NOTCH1 mutation, *TP53* alteration and myeloid antigen expression predict outcome heterogeneity in children with first relapse of T-cell acute lymphoblastic leukemia

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doi:10.3324/haematol.2016.157792

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SUPPLEMENTAL MATERIAL

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A Supplemental Methods

Patients and Samples

All patients were enrolled in the German multicenter relapse trials ALL-REZ BFM 95/96 and 2002 of the Berlin-Frankfurt-Münster study group between October 1995 and June 2012 including respective pilot trials (P99, P01 and P02). Patients were included in the study when they matched the following clinical criteria: first relapse of ALL, T-cell immunophenotype and bone marrow (BM) involvement. We excluded patients with isolated extramedullary relapse due to insufficient number of BM blasts for reliable analysis and frequent lack of extramedullary specimen. For the study of *TP53*, *NRAS*, *KRAS*, *PTEN*, *NOTCH1 and FBXW7* we further selected patients according to the availability of high quality DNA samples. In total, we were able to analyze *NRAS*, *KRAS*, and *PTEN* mutations and *TP53* mutations and deletions in 81 T-ALL relapse patients. *NOTCH1* and *FBXW7* mutations were analyzed in a subset of 74 patients. For the study of immunophenotype we selected patients according to availability of detailed immunophenotyping data from the respective reference laboratory of the Berlin-Frankfurt-Münster study group. Data was obtained from 74 patients of which 51 patients overlapped with the genetics cohort (48 patients in case of *NOTCH1/FBXW7*, Fig. 1).

The total cohort of the study (n=104) included 57 very early relapses (<18 months after initial diagnosis), 26 early relapses (≥18 months after initial diagnosis and <6 months after completion of primary treatment) and 21 late relapses (≥6 months after completion of primary treatment). Regrettably, systematic information on the authenticity of late relapses[1] cannot be provided as matched immunogenetic data from diagnosis and relapse was scarce. All patients were treated within the high-risk S4 arms of the ALL-REZ BFM trials, with an intensive multi-agent BFM induction and consolidation chemotherapy, cranial irradiation, followed by hematopoietic stem cell transplantation (HSCT) if a second remission was achieved. Initially, all patients were treated with polychemotherapy only, except patient 86 who received HSCT during frontline treatment. We tested the genetics cohort (n=81) and the immunophenotype cohort (n=74) for being representative of all patients enrolled in the trials and selected by the

same clinical criteria (n=150). We detected no significant differences in relevant clinical parameters between the study cohorts and the total trial cohort (Suppl. Tab. 1). The median follow-up time of patients without subsequent event was 10 years for the genetics and 9 years for the immunophenotype cohort. The median percentage of blasts in BM and peripheral blood specimen was 85% and 81% in genetics and immunophenotype cohort, respectively. Leukemic cells were enriched by ficoll density gradient centrifugation prior to genetic analysis and immunophenotyping.

Detection of Genetic Alterations

Mutations in hotspot regions of *NOTCH1* (exon 26, N-terminal region of heterodimerization domain; exon 27, C-terminal region of heterodimerization domain; exon 34, transcriptional activation and PEST domain), *FBXW7* (exons 9-10, C-terminal binding site), *TP53* (exons 5-8, DNA binding domain,), *NRAS* (exon 2 and 3, including key codons 12-13 and 61, respectively), and *KRAS* (exon 2, including key codons 12-13) were identified by Sanger sequencing as described previously.[2-5] *KRAS* exon 3 (including key codon 61) was sequenced using primers 5'-TCAAGTCCTTTGCCCATTTT-3' (forward) and 5'-TGCATGGCATTAGCAAA GAC-3' (reverse) at 57°C annealing temperature and *PTEN* exon 7 (part of C2 domain) using primers 5'-TCCATATTTCGTGTATATTGCTGA-3' (forward) and 5'-AGCAAAACACCTGCA GATCTAA-3' (reverse) at 59°C annealing temperature. Deletions of *TP53* were detected by multiplex ligation-dependent probe amplification using MLPA Kit P056 from MRC-Holland (Amsterdam, The Netherlands) according to the manufacturer's instructions, and confirmed by fluorescence in situ hybridization (FISH) if BM smear preparations were available (as described previously).[3] Loss of heterozygosity and uniparental disomy of the *TP53* locus on chromosome 17 was assessed using CytoScan™ HD arrays and the Chromosome Analysis Suite (ChAS) software, version 3.1.0.15 (both from Affymetrix, Santa Clara, CA, U.S.A.). Genetic alterations are summarized in Suppl. Tab. 2.

Immunophenotyping

Flow cytometry was performed according to guidelines of the European Group for the Immunological Characterization of Leukemias (EGIL) including methodology and marker panels described in Bene et al. 1995 and Ratei et al. 2013.[6, 7] Percentage levels of relevant immunological markers are given in Suppl. Tab. 3. Patients were classified by maturation stage into pro-/pre-T-ALL, cortical T-ALL or mature T-ALL according to the EGIL recommendations.[6] The presence of an early T-cell precursor (ETP) ALL immunophenotype was defined according to Coustan-Smith et al. 2009, i. e. CD1a and CD8 negative (<5%), weak CD5 (<75%) and co-expression (>25%) of at least one of the myeloid or stem cell markers CD13, CD33, CD65, CD11b, CD34, HLA-DR and CD117.[8] However, following the EGIL consensus, we used a less stringent cutoff for negativity of CD5 and CD8 (<20% positive lymphoblasts). Furthermore, we excluded CD11b from the ETP definition, as this marker was not assessed in our cohort. Myeloid antigen (MyAg) positivity was defined as expression of CD13 and/or Cd33 and/or CD65 in ≥ 20% of lymphoblast cells.

Statistical Analysis

Categorical parameters were compared using Pearson's chi-square test or Fisher's exact test if expected cell sizes were less than five. The probability of event-free survival (EFS) and overall survival (OS) was calculated from time of first relapse to subsequent event or to death, respectively, or in terms of continuous complete second remission (CCR), until May 6th 2013. Significant differences in survival between groups were assessed using a two tailed log-rank test. Nonresponse to treatment and death during induction therapy were regarded as early events at the time point cero. Statistical calculations were performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corporation, Armonk, NY, U.S.A.) and R.[9] Multivariate Cox regression modelling was done for EFS using backward stepwise selection to remove non-significant factors. Results with a probability of less than 5% to be wrong by chance (P-values < 0.05) were considered as significant results.

