ARTICLE

Dose-adjusted EPOCH and rituximab for the treatment of double expressor and double-hit diffuse large B-cell lymphoma: impact of *TP53* mutations on clinical outcome

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ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease, including one-third of cases overexpressing MYC and BCL2 proteins (double expressor lymphoma, DEL) and 5-10% of patients with chromosomal rearrangements of *MYC, BCL2* and/or *BCL-6* (double/triple-hit lymphomas, DH/TH). *TP53* mutations are detected in 20-25% of DEL. We report the efficacy of dose-adjusted EPOCH and rituximab (DA-EPOCH-R) in a series of 122 consecutive patients, including DEL (n=81, 66%), DEL-MYC (n=9, 7%), DEL-BCL2 (n=13, 11%), or high-grade lymphomas (DH/TH) (n=19, 16%). Central nervous system (CNS) prophylaxis included intravenous methotrexate (n=66), intrathecal chemotherapy (IT) (n=40) or no prophylaxis (n=16). Sixty-seven patients (55%) had high-intermediate or high International Prognostic Index (IPI) and 30 (25%) had high CNS-IPI. The 2-year progression-free survival (PFS) and overall survival (OS) for the entire study population were 74% and 84%, respectively. There was a trend for inferior OS for DH/TH (2-year OS: 66%, *P*=0.058) as compared to all the others. The outcome was significantly better for the IPI 0-2 *versus* IPI 3-5 (OS: 98% *vs.* 72%, *P*=0.002). DA-EPOCH-R did not overcome the negative prognostic value of *TP53* mutations: 2-year OS of 62% *versus* 88% (*P*=0.036) were observed for mutated as compared to wild-type cases, respectively. Systemic CNS prophylaxis conferred a better 2-year OS (94%) as compared to IT or no prophylaxis (76% and 65%, respectively; *P*=0.008). DA-EPOCH-R treatment resulted in a favorable outcome in patients with DEL and DEL with single rearrangement, whereas those with multiple genetic alterations such as DEL-DH/TH and *TP53* mutated cases still have an inferior outcome.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a clinically and biologically heterogeneous disease. Historically DLBCL patients have been uniformly treated with R-CHOP chemoimmunotherapy regimen (rituximab, cyclophosphamide, adri-

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amycin, vincristine, prednisone), leading to a long-lasting complete remission in approximately 60% of cases.[1,2] For these patients, the International Prognostic Index (IPI), is an easy and valid tool for prognostic stratification.[3]

In 2000 Alizadeh *et al*. used gene expression profiling (GEP) to identify the cell of origin (COO) and described prognostic subgroups according to diversity in gene expression patterns indicative of different stages of B-cell differentiation.[4] Immunohistochemical algorithms, used as surrogates of GEP for the identification of COO subgroups, have been extensively used but have a limited concordance with GEP when applied to patients with DLBCL treated with R-CHOP.[5]

Among other possible prognostic indicators for patients with DLBCL, tumor protein p53 (*TP53*) mutations seem to represent simple and attractive biomarkers to be used in the daily routine clinical practice. The *TP53* gene is involved in maintaining genomic stability in response to DNA damage by activating DNA repair programs and by triggering cell cycle arrest. The loss of *TP53* is associated with lymphomagenesis and resistance to chemotherapy.[6] *TP53* mutations are present in 10% of DLBCL patients and confer a poor prognosis in patients treated with R-CHOP.[7,8] Recently, Chapuy *et al*. performed whole genomic sequencing in 304 primary DLBCL patients and identified genetically different subgroups among germinal center B-cell like (GCB) and activated B-cell (ABC) lymphoma patients. Patients with *TP53* mutations were described as a distinct cluster with a very poor prognosis.[9]

MYC and *BCL2* represent other possible poor prognostic markers when rearranged or overexpressed in DLBCL and lymphomas harboring the double or triple translocation (DH or TH) represent new entities in the recently updated 2016 World Health Organization Lymphoma Classification. Most DH lymphomas are in the GCB category, they present with advanced stages and have a very poor prognosis when treated with the R-CHOP regimen.[10-12] Also, the *BCL2* translocation alone has been shown to play a significant prognostic role in GCB DLBCL patients treated with R-CHOP.[13,14] Additionally, the presence of *MYC* rearrangements seems to be a poor prognostic marker, although its role in the absence of either BCL2 or p53 alterations remains controversial.[15] The concomitant overexpression of MYC and BCL2 on the tumor cell surface observed in double expressor lymphomas (DEL), could be the result of other mechanisms, different from translocation such as copy gain/amplification. The prognosis of DEL patients treated with standard R-CHOP is worse than that of non-DEL patients,[16,17] and these individuals have a risk of central nervous system (CNS) relapse of 9.7% at 2 years.[18] Moreover, a consensus on the optimal treatment for these patients has yet to be established.

Since 2013 at our Institution, DEL patients have been treated with the intensive chemotherapy regimen dose-adjusted EPOCH and rituximab (DA-EPOCH-R), achieving a promising 2-year progression-free survival (PFS) and overall survival (OS) of 62% and 85%, respectively. These survival rates were better than those reported with R-CHOP in a historical cohort.[19]

The aim of the present study was to assess the incidence and prognostic role of *TP53* mutations and *BCL2*, *MYC* translocations in a large cohort of DEL patients consecutively treated with DA-EPOCH-R. The analysis of the cumulative risk of CNS relapse as well as the effect of the different CNS prophylaxis applied in this selected cohort of DEL patients was also assessed.

