## Risk adapted high-dose and dose-dense therapies modulate the impact of biological classification in diffuse large B-cell lymphoma prognosis

Diffuse large B-cell lymphoma (DLBCL) is a highly heterogeneous disease entity.[1] Young patients with high-intermediate and high aa-IPI score seem to be good candidates to receive alternative treatments to standard RCHOP-21 including EPOCH-R,[2] R-ACVBP+HDT-ASCT[3] and upfront autologous stem cell transplantation.[4] Other risk factors can be used to identify patients for the use of more dose-intense regimens including bulky disease, interim PET positivity and, importantly, molecular profiles.[5-7]

The aim of our study was to evaluate whether high-dose regimens in patients enrolled in clinical trials might modify the prognostic impact of already recognized biological markers in DLBCL (Cell of Origin (COO) classification, MYC, BCL2 and BCL6 translocations, microRNA expression, MYC and BCL2 protein overexpression).

We analyzed a series of 156 patient samples. Patients were enrolled in four clinical trials from GELTAMO, PETHEMA and GOTEL and treated using first-line dose-dense or high-dose regimens (R-CHOP 14: 76 patient samples from 209 (36%) patients; dose-adjusted EPOCH-R: 42 patient samples from 81 patients included (52%); and MegaCHOP-R plus stem cell transplantation based on interim PET results: 38 patient samples from 71 patients enrolled (53%)).[8] All samples received in the reference center were included in the study and biological marker analysis was only hampered by tissue limitations in specific cases.

A summary of the major clinical characteristics of the patients included in this study is shown in Table 1. The study was approved by the Institutional Ethical Committee of all participating institutions and by the national regulatory agency according to Spanish law, and complied with the Declaration of Helsinki. A retrospective cohort of 240 cases of DLBCL treated with chemoimmunotherapy was used for comparison.[9]

Centralized review of the histopathological diagnosis was performed by 2 hematopathologists (SMM, MAP) according to WHO classification.[1] All cases were classified according to the COO using the immunohistochemical algorithms by Hans, Choi and Visco-Young.[10-12]

**Table 1.** Clinical features of the series.

| Number of patients | 156 | OS*, PFS** pooled set | 76 | OS*, PFS** RCHOP-14 (76 patients) | 42 | OS*, PFS** EPOCH-R (42 patients) | 38 | OS, PFS Mega CHOP-R and BMT (38 patients) |
|---|---|---|---|---|---|---|---|---|
| IPI factors | | | | | | | | |
| Age | | *0.02, **0.003 | | *0.01,**0.0007 | | *ns, **ns | | *ns, ns** |
| ≤60 years | 90 (57%) | | 45 | | 18 | | 27 | |
| >60 years | 63 (40%) | | 28 | | 24 | | 10 | |
| Stage | | *ns,**ns | | *ns,**ns | | *ns, **ns | | *ns, ns** |
| I-II | 74 (47%) | | 31 | | 42 | | 1 | |
| III-IV | 79 (50%) | | 42 | | 0 | | 36 | |
| LDH | | *ns,**ns | | *ns,**ns | | *ns,**ns | | *ns, ns** |
| low | 49 (31%) | | 35 | | 4 | | 9 | |
| high | 100 (64%) | | 36 | | 38 | | 26 | |
| Performance status | | *ns, **0.04 | | *0.001,**0.01 | | *ns,**ns | | *ns, ns** |
| Ambulatory (0-1) | 107 (68%) | | 61 | | 24 | | 21 | |
| Not ambulatory (2-4) | 43 (27%) | | 10 | | 18 | | 15 | |
| Extranodal site involvement | | *ns,**ns | | *ns,**ns | | *ns,**ns | | *ns, ns** |
| ≤ 1 site | 101 (64%) | | 54 | | 30 | | 17 | |
| > 1 site | 49 (31%) | | 18 | | 12 | | 18 | |
| IPI score (number of IPI factors) | *ns, **0.03 | | *ns,**ns | | *ns,**ns | | *ns, 0.03** | |
| Low risk (0,1) | 34 (22%) | | 30 | | 3 | | 1 | |
| Low – intermediate risk (2) | 38 (24%) | | 22 | | 5 | | 11 | |
| High – intermediate risk (3) | 56 (36%) | | 15 | | 22 | | 18 | |
| High risk (4,5) | 25 (16%) | | 6 | | 12 | | 7 | |
| COO-CLASSIFICATION | | | | | | | | |
| GCB (according to Choi) | 81/144 (56%) | *ns,**ns | 42 | *ns,**ns | 22 | *ns,**ns | 15 | *ns,ns** |
| ABC | 63/144 (44%) | | 25 | | 18 | | 18 | |
| GCB (according to Hans) | 63/146 (43%) | *ns,**ns | 35 | *ns,**ns | 16 | *ns,**ns | 8 | *ns, ns** |
| NON-GCB | 83/146 (57%) | | 34 | | 24 | | 20 | |
| GCB (according to Visco-Young) | 75/145 (52%) | *ns,**ns | 39 | *ns,**ns | 22 | *ns,**ns | 12 | *ns, ns** |
| ABC | 70/145 (48%) | | 29 | | 18 | | 21 | |
| CONCURRENT MYC-BCL2 IHC EXPRESSION | | | | | | | | |
| | | *ns,**ns | | *ns,**ns | | *ns,**ns | | *ns, ns** |
| DP* | 31/117 (26%) | | 35 | | 29 | | 19 | |
| NON DP | 86/117 (73%) | | 17 | | 4 | | 9 | |

