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Original Article

Prognostic impact of tumor-associated macrophages, lymphocyte-to-monocyte and neutrophil-to-lymphocyte ratio in diffuse large B-cell lymphoma

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Abstract: Introduction: Microenvironment has a prognostic influence in diffuse large B-cell lymphoma (DLBCL); among its components, tumor-associated macrophages (TAM) play a leading role. TAM can be classified into M1 (anti-tumor) and M2 (pro-tumor). Another prognostic factor could be represented by lymphocyte-to-monocyte and neutrophil-to-lymphocyte ratio (LMR and NLR). Objective: The aim of the study is to evaluate the prognostic impact of M1 and M2 TAM subtypes, LMR and NLR in DLBCL. Methods: We analyzed 37 consecutive patients between 2009 and 2013. Out of 37 patients, 28/37 (75.6%) received R-CHOP/CHOP-like regimens, 9/37 (24.4%) less intensive therapies. Immunohistochemistry was performed with antibodies against CD68 and CD163. We divided our cohort into 2 categories according to the Steidl score. TAM who coexpressed CD68 and CD163 were considered as M2. For LMR and NLR we used previously published cut-offs of 2.71 and 2.81. Results: CR rate was 70.3%; we did not record a significant correlation between CD68+ TAM, CD163+ TAM, CD68+/CD163+ TAM, LMR, NLR and CR. We observed a reduced PFS in patients with IPI ≥ 2 and high M2 TAM expression and a trend between higher expression of CD68+ TAM and improved PFS. Conclusion: M2 TAM could have a prognostic role for IPI ≥ 2 DLBCL patients receiving R-CHOP, which thus warrants further investigation.

Keywords: Diffuse large B-cell lymphoma, TAM, prognosis

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL), characterized by aggressive behavior [1]. Anthracycline-based chemoimmunotherapy, such as rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP), can be curative in most cases; however, 20-40% of patients experience relapse or have refractory disease [1, 2]. International Prognostic Index (IPI) has been validated in the rituximab era, but it includes only clinical variables and it does not consider the well-known biological heterogeneity of the disease [3]. Cell-of-origin (COO), based on gene expression profiling (GEP) is relevant, dividing patients into 2 categories: germinal center B-cell-like (GCB, better prognosis) and activated B-cell-like (ABC, poor prognosis) [4]. Unfortunately, GEP is not always available and the use of immunohistochemistry (IHC) algorithms, even if acceptable, has reproducibility issues and is not uniformly reported to have prognostic utility [4-6].

Previous GEP-based studies showed that the microenvironment has a prognostic influence in DLBCL. Stromal-1 signature, characterized by an elevated number of macrophages and genes expressed by components of extracellular matrix, is associated with good prognosis, while stromal-2 signature is characterized by increased angiogenesis and dismal outcome [7].

Tumor microenvironment includes macrophages, dendritic cells, T-helper, T-cytotoxic and reactive B-lymphoid cells; it could promote neoplastic cell survival and could be associated with drug resistance [8]. Among its cellular components, tumor-associated macrophages

(TAM) surely play a leading role. TAM are characterized by remarkable plasticity; depending on the stimuli that trigger their activation, they are polarized towards form M1, leading to antitumor responses, or M2, leading to tumor growth and progression [8-10].

The role of TAM in lymphoproliferative malignancies, such as Hodgkin lymphoma (HL) and DLBCL, has been analyzed with conflicting results, owing to a wide heterogeneity in the choice of TAM-associated markers, antibodies used for IHC and best cut-offs [11-14]. Some studies demonstrated that the content of TAM CD68+ was associated with improved survival in DLBCL, while an alteration of CD163/CD68 ratio, due to an increase in TAM CD163+ cell number, suggestive of M2 polarization, was correlated with poor prognosis [15-25].

Another prognostic tool should be represented by lymphopenia and increased neutrophils count (ANC), a surrogate marker of immunological dysfunction and chronic inflammation, respectively [26, 27]. It has been hypothesized that an absolute low lymphocyte count (ALC), together with neutrophilia, could correlate with an impaired immunological response against lymphoid malignancies [26-31]. Some studies observed a prognostic role of ALC, lymphocyte/monocyte ratio (LMR) and neutrophils/lymphocytes ratio (NLR) in DLBCL patients receiving R-CHOP [23, 32, 33]. According to this background, in this study we would like to evaluate the prognostic influence of M1 and M2 TAM subtypes, LMR and NLR in newly diagnosed DLBCL patients.

