SUPPLEMENTARY APPENDIX

Prognostic influence of macrophages in patients with diffuse large B-cell lymphoma: a correlative study from a Nordic phase II trial

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Supplementary Material

Prognostic Influence of Macrophages in Patients with Diffuse Large B-cell Lymphoma. A Correlative Study from a Nordic Phase II Trial

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Supplementary Methods

Patients

The prospectively collected screening cohort consisted of DLBCL patients who were less than 65 years old and had primary high-risk (age-adjusted International Prognostic Index (aaIPI) score 2-3) disease. They were treated in the Nordic phase II NLG-LBC-04 protocol with six courses of R-CHOEP14 (rituximab, cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisone supported with G-CSF) followed by systemic CNS prophylaxis with one course of high-dose methotrexate and one course of high-dose cytarabine (1). The original trial included 156 eligible patients with DLBCL (n= 143) or grade 3 FL (n=13). Cases with FL were excluded from this study. Histological diagnosis was established from surgical or needle biopsies of the pretreatment tumor tissue according to current criteria of the World Health Organization classification (WHO) (2) by local pathologists followed by central pathology review. Classification into molecular GCB and non-GCB subtypes was performed immunohistochemically (IHC) according to Hans algorithm (3).

Gene expression validation cohorts represent subsets of CGCI (n=92) and LLMPP (n=233) study populations treated with R-CHOP like regimen (Table S2), and LLMPP prerituximab cohort (n=181) treated with CHOP. The materials are based on the availability of data on gene expression, baseline characteristics and follow-up.

Immunohistochemical validation cohorts consisted of an independent population-based series of DLBCL patients. The first set consisted of 72 primary DLBCL patients treated with chemoimmunotherapy at the Helsinki University Central Hospital between 2001 and 2006 was used. All patients received antracyclin-based regimens with rituximab. Of these, the majority was treated with R-CHOP (n=57) or R-CHOEP/CHOED (n=9). Other baseline characteristics are described in Table 2. In addition, 50 DLBCL patients treated with chemotherapy before rituximab was adapted into clinical routine served as a prerituximab control group (Table S3). They had a high-risk primary or relapsed DLBCL and were treated with induction chemotherapy followed by consolidation with autologous stem cell transplantation (ASCT) supported high dose therapy. The median follow-up times for the validation cohort and prerituximab control groups were 65 and 85 months, respectively. All samples were taken before treatments. Selection was based on the availability of FFPE tissue and clinical information.

Inclusion criteria in the TAM response cohort were age ≥ 18 years, CD20 positive DLBCL, primary disease, performance status WHO≤2, written informed consent, and tissue available for molecular studies. The patients were treated according to local practise with R-CHOP x 6-8 and participation to this molecular study did not affect the given treatment. To avoid risks, tissue biopsies were restricted to superficially located lymphoma lesions and performed as ultrasound-directed tru-cut biopsies.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections either as a part of a TMA (Nordic phase II cohort) or on individual slides (validation and prerituximab cohorts), and processed as previously described (4). Sections were stained for CD68 (monoclonal anti-CD68 antibody; 1:2000, clone KP1, Dako Cytomation, Denmark), CD163 (monoclonal anti-CD163 antibody; 1:70, clone 10D6, Thermo Fisher Scientific, Fremont, USA) and CCL18 (monoclonal anti-CCL18/PARC antibody; 1:200, R&D Systems, Minneapolis, USA). The number of CD68+, CD163+ and CCL18+ TAMs were counted manually as absolute cells numbers at 630x magnification (field of view 0,096mm^2) with Leica DM LB light-field microscope (Leica Microsystems GmbH). Five representative fields with the most abundant TAM infiltration (hot spots) without necrosis and fibrosis were counted and scoring results averaged. All scorings were performed blindly. These analyses were performed in Helsinki.

Serial sections were also stained for CD14 (monoclonal anti-CD14 antibody; 1:800, clone 7, Leica Biosystems, Newcastle, UK), CD3 (monoclonal anti-CD3 antibody, 1:600, clone SP7, Thermo Fisher Scientific), CD4 (monoclonal anti-CD4 antibody; 1:500, clone 4B12, Thermo Fisher Scientific), CD8 (monoclonal CD8-antibody; 1:600, clone 4B11, Leica Biosystems), CD21 (monoclonal anti-21 antibody; 1:100, clone 2G9, Leica Biosystems), CD57 (monoclonal anti-CD57 antibody, 1:200, clone NK-1, Leica Biosystems) and CD68 (monoclonal anti-CD68 antibody; 1:500, clone PG-M1, Dako Cytomation, Denmark) and GCET (monoclonal anti-GCET1 antibody;