Methods

Patients

All consecutive patients with a diagnosis of DEL treated since 2013 with DA-EPOCH-R at four hematological divisions in northern Italy were retrospectively identified. For inclusion in this observational study, the following inclusion criteria were required: i) histologically proven diagnosis of DEL with an age ≥18 years; ii) availability of formalin-fixed, paraffin-embedded (FFPE) samples; iii) no exposure to previous therapy except for the first cycle of R-CHOP that was allowed while waiting for immunohistochemistry (IHC) results and cytogenetic characterization. Exclusion criteria were HIV positivity, CNS involvement at diagnosis, and histology other than DEL. The ethics committees of the participating centers approved the study (INT 35/17). Written informed consent was obtained from all patients.

Immunohistochemistry and fluorescence *in situ* hybridization analysis

FFPE tissue samples were sectioned at 3-μm thickness. IHC was performed using the EnVision FLEX+, mouse, high pH method (Dako Denmark A/S Produktionsvej 42 DK-2600 Glostrup, Denmark) and a Dako Autostainer Link48 (Dako, Italia, SPA, Milano, Italy). Slides were stained with monoclonal antibodies against CD19, CD20, CD10, BCL2, BCL6, MUM1, MYC, and Ki67.

Fluorescence *in situ* hybridization (FISH) analyses for *BCL2, BCL6*, and *MYC* rearrangements were performed in all patients using "LSI BCL2 LSI BCL6 (ABR), C-MYC "break apart" probes (Vysis/Abbott Molecular, Illinois, USA) according to the manufacturer's instructions (detailed in the *Online Supplementary Appendix*).

TP53 mutations

TP53 is located on chromosome 17p13.1 and consists of 14 exons (1-11), 10 of which are coding sequences for the p53 protein.[20,21] *TP53* mutations were assessed according to Institutional practice, Sanger sequencing was used for samples collected from patients at Humanitas Cancer Center while next-generation sequencing followed by direct sequencing was used for all other samples (procedures described in the *Online Supplementary Appendix*).

Treatment and central nervous system prophylaxis

Patients received the DA-EPOCH-R regimen therapy every 21 days for six cycles. In all patients dose adjustment based on cell counts between cycles according to the NCI algorithm was applied.[22] At diagnosis, all the patients performed lumbar puncture for cytology and flow cytometry analyses of cerebrospinal fluid (CSF). During this first procedure, all patients received intrathecal chemotherapy with methotrexate (MTX) and cytarabine. Neuro-imaging was considered only in case of the presence of neurological signs or symptoms. In absence of definitive clinical guidelines on CNS prophylaxis, the choice was determined by the treating physician. High-dose MTX (HD-MTX: 3 g/m^2) was used as prophylaxis in 66 patients (for the majority at the end of the treatment). Intrathecal chemotherapy at day 1 of every cycle, including MTX/cytarabine/dexamethasone, was given to 40 patients.

Aim of the study and statistical analysis

Objectives of the study were assessment of the effect of biological variables (*TP53* mutations and *BCL2* and *MYC* translocations) on survival analyses at 2-years in a large cohort of DEL patients consecutively treated with DA-EPOCH-R. Analysis of the cumulative risk of CNS recurrence in the whole population and PFS and OS impact of different CNS prophylaxis were also evaluated. Fisher's exact test was performed to assess the association between *TP53* mutation status and patients' clinical characteristics. Statistical analyses were performed using R (version 3.5.0) (detailed in the *Online Supplementary Appendix*).

Table 1. Clinical characteristics of 122 consecutive diffuse large B-cell lymphoma patients overall and according to rearrangements.