Patients and methods

Patients

This retrospective study was based on a series of 37 newly diagnosed DLBCL patients managed at the divisions of Hematology and Pathology in Siena and requiring first-line treatment. The patients were recruited from March 2009 to August 2013. Inclusion criteria were: histopathological diagnosis made according to the WHO 2008 classification [34]; at least one evaluable lesion at baseline CT scan; adequate tumor tissues for IHC analysis; available follow-up records. Exclusion criteria were: HIV infection, transformed DLBCL, primary mediastinal DLBCL, primary central nervous system DLBCL, primary cutaneous DLBCL. All procedures performed in studies were in accordance with the ethical standards of the institutional review board. All patients gave their written informed consent in accordance with the Declaration of Helsinki. At pre-treatment evaluation, medical history, physical examination, complete blood cell count (CBC), a biochemistry panel including lactate dehydrogenase and beta2-microglobulin, HBV, HCV, HIV evaluation, echocardiogram, CT scan, PET scan and bone marrow biopsy were performed. Ann Arbor staging system was applied before starting treatment. In the study, we included DLBCL cases with sufficient diagnostic paraffin-embedded material to perform IHC analysis and with available clinical follow-up information after therapy to perform survival analysis.

Treatment regimen and concomitant medications

Treatment regimen consisted of standard dose R-CHOP (rituximab 375 mg/m^2 intravenously on day 1, cyclophosphamide 750 mg/m^2, doxorubicin 50 mg/m^2, vincristine 1.4 mg/m^2 with a maximum dose of 2 mg on day 2, prednisone 40 mg/m^2 on days 2-6) or CHOP-like (doxorubicin substituted by a non-pegylated liposomal formulation or mitoxantrone because of cardiac comorbidity or high cardiovascular risk) every 21 days for up to 6 cycles, providing that hematological recovery had occurred. Frail patients according to a comprehensive geriatric assessment (CGA) received less intensive regimens such as rituximab in association with cyclophosphamide, mitoxantrone, vincristine, etoposide, bleomycin and prednisone (VNCOP-B) or bendamustine [35-38]. Involved-field radiotherapy (IF-RT) was administered as consolidation in localized disease. Intravenous hydrocortisone (200 mg), intravenous chlorpheniramine (10 mg) and oral paracetamol (500 mg) were given before the first rituximab administration; if no adverse reactions occurred, hydrocortisone was not given before subsequent courses.

Concomitant medications included antimicrobial prophylaxis of Pneumocystis jirovecii with trimethoprim-sulfamethoxazole from beginning to at least 6 months after treatment and granulocyte colony-stimulating factor to patients who developed grade 3-4 neutropenia. HBV-positive

patients received lamivudine prophylaxis until 12 months after therapy. Treatment was discontinued if intermediate evaluation showed unsatisfactory response and at any time in case of unacceptable toxicity.

Assessment of response

Response to treatment was assessed after 4th cycle (R-CHOP) or 8th week (R-VNCOP-B) and at least 4 weeks after end of treatment according to 2007 Cheson's criteria [39]. All patients received CT scan and PET, while bone marrow biopsy was repeated only if positive before treatment. Patients not achieving CR were considered as treatment failures. During the follow-up period, CT scans were performed at 6, 12 and 24 months; gastric DLBCL cases also received endoscopy. Clinical follow-up was continued every 6 months until the 5th year and annually thereafter.

Immunohistochemistry and blood sample analysis

Formalin-fixed, paraffin embedded diagnostic specimens including tumor cells from each patient were selected for IHC analysis. Subsequently, 4-μm-thick paraffin sections were performed; one section was stained with hematoxylin and eosin, the other was stained with monoclonal antibodies against human CD68 (clone PG-M1, 1:50; Dako) and CD163 (1:200; Novocastra-Leica). IHC was then performed using an automatic staining system (BenchMark Ultra; Dako). The antibody used for CD163 was stained brown with 3,3' diaminobenzidine (DAB); CD68, on the other hand, was stained red with Permanent Red.

All cases were then evaluated in double blind by 2 professional pathologists, who did not know the clinical data. At least 3 high magnification fields were observed, firstly evaluating in the area with the most intensive expression the total amount of CD68-positive and CD163-positive macrophages and afterwards analyzing the various groups (CD163 or CD68 single-positive, CD163/CD68 double-positive), finally giving a score based on the percentage of their expression, as published by Steidl and colleagues [11]. Based on this percentage, patients were divided into three groups: < 5% (low expression, score 1), 5-25% (intermediate expression, score 2) and > 25% (high expression, score 3). With the aim of dividing our cohort into only 2 different categories, patient with score 1-2 were considered together. The Steidl score, although designed for HL, was chosen because it represents the best validated and reproducible score to date.

Remarkably, in our analysis CD68/CD163 double-positive macrophages were considered as M2 [15, 16].

For LMR and NLR evaluation, we used the discriminative cut-off values of 2.71 and 2.81, respectively, as previously published [23]. Patients were categorized as high-LMR (≥ 2.71) and low-LMR (< 2.71) or high LNR (≥ 2.81) and low-LNR (< 2.81).

