

Stratifying diffuse large B-cell lymphoma patients treated with chemoimmunotherapy: GCB/non-GCB by immunohistochemistry is still a robust and feasible marker

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ABSTRACT

Diffuse large B cell lymphoma (DLBCL) is a heterogeneous group of aggressive lymphomas that can be classified into three molecular subtypes by gene expression profiling (GEP): GCB, ABC and unclassified. Immunohistochemistry-based cell of origin (COO) classification, as a surrogate for GEP, using three available immunohistochemical algorithms was evaluated in TMA-arranged tissue samples from 297 patients with *de novo* DLBCL treated by chemoimmunotherapy (R-CHOP and R-CHOP-like regimens). Additionally, the prognostic impacts of MYC, BCL2, IRF4 and BCL6 abnormalities detected by FISH, the relationship between the immunohistochemical COO classification and the immunohistochemical expression of MYC, BCL2 and pSTAT3 proteins and clinical data were evaluated.

In our series, non-GCB DLBCL patients had significantly worse progression-free survival (PFS) and overall survival (OS), as calculated using the Choi, Visco-Young and Hans algorithms, indicating that any of these algorithms would be appropriate for identifying patients who require alternative therapies to R-CHOP. Whilst MYC abnormalities had no impact on clinical outcome in the non-GCB subtype, those patients with isolated MYC rearrangements and a GCB-DLBCL phenotype had worse PFS and therefore might benefit from novel treatment approaches.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most prevalent subtype of mature B-cell neoplasms worldwide, accounting for approximately 30% of all non-Hodgkin lymphomas. A combination of rituximab with an anthracycline-based chemotherapy regimen, such as R-CHOP, produces a favorable outcome for most patients. Nevertheless, one third of patients will still relapse, of whom a proportion will suffer from refractory disease [1].

Although the International Prognostic Index (IPI) has proved to be a very useful clinical tool, and is widely used to risk-stratify patients [2], it is exclusively based on clinical variables and therefore fails to capture the biological heterogeneity of DLBCL, and does not identify potential targeted therapies.

In recent years, biological studies have shown that DLBCL is not a single disease but a group of disorders with specific signaling programs [3], some of which have been shown to benefit from specific novel treatments. Specifically, gene expression profiling (GEP) has identified three DLBCL subtypes: the germinal center B-cell-like (GCB) and the activated B cell-like (ABC) DLBCL subtypes, and unclassified cases (~10%). GCB patients tend to have better survival than the ABC and unclassified DLBCL patients when treated by chemotherapy, with or without rituximab [4, 5]. New treatments including ibrutinib [6] or immunomodulators such as lenalidomide have shown very promising results in patients with an ABC or non-GCB DLBCL subtype [7], as determined by GEP and immunohistochemistry in separate clinical trials. Although preferential activity with bortezomib for cases with the refractory ABC/non-GCB subtype was suggested early on [8], recently published data concerning frontline VR-CAP do not indicate a significant difference with R-CHOP in the non-GCB type DLBCL [9], and the differential efficacy remains controversial.

For this reason, identification of cases that will not respond to conventional approaches but might benefit from targeted therapy has become a matter of considerable interest.

Several studies have demonstrated that immunohistochemical (IHC) algorithms can reproduce GEP classification and potentially be used for the prognostic stratification of DLBCL [10–12]. Of these, the Hans, Visco-Young and Choi algorithms have shown the highest concordance with the cell of origin (COO) as defined by GEP. However, several clinical studies investigating the ability of COO IHC algorithms to prognostically stratify DLBCL patients treated with anthracycline-based chemotherapy with or without rituximab have yielded conflicting results [13–18].

Recurrent non-random chromosomal rearrangements leading to the deregulated expression of MYC, BCL6 and BCL2 in DLBCLs have been reported to have mixed

associations with prognosis [17, 19–24]. Concurrent *MYC* and *BCL2* rearrangements [20, 25–27] are associated with poor outcome in DLBCL and are commonly found in GCB-type DLBCLs [28]. *IRF4* rearrangements are associated with a GCB phenotype in grade 3FL and DLBCL, and with younger age and favorable outcome [29]. Constitutive activation of STAT3 is preferentially detected in the non-GCB subtype of DLBCL, and seems to favor proliferation and survival of lymphoma cells [30]. However, the prognostic impact of this molecule is yet to be established.

In an attempt to confirm that IHC algorithms can be used to prognostically stratify DLBCL patients, we compared the Hans, Choi and Visco-Young algorithms in a cohort of 297 diagnostic samples from patients with R-CHOP-treated DLBCL. Also, to establish whether deregulation of specific oncogenes may further classify COO-based DLBCL subtypes, adding meaningful prognostic information, we performed FISH of *BCL6*, *BCL2*, *IRF4* and *MYC* and evaluated the expression of MYC, BCL2, BCL6 and pSTAT3 proteins in the same samples.