	Overall N=122	DEL N=81	*BCL2* N=13	*MYC* N=9	HG-BCL N=19
Age, continuous					
Median (years)	59.0	57.0	64.0	57.0	62.0
third/first quartile	(49.0; 65.0)	(46.0; 64.0)	(55.0; 67.0)	(37.0; 61.0)	(57.0; 67.0)
Age, categorical					
≤60 years old	66 (54.1)	48 (59.3)	5 (38.5)	5 (55.6)	8 (42.1)
>60 years old	56 (45.9)	33 (40.7)	8 (61.5)	4 (44.4)	11 (57.9)
Rearrangements					
None	81 (66.4)	81 (100)	–	–	–
DEL-BCL2	13 (10.7)	–	13 (100)	–	–
DEL-MYC	9 (7.4)	–	–	9 (100)	–
DEL-DH/TH	19 (15.6)	–	–	–	19 (100)
Ki67 (%)*					
Median	90.0	90.0	90.0	90.0	75.0
third/first quartile	(75.0; 90.0)	(80.0; 90.0)	(85.0; 95.0)	(81,3; 91.3)	(65.0; 87.5)
Sex					
Male	75 (61.5)	49 (60.5)	7 (53.8)	6 (66.7)	13 (68.4)
Female	47 (38.5)	32 (39.5)	6 (46.2)	3 (33.3)	6 (31.6)
Cell of origin					
GCB	55 (45.1)	22 (27.2)	10 (76.9)	5 (55.6)	18 (94.7)
Non-GCB	60 (49.2)	52 (64.2)	3 (23.1)	4 (44.4)	1 (5.3)
Not assessed	7 (5.7)	7 (8.6)	0 (0.0)	0 (0.0)	0 (0.0)
Staging					
I-II	27 (22.1)	18 (22.2)	2 (15.4)	4 (44.4)	3 (15.8)
III-IV	95 (77.9)	63 (77.8)	11 (84.6)	5 (55.6)	16 (84.2)
IPI					
0-2	55 (45.1)	40 (49.4)	4 (30.8)	6 (66.7)	5 (26.3)
3-5	67 (54.9)	41 (50.6)	9 (69.2)	3 (33.3)	14 (73.7)
CNS-IPI					
0-1	31 (25.4)	21 (25.9)	3 (23.1)	5 (55.6)	2 (10.5)
2-3	61 (50.0)	47 (58.0)	5 (38.5)	2 (22.2)	7 (36.8)
4-6	30 (24.6)	13 (16.0)	5 (38.5)	2 (22.2)	10 (52.6)
Extranodal site (at risk for CNS)					
Yes	16 (13.1)	10 (12.3)	0 (0.0)	4 (44.4)	2 (10 .5)
No	106 (86.9)	71 (87.7)	13 (100.0)	5 (55.6)	17 (89.5)
CNS prophylaxis					
None	16 (13.1)	12 (14.8)	0 (0.0)	1 (11.1)	3 (15.8)
IT MTX	40 (32.8)	24 (29.6)	5 (38.5)	3 (33.3)	8 (42.1)
IV MTX	66 (54.1)	45 (55.6)	8 (61.5)	5 (55.6)	8 (42.1)
Autologous SCT					
Yes	22 (18.0)	12 (14.8)	3 (23.1)	1 (11.1)	6 (31.6)
No	100 (82.0)	69 (85.2)	10 (76.9)	8 (88.9)	13 (68.4)

DEL: double expressor lymphomas; HG-BCL: high-grade B-cell lymphomas; DH/TH: double-hit/triple-hit; GCB: germinal center lymphomas; Non-GCB: non-germinal center lymphomas; IPI: International prognostic index; CNS: central nervous system; IT: intrathecal; MTX: methotrexate; IV: intravenous; SCT: stem cell transplantation. *10 missing values, 8 in DEL group and 1 each in DEL/BCL2 and DEL/MYC rearrangements group.

Table 2. Kaplan-Meier estimates of 2-year progression-free and overall survival according to patients and disease characteristics

	2-year PFS (95% CI)	P*	2-year OS (95% CI)	P*
Age		0.138		0.064
≤60	80.0 (70.4; 90.9)		88.4 (80.0; 97.7)	
>60	67.7 (55.6; 82.4)		78.2 (66.9; 91.3)	
Sex		0.012		0.094
Male	66.3 (55.9; 78.7)		80.7 (71.3; 91.3)	
Female	87.1 (77.0; 98.7)		88.9 (79.0; 100)	
Cell of origin**		0.544		0.907
Germinal central B cell	70.6 (59.1; 84.4)		81.0 (69.9; 93.9)	
Non-Germinal central B cell	74.6 (63.2; 87.9)		84.7 (75.4; 95.2)	
Rearrangements		0.203		0.058
DEL	74.8 (64.9; 86.3)		85.9 (77.6; 95.2)	
DEL-BCL2	69.2 (48.2; 99.5)		90.9 (75.4; 100)	
DEL-MYC	100		100	
DEL-DH/TH	63.2 (44.8; 89.0)		66.2 (47.4; 92.5)	
Staging		0.048		0.081
I-II	91.8 (81.6; 100)		95.0 (85.9; 100)	
III-IV	69.8 (60.6; 80.4)		81.0 (72.6; 90.3)	
International prognostic index		0.002		0.002
0-2	88.1 (79.6; 97.6)		97.8 (93.7; 100)	
3-5	62.2 (50.7; 76.3)		71.8 (60.5; 85.2)	
CNS prophylaxis		0.027		0.008
None	50.8 (28.6; 90.2)		64.9 (41.7; 100)	
Intrathecal methotrexate	69.5 (56.5; 85.5)		75.8 (63.2; 91.0)	
Intravenous methotrexate	81.8 (72.0; 93.0)		94.3 (88.2; 100)	
TP53 mutation***		0.033		0.036
Wild-type	79.9 (68.9; 92.8)		87.7 (78.0; 98.5)	
Mutated	58.3 (37.3; 91.1)		61.7 (39.8; 95.6)	

PFS: progression-free survival; OS: overall survival; CI: confidence interval; DEL: double expressor lymphomas; DH/TH: double-hit/triple-hit; CNS: central nervous system. *Log-rank test P-value; **Excluding 7 not-assessed patients; ***Excluding 53 not-assessed patients.

Results

Patients' characteristics and treatment

A total of 122 patients affected by DEL were consecutively treated with DA-EPOCH-R between November 2015 and March 2020. Patients' characteristics are summarized in Table 1. The median age was 59 years (range, 24-79 years), and 62% were male.

