SUPPLEMENTARY APPENDIX

High CD45 surface expression determines relapse risk in children with precursor B-cell and T-cell acute lymphoblastic leukemia treated according to the ALL-BFM 2000 protocol

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Online Supplementary Appendix

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Content:

Supplementary Methods

Patients

In accordance with institutional review board regulations, clinical samples were obtained from children with ALL before treatment. The study was approved by the institutional review board of the Hannover Medical School, Hannover, Germany, and informed consent obtained from patients and/or their legal guardians in accordance with the Declaration of Helsinki. Diagnostics, risk group assignment, and treatment were performed according to the ALL-BFM 2000 protocol.[1, 2] Risk-adapted treatment stratification (standard-, intermediate-, or high-risk) was performed using cytogenetic markers (t(9;22), t(4;11)) or their molecular equivalents (*BCR-ABL1* and *MLL-AF4*) and the *in vivo* response to treatment. Response was assessed cytomorphologically (blast reduction in peripheral blood after 7 days of treatment; blast clearance from the bone marrow after induction therapy on treatment-day 33), or molecularly by measurement of minimal residual disease (MRD) on treatment-day 33 and after induction consolidation at week 12. Measurement of MRD is based on the detection of clone-specific immunoglobulin and T-cell receptor gene rearrangements by PCR amplification. The level of sensitivity reached in most cases is 10^{-4} – 10^{-5} (detection of one leukemic cell per 10^4 – 10^5 cells).

SR patients were MRD-negative on both TP and HR patients had a persistent high MRD (≥10^{-3}) at TP2. MRD-IR patients had positive MRD detection at either one or both time points but at a level of <10^{-3} at TP2. Patients with poor prednisone response (PR) or induction failure (≥5% leukemic blasts in the bone marrow, BM) or positivity for translocations t(9;22) or t(4;11) or their molecular equivalents (*BCR/ABL1* or *MLL/AF4* rearrangement) were stratified into the HR group independent of their MRD results.

Patients consecutively enrolled from 04/2003 to 01/2008 in trial ALL-BFM 2000 were included in our present study.

Flow cytometric measurement and quantification of CD45 surface expression

CD45 expression was routinely assessed at diagnostic immunophenotyping, which was performed centrally in the immunodiagnostic reference laboratory of the ALL-BFM 2000 trial. Samples were taken prior to the initiation of treatment and shipping was routinely done overnight.

Bone marrow or peripheral blood mononuclear cells were isolated with ficoll gradient centrifugation, washed and stained with fluorochrome-conjugated antigen-specific monoclonal antibodies. After a final washing procedure cell preparations were resuspended in PBS. Acquisition of 10000 events was done with a FacsCalibur (Becton Dickinson, San Jose, California, USA) flow cytometer and analyzed with the CellQuest® (Becton Dickinson, San Jose, California, USA) software. Except for the cytoplasmic IgM which was analyzed by fluorescence microscopy, all other cytoplasmic antigen expressions were measured with flow cytometry. Further details on the methods applied were recently published[3]

Analysis and reporting was done according to the guidelines proposed by the European Group for the Immunological Characterization of Leukemias (EGIL).[4] Accordingly, antigen expression was quantified in terms of percentage of positive cells as compared to an antigen-negative subpopulation or, in case of CD45, in comparison to a negative control with an isotype- and fluorochrome-matched antibody. The CD45 antibody used throughout the study was purchased from BeckmanCoulter, Miami, FL (clone J33, conjugated with phycoerythrincyanin 5.1 (PC5)). As negative control, an isotype- and fluorochrome-matched (IgG1-PC5)

antibody (BeckmanCoulter) was used. The sample was qualified as antigen positive if the number of antigen-positive cells was equal to or more than 20%.