RESULTS

The COO of 272 (91%), 282 (95%) and 275 (92%) of the 297 DLBCL tumor samples could be classified by the Hans, Visco-Young and Choi algorithms, respectively (Figure 1). No significant differences were observed between the IHC phenotypes with respect to age or extranodal involvement. However, the non-GCB group of patients had a higher IPI score ($p \leq 0.011$) and more frequently presented with stage III or IV disease ($p \leq 0.001$). Non-GCB cases had higher LDH levels and worse performance status than GCB cases as classified by the Hans and Visco-Young algorithms (Table 1 and Supplementary Tables 1 and 2).

Comparing GEP with the results of the Hans, Choi and Visco-Young algorithms, revealed discrepancies in the COO classification in only 14%, 13% and 8% patients, respectively [12].

MYC, BCL2, BCL6 and pSTAT3 protein expression and correlation with COO phenotypes

45% of the evaluable samples (62/140) showed MYC overexpression. 72% and 64% of patients with MYC protein overexpression also had high LDH levels ($p = 0.011$) and high IPI scores ($p = 0.029$), respectively (Supplementary Table 3). MYC overexpression was not associated with a particular COO phenotype (Supplementary Table 4). MYC and BCL2 double-positivity was found in 34% of evaluable cases (42/123 cases). As previously described, MYC and BCL2 double-

positive (DP) cases determined by IHC were commonly found to have a non-GCB phenotype ($p=0.007$, $p=0.018$ and $p=0.002$ for the Visco-Young, Hans and Choi algorithms, respectively) (Supplementary Table 4). Most patients coexpressing the MYC and BCL2 proteins were aged over 60 years (74%), had stage III or IV disease (74%), and 36% of cases had two or more extranodal sites involved and were therefore associated with high-risk IPI ($p=0.003$; Supplementary Table 5). 15% and 24% of MYC and BCL2 double-positive cases had MYC and BCL2 rearrangements, respectively ($p=0.030$ and $p=0.938$, Supplementary Table 6).

Isolated BCL2 overexpression was not associated with the COO classification, and there were no associations between BCL2 protein expression and any of the clinical or biological features analyzed in our series (data not shown).

pSTAT3 protein expression was detected in 34% (47/139) of evaluable cases and was associated with advanced-stage disease ($p=0.003$) and high IPI score ($p=0.045$; Supplementary Table 5). Its expression was more frequent in cases with elevated MYC expression ($p=0.006$) (Supplementary Table 3).

MYC, BCL6, BCL2 and IRF4 abnormalities

MYC rearrangements were observed in 7% of patients (15 out of 206 cases) (Table 2 and Figure 1), 30% of which had additional cytogenetic abnormalities, BCL2 rearrangements and MYC gains being the most common added gene aberrancies (in 4 out of 13, and 2 out of 15 evaluable cases, respectively). MYC rearrangements were more frequent in patients younger than 60 years of age ($p=0.029$), with more than two extranodal localizations ($p=0.009$), and in the high-risk IPI group ($p=0.051$). We found no difference in the distribution of MYC rearrangements with respect to the COO phenotype. No significant correlation was observed between the presence

of MYC rearrangements and MYC protein expression (5 out of 63 cases with MYC protein overexpression had MYC rearrangements).

MYC gains (Supplementary Table 7) were observed in 19% of patients and were more frequently observed in the group of patients with normal LDH values ($p=0.032$). Cases with MYC gains expressed MYC, BCL2 and BCL6 protein in 31%, 56% and 84% of cases, respectively. MYC gains were associated with gains in BCL2 ($p=0.002$) and BCL6 ($p=0.002$), but there was no association with other cytogenetic, biological or clinical features.

BCL2 rearrangements were detected in 19% of patients (Table 2). There was no association between the presence of any type of BCL2 gene abnormality and the main clinical features studied. BCL2 rearrangements were almost entirely restricted to the GCB phenotype (Table 1, $p<0.001$). There was a close correlation between the presence of a BCL2 rearrangement and BCL2 protein overexpression ($p=0.003$; 95% of cases with the BCL2 rearrangement expressed the BCL2 protein; data not shown). BCL2 gains were observed in 14% of patients and were more frequently detected in the non-GCB phenotype group ($p=0.013$; 80% of cases with BCL2 amplification had a non-GCB phenotype; Table 1).

BCL6 rearrangements were observed in 30% (51 out of 171) of DLBCL patients, were associated with a non-GCB phenotype (Table 1, $p<0.001$; 70% of cases) [38] and were more frequent in the group of patients with elevated LDH ($p=0.021$) (Table 2). However, there was no association with other clinical features. BCL6 gains, observed in 17% of patients, showed no significant association with any of the clinical or phenotypic features analyzed. In contrast to the BCL6 rearrangements, there was a direct relation between the presence of BCL6 gains and BCL6 protein expression (Table 2; $p=0.02$).