Statistical analysis

This was a single arm, single-center study, patients' characteristics were analyzed with descriptive statistics. Categorical variables were analyzed using Chi-square or Fisher's exact test; Fisher's exact test was preferred for small sample size, when the expected frequency was less than 5. Partial remission (PR) was not considered a satisfactory result and grouped together with stable or progressive disease (SD/PD). For survival analysis, primary endpoint was progression-free survival (PFS), defined as the time from the first day of treatment until disease progression, relapse, death for any cause or last follow-up (censored); patients that did not achieve CR after induction therapy were censored at that point for the progression [39]. Overall survival (OS) was defined as the time from the first day of treatment until death for any cause. Survival curves were estimated using the method of Kaplan and Meier and log rank test for significant associations; a p value < 0.05 was considered statistically significant. All statistical analyses were made using software MedCalc, version 2.0.

Results

Characteristics of the patients

Clinical characteristics of patients are represented in **Table 1**. Median age at diagnosis was 61 years (range 22-89 years), male/female distribution was 16/21. Among them, 21/37 (56.7%) patients had early-stage disease (Ann-Arbor stage I-II), while 16/37 (43.3%)

Table 1. Patient's characteristics

Characteristic	Number of patients (%)
Age: median [range]	61 years [22-89]
Men	16/37 (43.2%)
Women	21/37 (56.8%)
Early-stage disease	21/37 (56.7%)
Advanced-stage disease	16/37 (43.3%)
B symptoms	10/37 (27%)
Bulky disease (≥ 7 cm)	4/37 (10.8%)
GCB-type	19/37 (51.3%)
ABC-type	18/37 (48.7%)
LDH elevated	27/37 (73%)
IPI score 0-1	15/37 (40.6%)
IPI score 2	11/37 (29.7%)
IPI score 3-5	11/37 (29.7%)
ECOG performance status 0-1	32/37 (81%)
CD68 expression low	19/37 (51.3%)
CD68 expression high	18/37 (48.7%)
CD163 expression low	12/37 (32.4%)
CD163 expression high	25/37 (67.6%)
M2 TAM CD68/CD163 low	16/37 (43.2%)
M2 TAM CD68/CD163 high	21/37 (56.8%)
LMR < 2.71	21/37 (56.8%)
LMR ≥ 2.71	16/37 (43.2%)
NLR < 2.81	11/37 (29.7%)
NLR ≥ 2.81	26/37 (70.3%)

Abbreviations: GCB, germinal center B-cell; ABC, activated B-cell; LDH, lactate dehydrogenase; IPI, international prognostic index; TAM, tumor associated macrophages; LMR, lymphocyte-to-monocyte ratio; NLR, neutrophil-to-lymphocyte ratio.

patients had advanced-stage disease (stage III-IV); 4/37 patients (10.8%) presented with bulky disease (diameter ≥ 7 cm). COO was retrospectively determined by IHC using Hans algorithm, 19/37 (51.3%) and 18/37 (48.7%) cases were GCB and ABC-type, respectively. IPI was low (0-1), intermediate (2) and high (3-4) in 15/37 (40.6%), 11/37 (29.7%) and 11/37 (29.7%) cases, respectively. Out of 37 patients, 28/37 (75.6%) received R-CHOP or CHOP-like regimens, while 9/37 (24.4%) were treated with less intensive regimens such as R-VNCOP-B (7 cases) or R-bendamustine (2 cases).

Overall, the number of CD163-positive cells exceeded that of CD68-positive cells, as previously reported by Nam and colleagues [16]. CD68 expression was low in 19/37 patients (51.3%) and high in 18/37 (48.7%), while CD163 expression was low in 12/37 patients (32.4%) and high in 25/37 (67.6%). CD68/CD163 double-positive M2 TAM expression was low and high in 16/37 (43.2%) and 21/37 (56.8%) cases, respectively. NLR was < and ≥ 2.81 in 11/37 (29.7%) and 26/37 cases (70.3%); LMR was < and ≥ 2.71 in 21/37 (56.8%) and 16/37 cases (43.2%), respectively. Characteristics of patients were well balanced in the different subgroups, as reported in **Table 2**.

Response to treatment

Responses to treatment are summarized in **Table 3**. Out of 37 patients, 26/37 (70.3%) achieved a CR, while 11/37 patients (29.7%) were considered as a treatment failure; in this group, 5/11, 4/11 and 2/11 patients achieved a PR, SD and PD, respectively. Two patients in PR received platinum-containing 2nd line therapy, all patients achieved a CR, 1/2 received autologous stem-cell transplantation (ABMT) as consolidation therapy; 1 patient with localized disease was successfully treated with IF-RT; 2 patients did not receive other therapies because of poor clinical conditions and pancreatic neoplasm (1 case each). Two patients in PD received platinum-containing 2nd line therapy, 1/2 achieved a CR and received ABMT as consolidation therapy, while the other was chemo-refractory. Out of 4 elderly patients with SD, 3/4 received gemcitabine and the other received palliative RT, none of them responded to treatment.