MYC and *BCL2* rearrangements by FISH were evaluated in all cases. *BCL6* rearrangement could be done for 95 out of 122 patients (78%). According to IHC and FISH testing, the study population was divided into three subgroups: i) DEL only without any rearrangements (n=81, 66%); ii) DEL single-hit (DEL-SH) with either *MYC* or *BCL2* rearrangement (n=9 *MYC*, n=13 *BCL2*); and iii) high-grade lymphomas (double-hit/triple-hit [DH/TH]) (n=10 *MYC/BCL2*, n=3 *MYC/BCL6*, n=6 *MYC/BCL2/BCL6*). The COO assignment according to Hans algorithm was analyzed in 115 of 122 patients (94%) of whom 55 (45%) were GCB and 60 (49%) non-GCB.

Ninety-five (78%) patients had an advanced stage and 67 (55%) presented an IPI score of 3-5. Moreover, the number of patients with limited disease was low (n=27) with only four cases with stage I (all these cases presented with bulky extranodal disease) (*Online Supplementary Table S1*).

The median number of chemotherapy cycles was 6 (range, 1-6 cycles). Due to the aggressive clinical presentation requiring urgent treatment, 19 (15%) patients received the first cycle of R-CHOP while waiting for complete FISH analyses. Response assessment following DA-EPOCH-R treatment was feasible in 117 patients: of these 84 (72%) and 16 (14%) achieved a complete or partial remission, respectively. Seventeen patients (14%) showed progressive disease. Five patients were not evaluable for a response after DA-EPOCH-R for toxicity (n=1, death of pneumonia), de-escalation therapy (n=2), consolidation with high-dose therapy before the sixth cycle (n=2). Overall, 22 of 122 (18%) patients (n=12 DE, n=3 SH-BCL2, n=1 SH-MYC, n=6 DH/TH) underwent autologous stem cell transplantation, in clinical remission, during treatment (n=2) and as consolidation after six cycles of DA-EPOCH-R (n=20) (*Online Supplementary Table S2*).

With the exclusion of one patient who died of pneumonia, other adverse events were manageable. We observed febrile neutropenia in 16 of 122 (13%) and infections requiring hospital admission (n=6 pneumonia, n=1 sepsis) in seven patients (6%).

Survival outcome

After a median follow-up of 24 months (interquartile range [IQR], 14-38 months), 110 patients were alive and 22

died (n=20 for disease progression, n=1 for toxicity, n=1 suicide). PFS (95% confidence interval [CI]) and OS at 2-year were 74% (66-83%) and 84% (77-91%), respectively (Figure 1A and B). The 2-year OS and PFS were not significantly different between DEL, DEL-MYC, DEL-BCL2 and, DEL-DH/TH, with a trend for inferior survival in this last subgroup (OS: 66% [range, 47-92%], *P*=0,058) (Figure 1C and D).

