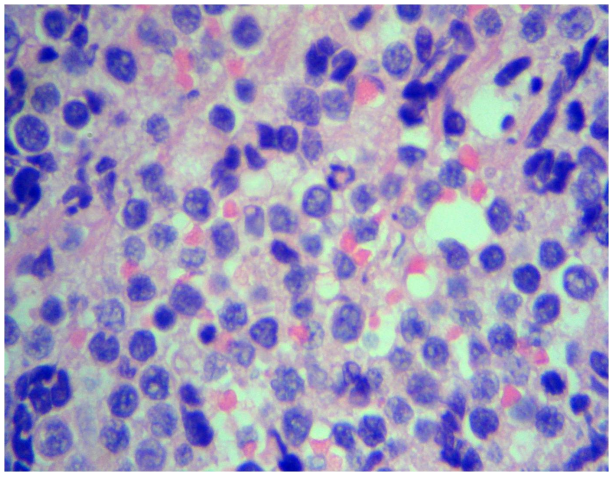
number of immunoblasts, centrocytes, and centrocytoid cells. Sixty-four (73.5%) cases were classified as polymorphic CB (**Figure 1(a)**) and 23 (26.4%) cases as monomorphic CB (**Figure 1(b)**). Eleven (10.1%) were classified as anaplastic and 10 (9.2%) as immunoblastic lymphomas (IB) (**Table 1**). Considering immunohistochemical marker expression, lymphomas that were positive for CD 10 and Bcl6 (28.7%) were considered as deriving from germinal center (GC) B-cells (**Figure 2(a)**), whereas those expressing Mum-1 (**Figure 2(b)**) and lacking CD 10 expression (58.3%) were considered as deriving from activated B-cells (ABC) or non-GC cells. Lymphomas expressing only bcl2 markers were considered as a third type and corresponded to 12.9% of cases. (**Table 2**).

Infiltration to bone marrow was documented in 30.6% of cases, and the mean age at detection of BM infiltration was 54.8 years. Infiltration was determined as discordant in 55% (**Figures 3(a)** and **(b)**) and concordant (**Figures 4(a)** and **(b)**) in 45% of cases. BM infiltration by site was most common in lymph node with 21 of 65 cases, followed by tonsils with 3 of 11 cases showing BM infiltration and digestive tube with 3 of 7 cases showing BM infiltration. DLBCL of primary sites corresponding to eyes, adnexa, and other sites (22 cases) showed BM infiltration in 6 cases (5.5%) ($P = 0.638$).

Regarding morphology and BM infiltration, we documented that the CB variant was most frequently associated to BM involvement (29 cases). The percentage of BM infiltration in the polymorphic (45.5%) and monomorphic subtypes (42.4%) was similar ($P = 0.0003$) (**Table 3**). Three cases of immunoblastic lymphomas (9%) and one case of anaplastic lymphoma (3%) showed BM infiltration (**Table 4**). Correlation between immunophenotype and BM infiltration showed that 10 out of 31



(a)



(b)

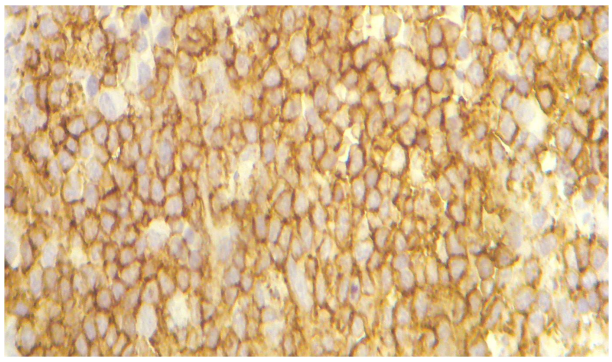
**Figure 1. Diffuse large B-cell lymphoma. H&E stains show a typical polymorphic centroblastic lymphoma (a) (40×) and the centroblastic, monomorphic (b) (H&E, 40×).**