B Supplemental Tables

Suppl. Tab. 1. Test on representativeness of the study cohorts for the total trial cohort

	Genetics Cohort		Immunophenotype Cohort		Total Trial Cohort[1]		P	P
	N	%	N	%	N	%	(Genetics)	(Immun)
Total	**81**	100	**74**	100	**150**	100		
Gender							0.914	0.541
male	61	75.3	58	78.4	112	74.7		
female	20	24.7	16	21.6	38	25.3		
Initial treatment							0.802	0.405
BFM protocol	60	74.1	63	85.1	115	76.7		
COALL protocol	18	22.2	9	12.2	28	18.7		
other protocol	3	3.7	2	2.7	7	4.7		
Age at relapse							0.474	0.581
<5 years	13	16.0	10	13.5	18	12.0		
≥ 5 and < 10 years	23	28.4	21	28.4	53	35.3		
≥ 10 years	45	55.6	43	58.1	79	52.7		
Time to relapse[2]							0.857	0.912
very early	42	51.9	43	58.1	83	55.3		
early	23	28.4	17	23.0	38	25.3		
late	16	19.8	14	18.9	29	19.3		
Site of relapse[3]							0.663	0.497
BM isolated	55	67.9	49	66.2	106	70.7		
BM combined	26	32.1	25	33.8	44	29.3		
Response							0.907	0.573
CR2 achieved	53	65.4	45	60.8	97	64.7		
CR2 not achieved	28	34.6	29	39.2	53	35.3		
HSCT in CR2							0.412	0.568
yes	37	45.7	41	55.4	76	51.4		
no	44	54.3	33	44.6	72	48.6		
(no data)	(0)		(0)		(2)			
Outcome							0.960	0.815
in CCR	20	24.7	18	24.3	32	21.3		
death in CR	9	11.1	4	5.4	15	10.0		
2nd malignancy	1	1.2	0	0.0	2	1.3		
second relapse	23	28.4	23	31.1	48	32.0		
non-response	24	29.6	24	32.4	42	28.0		
induction death	4	4.9	5	6.8	11	7.3		
Event-free survival	0.242±0.048		0.241±0.050		0.209±0.034		0.457	0.971
Overall survival	0.261±0.051		0.276±0.053		0.235±0.036		0.485	0.827

[1]Total trial cohort includes all German patients with first relapse of T-ALL and bone marrow involvement recruited by relapse trials ALL-REZ BFM 95/96 and 2002. [2]Time to relapse: very early (within 18 months after initial diagnosis of ALL), early (between 18 months and 30 months after initial diagnosis of ALL), late (more than 30 months after initial diagnose of ALL). [3]Site of relapse: BM isolated (no evidence of extramedullary disease), BM combined (more than 5% leukemia cells present in BM combined with extramedullary disease). *Abbreviations:* ALL, acute lymphoblastic leukemia; BM, bone marrow; CR, complete remission; CCR, continuous complete remission; HSCT, hematopoietic stem cell transplantation.

Suppl. Tab. 2. Genetic alterations in leukemic cells of children with first relapse of T-ALL.

Patient ID	*NOTCH1* status	*FBXW7* status	*TP53* status	*TP53* mutation	*TP53* CNA / LOH	*PTEN* status	*NRAS* status	*KRAS* status
1	ND	ND	wt			wt	wt	wt
2	mut (HDN)	mut (ex 9)	wt			wt	wt	[c.24 A>G, p.V8V]*
3	mut (HDN)	mut (ex 9)	wt			wt	wt	wt
4	mut (HDN)	wt	mut&del	c.524G>A, p.R175H; c.752T>A, p.I251N	del by MLPA only	wt	wt	wt
5	wt	wt	wt			wt	wt	wt
6	ND	ND	mut&del	c.743G>A, p.R248Q	nuc ish(TP53x1, CEP17x2)[298/332]	wt	wt	wt
7	mut (HDN)	mut (ex 10)	wt			wt	wt	wt
8	wt	wt	wt			wt	c.190T>G, p.Y64D	wt
9	mut (HDN)	mut (ex 9)	wt			wt	wt	wt
10	mut (TAD)	wt	wt			wt	wt	wt
11	wt	wt	wt			indel after c.696 (het)	wt	wt
12	wt	mut (ex 9)	wt			wt	wt	wt
13	mut (HDN)	mut (ex 9)	wt			wt	wt	wt
14	wt	wt	mut&del	c.638G>C, p.R213P	del by MLPA only	wt	c.35G>A, p.G12D	wt
15	mut (HDC&TAD)	wt	wt			wt	wt	wt
16	wt	mut (ex 9)	wt			wt	wt	wt
17	wt	wt	wt			wt	wt	wt
18	wt	wt	wt			wt	wt	wt
19	mut (HDN)	wt	wt			wt	wt	wt
20	mut (HDC)	wt	wt			wt	wt	wt
21	mut (HDN&PEST)	wt	wt			wt	wt	wt
22	mut (HDN)	wt	wt			wt	wt	wt
23	mut (PEST)	wt	wt			wt	wt	wt
24	mut (HDN)	wt	wt			wt	wt	wt
25	wt	wt	wt			wt	wt	wt
26	wt	wt	wt			c.696_697insG (het)	wt	wt
27	mut (HDN)	wt	wt			wt	wt	wt
28	mut (HDN)	wt	wt			wt	wt	wt
29	mut (HDN)	wt	wt			wt	wt	wt
30	mut (HDN&TAD)	wt	wt			c.694_700delinsCCG (hom)	wt	wt
31	wt	wt	wt			wt	wt	wt
32	wt	mut (ex 9)	mut	c.431A>G, p.Q144R	arr[hg19]17p13.3p11.2(18,900-19,977,614)x2 hmz	wt	wt	wt
33	wt	wt	wt			wt	wt	wt
34	wt	wt	wt			wt	wt	wt
35	wt	wt	wt			wt	wt	wt