In the study presented here, we re-analyzed the "raw" flow cytometric data and quantified CD45 expression in order to provide a full-scale estimation of CD45 expression. To control for technical variation, we normalized the measured expression values by use of normal mature B- and T-lymphocytes within the sample, which are known to stably express high levels of CD45.[5, 6] We first calculated the mean fluorescence indexes (MFI) from the corresponding mean fluorescence intensities of the subpopulations according to the formula: MFI=(mean CD45 fluorescence intensity of the subpopulation - mean fluorescence intensity of the negative control)/mean fluorescence intensity of the negative control. Next, we calculated the relative ratio of CD45 expression in leukemic cells as MFI of leukemic blasts/MFI of normal lymphocytes. Thus, the CD45 expression values were normalized by use of residual normal lymphocytes in the same samples as internal standard. The resulting expression values were described in percentage as relative ratio of CD45 expression in leukemic vs. normal cells.

Detection of *P2RY8-CRLF2* fusion

In 250 patients with BCP-ALL data on the *P2RY8-CRLF2* status were available. The presence of the fusion transcript *P2RY8-CRLF2* was analyzed by RT-PCR, as we have previously reported, using primer P2RY8F01 for *P2RY8* (5′-cacgaacaccttctcaagca-3′) and CRLF2R03 for *CRLF2* (5′- ggtagttggtgcactggtca-3′). PCR product was ~390 bp. Reference sequences are P2RY8-001 (ENST00000381297) and CRLF2-001 (ENST00000400841). HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) was used with the following thermal cycling

conditions: 1 cycle of 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C 60 s, followed by 1 cycle of 72°C for 10 min. [7, 8]

Analysis of *IL7R* mutations

In 107 patients with T-ALL data on the *IL7R* mutation status were available. Analysis was performed as previously described[9]: Intronic primers of human *IL7R* sequence (available from GenBank/EMBL/DDBJ under accession no. NM_002185.2) were used to amplify exons 1–8 of the gene with PCR. Fast start Taq DNA polymerase (Roche) was used with the following thermal cycling conditions: 1 cycle of 94°C for 5 min, followed by 5 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C 30 s, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C 30 s, followed by 1 cycle of 72°C for 7 min. Fragments were analyzed by denaturing high-performance liquid chromatography as described before.[8, 9]

Statistical analysis

Event-free survival (EFS) was calculated from the date of diagnosis to last follow-up or to the first event (no complete remission [CR] as event on day 0, relapse, secondary malignancy, or death of any cause). Rates were calculated according to Kaplan-Meier and compared by log-rank test. Cumulative relapse incidence (CRI) functions were constructed by the method of Kalbfleisch and Prentice and compared by the Gray test. Multivariate cox regression was used to calculate the hazard ratio for an event, mediated by CD45-high expression. Proportional differences between patient groups were analyzed by Chi^2 or Fisher's exact tests. Statistical analyses were carried out using the SPSS statistical package (IBM, Chicago, IL, USA; release XY) and two-sided P-values below 0.05 were considered to be statistically significant.

Supplementary Table S1. Patient characteristics and response to treatment in the study cohort of 1065 pediatric patients with ALL, in comparison to the remaining ALL-BFM 2000 patient cohort that was not studied.

Feature	Cohort not studied (%)	Study cohort (%)	P[1]
Number of patients	1175 (100)	1065 (100)	
Sex			0.71
Male	656 (55.8)	603 (56.6)	
Female	519 (44.2)	462 (43.4)	
Age at diagnosis (years)			0.89
1 – 9	869 (74.0)	785 (73.7)	
≥ 10	306 (26.0)	280 (26.3)	
Presenting WBC count ($x10^{10}$/L)			0.36
< 1	525 (44.7)	487 (45.7)	
1 – 4.99	404 (34.4)	363 (34.1)	
5 – 9.99	133 (11.3)	99 (9.3)	
≥ 10	113 (9.6)	116 (10.9)	
Prednisone response[2]			0.41
Good	1057 (90.0)	948 (89.0)	
Poor	106 (9.0)	107 (10.1)	
No information	12 (1.0)	10 (0.9)	
MRD[3]			<0.0001
MRD-SR	353 (30.0)	355 (33.4)	
MRD-IR	581 (49.5)	427 (40.0)	
MRD-HR	49 (4.2)	84 (7.9)	