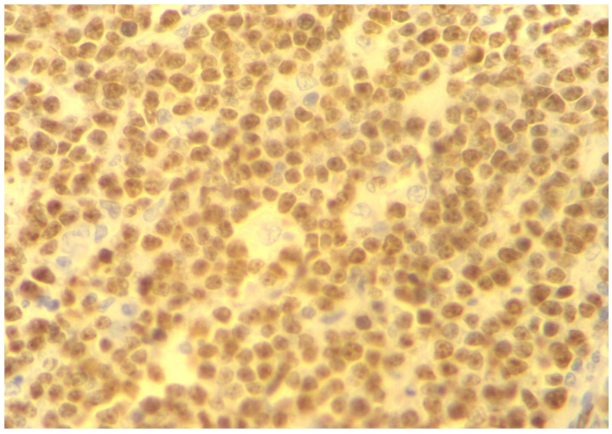
**Table 2. Correlation to the immunophenotype and morphological variants to the DLBCL.**

| Immunophenotype | Morphologic types | | | | Total |
|---|---|---|---|---|---|
| | CB poli | CB mo | IB | Anaplasic | |
| GC | 23(21.2%) | 5(5.6%) | 1(0.9%) | 2(1.8%) | 31(28.7%) |
| ABC | 36(33.3%) | 12(11.1%) | 8(7.4%) | 7(6.4%) | 63(58.3%) |
| 3th Type | 5(4.6%) | 6(5.5%) | 1(0.9%) | 2(1.8%) | 14(12.9%) |
| Total | 64(59.2%) | 23(21.2%) | 10(9.2%) | 11(10.1%) | 108(100%) |

$Xi^2$ 89.18 ($p = 0.178$).



(a)

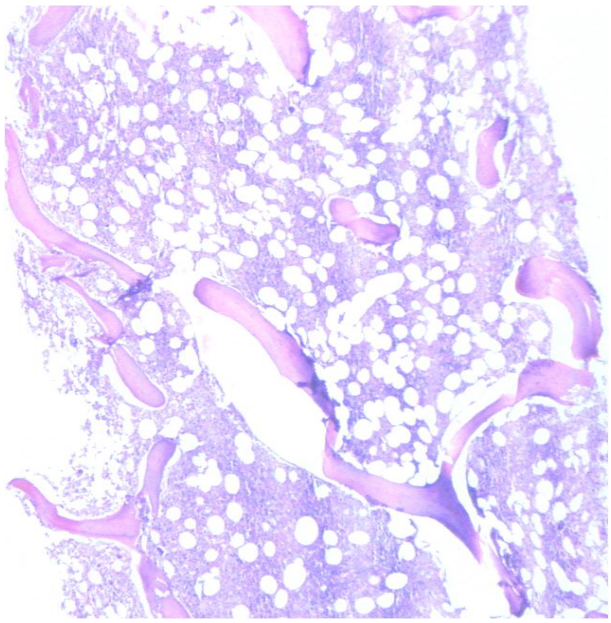


(b)

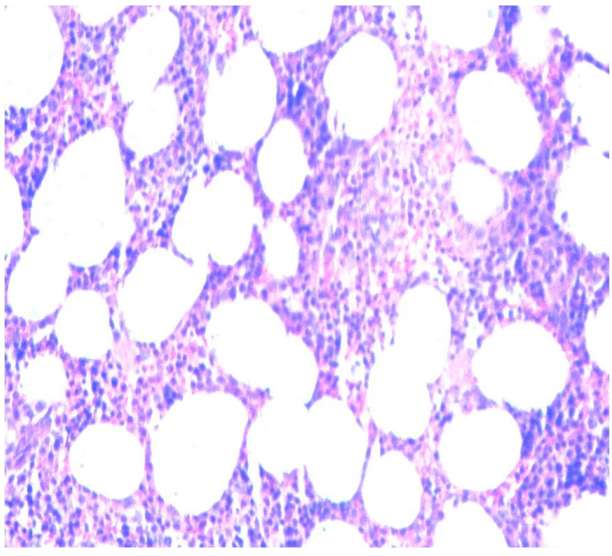
**Figure 2. Immunohistochemical stains for CD10+ (a) expression profiles to the GCB and MUM-1+ (b) protein expression in the DLBCL ABC.**

(9.2%) germinal center-originating lymphomas infiltrated the BM, whereas 17 of 63 cases deriving from B activated lymphocytes (15.7%) infiltrated the BM ($P = 0.49$) (**Table 4**). Six cases of the total 14 lymphomas expressing only bcl2 showed BM involvement. No statistically significant correlation was found between the expression of CD10, bcl6, bcl2, and MUM-1 markers and the type of infiltration ($P = 0.469$, $P = 0.685$, $P = 0.518$, and $P = 0.168$, respectively).