Double rearrangements were detected in 10 of 178 cases: there were concurrent BCL2/MYC rearrangements

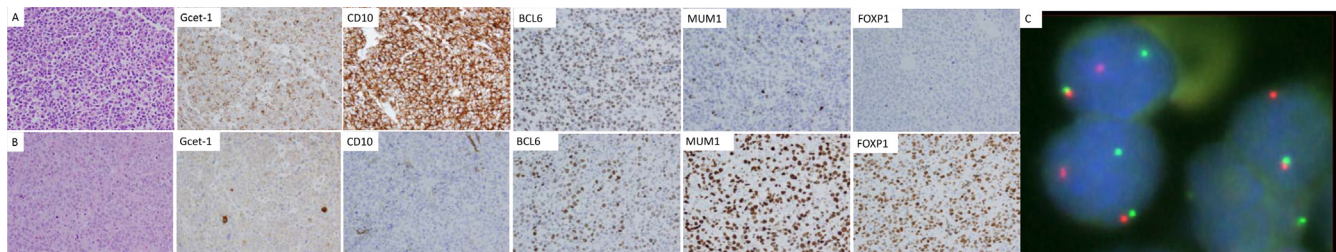


Figure 1: A. Immunohistochemistry was used to identify DLBCL subtypes. A prototypical example of GCB-type DLBCL is shown, as classified by the three algorithms considered. The case shows positive staining for GCET1, CD10, BCL6 and negative (or below the threshold) staining for MUM1 and FOXP1. B. A case of non-GCB/ABC subtype, classified by the three algorithms, is shown. The case has immunohistochemical expression of MUM1 and FOXP1 and negative (or below the threshold) staining for Gcet1, CD10 and BCL6. C. FISH using the LSI MYC break-apart probe on a tissue sample of a patient with a GCB DLBCL with rearrangement of the MYC gene.

Table 1: Clinical characteristics and differences in MYC, BCL2, BCL6, pSTAT3 and IRF4 changes according to cell of origin type

Cohort	Choi classification (n=275)					Visco-Young classification (n=282)					Hans classification (n=272)				
	GCB (n=120)		Non-GCB (n=155)		p	GCB (n=112)		Non-GCB (n=170)		p	GCB (n=115)		Non-GCB (n=157)		p
	No	%	No	%		No	%	No	%		No	%	No	%	
IPI score High (3-5)	43 of 116	37	77 of 146	53	0.011	39 of 107	36	83 of 159	52	0.011	38 of 112	34	84 of 151	56	<0.001
MYC															
<i>rMYC</i>	9 of 85	11	6 of 111	6	0.176	9 of 79	11.5	6 of 120	5	0.095	9 of 85	11	6 of 116	5	0.149
<i>gMYC</i>	20 of 81	25	17 of 102	17	0.179	21 of 70	28	16 of 109	15	0.03	21 of 80	26	16 of 108	15	0.051
BCL2															
<i>rBCL2</i>	30 of 79	38	5 of 104	5	<0.001	30 of 78	38	6 of 107	6	0.000	31 of 80	39	4 of 105	4	<0.001
<i>gBCL2</i>	5 of 77	7	20 of 101	20	0.011	5 of 76	7	20 of 104	19	0.015	5 of 77	7	20 of 103	19	0.013
BCL6															
<i>rBCL6</i>	11 of 72	15	38 of 92	41	<0.001	11 of 68	16	39 of 98	40	0.001	12 of 71	17	38 of 96	40	0.002
<i>gBCL6</i>	15 of 81	19	15 of 92	16	0.701	13 of 77	17	17 of 98	17	0.936	15 of 79	19	15 of 97	16	0.536
IRF4															
<i>rIRF4</i>	1 of 20	5	2 of 53	4	0.623	1 of 16	6	2 of 58	3	0.524	1 of 22	4.5	2 of 52	4	0.659
<i>gIRF4</i>	0 of 9	0	4 of 30	13	0.333	0 of 8	0	4 of 32	12.5	0.393	0 of 10	0	4 of 30	13	0.3
DHL															
MYC/BCL2+ (n=4)	4 of 55	7	1 of 68	1.5	0.123	4 of 55	7	1 of 70	1	0.117	4 of 55	7	1 of 70	1	0.117

Abbreviations: GCB: germinal center B-cell-like; DLBCL, diffuse large B-cell lymphoma; *rMYC*: rearrangement of *MYC*; *gMYC*: gain of the *MYC* locus; *rBCL2*: rearrangement of *BCL2*; *gBCL2*: gain of *BCL2* locus; *rBCL6* rearrangement of *BCL6*; *gBCL6*: gain of *BCL6* locus. IPI score ranges from 0 to 5, with 0 indicating the absence of prognostic factors and 5 indicating the presence of all prognostic factors. The IPI score was stratified by the proposed RIPI score. pSTAT3, phosphorylated STAT3 protein expression.

in 4 cases, *BCL2/BCL6* rearrangements in 5 cases and *MYC/BCL6* in 1 case. Taken together, 80% of double-rearrangement DLBCL cases had a GCB phenotype ($p=0.029$), and there was a higher absolute proportion of them in patients aged over 60 years ($p=0.073$) and with elevated LDH values ($p=0.053$), although these trends were not statistically significant (Supplementary Table 8). 37% of patients presented involvement of two or more extranodal sites, and 60% had stage III or IV disease. Considering only double-hit cases (*BCL2/MYC* and *BCL6/MYC* patients), no significant association with any specific clinical or biological feature was detected, probably due to the small number of cases available.