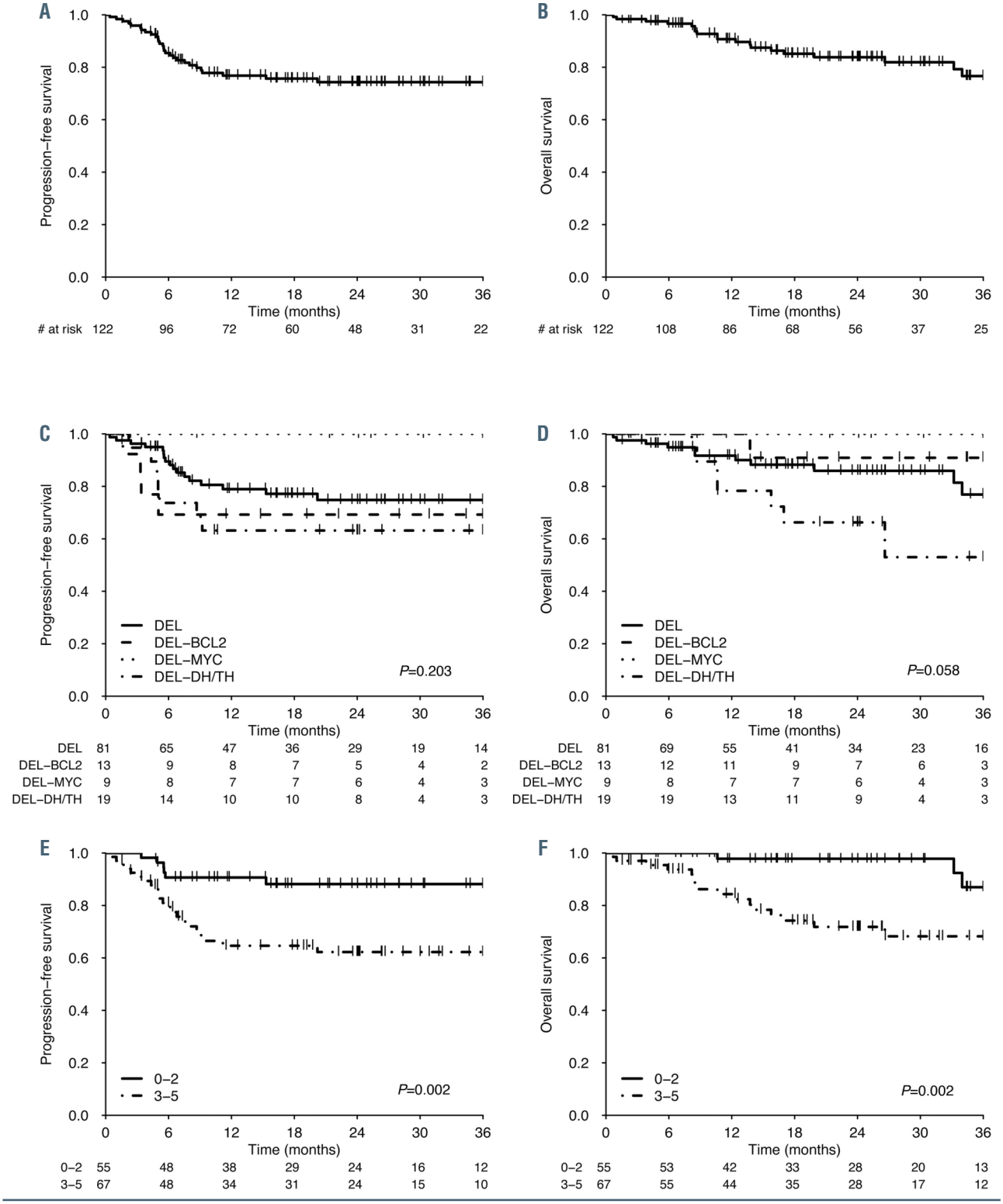

Figure 1. Kaplan-Meier estimates of progression-free survival and overall survival. Progression-free survival (A, C and E) and overall survival (B, D and F) for the whole cohort (A and B) and according to rearrangements (C and D). DEL: double expressor lymphomas only; DEL-MYC, DEL-BCL2: high-grade lymphomas (double-hit/triple-hit [DH/TH]) and International Prognostic Index (panels E and F).

Table 3. **Table 3. Clinical characteristics of 69 patients evaluated for *TP53* mutation status.**

	Mutated *TP53* N=16	Wild-type *TP53* N=53	*P**
Rearrangements			0.258
None	8 (50.0)	36 (67.9)	
DEL-BCL2	3 (18.8)	6 (11.3)	
DEL-MYC	3 (18.8)	3 (5.7)	
DEL-DH/TH	2 (12.5)	8 (15.1)	
Cell of origin**			0.776
Germinal central B cell	8 (53.3)	25 (48.1)	
Non-Germinal central B cell	7 (46.7)	27 (51.9)	
Staging			0.124
I-II	2 (12.5)	18 (34.0)	
III-IV	14 (87.5)	35 (66.0)	
International prognostic index			0.161
0-2	5 (31.2)	28 (52.8)	
3-5	11 (68.8)	25 (47.2)	
Systemic CNS therapy			0.241
None	0 (0.0)	5 (9.4)	
Intrathecal methotrexate	7 (43.8)	14 (26.4)	
Intravenous methotrexate	9 (56.2)	34 (64.2)	
Autologous stem cell transplantation			0.334
Yes	1 (6.2)	11 (20.8)	
No	15 (93.8)	42 (79.2)	
Progression-free survival			0.033***
2-year estimate (95% CI)	58.3 (37.3; 91.1)	79.9 (68.9; 92.8)	
Events	6 (37.5)	9 (17.0)	
CNS relapse-free probability			0.782***
2-year estimate (95% CI)	90.0 (73.2; 100)	92.5 (84.6; 100)	
Events	1 (6.3)	3 (5.7)	

DH/TH: double/triple hit; CNS: central nervous system. *Fisher Exact test *P*-value; **Excluding 7 not-assessed patients; ***Log-rank test *P*-value.

Age above 60 years did not affect outcome whereas the male sex was associated with a significantly shorter PFS (*Online Supplementary Table S3*). The COO did not show a significant impact either on PFS or OS. Isolated MYC (≥70%) or BCL2 (≥80%) as assessed by IHC did not impact PFS and OS (*data not shown*).

As expected, patients with a limited disease had a significantly higher 2-year PFS 92% (range, 81-100%) as compared to advanced stages (70% [range, 61-80%], *P*=0,048) (Table 2). The analysis of outcome by IPI score showed a 2-year PFS of 62% (range, 51-76%) and OS of 71% (range, 61-85%) for high-intermediate and high IPI score that was inferior compared to low-intermediate and low cases (88%, [range, 79-98%] and 98% [range, 94-100%]; *P*=0,002 and *P*=0,002 respectively) (Figure 1E and F). In those achieving a response, we did observe a significant difference in outcome between patients who did or did not receive autologous transplantation (*Online Supplementary Figure S1*). Complete results of univariable Cox models for PFS and OS according to the patients and disease characteristics are reported in the *Online Supplementary Table S4*.

Evaluation of *TP53* mutation

The *TP53* mutation could be retrospectively evaluated in 69 of 122 (57%) patients due to the absence of sufficient residual archival material or to poor quality of the genomic DNA extracted from paraffin-embedded tissues. The *TP53* mutation status was assessed in 44 DEL (64%), six DEL-MYC (9%), nine DEL-BCL2 (13%), and ten DEL-DH/TH (15%). Overall a pathogenic *TP53* mutation (as defined by the IARC TP53 database) was present in 16 patients (23%). We evaluated the outcome according to the presence or absence of TP53 mutation. The two groups were not statistically different for the main clinical characteristics (Table 3). The 2-year PFS was 58% (range, 37-91%) and 80% (range, 70-93%; *P*=0.033) and the 2-year OS was 62% (range, 40-96%) and 88% (range, 78-99%; *P*=0.036), for mutated and wild-type cases respectively (Figure 2A and B).