Presence of Epstein Barr virus (EBV) was positive in



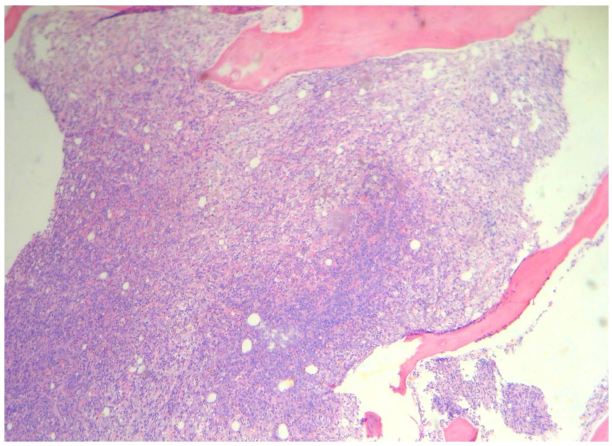
(a)



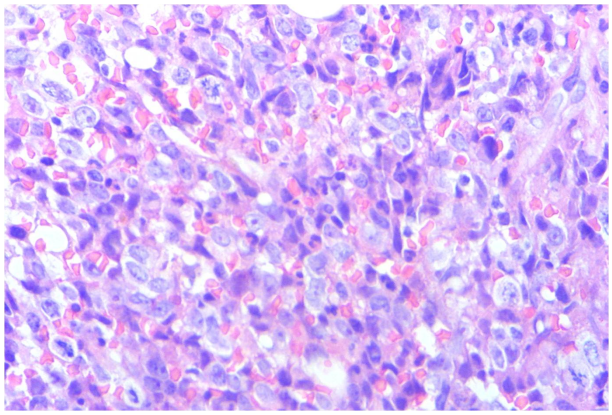
(b)

**Figure 3. Discordant infiltration to bone marrow (H&E) (a) Low power and (b) High power.**



(a)



(b)

**Figure 4. Concordant infiltration to bone marrow (H&E) (a) Low power and (b) High power.**

**Table 3. The morphologic type and bone marrow infiltration of the DLBCL.**

| Morphologic type of D LBCL | Bone marrow infiltration | | N |
|---|---|---|---|
| | Neg | Pos | |
| CB Poli | 48 | 15 | 63 |
| CB mo | 9 | 14 | 23 |
| IB | 7 | 3 | 10 |
| Anaplasic | 10 | 1 | 11 |
| Total | 75 | 33 | 108 |
| % | 69.2 | 30.6 | 100.0 |

$Xi^2$ 13.879 ($p = 0.0003$).

10 of the 66 patients over 50 years of age (15.1%) (**Figure 5**). Morphologically, eight of these cases were classified as CB and two as IB ($P = 0.207$) (**Table 5**). One case expressed CD10 and was classified as originating from the GC, whereas seven cases expressed MUM-1 and were considered as derived from ABC. Two cases

**Table 4. Type of infiltration to bone marrow and the immunophenotype of the DLBCL.**

| Infiltration type | Inmunophenotype of DLBCL | | | Total |
|---|---|---|---|---|
| | GC | ABC | Third type | |
| Concordant | 6 | 7 | 2 | 15 |
| Discordant | 4 | 10 | 4 | 18 |
| Non infiltration | 21 | 46 | 8 | 75 |
| Total | 31 | 63 | 14 | 108 |

$Xi^2$ 2.930 ($p = 0.570$).

**Table 5. Shows the morphologics variants of DLBCL and cases EBER positive in patients older than 50 years old.**

| | EBER | | |
|---|---|---|---|
| | Neg | Pos | Total |
| CB pol | 33 | 4 | |
| CB mo | 11 | 4 | |
| IB | 7 | 2 | |
| Anaplasic | 5 | 0 | |
| Total | 56 | 10 | 66 |

$Xi^2$ 8.446 ($p = 0.207$).