36	wt	wt	wt			indel after c.661 (het)	wt	wt
37	mut (HDC)	wt	wt			wt	wt	wt
38	wt	wt	wt			wt	wt	wt
39	mut (HDN)	wt	wt			wt	wt	wt
40	wt	mut (ex 9)	wt			wt	c.176C>A, p.A59D	wt
41	wt	wt	wt			wt	wt	wt
42	mut (HDN)	wt	wt			wt	wt	wt
43	mut (HDC)	mut (ex 9)	wt			wt	wt	wt
44	wt	wt	wt			wt	wt	c.40G>T, p.V14L
45	mut (HDN&PEST)	wt	wt			wt	wt	wt
46	mut (HDC&PEST)	wt	mut	c.736A>G, p.M246V	arr[hg19]17p13.3p11.2(18,900-16,185,122)x2 hmz	wt	wt	wt
47	mut (HDN)	mut (ex 10)	wt			wt	wt	wt
48	wt	mut (ex 9)	wt			wt	wt	wt
49	mut (HDN)	mut (ex 10)	wt			wt	wt	wt
50	mut (HDN&PEST)	wt	del		nuc ish(TP53x1, CEP17x2)[233/315]/(TP53x1,CEP17x1)[33/315]	wt	wt	wt
51	wt	wt	wt			indel after c.737 (het)	wt	wt
52	wt	wt	wt			wt	wt	wt
53	mut (HDN)	wt	wt			wt	wt	wt
54	mut (HDN)	mut (ex 9)	wt			wt	wt	wt
55	wt	wt	wt			wt	wt	wt
56	wt	wt	wt			wt	wt	wt
57	wt	wt	wt			wt	wt	wt
58	mut (PEST)	wt	wt			wt	wt	wt
59	wt	wt	wt			wt	wt	wt
60	mut (HDN)	mut (ex 9)	wt			wt	wt	wt
61	mut (HDC)	wt	wt			wt	wt	wt
62	wt	wt	wt			wt	c.35G>T, p.G12V	wt
63	mut (HDN)	wt	wt			wt	wt	wt
64	wt	wt	wt			wt	wt	wt
65	mut (HDN)	wt	wt			wt	c.35G>T, p.G12V	wt
66	wt	wt	wt			c.703_704insG (het)	wt	wt
67	mut (HDN)	wt	wt			wt	wt	wt
68	ND	ND	wt			indel after c.693 (het)	wt	wt
69	mut (HDN)	mut (ex 9)	wt			wt	wt	wt
70	ND	ND	wt			wt	wt	wt
71	ND	ND	wt			wt	c.34G>A, p.G12S	wt
72	ND	ND	wt			indel after c.700 (het)	c.35G>T, p.G12V	wt
73	ND	ND	wt			wt	wt	wt
74	wt	wt	wt			wt	wt	wt

75	wt	wt	wt	wt	wt	wt
76	mut (HDN)	mut (ex 9)	wt	wt	wt	wt
77	wt	wt	wt	wt	wt	wt
78	mut (PEST)	wt	wt	wt	c.181C>A, p.Q61K	wt
79	wt	mut (ex 9)	wt	wt	wt	wt
80	mut (HDN)		wt	wt	wt	wt
81	wt	wt	wt	wt	wt	wt

*KRAS p.V8V, synonymous change, known germline polymorphism (van Grieken et al. 2013, British Journal of Cancer 108, 1495–1501)

Abbreviations: aa, amino acid; ALL, acute lymphoblastic leukemia; CNA, copy number alteration; del, deletion; ex, exon; HDC, C-terminal heterodimerization domain; HDN, N-terminal heterodimerization domain; het, heterozygous; hom, homozygous; ID, identifier; LOH, loss of heterozygosity; MLPA, multiplex ligation-dependent probe amplification; mut, mutation; ND, not defined; PEST, polypeptide enriched in proline, glutamate, serine and threonine domain; pt, patient; TAD, transactivation domain; wt, wild type.

Suppl. Tab. 3. Expression of immunological markers in leukemic cells of children with first relapse of T-ALL

PATIENT ID	EGIL CLASS	ETP-ALL	CD1A	CD2	CD3	CD3 CYTO	CD4	CD5	CD7	CD8	CD13	CD33	CD34	CD65	CD117	HLADR	TDT
2	mature T	no	4	87	61	ND	33	83	66	56	6	13	1	4	2	7	24
4	pre T	no	5	3	3	79	37	87	92	15	62	3	1	3	0	4	47
5	pre T	no	13	29	5	77	1	95	99	19	12	12	26	0	29	0	60
8	pro T	yes	0	3	3	79	17	8	95	20	25	82	95	1	0	67	6
9	mature T	no	0	1	48	81	35	83	91	60	87	1	0	0	0	0	87
10	pre T	no	2	3	2	93	1	97	99	1	0	6	94	0	58	0	87
12	mature T	no	6	5	97	ND	1	90	98	11	78	0	46	26	31	1	16
13	cortical T	no	74	99	97	95	47	98	98	86	1	0	62	1	0	1	90
14	pre T	no	5	23	19	81	38	80	89	29	5	9	34	6	14	17	39
15	cortical T	no	79	1	28	94	94	99	98	84	16	4	0	0	0	0	94
17	cortical T	no	36	14	4	98	28	96	99	48	14	93	81	2	12	0	54
18	mature T	no	0	2	88	ND	1	76	97	49	0	0	8	0	14	1	19
21	mature T	no	8	3	88	91	3	92	93	57	7	5	5	10	5	4	42
23	cortical T	no	55	10	2	95	2	97	98	39	96	2	2	3	9	3	87
24	cortical T	no	98	99	26	94	25	98	99	96	6	0	0	16	14	5	89
26	cortical T	no	51	63	31	83	63	93	92	6	0	1	0	0	83	3	59
27	mature T	no	12	3	94	90	7	94	95	19	6	2	1	6	2	6	52
28	cortical T	no	88	22	79	98	79	98	95	96	0	0	0	2	0	3	93
29	cortical T	no	93	99	44	98	97	97	99	97	0	0	2	0	0	2	64
30	cortical T	no	63	99	45	91	23	99	99	98	0	0	0	1	0	1	25
35	mature T	yes	0	1	99	98	22	1	99	13	75	97	54	0	2	1	93
37	cortical T	no	41	97	31	88	85	91	92	88	5	4	3	5	3	40	67
38	mature T	no	1	54	98	97	6	97	97	71	1	1	0	2	28	3	1
40	pre T	yes	1	14	12	93	5	65	98	8	76	74	86	0	16	2	61
44	pre T	no	17	16	11	94	12	98	98	84	3	70	93	1	22	7	81
45	cortical T	no	67	97	20	85	89	97	96	17	2	25	10	0	0	3	73
47	pre T	no	3	68	2	94	0	98	97	81	1	2	49	0	0	71	75
48	cortical T	no	38	75	19	97	28	98	71	58	3	0	25	1	0	0	83
49	cortical T	no	92	60	83	95	58	97	98	95	6	0	0	0	0	3	88
51	cortical T	no	38	98	14	95	91	98	98	96	1	1	0	4	0	21	33
53	cortical T	no	50	98	7	97	92	91	91	95	2	0	0	3	0	0	74
54	cortical T	no	54	97	14	97	87	97	97	96	0	0	0	1	0	0	17
55	pre T	yes	0	98	11	98	0	17	99	0	37	0	0	0	7	82	62
56	cortical T	no	29	47	96	97	4	92	97	6	12	2	0	0	9	3	0
57	cortical T	no	21	47	7	89	4	92	91	6	7	4	2	0	54	10	21
59	cortical T	no	22	5	87	92	77	95	80	53	1	2	2	1	1	2	67
60	cortical T	no	23	13	1	96	2	98	98	46	1	4	7	0	0	5	99

