



CD163 and c-Met expression in the lymph node and the correlations between elevated levels of serum free light chain and the different clinicopathological parameters of advanced classical Hodgkin's lymphoma

Magdy Bedewy¹, Shereen El-Maghraby², Ahmed Bedewy²

¹Military Medical Academy, Cairo, ²Hematology Department, Medical Research Institute, Alexandria, Egypt

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Correspondence to
Ahmed Bedewy, M.D.
Hematology Department, Medical
Research Institute, 8 Mahmoud Younes
Street, Smouha 21615 Alexandria, Egypt
Tel: +201000040511
Fax: +2035222602
E-mail: dr_ahmed_bedewy@yahoo.com

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Background

Advances in the understanding of Hodgkin's lymphoma (HL) show various functions of infiltrating immune cells and cytokines in relation to clinical outcomes. The expression of CD163 and c-Met has been suggested to have a role in lymphoid malignancy. Thus, we evaluated the expressions of CD163, c-Met, and serum free light chain (sFLC) in relation to the clinicopathological features of patients with advanced classical HL (cHL).

Methods

We assessed the expression of CD163 and c-Met in 34 patients with cHL through immunohistochemistry on the lymph node biopsy sections and the levels of pretreatment sFLC were estimated using ELISA.

Results

High CD163 expression correlated with increased age, B symptoms, International Prognostic Score (IPS) ≥ 3 , mixed cellularity subtype, and low response to treatment. Further, high c-Met expression correlated with increased age at diagnosis, leukocytosis, B symptoms, and lower chance to achieve complete remission. The sFLC levels correlated with increased age at diagnosis, lymphopenia, IPS ≥ 3 , B symptoms, and lower complete remission rates.

Conclusion

In advanced cHL, increased expression of CD163 and c-Met showed a significant association with adverse prognostic parameters and poor response to treatment. Pretreatment high sFLC level also correlated with poor risk factors, suggesting its use as a candidate prognostic marker. A comprehensive approach for prognostic markers might represent a step towards developing a tailored therapeutic approach for HL.

Key Words Hodgkin, c-Met, CD163, Free light chain

INTRODUCTION

Although the treatment for Hodgkin's lymphoma (HL) can be considered a successful paradigm of modern treatment strategies, approximately 20% of patients with advanced-stage HL still die following relapse or progressive disease. A similar proportion of patients are over-treated leading to treatment-related late sequelae including solid tumors and end-organ dysfunction [1, 2].

Lymphoma-associated macrophages have been suggested

to play a prognostic role in several different lympho-proliferative entities, including classical HL (cHL). Gene expression profiling data showed a correlation between up-regulation of genes related to intra-tumor macrophage infiltration and poor response to treatment, suggesting an underlying tumor-promoting role for these cells [3].

CD68, the most commonly used macrophage marker, is widely synthesized by a variety of normal and neoplastic cells, and it is therefore non-specific for the monocyte/macrophage lineage [4]. CD163 appears to be involved in anti-inflammatory functions predominantly associated with M2

macrophages, which promote tumor cell growth and metastasis, unlike M1 macrophages that kill tumor cells [5]. Unlike CD68, CD163 staining pattern is reported to show a cleaner background with less non-specific staining of Hodgkin's Reed-Sternberg (RS) cells and other inflammatory elements [6].

CD163 is a glycoprotein belonging to the scavenger receptor cysteine-rich superfamily [7]. It is controlled by various inflammatory mediators, such as interferon γ , interleukins 6, interleukin 10, and glucocorticoids [8].

The *MET* proto-oncogene located on the 7q31 locus encodes the receptor tyrosine kinase MET and is also known as the hepatocyte growth factor (HGF) receptor [9]. The binding of HGF to its receptor MET results in the phosphorylation of the tyrosine at the C-terminus of the receptor, leading to its activation and triggering downstream signaling [10]. This signaling pathway is involved in cellular proliferation, survival, and migration. [11] Furthermore, c-Met has been shown to have a prognostic significance in numerous malignancies including Non-Hodgkin's lymphoma [12].