In responders, CD68 was high in 14/26 (53.8%) cases and low in 12/26 (46.2%); in patients who had treatment failure CD68 was high in 4/11 (36.4%) cases and low in 7/11 (63.6%) (P=0.47). CD163 was high in 17/26 (65.4%) patients in CR and low in 9/26 (34.6%); in patients who had treatment failure CD163 was high in 8/11 (72.7%) cases and low in 3/11 (27.3%). CD68/CD163 M2 TAM expression in responders was high in 13/26 (50%) cases and low in 13/26 (50%) cases; in patients who experienced treatment failure was high in 8/11 (72.7%) and low in 3/11 (27.3%) cases (P=0.49).

LMR was ≥ 2.71 in 12/26 (46.1%) responders and < 2.71 in 14/26 (53.9%), while it was ≥ 2.71 in 4/11 (36.3%) non-responders and <

Table 2. Characteristics of patients according to the CD68 and CD163 expression

Characteristic	CD68			CD163			CD68/CD163		
	Low	High	P	Low	High	p	Low	High	P
Age < 60	6/19	7/18	0.9	5/12	8/25	0.83	7/16	6/21	0.54
Men	8/19	8/18	0.88	6/12	10/25	0.82	8/16	8/21	0.69
Women	11/19	10/18		6/12	15/25		8/16	13/21	
Early-stage	12/19	9/18	0.63	7/12	14/25	0.89	10/16	11/21	0.77
Advanced-stage	7/19	9/18		5/12	11/25		6/16	10/21	
B symptoms	7/19	4/18	0.47	4/12	6/25	0.69	3/16	7/21	0.53
GCB-type	11/19	8/18	0.62	7/12	12/25	0.81	9/16	10/21	0.85
ABC-type	8/19	10/18		5/12	13/25		7/16	11/21	
LDH elevated	12/19	15/18	0.26	8/12	19/25	0.69	10/16	17/21	0.27
IPI score 0-1	9/19	6/18		4/12	11/25		6/16	9/21	
IPI score 2	6/19	5/18	0.47	4/12	7/25	0.82	6/16	5/21	0.7
IPI score 3-5	4/19	7/18		4/12	7/25		4/16	7/21	
ECOG PS 0-1	15/19	17/18	0.33	9/12	24/25	0.09	13/16	19/21	0.63
LMR < 2.71	12/19	9/18	0.63	6/12	15/25	0.82	9/16	12/21	0.95
LMR ≥ 2.71	7/19	9/18		6/12	10/25		7/16	9/21	
NLR < 2.81	7/19	4/18	0.47	3/12	8/25	1.0	5/16	6/21	0.85
NLR ≥ 2.81	12/19	14/18		9/12	17/25		11/16	15/21	

Abbreviations: GCB, germinal center B-cell; ABC, activated B-cell; LDH, lactate dehydrogenase; IPI, international prognostic index; PS, performance status; LMR, lymphocyte-to-monocyte ratio; NLR, neutrophil-to-lymphocyte ratio.

2.71 in 7/11 (63.7%) (P=0.72); NLR was ≥ 2.81 in 19/26 responders (73.1%) and < 2.81 in 7/26 (26.9%), while it was ≥ 2.81 in 7/11 (63.6%) non-responders and low in 4/11 (36.4%) (P=0.69).

Survival analysis

After a median follow-up of 60 months, 23/37 (62.2%) patients were alive while 14 patients died (37.8%), 7 because of PD, 2 because of secondary malignancies, 3 because of infectious complications and 2 because of cardiac failure in CR. IPI maintained its prognostic value and was associated with both PFS and OS (Figures S1, S2, available on request). There was a trend between high CD68, low M2 TAM expression and increased PFS, while CD163, LMR and NLR were not associated with PFS (**Figure 1A-E**). Comparable results were obtained with OS analysis (**Figure 2A-E**). Median PFS and OS were not reached in the entire cohort; 5-y PFS and OS were 61% and 64%, respectively (**Figure 3A**, **3B**). Interestingly, when we tried to separately analyze patients with IPI ≥ 2, we observed a significant association between high M2 TAM and inferior PFS (**Figure 4**, P=0.04), while there was no association between CD68, CD163, LMR, NLR and PFS. Moreover, there was a trend between high M2 TAM and inferior OS, even if it did not reach statistical significance (**Figure 5**), while there was no association between CD68, CD163, LMR, NLR and OS.

Discussion

In this analysis we observed that i) there was no correlation between TAM CD68+, TAM CD163+, TAM M2, LMR, NLR and the attainment of CR, ii) for patients with IPI ≥ 2, there is a significant association between high M2 TAM and inferior PFS, iii) there was no association between CD68, CD163, LMR, NLR and outcome, while IPI maintained its prognostic value in our cohort of patients treated in the rituximab era.

Prognostic factors in DLBCL are still a matter of debate and treatment strategy is principally determined by IPI [3]. COO assessment with nanostring is not yet available in the majority of laboratories and IHC analysis produced conflicting results [4, 5].