No information	192 (16.3)	199 (18.7)	
Immunology			0.46
Non-T-ALL	1008 (85.8)	925 (86.9)	
T-ALL	167 (14.2)	140 (13.1)	
ETV6/RUNX1			0.72
Negative	880 (75.0)	757 (71.1)	
Positive	253 (21.4)	226 (21.2)	
No information	42 (3.6)	82 (7.7)	
BCR/ABL			0.97
Negative	1151 (98.0)	1043 (97.9)	
Positive	24 (2.0)	22 (2.1)	
MLL/AF4			0.86
Negative	1169 (99.5)	1059 (99.4)	
Positive	6 (0.5)	6 (0.6)	
Final risk group			0.008
SR	341 (29.0)	348 (32.7)	
IR	677 (57.6)	545 (51.2)	
HR	157 (13.4)	172 (16.2)	

[1]X^2 test comparing study cohort and patients not studied, patients with no information excluded from test; [2]good: less than 1000 leukemic blood blasts / µl on treatment day 8, poor: more than 1000 / µl.

[3]MRD risk groups[1,3]: MRD-SR: TP1+2 negative, MRD-IR: TP1 and/or TP2 $<10^{-3}$, MRD-HR: TP2 $\geq 10^{-3}$.

Supplementary Table S2. CD45 expression according to immunophenotypic subtype in T-ALL

P<0.0001*	Pro-T-ALL	Pre-T-ALL	Intermediate T-ALL	Mature T-ALL
Mean	37.04%	46.18%	53.51%	54.33%
SD[#]	32.663%	37.637%	26.893%	30.124%
Median	30.35%	37.46%	50.11%	57.20%
1st Quartile	13.6%	20.8%	31.9%	36.9%
3rd Quartile	37.9%	51.5%	73.7%	66.9%

*Kruskal-Wallis test comparing all subtypes

Supplementary Table S3. CD45 expression according to immunophenotypic subtype in BCP-ALL

P<0.0001*	Pro-B ALL all cases	*Pro-B ALL MLL/AF4+*	*Pro-B ALL MLL/AF4-*	Common ALL	Pre-B ALL
Mean	17.55%	*21.91%*	*16.24%*	7.55%	7.57%
SD[#]	10.121%	*12.087%*	*9.408%*	7.954%	7.179%
Median	15.00%	*22.98%*	*14.52%*	5.39%	6.01%
1st Quartile	11.4%	*14.9%*	*11.3%*	1.9%	2.1%
3rd Quartile	23.1%	*32.4%*	*20.7%*	10.9%	11.1%

*Kruskal-Wallis test comparing pro-B ALL (all cases) and common ALL and pre-B ALL

[#]SD, standard deviation

Supplementary Table S4. CD45 expression according to genetic subtype in BCP-ALL

	ETV6/RUNX1	High hyperdiploidy	*BCR/ABL*	*MLL/AF4*	B-other
Mean	6.33%	3.60%	12.15%	24.15%	10.65%
SD[#]	4.634%	5.482%	8.912%	8.635%	9.358%
Median	5.77%	1.69%	12.08%	22.98%	9.02%
1st Quartile	2.9%	0.6%	4.4%	18.8%	3.9%
3rd Quartile	8.8%	4.0%	16.4%	29.5%	15.4%
*P**	<0.0001	<0.0001	n.s.	0.0065	-

[#]SD, standard deviation

*Kruskal-Wallis test, each genetic subtype compared to 'B-other'