The serum free light chains (sFLC) are kappa (κ) and lambda (λ) light chains, which are produced by monoclonal and/or polyclonal B-cell populations [13]. The diagnostic and prognostic values of elevated sFLC levels are established in monoclonal gammopathy of undetermined significance, multiple myeloma, solitary plasmacytoma, and AL-amyloidosis [14].

sFLC abnormalities can occur in different ways: monoclonal elevated sFLC (elevated κ and/or λ with abnormal FLC ratio), polyclonal elevated sFLC (elevated κ and/or λ with normal sFLC ratio), and ratio-only sFLC abnormality (normal range κ and λ with abnormal sFLC ratio). The polyclonal increases in sFLCs are not associated with an abnormal sFLC ratio and may be indicative of renal dysfunction [15], older age [16], autoimmune disease [17], and chronic inflammation [18].

In the current study, we used immunohistological staining to assess the CD163 and c-Met expression in lymph node biopsy sections from newly diagnosed cases of advanced cHL, in addition to estimation of pretreatment sFLC levels, and determined their correlation with the clinicopathological features and the response to treatment.

MATERIALS AND METHODS

Subjects

In the present work, we studied 34 newly diagnosed patients with advanced cHL (stages from IIB to IV on Ann Arbor scale), who presented to the Hematology Department, Medical Research Institute, Alexandria University and Mustafa Kamel Military Hospital, Alexandria. Patients were diagnosed according to the results of the routine morphological and immunohistochemical examination of lymph node biopsy materials. The histopathological classification was based on the World Health Organization (WHO) criteria [19].

Patients were staged according to the Ann Arbor staging

system [20]. Staging evaluation for each patient involved a physical examination; CT scans of the thorax, abdomen, and pelvis, bilateral bone marrow aspiration, and trephine biopsy. Laboratory evaluation included complete blood cell count, erythrocyte sedimentation rate, and liver and kidney function tests. Presence or absence of B symptoms and bulky disease were reported. The patients were scored on the basis of the International Prognostic Score (IPS), which is based on age, gender, stage, and presence of hypoalbuminemia, lymphopenia, and leukocytosis [21].

Patients were treated with 6 cycles of adriamycin, bleomycin, vinblastine, and dacarbazine (ABVD), which was complemented by radiation therapy in patients with bulky disease or localized residual masses. Treatment response was assessed using standardized guidelines [22].

Complete remission (CR) was defined as disappearance of all clinical evidence of disease and normalization of all laboratory values and radiographic results lasting for at least 4 weeks. Partial response (PR) was defined as a reduction of 50% or more in the sum of the products of the cross-sectional diameters of all known lesions lasting for at least 4 weeks [23].

Immunohistochemistry

Paraffin sections of the lymph nodes were stained according to the manufacturer's instructions using monoclonal antibodies against CD163 (Thermo Fisher Scientific, Kalamazoo Michigan) and c-Met (Santa Cruz biotechnology, Santa Cruz, California). Staining was performed using EconoTek HRP anti-polyvalent (DAB) kit (Scytek laboratories, Logan, Utah). The slides were counterstained with hematoxylin and covered using a coverslip. The cut-off value for CD163 was 20%, whereas that for c-Met was 30%. These cut-off points for CD163 and c-Met were adopted based on the studies by Yoon *et al.* [24] and Xu *et al.* [11], respectively.

The slides were evaluated by 2 hematologists. CD163 was assessed on the basis of a membranous staining pattern, while the c-Met immunoreactivity was assessed on the basis of both membranous and cytoplasmic staining patterns.

sFLC ELISA

Frozen pretreatment serum samples with normal renal function were assayed to detect κ and λ light chains by using human immunoglobulin FLC κ and λ ELISA (Biovendor Research and diagnostic products, Candler, North Carolina, USA).

The assay separately measures κ FLCs (normal range, 0.225–3.45 mg/dL) and λ FLCs (normal range, 0.45–5.42 mg/dL). In addition, the assay provides the FLC ratio (normal range, 0.23–1.85). Elevated FLC was defined as the κ or λ concentrations above the normal range.

Statistical analysis

Data was analyzed using the SPSS software package version 20.0. Qualitative data were described using number and percentage. Comparison between different groups regarding categorical variables was tested using the chi-square test.

When more than 20% of the cells had expected counts less than 5, correction for chi-square was conducted using Fisher's exact test or Monte Carlo correction. The distributions of quantitative variables were tested for normality using the Shapiro-Wilk test and D'Agostino test; in addition, histogram and QQ plot were used for the vision test. If the data displayed normal distribution, parametric tests were applied. If the data displayed non-normal distribution, non-parametric tests were used. Quantitative data were described using mean and standard deviation for normally distributed data. Non-normal distributed data was expressed using median, minimum and maximum range, and Mann-Whitney Test (used to analyze 2 independent populations). Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

Table 1. Clinical and laboratory characteristics of the study cohort.