*LDH: lactic dehydrogenase levels, IPI: International Prognostic Index; COO: cell of origin classification. GCB: germinal center B-cell type; ABC: activated B-cell type; NON-GCB: non-germinal center B-cell type; DP: double-positive for MYC and BCL2 immunohistochemical expression. NON-DP: non-double positive.*

Immunohistochemical expression of BCL2 and C-MYC was also scored. Interphase FISH analysis was performed using Dual Color Break Apart DNA probes against BCL2, BCL6 and C-MYC. microRNA expression signatures based on RT-PCR were calculated and miRNA-based survival models[9] were applied for OS and PFS in this series of patients. ROC analysis was performed to identify the higher sensitivity and specificity cut-off value for each survival

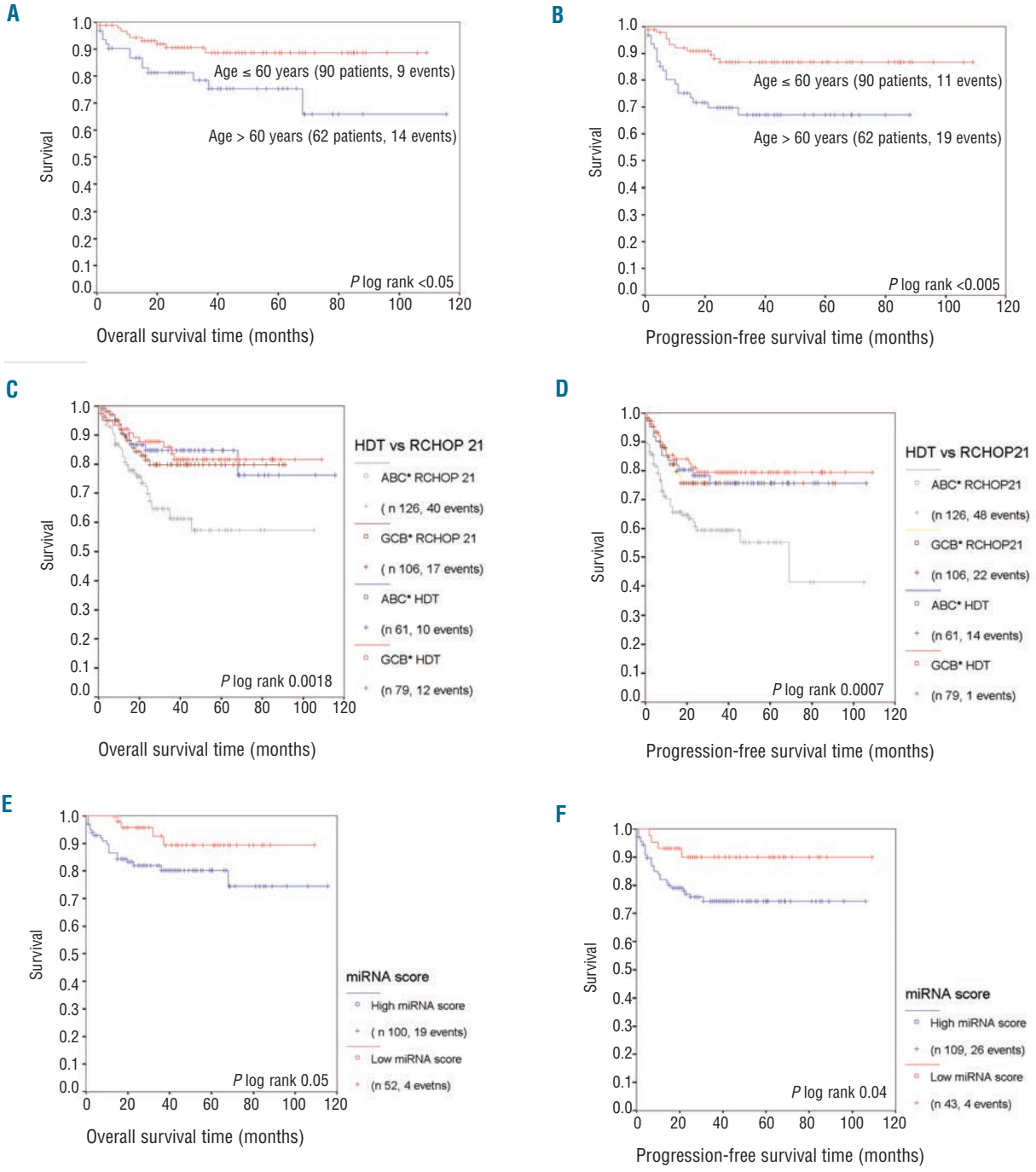


**Figure 1.** (A, B) Overall survival (OS) and progression-free survival (PFS) Kaplan Meier estimates according to advanced age (>60 years old). Patients under 60 years old had a probability of OS and PFS over 85% in the median follow-up time. (C, D). IPI risk stratification into Low, Low-intermediate, High-intermediate and High IPI was predictive for PFS ($P < 0.05$) but not OS. Patients with low IPI risk had an excellent outcome with survival probabilities over 90% in the median follow-up time. However no differences were found among Low-intermediate, High-intermediate and High IPI risk. (C, D). OS and PFS Kaplan Meier estimates according to cell of origin classification: ABC type DLBCL prognosis was significantly improved when compared with the R-CHOP 21 treated control cohort ($P < 0.005$), while GCB type DLBCL prognosis was not altered. Patients treated with high-dose and dose-dense therapies had an OS and PFS equivalent for both GCB and ABC subtypes (81% *vs.* 84% for OS and 79% *vs.* 75% for PFS). (E, F). miRNA expression signatures were found of prognostic value for OS ($P$ log rank = 0.05) and PFS ($P$ log rank 0.04). Cases with low miRNA score had a 90% ± 4 % PFS probability compared to 74% ± 4 % for cases with high miRNA score.

function and cases were categorized into low miRNA score and high miRNA score accordingly. Kaplan Meier survival curves and multivariate analysis were made using SPSS v11.

Overall survival and PFS at the median follow-up time (46 months) were 83% (±3.3%) and 79% (±3.4%), respectively. Median survival time for OS was 97.26 months (±3.57) and median PFS were 88.04 months (±3.42). Median age was 57 years old (range 17-80 years). No significant differences were found in OS or PFS according to the study protocol (*Online Supplementary Figure S1*). Advanced age (>60 years) was predictive of poor OS and PFS in the univariate analysis ($P$<0.05) for the pooled set of patients and the RCHOP14 cases. Poor performance status was predictive of poor PFS ($P$<0.05) (Table 1) for the pooled set of cases and poor OS and PFS in the RCHOP14 cases ($P$=0.001 and $P$=0.01, respectively). IPI risk stratification was predictive for PFS ($P$<0.05) but not OS (Table 1 and Figure 1).