TAM could reflect tumor-associated inflammation and could represent the most important component of microenvironment; its prognos-

Table 3. Response to treatment

	Number of patients	%	p
CR	26/37	70.3%	
PR	5/37	13.5%	
SD	4/37	10.8%	
PD	2/37	5.4%	
Responders (CR)			
CD68 high	14/18	77.7%	0.47
CD68 low	12/19	63.1%	
CD163 high	17/25	68%	1.0
CD163 low	9/12	75%	
CD68/CD163 M2 high	13/21	61.9%	0.28
CD68/CD163 M2 low	13/16	81.2%	
LMR ≥ 2.71	12/16	75%	0.72
LMR < 2.71	14/21	66.6%	
NLR ≥ 2.81	19/26	73.1%	0.69
NLR < 2.81	7/11	63.6%	
Treatment failure (PR+SD+PD)			
CD68 high	4/18	22.2%	0.47
CD68 low	7/19	36.8%	
CD163 high	8/25	32%	1.0
CD163 low	3/12	25%	
CD68/CD163 M2 high	8/21	38.1%	0.28
CD68/CD163 M2 low	3/16	18.8%	
LMR ≥ 2.71	4/16	25%	0.72
LMR < 2.71	7/21	33.3%	
NLR ≥ 2.81	7/26	26.9%	0.69
NLR < 2.81	4/11	36.4%	

Abbreviations: CR, complete remission; PR, partial response; SD, stable disease; PD, progressive disease; LMR, lymphocyte-to-monocyte ratio; NLR, neutrophil-to-lymphocyte ratio.

tic relevance was demonstrated in both solid and hematological malignancies [8]. In our opinion, the comprehension of the contribution of tumor microenvironment to treatment failure in DLBCL could represent a critical hurdle to overcome with the aim to design a targeted-therapy and to finally improve survival.

Riihijarvi and colleagues described the association between CD68 mRNA levels assessed with GEP, CD68 protein expression in IHC and DLBCL outcome. The study was conducted on 59 cases, using the anti-CD68 KP1 antibody and the positive cells were counted manually [18]. With the limitations related to the arbitrary choice of cut-off, CD68 was associated with favorable prognosis in patients treated with rituximab plus chemotherapy, while it was related to an unfavorable outcome in patients receiving only chemotherapy. Moreover, CD163 expression in GEP and IHC did not show any prognostic relevance [18]. Nam and colleagues analyzed 165 patients receiving R-CHOP and demonstrated that high CD68 expression was associated with OS improvement, while PFS and OS were reduced in cases with an increased CD163/CD68 ratio [17]. Conversely, Cai and colleagues showed that CD68 was a marker of poor prognosis, while the studies by Hasselblom and colleagues and Coutinho and colleagues did not report any significant correlation between CD68 and survival [19, 24, 25]. Gomez-Gelvez and colleagues enrolled 115 DLBCL patients receiving R-CHOP and designed a prognostic score including ABC subtype, FOXP3 > 17%, microvessel density < 800/mm^2 and CD68 < 2%; worse PFS was observed in the high-risk group [22].

These conflicting results could be due to wide heterogeneity in the choice of IHC antibodies (KP1 vs PG-M1), TAM markers, threshold (median vs best cut-off), scoring methods (manual vs automated) and treatment received (with or without rituximab) [14].

According to promising findings reported in non-hematological malignancies, major efforts should be focused on M2-TAMs, characterized by a pro-tumoral phenotype. In our opinion and as previously published, double staining for CD68 and CD163 could better identify a M2 phenotype, that was associated with inferior OS, while M1 phenotype did not show any prognostic relevance [15, 16]. Wang and colleagues retrospectively analyzed 355 DLBCL patients treated with R-CHOP and observed lower LMR, higher NLR and CD163+ M2 TAM ≥ 9.5% were related to shorter PFS and OS [23]. A recently published systematic review and meta-analysis confirmed that both CD68 and CD163 high density are associated with poor OS, but the highest hazard ratio is reached when CD163/CD68 TAM ratio is analyzed, further confirming that M2 TAM represent a robust predictor of outcome in NHL, including DLBCL, follicular lymphoma, mantle-cell lymphoma and T-cell lymphomas [40].

NLR demonstrated a prognostic role in lymphoproliferative disorders and we find merit in the attempt to identify a simple and reproducible