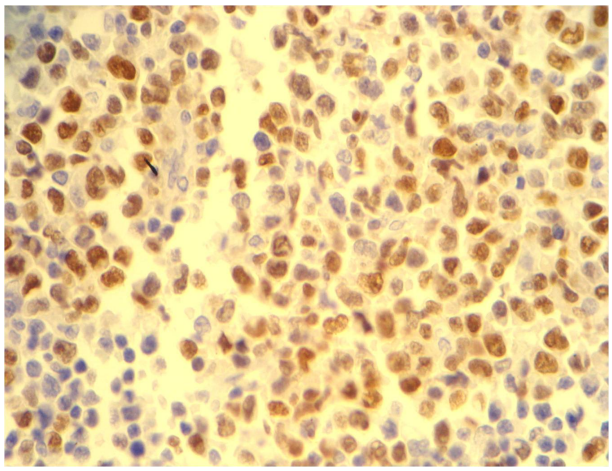


**Figure 5. Dako *in situ* hybridization for Epstein-Barr virus positive case showing brown intranuclear sgnals in DLBCL patients older ≥ 50 years old.**

did not express any of the aforementioned markers. Four of these 10 cases showed infiltration to BM, which was of the concordant subtype in one case and discordant in three cases ($P = 0.585$).

## 4. Discussion

The bone marrow involvement by large-B cell lymphoma

usually occurs in patients with widely disseminated disease. In this study, we associated site (nodal vs. extranodal), morphological variants, and immunohistochemical marker expression with infiltration to BM in patients at the time of diagnosis of the "novo" diffuse large B-cell lymphomas. Our results show that DLBCLs were more frequent in men and mean age at diagnosis was 52 years, which is younger than the age reported for another studies [1,2,3,15]. The primary sites most frequently affected were lymph nodes, followed by Waldeyer's ring, which is also in contrast to reports in the article of Höller and cols, where the digestive tube is mentioned as the most frequent extranodal site [15]. The mean age at diagnosis in patients with DLBCL of the digestive tract was 65 years, which proved older than the mean age of 52 years at diagnosis in other primary sites (lymph nodes, Waldeyer's ring, and others). And the mean age at diagnosis of primary DLBCL of the superior aerodigestive tract and others was of 51 and 53 years reported by Wong *et al*. [16]. BM infiltration was documented in 30.6% of cases. There are controversial data on the prognostic signifycance of BM involvement in DLBCLs evaluated by conventional histology methods, as it ranges from 11% to 35% [11,12]. This variability may be related to adequacy of obtained hematopoietic tissue and precision of histopathological reporting [14]. Our results regarding primary site and presence of BM infiltration were not significantly different from findings in other reports. The sites of extranodal lymphomas reported in countries such as Korea, Malaysia, and China are similar to our findings [17,18]. Morphologically, the predominant type was the CB variant. Centroblastic morphology was the only morphological variable significantly correlated with BM infiltration ($P = 0.0003$) and infiltration rates were similar in both CB subtypes. The second morphological variant was anaplastic lymphoma, which was present in 10% of cases and showed BM infiltration in one patient. IB subtype was the least frequent morphological variant (9%) in our study, which is slightly higher than the 4% frequency reported by a German study group showing poor survival rates in these patients [19]. Plasmacytoid differentiation in immunoblastic lymphomas has been identified as a risk factor [20]. Three of our IB cases showed BM infiltration. Using the antibodies recommended by Hans *et al*., DLBCLs were classified, on the basis of expression markers, in three immunophenotypes. Germinal center immnunophenotype was found in 29% of cases, which proved lower than found in other series using the same classification method and reporting 39% to 49% cases of GC phenotype [6,21]. Lymphomas originating from ABC were the most common (58%) in our series. Chen *et al.* found this phenotype in 78.2% of a 124 patient cohort using two classification algorithms. In this cohort they also found that BCL2 expression was associated with gain of a chromosome region 18/18q and 3/3q [22].

Some studies [23,24] have shown that the ABC phenotype is more common in DLBCLs of extranodal origin and the presence of this phenotype might account for differences in the development of nodal or extranodal DLBCLs. In our study, as in the study by Chen *et al*., DLBCLs were more frequently of primary nodal origin. Lymphomas that did not express CD 10, Bcl6, and Mum-1 corresponded to 12.9% of cases.