Characteristic	N (percentage)
Age	
≥ 45 yrs	14 (41.0)
< 45 yrs	20 (59.0)
Gender	
Male	18 (53.0)
Female	16 (47.0)
WBCs count ($\times 10^9/L$)	
< 15	27 (79.0)
≥ 15	7 (21.0)
Absolute lymphocytic count ($\times 10^9/L$)	
> 0.6	26 (76.0)
≤ 0.6	8 (24.0)
Hemoglobin level (g/dL)	
≥ 10.5	23 (68.0)
< 10.5	11 (32.0)
Albumin level (g/dL)	
≥ 4	13 (38.0)
< 4	21 (62.0)
Ann Arbor stage	
II b	15 (44.0)
III	11 (32.0)
IV	8 (24.0)
International prognostic score (IPS)	
< 3	22 (65.0)
≥ 3	12 (35.0)
Bulky disease	7 (21.0)
B symptoms	16 (47.0)
Subtype	
Nodular sclerosis	22 (65.0)
Mixed cellularity	11 (32.0)
Lymphocytes-rich	1 (3.0)
CD163 expression	
< 20%	19 (56.0)
≥ 20%	15 (44.0)
c-Met expression	
< 30%	15 (44.0)
≥ 30%	19 (56.0)
sFLC	
Normal	20 (59.0)
Elevated	14 (41.0)
Response	
Complete remission	20 (59.0)
Partial remission	14 (41.0)

RESULTS

Clinicopathological features of the study cohort

The main clinical and laboratory features of the study cohort at presentation are summarized in Table 1. The median age of the subjects was 30 years (range, 15–62 yrs). The male to female ratio was 1:1 with 18 males and 16 females. Sixteen patients (47%) exhibited B-symptoms and 7 patients (21%) presented with bulky disease. Stage II b was found in 15 (44%) out of the 34 patients, whereas 32% of patients were in stage III and 24% of patients were in stage IV. IPS was more than or equal to 3 in 12 patients (35%). The majority of the patients had histological features consistent with the nodular sclerosis subtype, approximately one-third of the patients had mixed cellularity, whereas only 1 patient had the lymphocyte-rich subtype.

The white blood cells count was $\geq 15 \times 10^9/L$ in 21% of patients, and the absolute lymphocytic count was $\leq 0.6 \times 10^9/L$ in 24% of patients. The hemoglobin level was less than 10.5 g/dL in 11 patients (32%) and 62% of patients had serum albumin levels less than 4 g/dL. Erythrocyte sedimentation rate was higher than 40 mm/h in 60% of the patients. Marrow involvement was detected in 6 patients (18%). Following 6 cycles of ABVD therapy, 20 (59%) out of the 34 patients showed CR, whereas 14 patients (41%) showed PR (Table 1).

CD163 expression and the clinicopathological features

CD163 expression varied widely from 5% to 85% with a median of 15%. The expression of CD163 was categorized into 2 groups based on the 20% cut-off. Fifteen patients (44%) had CD163 expression of $\geq 20\%$. The pattern of macrophage infiltration was membranous (Fig. 1). The expression of CD163 was higher in mixed cellularity cases than in nodular sclerosis cases ($P=0.006$).

Correlations between CD163 expression and the clin-

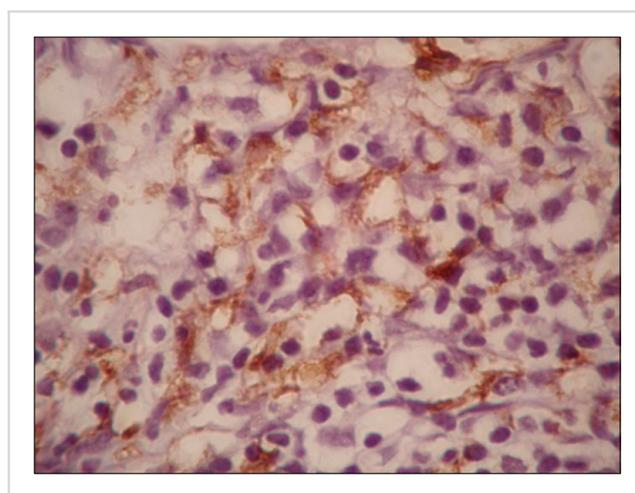


Fig. 1. Representative images showing immunohistochemical staining for CD163 in CHL tissue ($\times 1,000$).