IRF4 rearrangements and gains were observed in 3 out of 76, and 4 out of 41 assessable patients, respectively. 67% and 100% of *IRF4* rearrangement and gain cases, respectively, had a non-GCB phenotype (Table 1). There was no association between the *IRF4* rearrangement and the clinical variables. An association between *IRF4* and *BCL2* gains was observed ($p=0.035$). In contrast to previously published data [29], in our series, 60% of patients with *IRF4* rearrangements were over 60 years old. It should be noted, however, that 60% of the patients in this study were older than 60 years of age.

Survival analysis

All clinical and biological variables were evaluated for their ability to predict response and outcome. In the univariate analysis, stage III-IV disease was associated with worse OS ($p=0.016$) and PFS ($p<0.001$), as were elevated LDH levels, age ≥ 60 years, poorer performance status, extranodal involvement and high IPI score. As in previous studies, the independent prognostic impact of all IPI variables was maintained in the multivariate analysis.

Considering the Visco-Young, Choi and Hans algorithms, the non-GCB subgroup was associated with significantly worse PFS ($p=0.001$, $p=0.005$ and $p=0.003$, respectively) and OS ($p=0.002$, $p=0.001$ and $p=0.001$, respectively) (Table 3 and Figure 2A and 2B). In the multivariate analysis (Table 4), COO classification remained an independent prognostic factor of PFS and OS for all the algorithms (Table 4, hazard ratio [HR] 1.7-2.1; $p<0.03$). The algorithms showed close agreement with each other, with a level of concordance of 88.6-90.3 % (multi-rater kappa, $\kappa=0.8$).

As seen in a previous study [39], patients with *MYC* rearrangements and GCB phenotype had a significantly shorter PFS ($p=0.035$, $p=0.004$ and $p=0.021$, for the

Table 2: Clinical features with respect to the presence of *MYC*, *BCL2* and *BCL6* rearrangements

Patients, No. (%)	MYC (n=15/206) Rearranged		BCL2 (n=36/187) Rearranged		BCL6 (n=51/171) Rearranged	
		P		P		P
Age, years						
> 60	5 (36)	0.029	20 (62)	0.920	32 (65)	0.581
≤ 60	9 (64)		12 (38)		17 (35)	
Ann Arbor stage						
I-II	3 (21)	0.153	14 (44)	0.381	18 (37)	0.735
III-IV	11 (78)		18 (56)		30 (62)	
LDH						
Low	3 (23)	0.097	10 (38)	0.835	12 (30)	0.021
> upper limit of normal	10 (77)		16 (62)		28 (70)	
Extranodal sites						
≤ 1	5(35)	0.009	20 (74)	0.656	28 (72)	0.695
≥ 2	9(64)		7 (26)		11 (28)	
ECOG †						
PS ≤ 1	11(79)	0.473	18 (69)	0.780	25 (64)	0.659
PS > 1	3 (21)		8 (31)		14 (36)	
IPI score ‡						
Low (0-2)	4 (29)	0.051	19 (58)	0.268	24 (50)	0.533
High (3-5)	10 (71)		14 (42)		24 (50)	

Abbreviations: IPI, International Prognostic Index; LDH, lactate dehydrogenase; †ECOG PS, ranging from 0 to 4, where a higher score indicates greater impairment. ‡ IPI score, ranging from 0 to 5, where 0 indicates the absence of prognostic factors, and 5 indicates the presence of all prognostic factors. The IPI score was stratified by the proposed RIPI score.

Hans, Choi and Visco-Young algorithms, respectively) (Figure 2G, Supplementary Table 9). None of the algorithms was of prognostic value with respect to OS, although with the Visco-Young algorithm, patients with a GCB phenotype and *MYC* rearrangements showed a non-significant tendency towards a lower OS ($p=0.098$), (Supplementary Table 10). Neither *BCL2* nor *BCL6* abnormalities had a clear impact on outcome, either in the series as a whole or in the specific COO subtypes. In contrast, in the entire series, *BCL6* expression tended to be associated with better PFS ($p=0.077$) and OS ($p=0.048$).

In our series, patients coexpressing *MYC* and *BCL2* did not show worse PFS or OS. The presence of *MYC*, *BCL2* and/or pSTAT3-positive protein expression was not associated with poorer PFS or OS. This might be due to the limited number of cases with available IHC data for these markers. Unavailability was most commonly due to tissue exhaustion.