Multivariable analysis

Cox multivariate models concerning the PFS and OS of the patients were performed as summarized in Table 4. *TP53* mutation, IPI 3-5, and absence of CNS prophylaxis had a negative prognostic impact on OS whereas the female sex was associated with a significantly improved PFS.

Outcome of relapsed patients

Among 28 patients who relapsed (n=17 DEL, n=4 DEL-SH [only with *BCL2* translocation], n= 7 DEL-DH/TH), 19 (68%) died of lymphoma whereas nine patients are still alive (n=6 DEL, n=3 DEL/BCL2). Five of nine patients are in complete remission after receiving different salvage

Table 4. Results of the multivariable Cox models for progression-free and overall survival.

Model	Progression-free survival		Overall survival	
	Hazard ratio (95% CI)	P*	Hazard ratio (95% CI)	P*
Rearrangements		0.880		0.408
DEL-BCL2 *vs.* DEL	1.49 (0.48; 4.64)		0.16 (0.02; 1.48)	
DEL-MYC *vs.* DEL**	–		–	
DEL-DH/TH *vs.* DEL	1.15 (0.46; 2.87)		1.00 (0.37; 2.75)	
TP53 mutation		0.072		0.002
Mutated *vs.* wild-type	3.13 (1.04; 9.40)		8.90 (2.14; 36.99)	
Not performed *vs.* wild-type	0.98 (0.41; 2.35)		0.75 (0.22; 2.53)	
International prognostic index		0.063		0.018
0-2 *vs.* 3-5	0.36 (0.12; 1.06)		0.18 (0.04; 7.4)	
Systemic CNS therapy		0.062		0.019
None *vs.* intravenous MTX	3.74 (1.22; 11.41)		8.49 (1.82; 39.57)	
Intrathecal MTX *vs.* intravenous MTX	1.99 (0.83; 4.76)		4.25 (1.2; 15.02)	
Staging		0.847		–
III-IV *vs.* I-II	1.19 (0.21; 6.69)		–	
Sex		0.045		–
Female *vs.* male	0.36 (0.14; 0.98)		–	
Age***		–		0.752
65 *vs.* 49	–		1.52 (0.51; 4.56)	

CI: confidence interval; DEL: double expressor lymphomas; DH/TH: double-hit/triple-hit; CNS: central nervous system; MTX, methotrexate. *Wald test *P*-value; **No survival or progression events observed; ***Modeled as restricted cubic spline and reporting result of 65 *vs.* 49 years comparison.

therapies (n=2 auto-stem cell transplantation [auto-SCT], n=2 allo-SCT, n=1 lenalidomide in combination with radiotherapy).

Central nervous system prophylaxis and central nervous system relapse

At diagnosis, only two patients had cerebrospinal fluid involvement: one died early of systemic progressive disease and the other is still alive after therapy including high-dose methotrexate.

The CNS prophylaxis was chosen at the discretion of the treating physician. Sixty-six (54%) patients received systemic HD-MTX, 40 (33%) underwent intrathecal chemotherapy with methotrexate and cytarabine and 16 (13%) did not receive any CNS prophylaxis at all. In particular, patients not receiving CNS prophylaxis had less extranodal involvement at risk for CNS relapse. All characteristics are detailed in the *Online Supplementary Table S5*.

Systemic methotrexate-based CNS prophylaxis conferred a better 2-year OS (94%, [range, 88-100%]) as compared to intrathecal or no CNS prophylaxis (75%, [range, 63-91%] and 65%, [range, 42-100%] respectively; P=0.008) (Figure 2C and D). A significant advantage in OS was observed even after exclusion of DH/TH patients (2-year percentage OS 96% *vs.* 81%, *vs.* 63%, respectively, [P<0.001; Figure 2E and F]) that was the subgroup with the worst outcome.

Overall, we observed five CNS relapses, and the cumulative incidence of relapse at 1- and 2-year was 2% (range, 1-9%) and 5% (range, 2-13%) respectively in the entire cohort. All patients with CNS relapse were DEL only, all but one were non-GCB. Four of five patients died of CNS lymphomas. The CNS relapse occurred even in patients who received CNS prophylaxis (3 of 5 patients) and in four out five with low CNS-IPI.

Discussion

In the present retrospective study, we collected a large number of consecutive DEL patients (n=122) who were treated with the DA-EPOCH-R regimen to test the hypothesis that an intensive regimen could overcome poor clinical, and biological prognostic factors. To the best of our knowledge, this study includes the largest series of DEL patients exposed to an intensified regimen. All DEL patients were analyzed for *MYC* and/or *BCL2* rearrangements and, partly, for *TP53* mutational status. Indeed, the 2-year PFS and OS of 74% and 84%, respectively, for the entire cohort seem promising. Further, in patients characterized by IPI score of 3-5, the results are comparable to those achieved with other intensified treatments (R-CODOX/IVAC and R-ACVB).[23,24]

Currently, the treatment of DEL without any gene rearrangement remains an unmet clinical need. The phase III trial performed by the Alliance group comparing the R-CHOP and DA-EPOCH-R regimens in newly diagnosed DLBCL included only a limited number of DEL, thus preventing the possibility of drawing definitive conclusions on the role of DA-EPOCH-R in this subtype.[25] Our series of 81 DEL patients without any rearrangement showed a 2-year PFS and OS of 75% and 86%, respectively, suggesting a potential role of the intensive regimen. Recently, Morschhauser *et al*. evaluated the combination of venetoclax with standard R-CHOP or obinutuzumab-CHOP in DEL patients or those with high expression of *BCL2*, showing a similar 2-year PFS of 72% and 77%, respectively.