Table 2. CD163 expression, c-Met expression and serum FLC levels in relation to the clinical and laboratory parameters.

	CD 163		P	c-Met		P	Serum FLC		P
	<20%	≥20%		<30%	≥30%		Normal	High	
Age median (range)	27 (15–59)	49 (25–62)	0.008 ^{a)}	27 (15–60)	46 (25–62)	0.015 ^{a)}	27 (15–46)	54 (35–62)	<0.001 ^{a)}
Gender									
Male N (%)	8 (44)	10 (56)	0.428	7 (39)	11 (61)	0.561	11 (61)	7 (39)	0.870
WBCs count									
≥15×10 ⁹ /L N (%)	2 (29)	5 (71)	0.133	0 (0.0)	7 (100)	0.046 ^{a)}	3 (43)	4 (57)	0.358
ALC									
≤0.6×10 ⁹ /L N (%)	2 (25)	6 (75)	0.056	3 (38)	5 (62)	0.661	2 (25)	6 (75)	0.023 ^{a)}
Hb									
<10.5 g/dL N (%)	8 (73)	3 (27)	0.234	7 (64)	4 (36)	0.389	5 (45)	6 (55)	0.667
Albumin									
<4 g/dL N (%)	13 (62)	8 (38)	0.622	10 (48)	11 (52)	1.000	13 (62)	8 (38)	1.000
Stage									
IV N (%)	4 (50)	4 (50)	1.000	4 (50)	4 (50)	0.774	5 (63)	3 (37)	0.768
IPS median (range)	2 (1–3)	3 (1–5)	0.033 ^{a)}	2 (1–5)	2 (1–5)	0.424	2 (1–4)	3 (1–5)	0.030 ^{a)}
Bulky disease									
No N (%)	16 (59)	11 (41)	0.623	13 (48)	14 (52)	0.341	16 (59)	11 (41)	1.000
Yes N (%)	3 (43)	4 (57)		2 (29)	5 (71)		4 (57)	3 (43)	
B symptoms									
No N (%)	16 (89)	2 (11)	<0.001 ^{a)}	12 (67)	6 (33)	0.015 ^{a)}	15 (83)	3 (17)	0.015 ^{a)}
Yes N (%)	3 (19)	13 (81)		3 (19)	13 (81)		6 (38)	10 (62)	
Subtype N (%)									
Nodular sclerosis	17 (77)	5 (23)	0.006 ^{a)}	11 (50)	11 (50)	0.294	14 (64)	8 (36)	0.804
Mixed cellularity	2 (18)	9 (82)		3 (27)	8 (73)		6 (55)	5 (45)	
Lymphocytes-rich	1 (100)	0 (0)		1 (9.1)	0 (0.0)		1 (100)	0 (0)	
Response									
CR N (%)	16 (80)	4 (20)	0.005 ^{a)}	13 (65)	7 (35)	0.012 ^{a)}	16 (80)	4 (20)	0.034 ^{a)}
PR N (%)	3 (21)	11 (79)		2 (14)	12 (86)		4 (29)	10 (71)	

^{a)}Statistically significant at $P \leq 0.05$.

Abbreviations: IPS, international prognostic index; CR, complete remission; PR, partial remission; ALC, absolute lymphocyte count.

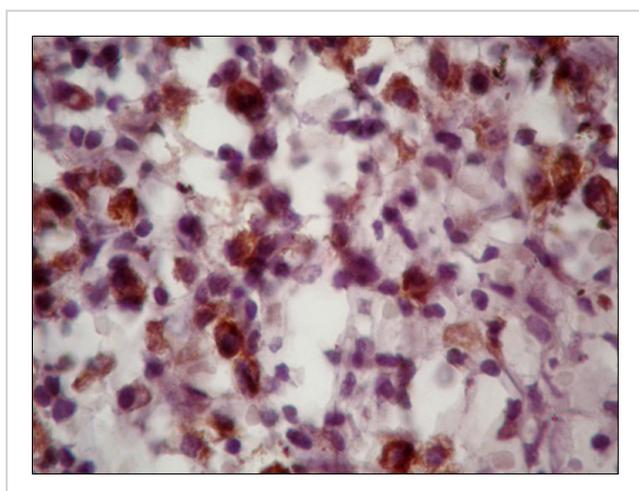


Fig. 2. Representative images of immunohistochemical staining for expression of c-Met in cHL tissue ($\times 1,000$).