Supplementary Figures

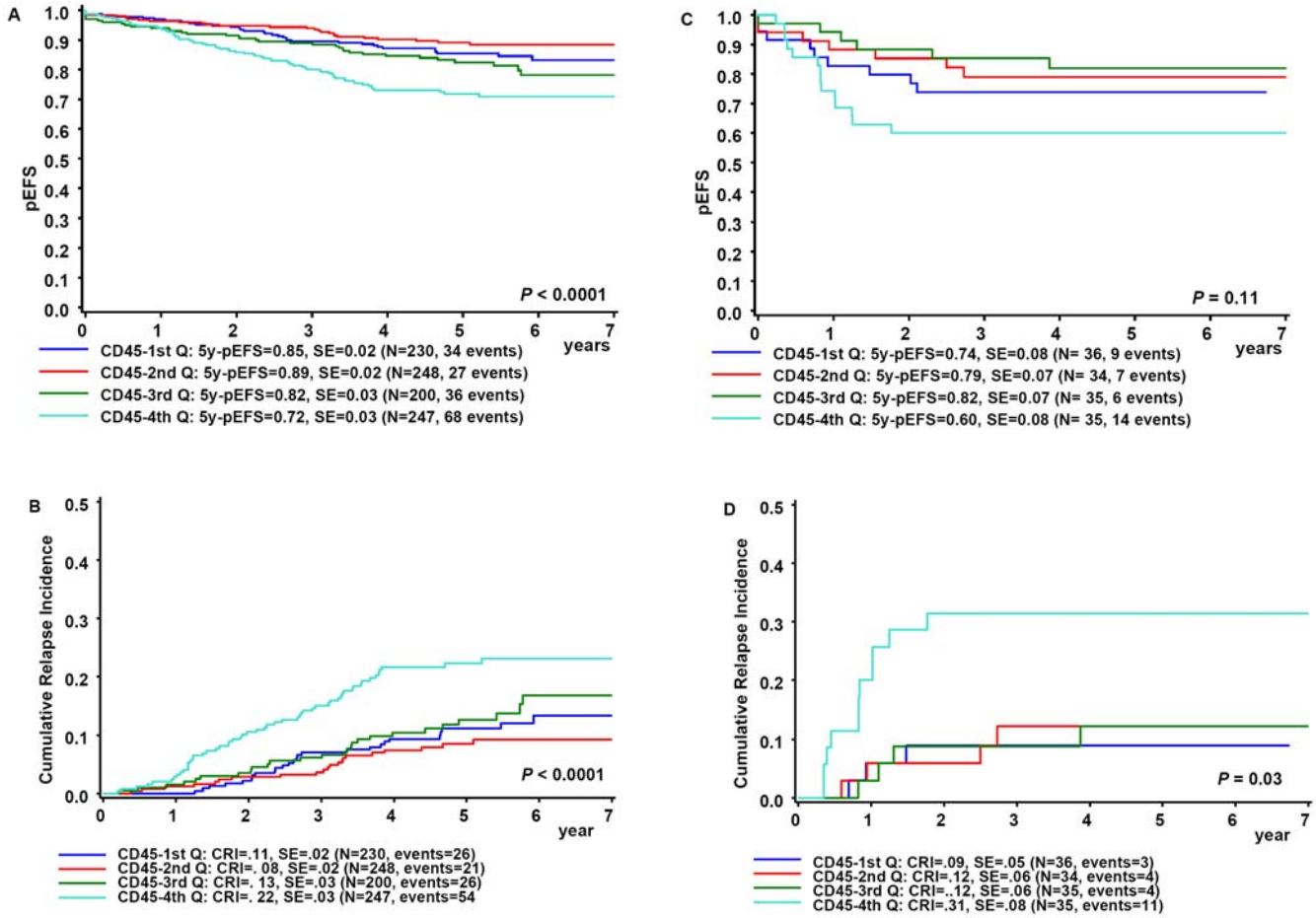
Figure legend

Supplementary Figure S1. Treatment outcome according to CD45 expression in quartiles. Kaplan-Meier estimates are shown for BCP-ALL on the left side (A+B) and T-ALL on the right side (C+D). A and C: event free survival (EFS) at 5 years, C+D: cumulative relapse incidence (CRI) at 5 years.