None of the IHC algorithms for COO subclassification was predictive for OS or PFS in the whole series or after treatment protocol stratification (Table 1). Interestingly, ABC type DLBCL (according to Choi´s algorithm) prognosis was significantly improved in this series when compared with the R-CHOP21 treated control cohort ($P$<0.005), while there was no change in GCB type DLBCL prognosis (Figure 1).

MYC and BCL2 protein expression was quantified according to previously published cut offs.[9,13,14] Median MYC protein expression was 30% and 54 of 116 (46%) evaluable cases had an expression of 40% or over. Median BCL2 protein expression was 50%. Sixty-seven of 124 (54%) evaluable cases had an expression of 50% or over and 53 of 124 cases (42%) of 70% or over. Neither single MYC nor BCL2 expression had an independent relationship with OS or PFS. Furthermore, no significant statistical differences were found according to concurrent expression of MYC and BCL2 protein (so-called double positive cases), irrespective of the cut off of positivity used (*data not shown*).

Thirty-three of 117 (28%) cases had concurrent expression of MYC and BCL2 (>50%). Thirty-one of 117 cases (26%) had co-expression of MYC and BCL2 (>70%). An association between DP cases and high-stage (III-IV) disease ($P$<0.05) and ABC-non-GCB molecular subgroup ($\chi^2$ <0.05 using V-Y algorithm) was found. By FISH, 6 (3.8%), 19 (12.1%) and 27 (17.2%) of the evaluable cases had MYC, BCL2 and BCL6 translocations, respectively. Only 3 cases of double hit lymphoma were found (1 with MYC and BCL2 translocations, 2 cases with MYC and BCL6 translocations). None of these genetic alterations was predictive for OS or PFS.

miRNA expression signatures were found of prognostic value for OS (P=0.05; log rank) and PFS ($P$=0.04; log rank). Cases with low miRNA score had a 90% ± 4% PFS probability compared to 74% ± 4% for cases with high miRNA score (Figure 1). This effect was independent of advanced age in the multivariate analysis for OS (RR for age of 2.96 and RR for miRNA score of 3.31; $P$=0.005) and PFS (RR for age of 3.53 and RR for miRNA score of 3.76; $P$<0.001). Patients aged under 60 years and those with a low miRNA score had a probability of OS and PFS of over 85% and 90% in the median follow-up time, respectively.

In summary, our results suggest that high-dose or dose-dense therapies as a primary treatment for patients aged under 60 years may overcome the negative impact of ABC-type aggressive molecular subgroup and concurrent MYC and BCL2 overexpression by IHC. These results are in keeping with recent data that demonstrate a benefit for high-intermediate and high risk patients with MYC positive DLBCL or BCLU treated with autologous stem cell transplantation as consolidation therapy.[4,15]

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