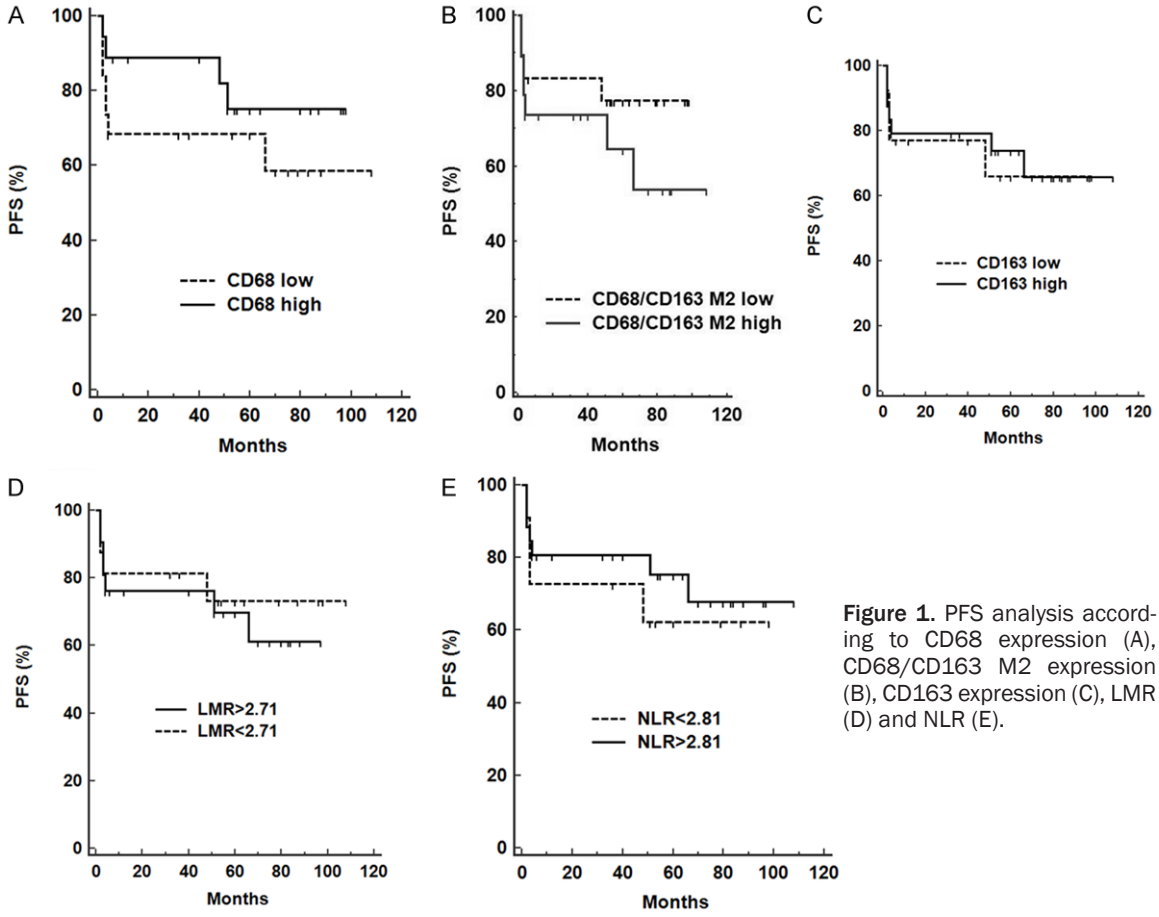

Figure 1. PFS analysis according to CD68 expression (A), CD68/CD163 M2 expression (B), CD163 expression (C), LMR (D) and NLR (E).

prognostic tool, that could represent a marker of immunosuppression and systemic inflammation [30, 31, 33]. However, at least 2 relevant biases could be identified, such as the administration of pre-phase steroid and a misdiagnosed infection that could increase ANC. Increased monocytes and decreased lymphocytes count could both promote angiogenesis and immunosuppression, finally favoring tumor growth and progression [23, 32]. Conflicting results have been achieved with regard to LMR and NLR prognostic value and the main issue remains the choice of an optimal cut-off [26-33].

In a recently published study, in 221 DLBCL cases receiving CHOP or R-CHOP, TAM prognostic value persists when patients characterized by different IPI (IPI 0-1, IPI 2-3, IPI 4-5) were further stratified according to CD68 or CD163 expression [41].

In our study, 37 consecutive DLBCL patients were analyzed, all of them received R-containing therapies. Characteristics of patients and response to treatment are consistent with previously published data, with a CR rate of 70.3%, PFS and OS at 5 years of 61% and 64%, respectively. We observed a trend between low CD68 expression, high M2 TAM expression and reduced PFS and OS, even if a statistical significance was not achieved, while CR rate was not influenced by TAM. LMR and NLR did not influence treatment response and survival. Overall, IPI maintained its prognostic value, patients with IPI 0-1 experienced a more favorable outcome compared to patients with IPI $\geq$ 2. When we have further stratified cases with IPI $\geq$ 2, we have observed a significant association between high M2 TAM expression, reduced PFS and a trend to reduced OS. We can argue that patients with intermediate or high IPI could represent a subgroup in which TAM evaluation could increase the prognostic power of IPI.