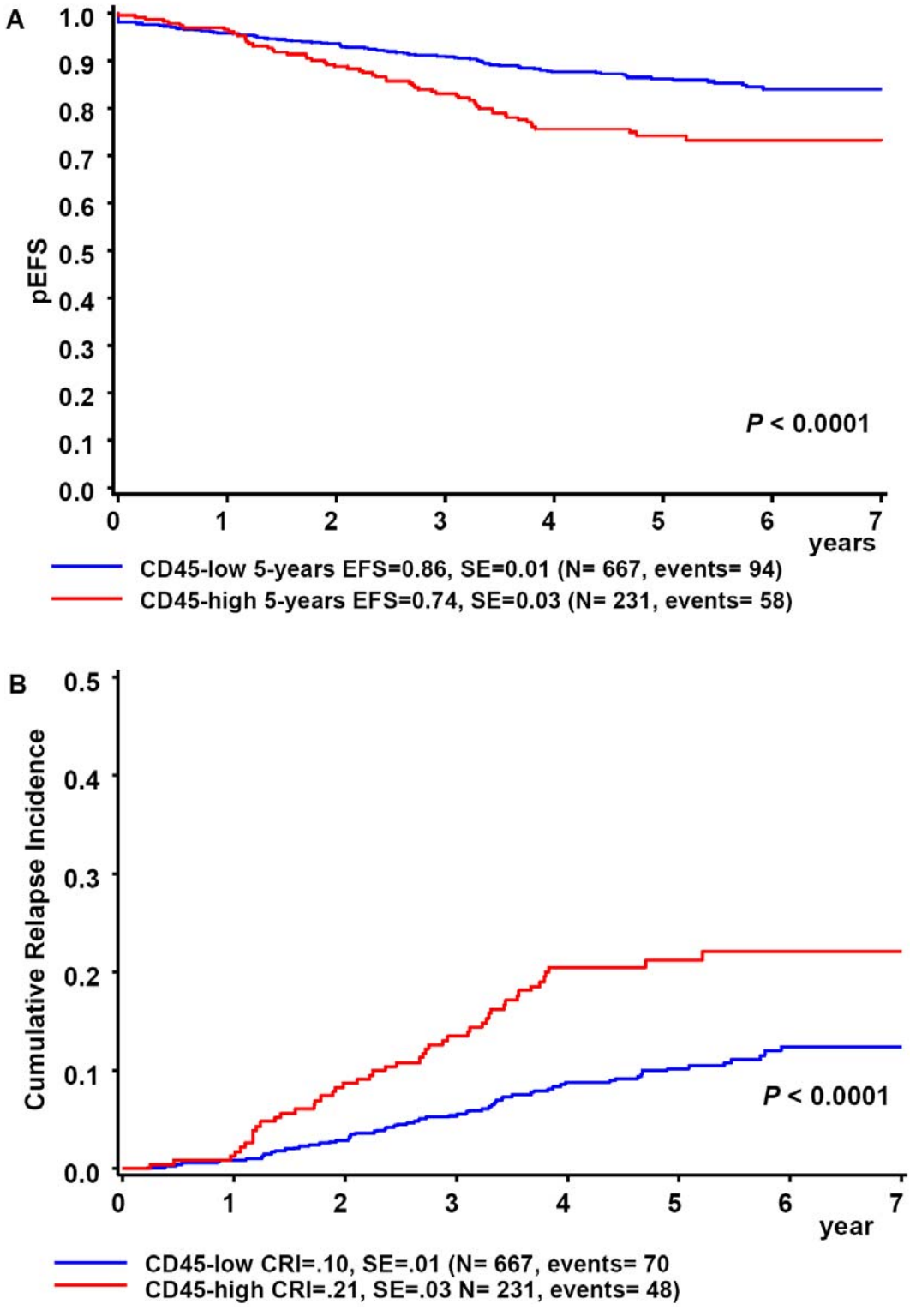
Supplementary Figure S2. Treatment outcome according to CD45 expression in BCP-ALL after exclusion of *MLL/AF4* and *BCR/ABL1*-positive cases. A: event free survival (EFS) at 5 years, B: cumulative relapse incidence (CRI) at 5 years.

Supplementary Figure S3. Treatment outcome according to CD45 expression in those BCP-ALL without known prognostic relevant genetic aberrations. Kaplan-Meier estimates are shown: A: event free survival (EFS) at 5 years, B: cumulative relapse incidence (CRI) at 5 years. As CD45 expression is significantly higher in this subgroup of BCP-ALL as compared to whole cohort, the 75th percentile cut-off to distinguish a high from low expression group was adjusted to that group (cut-off=16%).