61	pre T	no	0	4	1	89	1	89	96	1	4	94	52	3	0	34	14
62	cortical T	no	43	83	1	95	11	98	98	3	1	1	85	0	0	1	93
64	cortical T	no	82	98	98	96	4	98	99	99	0	0	0	0	0	0	0
65	cortical T	no	40	4	3	93	2	68	98	2	0	0	53	0	82	2	81
66	pre T	no	5	97	13	93	4	97	95	4	1	1	17	1	1	3	23
69	cortical T	no	91	97	73	67	90	98	98	88	0	0	0	0	0	0	75
70	pre T	no	0	0	1	75	0	84	92	0	91	5	94	0	8	0	73
71	pre T	no	4	92	18	76	7	94	98	18	59	44	70	15	8	34	64
73	cortical T	no	28	89	4	74	10	91	89	74	1	3	7	8	1	4	34
75	pre T	yes	0	3	3	86	44	12	92	4	24	94	93	2	0	61	57
77	mature T	no	4	99	96	98	57	98	98	94	4	0	0	43	0	2	90
78	cortical T	no	97	99	5	99	98	99	99	74	0	0	0	1	0	0	43
79	cortical T	no	31	92	17	87	7	95	95	67	3	3	0	2	6	6	4
81	pre T	yes	1	8	4	82	8	54	92	2	1	96	76	7	73	9	25
83	cortical T	no	47	29	41	73	49	90	90	63	2	2	0	0	0	4	62
86	pre T	no	7	92	4	92	20	93	95	81	32	3	4	0	1	6	47
88	cortical T	no	73	98	3	89	88	98	99	86	0	32	45	0	0	2	78
90	mature T	no	1	94	70	86	16	90	91	89	11	2	1	5	1	5	80
93	mature T	no	1	98	77	98	7	98	98	36	1	1	85	1	0	2	97
97	pre T	no	0	67	3	86	2	68	97	2	1	1	0	20	2	2	0
98	cortical T	no	72	91	44	89	84	92	85	3	2	0	1	2	1	5	0
99	cortical T	no	52	70	6	77	86	91	98	78	1	4	6	4	29	4	53
100	mature T	no	0	91	95	91	4	93	95	70	5	4	1	2	2	3	14
655	pre T	no	1	85	2	87	2	95	96	7	42	15	19	2	0	15	62
656	mature T	no	2	97	97	ND	2	86	98	8	65	1	0	3	1	2	76
657	mature T	no	2	1	85	95	1	71	82	2	2	3	0	18	2	1	0
658	cortical T	no	95	88	4	92	65	96	96	94	0	0	0	11	11	3	65
659	mature T	no	1	41	87	ND	15	86	85	37	24	9	28	0	37	36	33
660	mature T	no	1	97	97	ND	8	96	90	81	3	4	13	2	2	8	32
661	pre T	no	1	87	5	98	3	97	98	62	95	29	88	1	1	4	60
662	pre T	no	5	99	1	86	2	97	99	45	2	1	0	3	0	2	18
663	pre T	yes	0	38	25	90	30	30	90	17	1	57	59	5	16	55	50
664	pre T	no	0	5	3	86	2	92	99	47	85	65	96	6	0	17	85
665	pre T	no	13	1	1	97	48	97	98	14	7	3	8	0	84	15	67
666	cortical T	no	30	97	6	82	32	97	88	5	1	2	2	1	2	3	64
667	cortical T	no	25	2	77	75	2	79	74	12	10	10	82	9	4	11	35
668	pre T	yes	0	48	3	80	22	72	53	17	3	11	0	37	11	0	5

Abbreviations: ALL, acute lymphoblastic leukemia; cyto, cytoplasmatic; EGIL, European Group for the Immunological Characterization of Leukemias; ETP, early T-cell precursor; ID, identifier; ND, not defined

Suppl. Tab. 4. Clinical characteristics of children with first relapse of T-ALL by genetics and immunophenotype

Parameter	*NOTCH1*					*FBXW7*					*TP53*					*PTEN*					*NRAS / KRAS*				
	wild type		*mutation*			*wild type*		*mutation*			*wild type*		*alteration*			*wild type*		*mutation*			*wild type*		*mutation*		
	N	**%**	**N**	**%**	**P**	**N**	**%**	**N**	**%**	**P**	**N**	**%**	**N**	**%**	**P**	**N**	**%**	**N**	**%**	**P**	**N**	**%**	**N**	**%**	**P**
Total	35	100.0	39	100.0		55	100.0	19	100.0		75	100.0	6	100.0		73	100.0	8	100.0		72	100.0	9	100.0	
Time to relapse[1]					0.868					1.000					0.632					0.332					0.120
very early	18	51.4	21	53.8		29	52.7	10	52.6		38	50.7	4	66.7		38	52.1	4	50.0		38	52.8	4	44.4	
early	9	25.7	11	28.2		15	27.3	5	26.3		21	28	2	33.3		22	30.1	1	12.5		18	25.0	5	55.6	
late	8	22.9	7	17.9		11	20.0	4	21.1		16	21.3	0	0		13	17.8	3	37.5		16	22.2	0	0.0	
Site of relapse[2]					0.286					0.603					0.658					0.707					1.000
BM isolated	22	62.9	29	74.4		37	67.3	14	73.7		50	66.7	5	83.3		50	68.5	5	62.5		49	68.1	6	66.7	
BM combined	13	37.1	10	25.6		18	32.7	5	26.3		25	33.3	1	16.7		23	31.5	3	37.5		23	31.9	3	33.3	
Response					**0.022**					0.460					0.411					0.252					1.000
CR2 achieved	18	51.4	30	76.9		37	67.3	11	57.9		50	66.7	3	50		46	63.0	7	87.5		47	65.3	6	66.7	
CR2 not achieved	17	48.6	9	23.1		18	32.7	8	42.1		25	33.3	3	50		27	37.0	1	12.5		25	34.7	3	33.3	
Outcome					0.084					0.432					0.300					0.136					0.821
in CCR	5	14.3	14	35.9		12	21.8	7	36.8		20	26.7	0	0		18	24.7	2	25.0		18	25.0	2	22.2	
death in CR	4	11.4	3	7.7		6	10.9	1	5.3		9	12	0	0		7	9.6	2	25.0		8	11.1	1	11.1	
2nd malignancy	1	2.9	0	0.0		1	1.8	0	0.0		1	1.3	0	0		0	0.0	1	12.5		1	1.4	0	0.0	
second relapse	8	22.9	13	33.3		18	32.7	3	15.8		20	26.7	3	50		21	28.8	2	25.0		20	27.8	3	33.3	
non-response	14	40.0	8	20.5		16	29.1	6	31.6		22	29.3	2	33.3		23	31.5	1	12.5		22	30.6	2	22.2	
induction death	3	8.6	1	2.6		2	3.6	2	10.5		3	4	1	16.7		4	5.5	0	0.0		3	4.2	1	11.1	
Event-free survival	0.114±0.063		0.359±0.077		**0.034**	0.203±0.058		0.368±0.111		0.407	0.261±0.052		0±0		**0.051**	0.247±0.050		0.188±0.158		0.630	0.246±0.051		0.222±0.139		0.750
Overall survival	0.133±0.071		0.379±0.079		**0.026**	0.227±0.063		0.368±0.111		0.607	0.285±0.054		0±0		0.119	0.271±0.052		0.188±0.158		0.946	0.269±0.054		0.222±0.139		0.593