DISCUSSION

Prognostic stratification of DLBCL patients has customarily been based on the clinical parameters comprising the International Prognostic Index (IPI). This

classification continues to be the standard clinical tool for predicting the outcome of DLBCL patients in the rituximab era [40]. However, it does not capture the biological heterogeneity of the disease and does not identify potential targets for therapy. GEP studies have identified three subtypes of DLBCL with distinct biological signatures: the GCB, the ABC and the unclassified groups. Although gene expression in formalin-fixed paraffin-embedded tissue [41] is probably the preferred method for differentiating these two DLBCL subtypes, it is not yet available in most centers. Classification using digital PCR assay has shown promising results [41, 42]. Nevertheless, a subgroup of ~10% of cases would remain unclassified even after using these methods.

Several studies have shown that IHC algorithms can discriminate GCB and non-GCB DLBCL subtypes with a high concordance with GEP, but there is still a reluctance to using them in the clinical setting. In this study, the Hans, Visco-Young and Choi algorithms were used as surrogates for GEP-based COO molecular classification. Variation due to the technique was reduced by centralized staining and scoring. All the IHC approaches used were able to distinguish the two prognostic subgroups, and showed substantial agreement among themselves,

Table 3: Progression-free survival (PFS) and overall survival (OS) with respect to the IPI, cell of origin, immunohistochemical double-positive cases and gene abnormalities of the *MYC*, *BCL2* and *BCL6* loci

	PFS		OS	
	RR (95% CI)	p	RR (95% CI)	p
Age, years				
> 60	13 (0.8-2.1)	0.198	2.2 (1.3-3.7)	0.003
≤ 60				
Ann Arbor stage				
I-II	2.4 (1.5-3.9)	<0.001	1.8 (1.1-2.9)	0.019
III-IV				
LDH				
Low	2.5 (1.5-4.1)	<0.001	2.4 (1.4-4.2)	0.002
> upper limit of normal				
Extranodal sites				
≤ 1	2.1 (1.3-3.2)	0.001	2.0 (1.3-3.3)	0.003
≥ 2				
ECOG †				
PS ≤ 1	2.3 (1.5-3.6)	<0.001	2.6 (1.6-4.2)	<0.001
PS > 1				
IPI score ‡				
Low (0-2)	2.5 (1.6-3.9)	<0.001	2.7 (1.7-4.4)	<0.001
High (3-5)				
Cell of origin subtype				
Hans Classification				
Non-GCB	1.9 (1.2-3.0)	0.003	2.3 (1.3-3.8)	0.001
GCB				
Choi Classification				
Non-GCB	1.8 (1.2-2.9)	0.005	1.9 (1.1-3.1)	0.010
GCB				
Visco-Young Classification				
Non-GCB	2.3 (1.4-3.7)	0.001	2.3 (1.3-4.0)	0.002
GCB				
MYC				
r <i>MYC</i>	1.5 (0.6-3.7)	0.279	1.1 (0.4-3.2)	0.738
g <i>MYC</i>	0.5 (0.2-1.2)	0.173	0.6 (0.3-1.4)	0.273
MYC protein expression	0.9 (0.5-1.6)	0.803	0.9 (0.5-1.8)	0.979
BCL2				
r <i>BCL2</i>	0.7 (0.4-1.6)	0.477	0.3 (0.1-1.0)	0.067
g <i>BCL2</i>	0.8 (0.3-1.8)	0.648	1.1 (0.5-2.5)	0.687
BCL2 protein expression	1.2 (0.7-2.2)	0.410	1.0 (0.5-2.0)	0.918
BCL6				
r <i>BCL6</i>	1.0 (0.6-1.9)	0.784	1.2 (0.6-2.1)	0.514
g <i>BCL6</i>	0.7 (0.3-1.5)	0.384	0.8 (0.4-1.6)	0.565
BCL6 protein expression	0.6 (0.4-1.0)	0.077	0.6 (0.4-1.0)	0.048
pSTAT3 protein expression	0.9 (0.5-1.5)	0.739	0.8 (0.4-1.5)	0.484
IRF4				
r <i>IRF4</i>	-	0.450	-	0.500
g <i>IRF4</i>	0.9 (0.1-7.4)	0.981	0.4 (0.1-3.4)	0.440
Double-HIT (FISH)	0.8 (0.2-4.6)	0.874	0.6 (0.1-4.7)	0.646
c- <i>MYC</i> +/ <i>BCL2</i> +(IHC)	1.1 (0.6-2.0)	0.625	0.9 (0.4-1.8)	0.813

Abbreviations: r*MYC*: rearrangement of *MYC*; g*MYC*: gain of *MYC* locus; r*BCL2*: rearrangement of *BCL2*; g*BCL2*: gain of *BCL2* locus; r*BCL6* rearrangement of *BCL6*; g*BCL6*: gain of *BCL6* locus; DLBCL, diffuse large B-cell lymphoma; ECOG PS, Eastern Cooperative Oncology Group performance status; GCB, germinal center B-cell-like; GEP, gene expression profiling; IPI, International Prognostic Index; DHL, double-hit lymphoma; IHC, immunohistochemistry. ‡ The IPI score ranges from 0 to 5, with 0 indicating the absence of prognostic factors, and 5 indicating the presence of all prognostic factors. The IPI score was stratified by the proposed RIPI score.