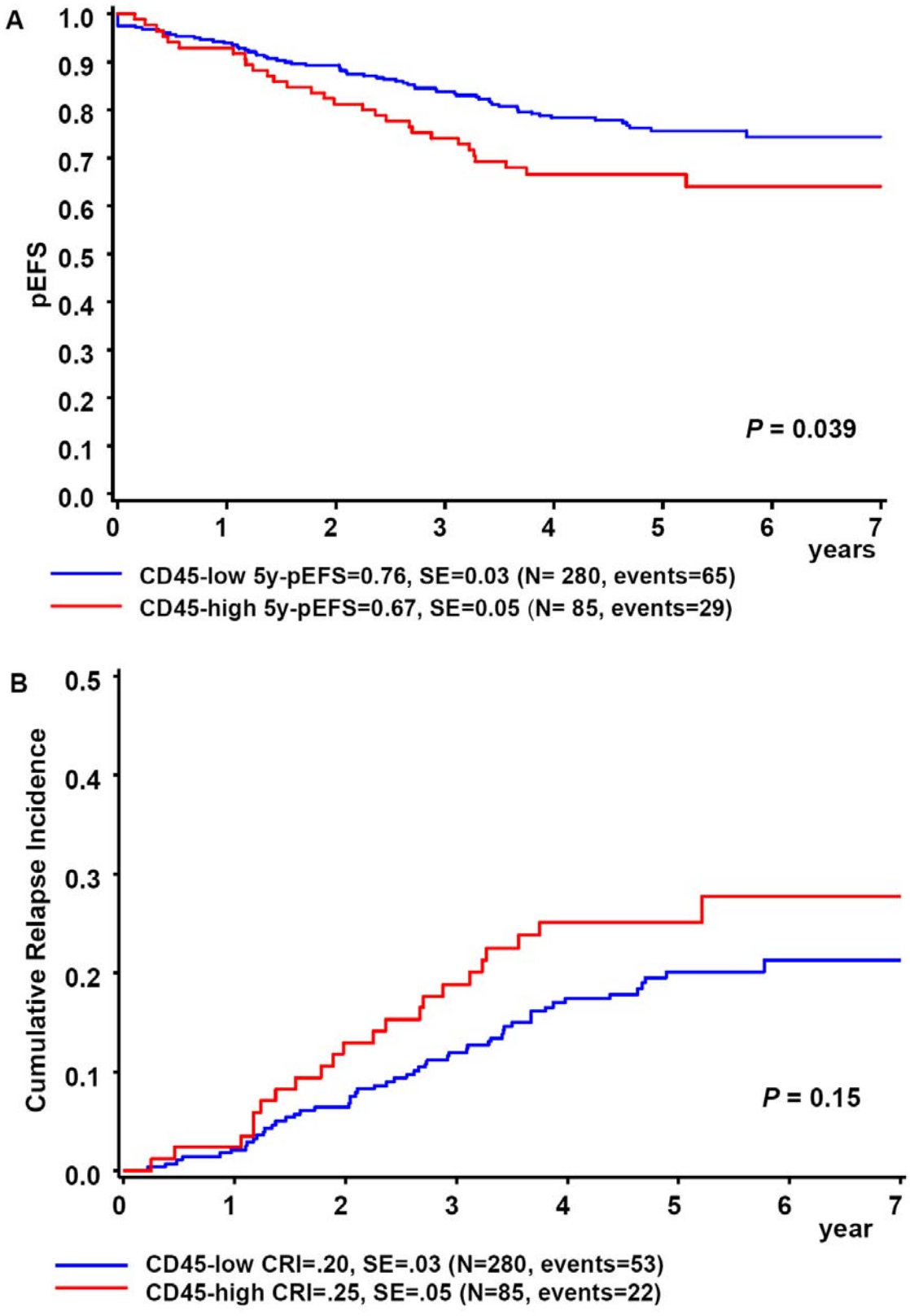
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Appendix - References

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