1:100, clone RAM341, Abcam, Cambridge) and analysed in Oslo. Number of CD14+ cells was counted as absolute cell numbers in 5% percentiles, and CD3+, CD4+ and CD8+ lymphocytes, CD57+ NK cells, and CD68+ TAMs in 10% percentiles. For GCET1 positivity, a 30% cut off level was used (5). CD21 positive dendritic cells were counted as present or not present. The counting was performed at 630x magnification with Olympus BX50 light field microscope (Olympus, Germany). Whole TMA cores were scored. If the cores showed areas with different percentage of positive cells, two to three fields were counted and scoring results averaged.

Reproducibility of the immunohistochemical data was tested by comparing CD68 stainings on a TMA subset of 59 samples performed both in Helsinki and Oslo. A comparison of the results demonstrated a significant level of agreement between two laboratories (r_s 0.770, $p<0.001$).

Statistics

Spearman correlation coefficient was calculated to evaluate the correlation between cell counts. The X^2 test was used to assess the differences in the frequency of the prognostic factors. Cox univariate and multivariate analyses were performed to study the prognostic value of the factors. Kaplan-Meier method was used to estimate survival rates and the differences in these rates were compared with log-rank test. Overall survival (OS) was determined from the date of study entry or diagnosis until last follow up or death from any cause. Progression free survival (PFS) was measured as the period between the date of registration or diagnosis and relapse or death. OS and PFS were reported in months. A web based cutoff finder

tool at http://molpath.charite.de/cutoffanalysis was used to determine the most prognostic cutoff level for survival outcomes. (6). The exploratory analyses of the modification of biomarkers were performed using paired-samples t-test or Wilcoxon's test for paired samples, as appropriate. Probability values below 0.05 were considered statistically significant and all p values were two-tailed. Data were analyzed using PASW Statistics 18.0 (SPSS, Inc.).

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Supplementary Results

Table S1. Baseline characteristics and outcome of exon array and TMA cohorts from the Nordic phase II study according to low and high *CD68* mRNA levels and low and high CD68+ TAM counts.

Characteristic	**Low CD68 gene expression (exon array)**	**High CD68 gene expression**	**p-value**	**Low CD68 IHC (TMA)**	**High CD68 IHC (TMA)**	**p-value**
Number of patients	14	24		10	49	
Median age (range)	54 (20-64)	54 (25-63)		50 (38-62)	54 (18-65)	
Age						
<60	11(79)	18 (75)	1.000	9 (90)	35 (71)	0.426
≥60	3 (21)	6 (25)		1 (10)	14 (29)	
Gender						
Male	8 (57)	16 (67)	0.729	7 (17)	34 (83)	1.000
Female	6 (43)	8 (33)		3 (17)	15 (83)	
aa-IPI						
2	7 (50)	20 (83)	0.061	6(60)	36 (73)	0.453
3	7 (50)	4 (17)		4 (40)	13 (27)	
DLBCL molecular subgroup						
GCB	9 (65)	15 (63)	0.347	6 (60)	24 (49)	0.438
nonGCB	2 (14)	7 (29)		3 (30)	19 (39)	
PMBL	1 (7)	1 (4)		0 (0)	3 (6)	
Unknown	2 (14)	1 (4)		1 (10)	3 (6)	
Relapses	7	3	0.021	6	10	0.018
Deaths	5	4	0.245	4	7	0.079

Table S2. Baseline characteristics and outcome of LLMPP and CGCI validation cohorts.