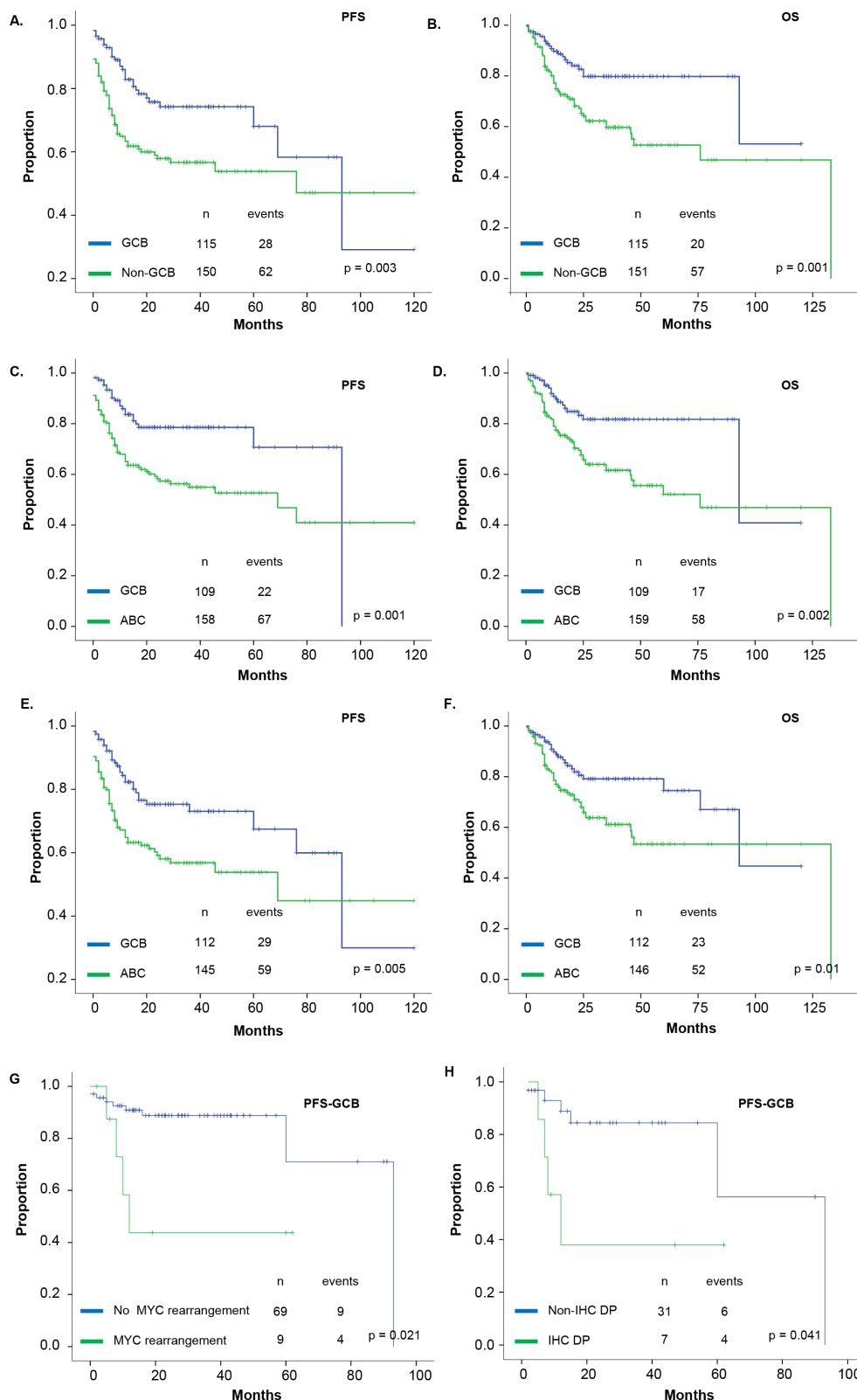


Figure 2: Univariate prognostic analysis of progression-free survival (PFS) and overall survival (OS). PFS and OS of GCB and non-GCB DLBCL using the Hans (A and B), Visco-Young (C and D), and Choi (E and F) algorithms. (G-I) PFS of MYC rearrangements in GCB-DLBCL patients using the Visco-Young, Han and Choi algorithms, respectively.

Abbreviations: IPI, International Prognostic Index; ‡ The IPI score ranges from 0 to 5, with 0 indicating the absence of prognostic factors and 5 indicating the presence of all prognostic factors. The IPI score was stratified by the proposed RIPI score. IHC DP: Immunohistochemical double-positive.