Our study has some strengths, such as the choice of double CD68/CD163 staining to investigate M2 TAM, the administration of rituximab to all patients and the use of the Steidl

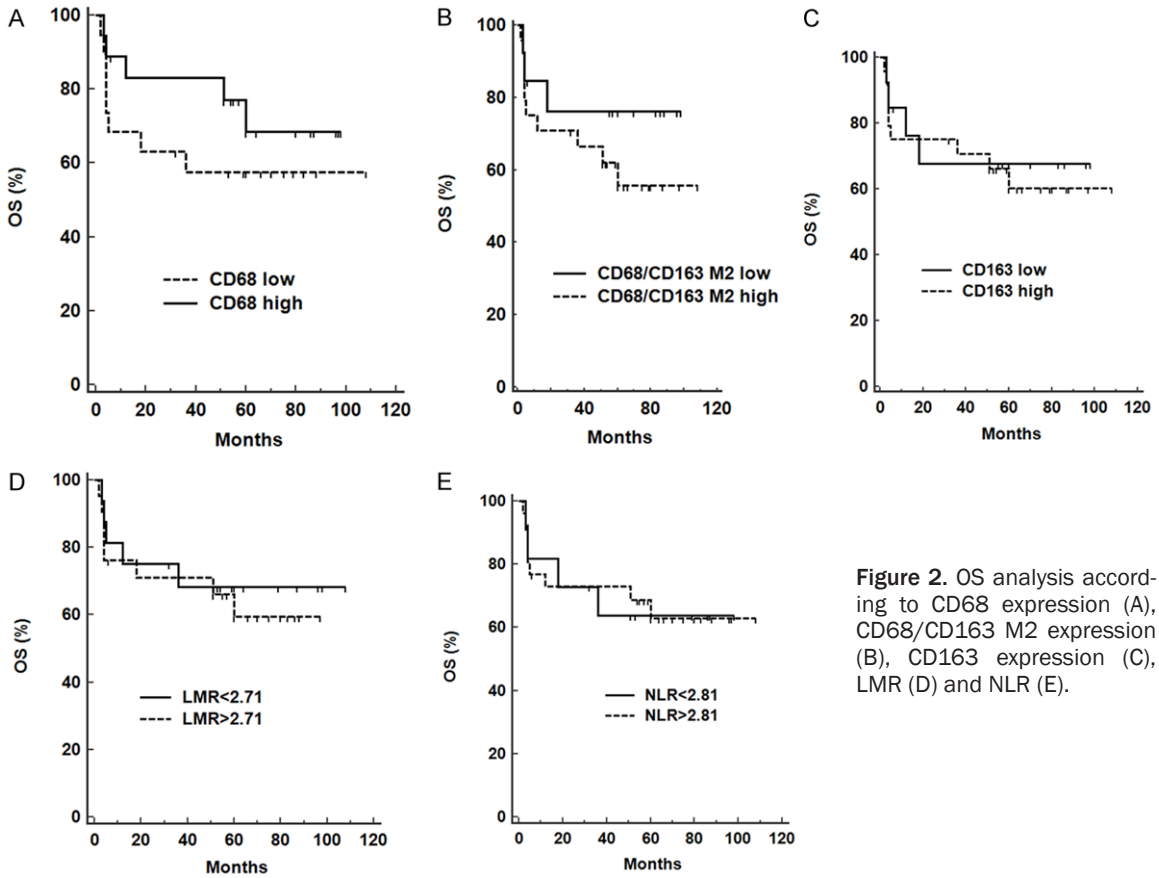

Figure 2. OS analysis according to CD68 expression (A), CD68/CD163 M2 expression (B), CD163 expression (C), LMR (D) and NLR (E).

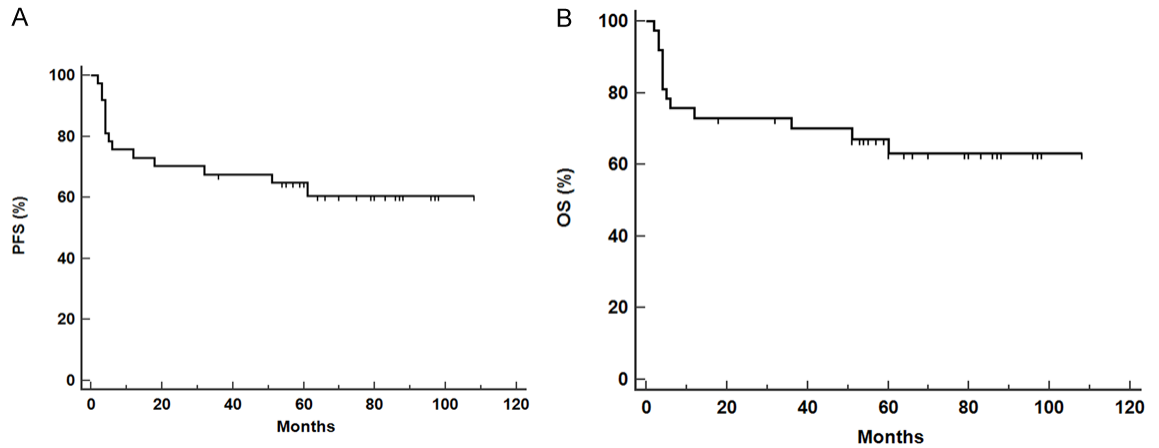

Figure 3. PFS (A) and OS (B) analysis in the entire cohort.

score that, even if designed for HL, could guarantee greater reproducibility compared to best cut-off methods used in single-cohort studies. However, our study has some limitations, most notably the retrospective design and small sample size (although comparable to previously published experiences) [20, 42].