Patients with a DEL-DH/TH status showed mainly an intermediate and high-risk IPI (75%) and had a trend for inferior OS at 2 years (66%) as compared to DEL only (86%), whereas the observed 2-year PFS of 63% was not

statistically different among these three subgroups. In addition, we have to consider that the prognosis of high-grade B-cell lymphomas (HGBCL) with overexpression of MYC or BCL2 proteins seems poor as compared to DH/TH, not DEL.[27] This finding suggests that more than 50% of DEL-DH/TH could be cured with an intensive regimen but that in case of relapse they have a trend for poor overall survival. Likely, the recent Food and Drug Administration and European Medicines Agency approval of new therapies such as CAR-T cells will have an impact on the OS of relapsed DEL-DH/TH HGBCL.[28,29]

A total of 27 patients with limited-stage disease (stage I,

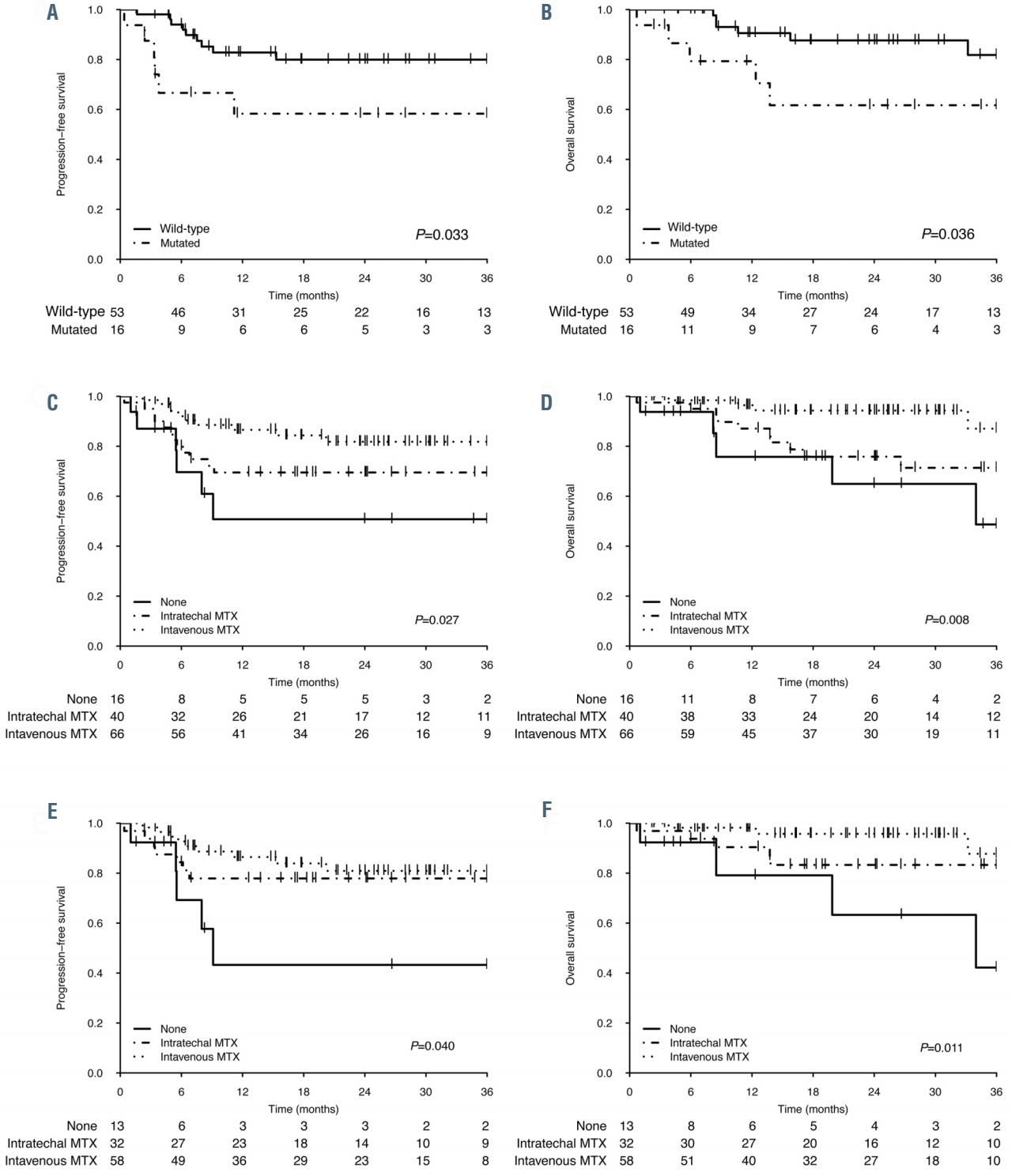

Figure 2. Kaplan-Meier estimates of progression-free survival and overall survival according to *TP53* mutation and central nervous prophylaxis. Progression-free survival (A, C and E) and overall survival (B, D and F) according to *TP53* mutational status (A and B) and central nervous system prophylaxis (none, intrathecal, intravenous methotrexate) including (C and-D) and excluding (E and F) high-grade B-cell lymphomas (double-hit/triple-hit [DH/TH]).