Table 4: Multivariate prognostic analysis of progression-free survival (PFS) and overall survival (OS) according to COO classification algorithms

	PFS		OS	
	RR (95% CI)	p	RR (95% CI)	p
IPI score ‡	2.3 (1.5-3.6)	<0.001	2.49 (1.5-4.0)	<0.001
Choi classification	1.7 (1.1-2.7)	0.016	1.7 (1.1-2.8)	0.027
IPI score ‡	2.2 (1.4-3.4)	<0.001	2.3 (1.4-3.7)	0.001
Hans classification	1.7 (1.1-2.8)	0.015	2.0 (1.2-3.4)	0.007
IPI score ‡	2.2 (1.4-3.4)	<0.001	2.4 (1.5-4.0)	<0.0001
Visco-Young classification	2.1 (1.3-3.5)	0.003	2.1(1.2-3.6)	0.006

Abbreviations: IPI, International Prognostic Index; ‡ The IPI score ranges from 0 to 5, with 0 indicating the absence of prognostic factors, and 5 indicating the presence of all prognostic factors. The IPI score was stratified by the proposed RIPI score.

and a similar correlation with GEP [12]. The results were comparable to those we and other researchers had obtained in previous studies [12, 43]. In agreement with previous reports [10, 44, 45], the non-GCB subgroup had a worse outcome, with a shorter PFS and OS, and this prognostic impact was maintained in the multivariate analysis. These results indicate that non-GCB patients require novel treatment approaches, including the addition of novel compounds, in order for the responses to be sustained. Indeed, a number of different new drugs including lenalidomide, some proteasome inhibitors, and the BTK inhibitor ibrutinib (PCI-32765) have recently shown promising results, especially in non-GCB/ABC DLBCL patients [7, 46–49]. Whether IHC and GEP are equally capable of distinguishing patients who could benefit from these novel compounds requires further investigation since the results published so far emerged from different methods of determination.

To refine the prognostic stratification of patients after GCB/non-GCB IHC classification, we analyzed the effect of *MYC*, *BCL2*, *BCL6* and *IRF4* abnormalities as detected by FISH. We detected *MYC* rearrangements in 7% of the cases, only 30% of which had additional cytogenetic abnormalities. The negative impact on prognosis for the isolated *MYC* rearrangement, as a single cytogenetic abnormality, was restricted to patients with a GCB phenotype and was associated with a shorter PFS. Thus, although the prognostic implication of the finding was not confirmed in the multivariate analysis, probably because of the small number of patients with this abnormality, our results [39] confirm that the favorable prognosis of GCB-type DLBCL is overcome by the negative effect of *MYC* rearrangements. For this reason, a systematic investigation of *MYC* rearrangements, at least within the GCB subgroup of patients, would be worthwhile. Despite their lower PFS, these patients did not have a significantly

worse OS, indicating that these particular cases may be more aggressive but can be rescued with savage therapies. Together, these findings suggest that GCB-type DLBCL patients carrying a *MYC* rearrangement would probably benefit from treatment strategies other than R-CHOP, for example, more aggressive chemotherapy schemes such as R-DAEPOCH, or the addition of *MYC* inhibitors. Indeed, several small molecules have been identified as *MYC* inhibitors, although none is currently in clinical use [50]. In a recently employed approach, *MYC* expression is blocked by impairing a BET bromodomain protein, a strategy that has proved effective both *in vitro* and in xenograft models in a range of hematological neoplasms, including Burkitt lymphoma [51–53]. It should be noted that *MYC* rearrangements and *MYC* expression were not correlated in this study, indicating that *MYC* expression might not be the best screening method for detecting *MYC* rearrangements in cases of DLBCL NOS. Almost half of the patients expressing *MYC* protein also had a high level of pSTAT3 expression. pSTAT3 was preferentially, but not exclusively, overexpressed in the non-GCB type DLBCL cases [30, 34], but, in contrast to other groups [54, 55], was not associated with an unfavorable outcome. Thus, our findings are consistent with those of previous studies demonstrating that pSTAT3 signaling is involved in regulating *MYC* expression [56], so pSTAT3 inhibitors could also be used in those cases [57, 58].

As in previous studies, double-hit DLBCLs seem to be restricted to GCB-type DLBCLs. However, the small number (5/100) and the short follow-up of these cases prevented us from determining the prognostic impact in terms of OS and PFS. Most patients with concurrent *MYC* and *BCL2* overexpression had high-risk IPI features and, consistent with previous findings, this phenotype was found more frequently than the double-hit with FISH and, unlike with true double-hits, was associated with a non-

GCB phenotype [33, 59, 60]. Notably, only 15% and 24% of double-IHC cases had *MYC* and *BCL2* rearrangements, respectively, suggesting that there must be other mechanisms that induce *MYC* and *BCL2* overexpression.

In our cohort, *BCL2* rearrangements, which were observed in 19% of the patients, were also mainly restricted to the GCB phenotype [22]. However, in contrast to the results from previously published series [22, 61], we found no clear negative effect on those patients' outcome, possibly due to the small number of cases featuring the abnormality. Also, the adverse prognostic implication of *BCL2* rearrangements might apply only to younger patients, so the different selection criteria used in the studies might explain the observed differences [62]. Nevertheless, *BCL2* inhibition is an attractive target for therapy, as illustrated by the fact that at least three *BCL2* antagonists are currently undergoing clinical trials for cancer treatment [63].