Suppl. Tab. 4, continued

Parameter	*Maturation stage according to EGIL*							*Early T-cell precursor ALL*					*Myeloid antigen expression*				
	mature T		*pre/pro T*		*cortical T*			*no*		*yes*			*negative*		*positive*		
	N	**%**	**N**	**%**	**N**	**%**	**P**	**N**	**%**	**N**	**%**	**P**	**N**	**%**	**N**	**%**	**P**
Total	16	100.0	24	100.0	34	100.0		66	100.0	8	100.0		47	100.0	27	100.0	
Time to relapse[1]							**0.002**					0.091					0.370
very early	15	93.8	8	33.3	20	58.8		41	62.1	2	25.0		30	63.8	13	48.1	
early	1	6.3	7	29.2	9	26.5		14	21.2	3	37.5		10	21.3	7	25.9	
late	0	0.0	9	37.5	5	14.7		11	16.7	3	37.5		7	14.9	7	25.9	
Site of relapse[2]							0.121					0.100					0.654
BM isolated	14	87.5	15	62.5	20	58.8		44	66.7	5	62.5		32	68.1	17	63.0	
BM combined	2	12.5	9	37.5	14	41.2		22	33.3	3	37.5		15	31.9	10	37.0	
Response							0.954					0.704					0.231
CR2 achieved	10	62.5	14	58.3	21	61.8		41	62.1	4	50.0		31	66.0	14	51.9	
CR2 not achieved	6	37.5	10	41.7	13	38.2		25	37.9	4	50.0		16	34.0	13	48.1	
Outcome							0.483					0.069					**0.012**
in CCR	2	12.5	4	16.7	12	35.3		18	27.3	0	0.0		17	36.2	1	3.7	
death in CR	0	0.0	2	8.3	2	5.9		3	4.6	1	12.5		2	4.3	2	7.4	
2nd malignancy	0	0.0	0	0.0	0	0.0		0	0.0	0	0.0		0	0.0	0	0.0	
second relapse	8	50.0	8	33.3	7	20.6		20	30.3	3	37.5		12	25.5	11	40.7	
non-response	5	31.3	8	33.3	11	32.4		22	33.3	2	25		14	29.8	10	37.0	
induction death	1	6.3	2	8.3	2	5.9		3	4.6	2	25		2	4.3	3	11.1	
Event-free survival	0.125±0.083		0.167±0.076		0.350±0.082		0.180	0.271±0.055		0.00±0.00		0.173	0.360±0.070		0.037±0.036		**0.004**
Overall survival	0.125±0.083		0.250±0.088		0.370±0.085		0.214	0.296±0.057		0.125±0.117		0.292	0.375±0.072		0.111±0.061		**0.010**

[1]Time to relapse: very early, <18 months after initial diagnosis of ALL;, early, ≥18 months after initial diagnosis of ALL and <6 months after completion of primary treatment, late, ≥6 months after completion of primary treatment). [2]Site of relapse: isolated BM (no evidence of extramedullary disease), combined BM (more than 5% leukemia cells present in BM combined with extramedullary disease. *Abbreviations*: ALL, acute lymphoblastic leukemia; BM, bone marrow; CR, complete remission; CCR, continuous complete remission; EGIL, European Group for the Immunological Characterization of Leukemias

C Supplemental Figures

Supplemental Figure 1

	***FBXW7* mut**	***TP53* alt**	***PTEN* mut**	***RAS* mut**	**cortical T**	**pre/pro T**	**mature T**	**ETP**	**MyAg pos**
***NOTCH1* mut**	13/74 (33,68)	3/74 (8,60)	1/74 (3,17)	2/74 (5,29)	16/48 (67,62)	4/48 (17,31)	4/48 (17,44)	0/48 (0,0)	5/48 (21,33)
	***FBXW7* mut**	1/74 (5,20)	0/74 (0,0)	1/74 (5,14)	7/48 (58,27)	2/48 (17,15)	3/48 (25,33)	1/48 (8,17)	3/48 (25,20)
		***TP53* alt**	0/81 (0,0)	1/81 (17,11)	0/51 (0,0)	2/51 (100,13)	0/51 (0,0)	0/51 (0,0)	1/51 (50,6)
			***PTEN* mut**	1/81 (13,11)	3/51 (75,11)	1/51 (25/7)	0/51 (0,0)	0/51 (0,0)	0/51 (0,0)
				***RAS* mut**	3/51 (38,11)	5/51 (63,33)	0/51 (0,0)	2/51 (25,33)	4/51 (50,24)
					cortical T	NA	NA	NA	4/74 (12,15)
						pre/pro T	NA	7/74 (29,88)	17/74 (71,63)
							mature T	1/74 (6,13)	6/74 (38,22)
								ETP	8/74 (100,30)

exclusion overlap

p<0.1≥0.05 (trend)