In conclusion, we can suggest that high M2 TAM content at diagnosis, especially in association with IPI, could contribute to identify DLBCL patients characterized by poor prognosis. A TAM-targeted strategy could be associated with a COO-targeted strategy in future clinical trials focused on DLBCL with IPI ≥ 2,

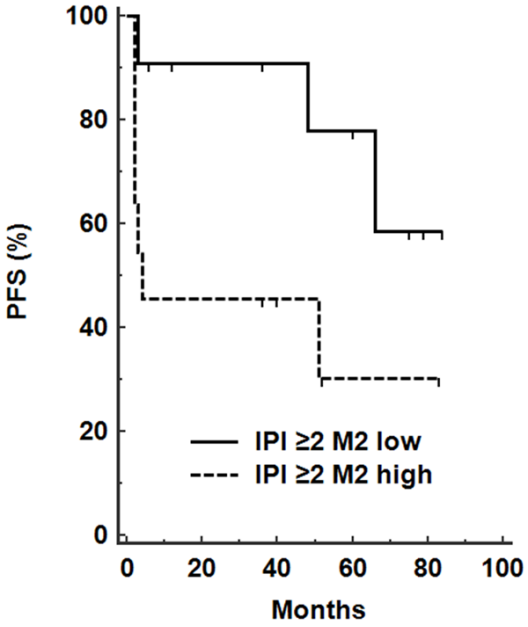

Figure 4. PFS analysis in patients with IPI ≥ 2, according to the CD68/CD163 M2 expression.

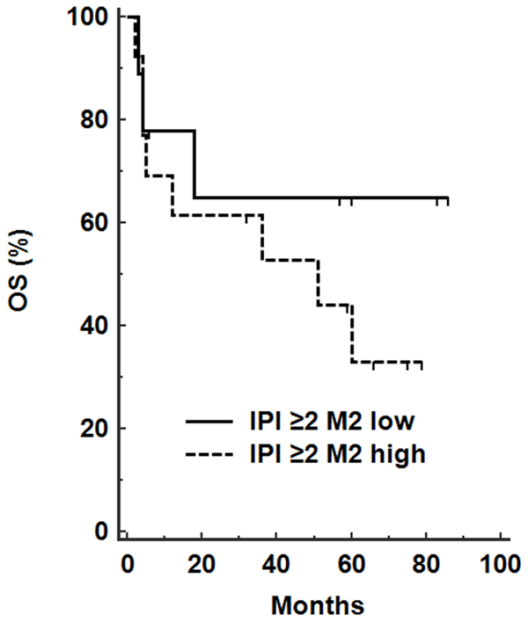

Figure 5. OS analysis in patients with IPI ≥ 2, according to the CD68/CD163 M2 expression.

with the aim to investigate a novel therapeutic approach for poor-risk patients.

Disclosure of conflict of interest

None.

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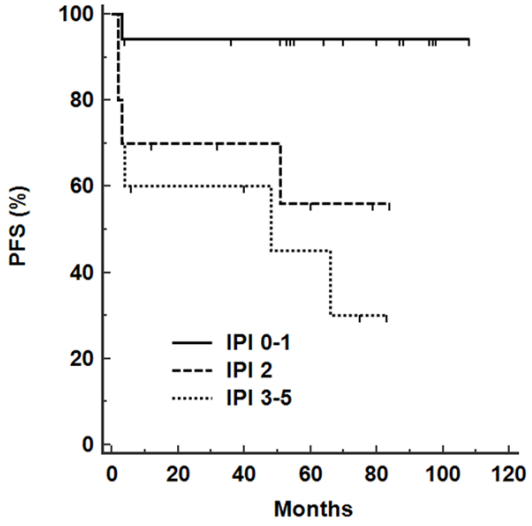

Figure S1. PFS analysis according to IPI.

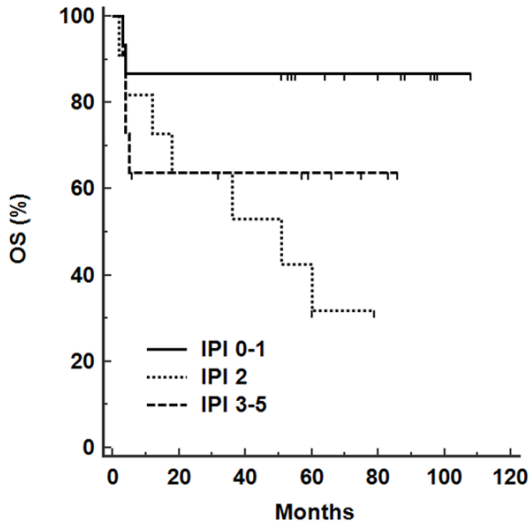

Figure S2. OS analysis according to IPI.