Correlation of immunophenotype and BM infiltration showed that the ABC phenotype was most frequently associated with BM infiltration (15.7%), followed by GC phenotype (9.2%), and the third type (5.5%). Infiltration patterns in bone marrow range from focal infiltrates to complete substitution of hematopoietic elements. Discordant involvement with presence of large cells in the primary tissue and small cells in bone marrow infiltrates has been recognized in DLBCL, and is encountered in 50 to 70% of cases in different series [12,21,25]. Routine stains identified the lymphoid infiltrate, although eight cases required immunohistochemical technique with the follow antibodies: CD 20, CD2 and CD5. The impact of bone marrow involvement varies and has prognostic value in cases of DLBCLs; it has been suggested that bone marrow involvement affects the international prognostic index (IPI) as it places the disease in clinical stage IV [25]. Immunohistochemistry and flow cytometry results from bone marrow aspirates have documented the presence of up to 11% of lymphoid neoplastic cells of unclear morphology [26,27]. The prognostic differences of the GC and ABC phenotypes must be reevaluated, since they were assed in the era before rituximab [28]. In the near future, it will be important to study correlations between immunohistological markers, bone marrow involvement at the time of diagnosis, and response to clinical treatment. Molecular and genetic alterations encountered in these types of lymphomas include chromosomic translocations that frequently involve the immunoglobulin locus, resulting in dysregulation of proto-ocogenes (c-MYC, BCL2, BCL6 and others) and gene mutations that involve key functions of normal B-cells. These mutations resist a characteristic pattern of somatic hypermutations, a process involving Ig genes in the differenttiation of B cells. These alterations are observed at the origin of the errors occurring during physiological events involved in the diversification repertoire of the germinal center, during class switch recombination (CSR) or somatic hypermutation (SHM) [28,29]. Experimental studies on animal models have confirmed the critical roll played by the induced activation of the cytidine deaminase enzyme in the physiology of CSR and SHM in the development of lymphoid tumors [30,31].

Epstein-Barr virus-positive DLBCL of the elderly has been separated from DLBCL, not otherwise specified, in the current 2008 WHO classification [1], and occurs in patients with no known history of immunodeficiency or other lymphoproliferative process. An 8% to 10% frequency has been reported in Asian series [32] but there are few data in westerns countries. In this study, *in-situ* hybridization studies were performed in 66 patients older than 50 years and presence of nuclear Epstein Barr Virus was detected in 10 patients (15.1%). These results are similar to those found in Asian literature and in a recent study by Quintanilla *et al*., who reported a 7% prevalence in Mexican patients [33]. The proportion of Epstein Barr positive cases increases with age; in a recent study in a Latin American country the mean onset age was 75 years and cases were classified as DLBCL of germinal center phenotype [34]. It is suggested that this increased incidence may be related to immunological damage or senescence as part of the aging process. Seventy percent of patients with EBV associated DLBCLs have extranodal disease (skin, lung, tonsils, and stomach) and 30% have primarily nodal disease. In this study, 9 patients presented lymph nodes as the primary site, whereas in one patient the primary site was the digestive tract. Regarding morphology, Epstein Barr-positive DLBCL cases were of the polymorphic centroblastic subtype. There was BM infiltration in four cases, three of them of the discordant type and one case had concordant involvement. It has been observed that the presence of EBV in DLCBLs is related to poor clinical outcome [35]. In this study the presence of EBV was related to DLBCLs of poor prognosis. In an another recent paper of Peru the authors reports 7 patients with de novo DLBCL and infection with HTLV-1carriers and EBV infection, is it interesant by the rare association [36].

In conclusion, there is scarce information in Latin America countries on the morphology and immunophenotypes of DLBCLs. Furthermore, there are few reports on the incidence and type of bone marrow involvement, and our study could have an impact on the initial diagnosis as well as for the clinical stratification of patients, since BM infiltration may have a negative impact on survival rate in patients with this type of lymphomas and recent data support the concept that molecular subtyping of diffuse large B-cell lymphoma is clinically relevant.

## 5. Acknowledgements

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## Abbreviations, Nomenclature, and Symbols

| | |
|---|---|
| DLBCL | Diffuse large B cell lymphoma |
| EBER | *In situ* hibridization techique to detect Epstein-Barr virus |
| PCR | Polymerase chain reaction |
| BM | Bone marrow |
| CB | Centroblastic |
| IB | Immunoblastic |
| GC | Germinal center |
| ABC | Activated B cells |
| CB pol | polymorphic centroblastic |
| CB mo | monomorphic centroblastic |
| Neg | negative |
| Pos | positive |

 