p<0.05≥0.001

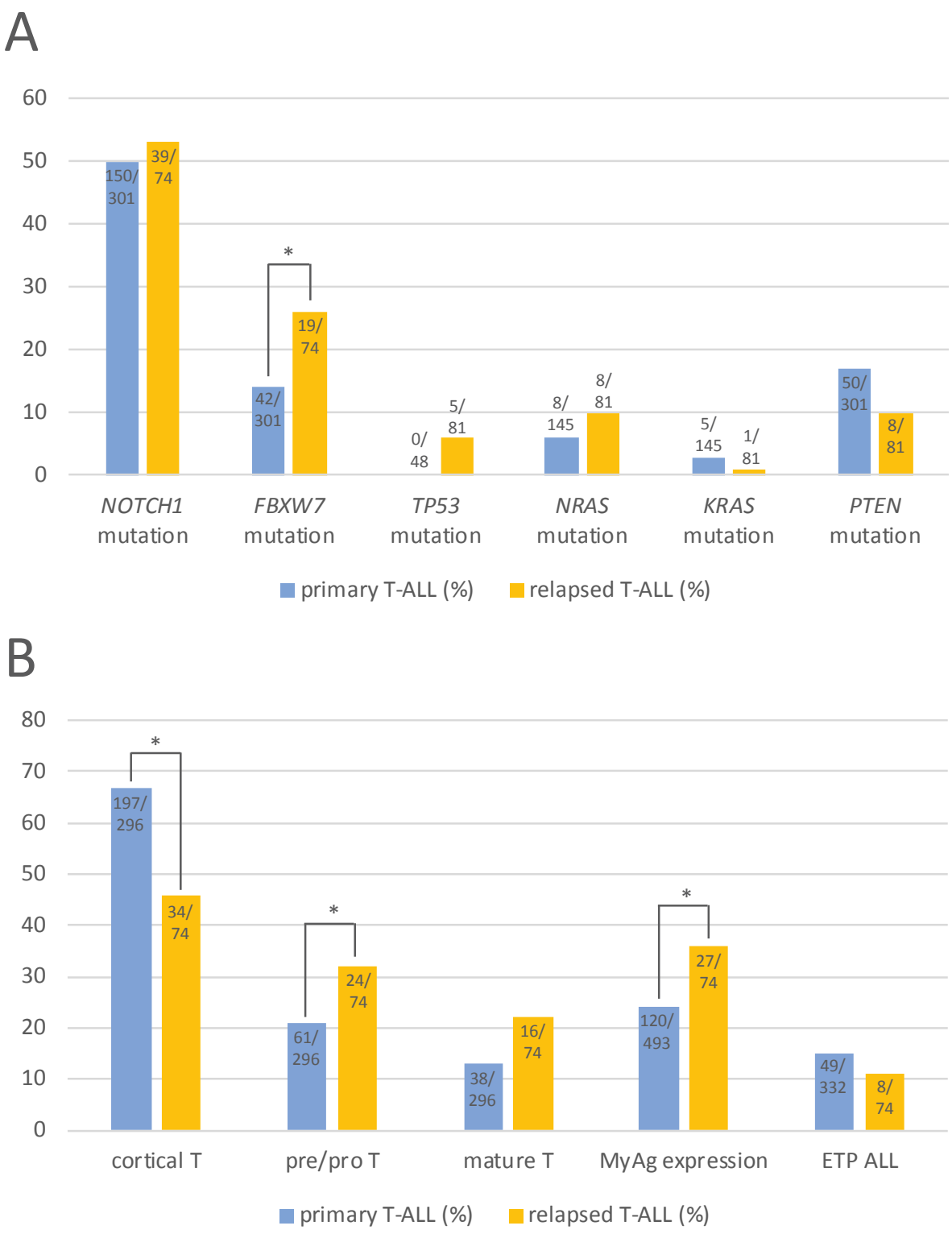
p<0.001

Suppl. Fig. 1. Overlap between genetic alterations and immunophenotypes

Overlap or exclusion of genetic alterations and immunophenotype subgroups in pediatric T-ALL relapses was assessed by cross-tabulation analysis. Cells show absolute numbers of patients positive for two given parameters versus the number of all patients included in the respective cross-tabulation analysis (the latter depending on data availability, see Fig. 1). Relative frequencies of double positive patients within rows and columns are given in brackets. Overlap of groups that were mutually exclusive by definition was not assessed, i. e. of immunophenotypes cortical T, pre/pro T and mature T and of early T-cell precursor ALL with the cortical T subtype. Green and red color indicate statistical significance of overlap and exclusion, respectively

Abbreviations: ALL, acute lymphoblastic leukemia; alt, alteration; ETP, early T-cell precursor; mut, mutation; MyAg, myeloid antigen, NA, not assessed; *RAS*, *NRAS* and *KRAS*.