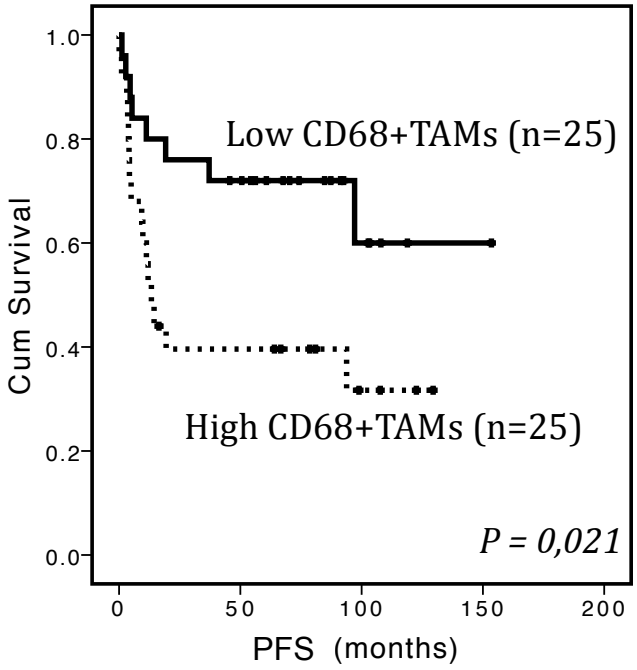
Characteristic	LLMPP-cohort	CGCI-cohort
Number of patients	233	92
Median age (range)	61 (17-92)	61 (16-92)
Age		
<60	113 (48,5)	39 (42)
≥60	120 (51,5)	53 (58)
Gender		
Male	134 (57,5)	61 (66)
Female	99 (42,5)	31 (34)
IPI		
0-2	ND	40(44)
3-5	ND	52 (56)
DLBCL molecular subgroup		
GCB	107 (46)	51 (55)
ABC	93 (40)	32 (35)
Unclassified	33 (14)	9 (10)
Relapses	ND	24
Deaths	60	21
Lymphoma specific	ND	18
Other	ND	3
5-year PFS	ND	73%
5-year OS	69%	78%

Table S3. Characteristics of the prerituximab cohort according to TAM content.

Characteristic	**All n (%)**	**High CD68+ TAM n (%)**	**Low CD68+ TAM n (%)**	**p-value**
Number of patients	50 (100)	25 (100)	25 (100)	NA
Median age (range)	44 (21-63)	46 (24-60)	42 (21-63)	NA
Gender				
Female	26 (52)	14 (56)	12 (48)	0.778
Male	24 (48)	11 (44)	13 (52)	
State of the disease				
Primary	30 (60)	14 (56)	16 (64)	0.773
Relapsed	20 (40)	11 (44)	9 (36)	
IPI				
0-2	36 (72)	21 (84)	15 (60)	0.071
3-5	9 (18)	2 (8)	7 (28)	
Not classified	5 (10)	2 (8)	3 (12)	
Molecular subgroup				
GCB	26 (52)	11 (44)	15 (60)	0.149
Non-GCB	20 (40)	13 (52)	7 (28)	
Not classified	4 (8)	1 (4)	3 (12)	
Induction treatment				0.817
CHO(E)P	22 (44)	10 (40)	12 (48)	
CHOP-like	10 (20)	5 (20)	5 (20)	
Other	18 (36)	10 (40)	8 (32)	
High dose therapy				
BEAC	34 (68)	20 (80)	14 (56)	0.111
BEAM	13 (26)	4 (16)	9 (36)	
Other	3 (6)	1 (4)	2 (8)	

Figure S1. Outcome of patients in the prerituximab cohort according to CD68+ TAM content. a. OS of DLBCL-patients according to high and low CD68+ TAM content. b. PFS of DLBCL-patients according to high and low CD68+ TAM content.

A.



B.

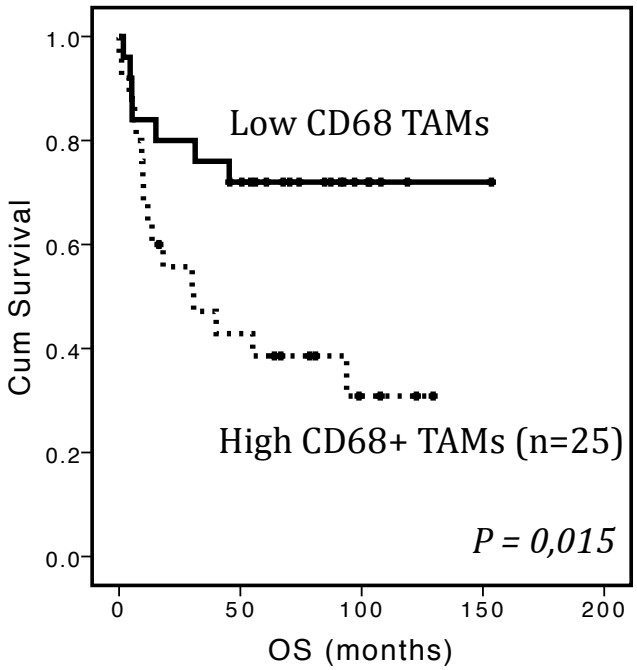


Figure S2. OS of patients in the LLMPP CHOP cohort according to CD68 gene expression.