icopathological features are shown in Table 2. High CD163 expression was associated with increased age ($P=0.008$), presence of B symptoms ($P<0.001$), IPS ≥ 3 ($P=0.033$), and lower CR rates ($P=0.005$).

c-Met expression and the clinicopathological features

c-Met expression was 15–90% with a median of 35% (Fig. 2). c-Met was expressed in more than 30% of the tumor cells in 19 (56%) of the 34 patients. High expression of c-Met was associated with increased age ($P=0.015$), leukocytosis ($P=0.046$), presence of B symptoms ($P=0.015$), and lower CR rates ($P=0.012$), but was not associated with gender, histological subtype, or other known clinical prognostic factors such as bulky disease and IPS ≥ 3 (Table 2).

sFLC levels and clinicopathological features

Median κ and λ FLC levels were 3 mg/dL (range, 1–9 mg/dL) and 2.2 mg/dL (range, 0.6–9 mg/dL), respectively. In 14 patients (41%), serum κ and/or λ FLC levels were elevated. This elevation was associated with increased age ($P<0.001$), lymphopenia ($P=0.023$), IPS ≥ 3 ($P=0.03$), presence of B-symptoms ($P=0.015$), and lower CR rates ($P=0.034$), but not with other risk factors such as gender, leukocytosis, stage, bulky disease, subtype, and albumin levels (Table 2).

DISCUSSION

The tumor microenvironment is an important factor in the development and progression of cancer. Recent evidence

suggests that the cellular composition of the tumor microenvironment can significantly modify the clinical outcome in hematologic malignancies, particularly in follicular lymphoma and cHL [25].

Tumor-associated macrophages are now accepted to play major roles in inflammation and cancer through a number of functions (e.g., promotion of tumor cell proliferation and angiogenesis, incessant matrix turnover, repression of adaptive immunity), which ultimately have significant impact on the disease progression [26].

In the present study, tumor-associated macrophages comprised 20% or more of the HL tissue, as per CD163 positivity, in 44% of our patients, and this was associated with older age at presentation, IPS ≥ 3 , presence of B-symptoms, mixed cellularity subtype, and lower CR rates. On the other hand, a recent study by Azambuja *et al.* found no association between clinical characteristics and CD163 expression [27]. However, in concordance with our work, several published researches noted the inferior prognosis pertained with cases having increased tumor-associated macrophages.

Steidl *et al.* reported that the increased number of tumor-associated macrophages as per immunohistochemical positivity for CD68 was correlated with inferior outcome in cHL patients [28]. Two recent studies reported similar results. Tan *et al.* in a multicenter study stated that increased CD163 expression was significantly associated with increased age, mixed cellularity subtype, inferior failure-free survival, and overall survival in locally extensive and advanced-stage cHL [29]. Yoon *et al.* reported the association between high CD163 indices ($\geq 20\%$) on one side and older age, male gender, presence of B symptoms, higher IPS (≥ 4), lower CR rates, and shorter duration of CR on the other side in cHL patients of any stage [24]. Kamper *et al.* [30] found that in cHL, a high number of intratumoral CD163⁺ monocytes/macrophages correlates with adverse outcome and clinical parameters reflecting underlying aggressive disease biology.

Lymphoid malignancies such as multiple myeloma and several B cell lymphomas were found to express c-Met, suggesting that c-Met is involved in the pathogenesis of these diseases [31]. We reported high c-Met expression (positivity in $\geq 30\%$ of cells in HL tissue) in 56% of our patients, and the c-Met levels are associated with older age at diagnosis, leukocytosis (WBC $> 15 \times 10^9/L$), presence of B symptoms, and lower chance of CR. Teofili *et al.* [32] found similar association, and they hypothesized that HGF/c-Met interaction stimulates cytokine release by c-Met-positive RS cells, leading to some of the disease characteristics, namely, the B symptoms. Moreover, c-Met could also have an anti-apoptotic role due to its association with the anti-apoptotic protein BAG-1 and its functional partner Bcl-2 [33].