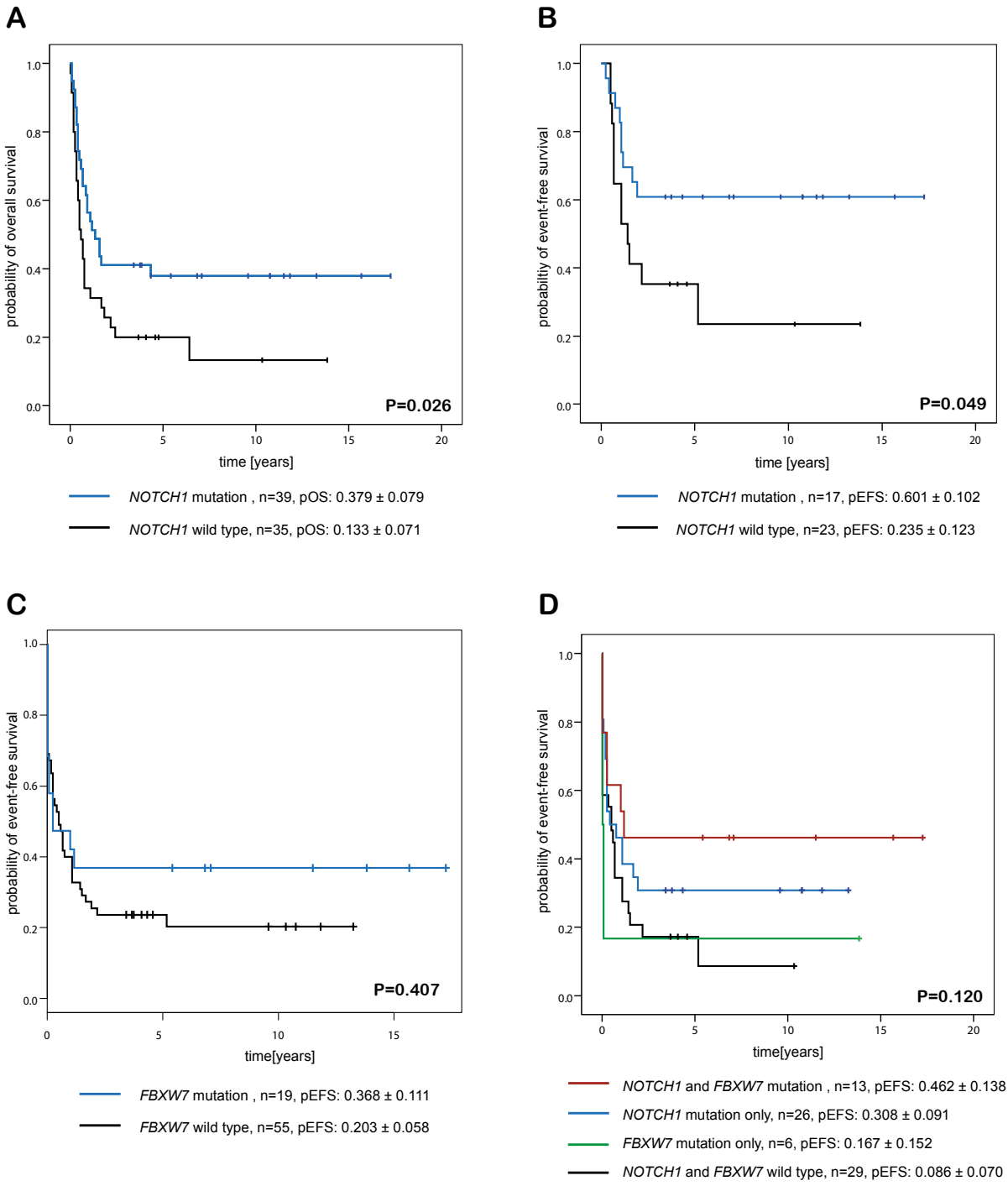
Supplemental Figure 2



Suppl. Fig. 2. Comparison of mutation frequencies between primary and relapsed T-ALL

Frequencies of gene mutations (A) and immunophenotypes (B) in relapsed T-ALL patients of the present study were compared to frequencies in primary T-ALL. To account for age and ethnicity related differences, we preferably used primary T-ALL studies from pediatric German or European trials for comparison (*NOTCH1/FBXW7* mutation, Kox et al. 2010, Germany, ALL-BFM 2000 ALL trial;[4] *TP53* mutation, Wada et al. 1982, Germany, ALL-BFM 1987-90 trial;[10] *NRAS/KRAS* mutation, Jenkinson et al. 2016, United Kingdom, UKALL2003 trial;[11] *PTEN* mutation and maturation stages, Bandapalli et al. 2013, Germany, ALL-BFM 2000 trial;[12] MyAg expression, Ratei et al. 2013, Germany, ALL-BFM 2000 trial;[7] ETP, Conter et al. 2016, Italy, AIEOP-BFM 2006 & 2009 trials[13]). The bar chart depicts percentage of positive patients. Each bar is labelled with the underlying absolute numbers of patients. Significant differences between primary and relapsed ALL at $p<0.05$ are indicated by asterisks (*FBXW7* mutation, P=0.014; cortical T, P=0.001; pre/pro T, P=0.031 and MyAg expression, P=0.026). Abbreviations: ETP, early T cell precursor; MyAg, myeloid antigen.

Supplemental Figure 3



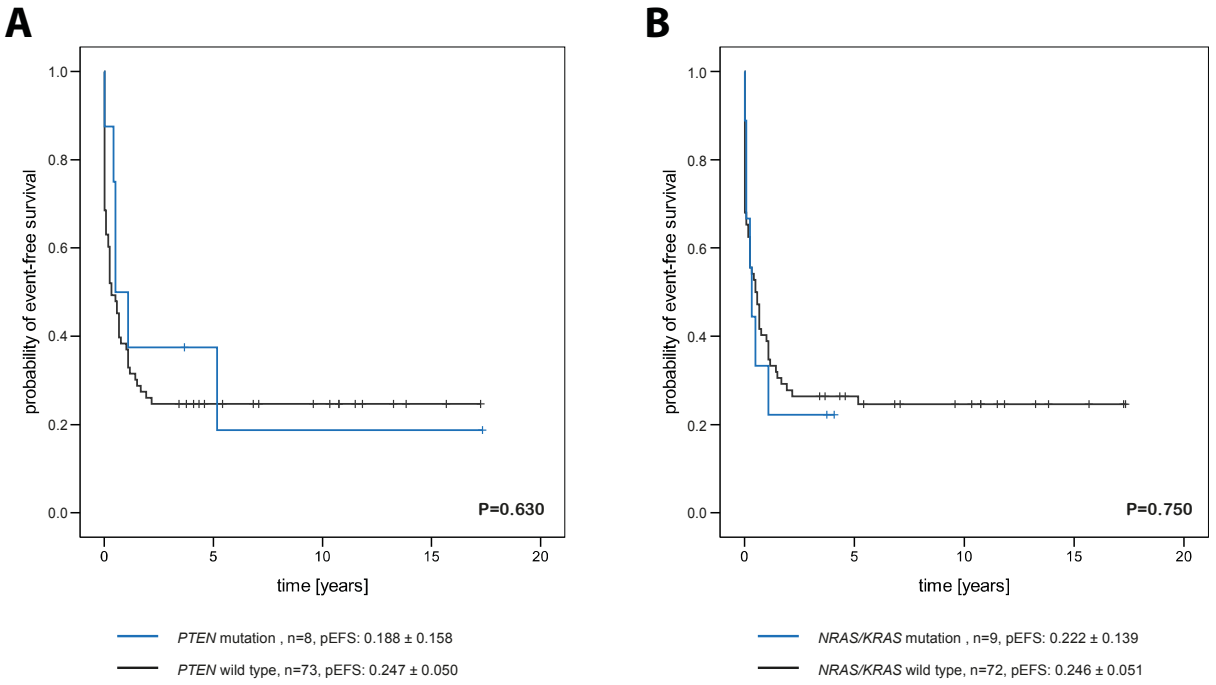
Suppl. Fig. 3. Survival of children with first relapse of T-ALL by *NOTCH1* and/or *FBXW7* mutations

(**A**) Kaplan Meier analysis for the probability of OS of patients with and without leukemic *NOTCH1* mutation, P=0.026 by log-rank test. *NOTCH1* mutation: n=39, censored n=15; *NOTCH1* wild type: n=35, censored n=6. (**B**) Kaplan Meier analysis for the probability of EFS

of patients who received HSCT in CR2, separated by leukemic *NOTCH1* mutation status, P=0.049 by log-rank test. *NOTCH1* mutation: n=17, censored n=14; *NOTCH1* wild type: n=23, censored n=5. (**C**) Kaplan Meier analysis for the probability of EFS of patients with and without leukemic *FBXW7* mutation, P=0.407 by log-rank test. *FBXW7* mutation: n=19, censored n=7; *FBXW7* wild type: n=55, censored n=12. (**D**) Kaplan Meier analysis for the probability of EFS by *NOTCH1* and *FBXW7* mutation status, P=0.120 by log-rank test. *NOTCH1* and *FBXW7* mutation: n=13, censored n=6; *NOTCH1* mutation only: n=26, censored n=8; *FBXW7* mutation only: n=6, censored n=1; *NOTCH1* and *FBXW7* wild type: n=29, censored n=4.