n=4 with extensive extranodal localization; stage II, n=23 (DEL, n=18; SH, n=6; DH/TH, n=3) were treated with DA-EPOCH-R and experienced an impressive 2-year PFS and OS of 92% and 95%, respectively. Interestingly, the majority of limited disease patients did not have *TP53* mutation (mutated, n=2; wild-type, n=18; not tested, n=7). Only a handful of studies, mainly of retrospective nature, have been focused on the prognosis of single hit or DH/TH lymphomas with limited-stage disease. Torka and colleagues analyzed the outcome of 81 patients carrying MYC rearrangement, including 40 DH, who received the standard R-CHOP regimen or intensive chemotherapy.[30] The authors did not find any statistical difference in PFS and OS across treatment strategies but in the subgroup of DH, the intensive therapy was associated with a higher CR rate.

Over the past years, the combination of CNS-IPI and biological factors has been considered the best way to estimate the CNS relapse risk. In a retrospective study of newly diagnosed DLBCL patients treated with R-CHOP, without any CNS systemic prophylaxis, Savage and colleagues reported that the DEL/non-GCB subgroup had a significantly higher risk of CNS relapse as compared to the non-DEL/non-GCB subgroup (15% *vs.* 3%).[31] In contrast, in the prospective Goya trial, COO and not *MYC/BCL2* double expression impacted the risk of CNS relapse.[32] More recently, the Nordic group has suggested the efficacy of the early administration of HD-MTX in newly diagnosed DLBCL at high risk of CNS relapse.[33] Our study is the first in which the risk of CNS relapse has been evaluated following the administration of a DA-EPOCH-R regimen and HD-MTX in 66 of 122 (54%) patients. Our population included 24% of patients with high-risk CNS-IPI. Despite the high-risk population analyzed, systemic CNS prophylaxis was associated with a very low cumulative incidence of CNS relapse (5%) and a significant advantage in PFS and OS. The advantage on the outcome can be influenced by other untested factors and should be confirmed in a larger trial.

Xu-Monette *et al.*[6] reported a 21% incidence of *TP53* mutations in a large population of DLBCL patients treated with R-CHOP and demonstrated that *TP53* disruption was associated to poor PFS and OS in both GCB and non-GCB subtypes. Following a better characterization of DLBCL beyond the COO, the frequency of this mutation was tested in DEL and did not result to be different from non DEL (25% *vs.* 22%, respectively). The role of genomic alterations, including *TP53* mutations, has recently been investigated in several studies by applying novel molecular techniques.[9] Interestingly, Meriranta *et al.* found a frequent association between *TP53* alterations and *MYC* overexpression or translocations and, in those with *TP53* mutation, a worse outcome in DEL as compared to non-DEL was reported.[34] Recently, Song *et al.* reported a poor prognosis in DLBCL carrying double-hit signature and *TP53* inactivation.[35] In our study, the prevalence of the *TP53* mutation was in keeping with previous studies, and the PFS/OS following DA-EPOCH-R treatment in *TP53*-mutated patients remained significantly lower than that observed in non-mutated patients suggesting the failure of intensive therapy to overcome the mutation adverse effect. The observed 2-year OS of 62% seems better when compared to the OS of 48% reported by Xu Monette[6] with R-CHOP and of 27% described by Chiappella *et al.*[36] following intensified therapy. These are indirect comparisons requiring a clinical study to be validated.

The intensified treatment with DA-EPOCH-R was well tolerated considering that 56% of patients were older than 60 years. We observed a limited incidence of severe toxicities, with one death for pneumonia and two patients de-escalated to R-CHOP for repeated infections. Other observed adverse events were manageable and did not compromise the completion of the therapeutic program.

Our data are promising, but we have to consider some limitations including: i) the retrospective nature; ii) the absence of information about MYC translocation partners (IG *vs.* non-IG); iii) the determination of cell of origin performed according to the Hans algorithm and not by the nanostring technology; iv) the absence of a control series of non-DEL patients with a single rearrangement or with DH/TH genotype.

In conclusion, we show a good outcome for DA-EPOCH-R in combination with HD-MTX in DEL and DEL-SH lymphomas without *TP53* mutations, but the lower survival of patients DEL-DH/TH subtype or DEL with *TP53* mutations requires further clinical studies aimed at testing novel agents combined with chemotherapy.

Disclosures

No conflicts of interests to disclose.

Contributions

AD, AG designed research, analyzed data, and wrote the manuscript; FM, AT, AR, MB, AC, MP, LD, FR, FC and LF collected data; FB and RM performed statistical analyses; CC, CM and DR performed TP53 mutations; AC, DR, VM and FF performed histological diagnosis and FISH analysis; PC and CC-S supervised the study.

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