On the other hand, Xu *et al.* [11] reported that the expression of c-Met in tumor cells in patients with cHL strongly correlated with higher progression-free survival, whereas lack of c-Met expression was correlated with lower progression-free survival in the combined cohort. Notably, the immunohistochemical reporting of c-Met positivity was lim-

ited to RS cells in the study of Xu *et al.* [11], whereas in our study it refers to the HL tissue as a whole. We selected this way of evaluation, because it is impractical to limit the immunohistochemical evaluation to RS cells, which occur infrequently. Moreover, atypical forms of these malignant cells are present in certain subtypes of HL. Furthermore, in the study of Xu *et al.* [11], the reactive cells in HL tissue also reacted to c-Met. Finally, bidirectional cross-talk between the malignant cells and the reactive cells in the HL tissue is well known; thus, it is not justified to ignore the role of the reactive cells in the tumor behavior. Consequently, this difference in the reporting can be expected to explain the discordant results.

Nevertheless, recent studies by Scott *et al.* [34] and Steidl *et al.* [35] contradict this claim. These 2 groups recently published their work on the correlation between the gene expression profiling and prognosis in cHL. Scott *et al.* [34] conducted their research on formalin-fixed paraffin-embedded biopsies containing HL tissue, whereas Steidl *et al.* [35] used microdissected Hodgkin RS cells, thereby restricting the sample to the malignant cells. The observed effect of the profiling signature retained its significance in both studies despite the difference in the studied samples.

Moreover, contradicting results about the prognostic significance of c-Met have been reported in multiple studies on different tumors. Moreover, a favorable prognostic impact of c-Met expression has been shown in the small-scale study on breast cancer of Nakopoulou *et al.* [36] and in diffuse large B-cell lymphoma by Uddin *et al.* [37]. However, studies by Kawano *et al.* [12] and Lengyel *et al.* [38] showed opposite effects for both types of tumors.

Lymphoid malignancies often have a polyclonal B-cell infiltrate that can secrete immunoglobulins [39]. In the present study, 10 patients (40%) had elevated sFLC level, and the elevation was polyclonal. Elevated sFLC levels may reflect increased polyclonal B cell activity in cHL microenvironment. High sFLC levels correlated with increased age at diagnosis, lymphopenia ($< 0.6 \times 10^9/L$), unfavorable IPS (≥ 3), presence of B symptoms, and lower CR rates, but not with other risk factors such as sex, leukocytosis, stage, bulky disease, subtype, and serum albumin level.

In concordance with our results, Thompson *et al.* reported elevated sFLC in 30% of their cohort of cHL patients. sFLC elevation was significantly associated with age (> 45 yrs) at diagnosis, advanced stage, presence of B symptoms, elevated erythrocytes sedimentation rate, and unfavorable IPS. There was no association of FLC elevation with lymphopenia or bulky disease [39].

On the other hand, Rosaria *et al.* [40] stated that high sFLC levels were correlated with lymphopenia, leukocytosis (WBC $> 15 \times 10^9/L$), elevated erythrocytes sedimentation rate (> 50 mm/h), and unfavorable IPS (≥ 3), but not with stage, presence of B symptoms, bulky and extra nodal disease, serum LDH level, serum albumin level, and response rate.

Moreover, Rosaria *et al.* [41] reported that combining sFLC and Interim 2-[¹⁸F] Fluoro-2-deoxy-D-glucose positron emission tomography can identify poor risk HL patients,

who may benefit from upfront treatment escalation or early salvage.

In agreement with our results regarding response to treatment, Ogura *et al.* [42] stated that CR rate after ABVD therapy was 58.2% for III or IV, but after radiation therapy, CR rate increased to 78.5%.

In summary, in advanced cHL, increased tumor-associated macrophage levels correlated with adverse prognostic parameters and poor response to treatment, reflecting underlying aggressive disease. Therefore, it might be a reliable marker to assess prognosis. While c-Met expression correlated with some bad prognostic parameters and affected the response to treatment, published reports presented contradicting results on c-Met expression. This invites researchers to conduct further work to validate the importance of c-Met expression in cHL. Pretreatment high sFLC level correlated with poor risk factors, suggesting its use as a candidate prognostic marker. A comprehensive approach to define prognostic markers reflecting diverse pathophysiologic concerns might therefore represent a step towards developing a tailored therapeutic approach best-suited to each patient's needs.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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