Abbreviations: ALL, acute lymphoblastic leukemia; CR, complete remission; EFS, event-free survival; HSCT, hematopoietic stem cell transplantation; OS, overall survival.

Supplemental Figure 4

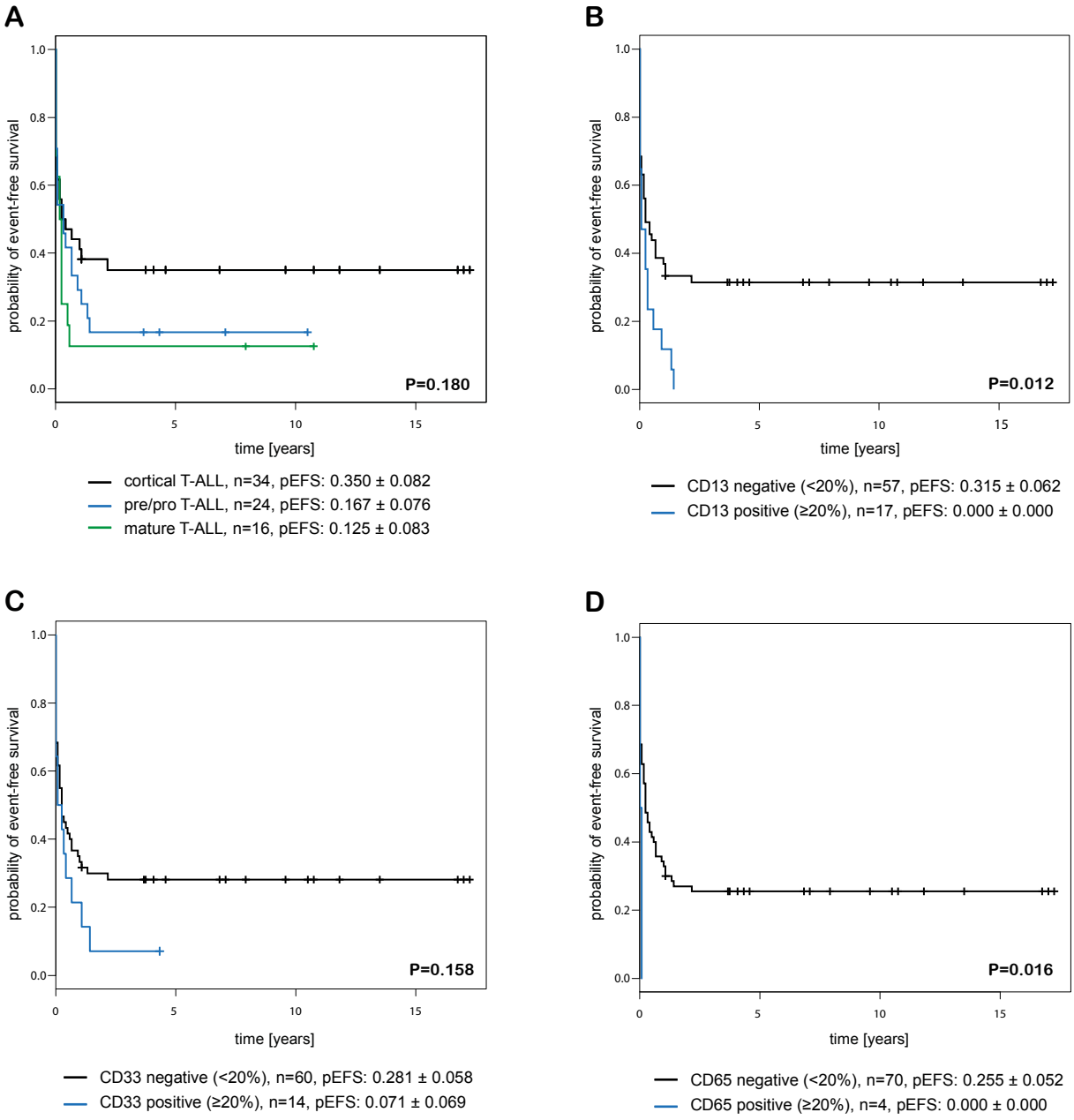


Suppl. Fig. 4. Survival of children with first relapse of T-ALL by *PTEN* and *NRAS/KRAS* mutations

(**A**) Kaplan Meier analysis for the probability of EFS of patients with and without leukemic *PTEN* mutation, P=0.630 by log-rank test. *PTEN* mutation: n=8, censored n=6; *PTEN* wild type: n=73, censored n=55. (**B**) Kaplan Meier analysis for the probability of EFS of patients with and without leukemic *NRAS/KRAS* mutation, P=0.750 by log-rank test. *NRAS/KRAS* mutation: n=9, censored n=7; *NRAS/KRAS* wild type: n=72, censored n=54.

Abbreviations: ALL, acute lymphoblastic leukemia; EFS, event-free survival

Supplemental Figure 5



Suppl. Fig. 5. Survival of children with first relapse of T-ALL by immunophenotype

(**A**) Kaplan Meier analysis for the probability of EFS by EGIL defined immunophenotype subgroup, P=0.180 by log-rank test. Cortical T-ALL: n=34, censored n=12; pre/pro T-ALL: n=24, censored n=4; mature T-ALL: n=16, censored n=2. (**B**) Kaplan Meier analysis for the probability of EFS of patients with and without expression of myeloid marker CD13, P=0.012 by log-rank test. CD13 positive (≥20%): n=17, censored n=0; CD13 negative (<20%): n=57, censored n=18. (**C**) Kaplan Meier analysis for the probability of EFS of patients with and

without expression of myeloid marker CD33, P=0.158 by log-rank test. CD33 positive (≥20%): n=14, censored n=1; CD33 negative (<20%): n=60, censored n=17. (**D**) Kaplan Meier analysis for the probability of EFS of patients with and without expression of myeloid marker CD65, P=0.016 by log-rank test. CD65 positive (≥20%): n=4, censored n=0; CD65 negative (<20%): n=70, censored n=18.

Abbreviations: ALL, acute lymphoblastic leukemia; EFS, event-free survival; EGIL, European Group for the Immunological Characterization of Leukemias.

D References for Supplemental Material

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