In accordance with previous findings, *BCL6* rearrangements that were usually detected in the non-GCB patient group [38] were not clearly associated with *BCL6* protein overexpression, and their detection was of no independent prognostic consequence with respect to OS or PFS. The prognostic contribution of *BCL6* protein expression remains controversial [64–66] and, as with some previous studies, we have shown that *BCL6* expression is associated with a better OS in the rituximab era. Since *BCL6* expression is almost completely restricted to germinal center cells and some lymphomas, *BCL6* is an attractive target for therapy with several specific *BCL6* inhibitors currently being investigated [67].

In summary, our results confirm that COO classification based on IHC is a reproducible and practical approach to stratifying DLBCL patients into GCB and non-GCB phenotypes, the latter being associated with a higher risk of progression and reduced survival when conventional R-CHOP regimens are used. These patients might benefit from treatment with novel compounds that target signaling pathways known to be constitutively activated in this particular lymphoma subtype.

Identification of *MYC* rearrangements enables GCB-DLBCLs to be stratified into prognostic groups. GCB patients with isolated *MYC* rearrangements have a higher risk of progression and might benefit from adding new therapeutic approaches, including drugs that inhibit *MYC* function. Despite the lack of prognostic information, identification of certain proteins, such as *BCL6* or pSTAT3, might be of interest as potential druggable target molecules.

PATIENTS AND METHODS

Patient selection

The study population consisted of a retrospective series of 297 unselected *de novo* cases of DLBCL

obtained from various centers in Spain (153 cases), and other institutions involved in the International DLBCL Rituximab-CHOP Consortium Program Study in the USA (144 cases). Some of these cases have already been published [31, 32]. The study was reviewed and approved by each of the participating Institutional Review Boards. The overall collaborative study and the study protocol and sampling methods were approved by the Institutional Review Board of the Hospital Universitario Marqués de Valdecilla/IDIVAL, Spain. Histopathological criteria used for their diagnosis and classification were those of the WHO Classification [3]. Cases associated with HIV or HCV infections or previous immunosuppressive treatments were excluded, as were cases diagnosed as T-cell histiocyte-rich B-cell lymphoma, primary mediastinal B-cell lymphoma cases, cutaneous LBCL, intravascular LBCL, EBV+DLBCL and those histologically associated with a follicular lymphoma component. All patients were treated as part of their routine care with standard treatment protocols using a combination of anthracycline-based regimens (6-8 cycles in most cases) and rituximab. Follow-up data on progression-free survival (PFS) and overall survival (OS) were available for 279 (94%) and 283 (95%) patients, respectively.

Immunohistochemistry, TMA construction and FISH analysis

Histopathological evaluation, immunohistochemistry and FISH analysis were performed in a central laboratory by experienced hematopathologists and cytogeneticists. All DLBCL cases were histologically confirmed and representative areas were selected. Detailed IHC staining procedures are described in the Supplementary Data. The cut-offs for the IHC markers were *BCL2* >70%, *MYC* >40% and pSTAT3 >10%, based on published data [33, 34]. Scoring of GCET1, MUM1, CD10, *BCL6*, and FOXP1 was based on the cut-offs used by Choi et al [11], Hans et al [10] and Visco Young [35]. Immunoreactivity was scored by two independent pathologists (SMM and SMU) and the percentage of tumor-cell staining was estimated by visual inspection and categorized into the appropriate decile. Disagreements were resolved by joint review using a multiheaded microscope. For each case the most representative core, with the highest percentage of neoplastic cells, was selected.

FISH analyses were performed on 3- μ m TMA tissue sections following standard procedures [36, 37] (see Supplementary Data for details). At least 100 intact, non-overlapping nuclei were analyzed from each TMA core. Discordant duplicates were reevaluated by two observers (AB, SG). Nuclei were scored as rearranged if at least one split orange-green signal was observed. Gains were reported when three or more fusion signals were observed.

Control values were previously established on the basis of samples of 10 controls (mean plus or minus three standard deviations). The cut-off values for chromosome gain or rearrangement were 15% in each case.

Statistical analysis

Patient characteristics and response rates were compared using the X^2 or Fisher's exact test, depending on the number of observations. PFS was defined as the time from initial diagnosis to progressive disease under therapy, failure to achieve complete remission, additional therapy, relapse, or death from any cause. OS was defined as the time from initial diagnosis to death. PFS and OS were analyzed using the log-rank test and illustrated as Kaplan-Meier plots. A Cox proportional hazards multivariate regression model was derived. Differences between the results of comparative tests were considered significant for two-sided values of $p < 0.05$. The multi-rater kappa was used to assess the concordance between the algorithms.

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CONFLICTS OF INTEREST

There are no conflicts of interest to disclose

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