Supplemental Data

RAS pathway mutations as predictive biomarker for treatment adaptation in pediatric BCP-ALL

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Full online Material and Methods

Patient material, patient-derived xenografts (PDX), cell lines, and subtype classification

This study comprised children with newly diagnosed BCP-ALL enrolled in consecutive Dutch Childhood Oncology Group trials (DCOG ALL-8, ALL-9 and ALL-10) and German Cooperative ALL trials (COALL 06-97 and 07-03). Written informed consent was obtained from parents or guardians and institutional review boards approved the use of excess of diagnostic material for research purposes. These studies were conducted in accordance with the Declaration of Helsinki. Mononuclear cells were isolated using density gradient centrifugation with Lymphoprep (Axis Shield, Norway) as described previously[1]. If not indicated otherwise, samples contained at least 90% leukemic blasts evaluated by morphological examination. In some cases this was achieved by negative selection using immunomagnetic beads. Animal experiments were approved by the animal ethics committee (EMC 2863 (103-12-08)). Xenografts of primary patient material were established in three 7-12 week-old female NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice (Charles River, France) per patient, using intra-femoral injection. Animals were randomly assigned to receive leukemia cell injections.Mice were sacrificed upon signs of overt leukemia or 180 days after transplantation. Leukemic cells were isolated from bone marrow and spleen, and subsequently used for sequencing, western blot, and trametinib cytotoxicity assays. Blast percentage was above >90% by morphological evaluation in all samples, in one case achieved by positive selection using immunomagnetic beads directed to human CD19 (Miltenyi Biotec, Germany). The leukemic cell line 697 was obtained from the German Collection of Microorganisms and Cell Cultures, regularly checked for mycoplasma contamination, and its identity verified using DNA fingerprinting (DSMZ, Germany).

Subtypes were determined by karyotype, fluorescence *in-situ* hybridization (FISH) and/or fusion-gene specific PCR, subdividing patients into *ETV6-RUNX1*-rearranged, *BCR-ABL1*-rearranged, high hyperdiploid karyotype (51-65 chromosomes and/or DNA index $\geq$1.16 and <1.5), *MLL*-rearranged (t(4;11)), *TCF3-PBX1*-rearranged, and B-lineage other (B-other). *BCR-ABL1*-like cases were identified using microarray gene expression profiling by the means of a 110 probeset classifier with proven independent prognostic relevance.[2, 3] In 11 cases all subtypes have been excluded, except the extremely rare *TCF3-HLF* fusion. As this is extremely rare, we consider it safe to allocate these cases to the B-other group.

Cell culture

Primary cells were cultured in RPMI-1640 Dutch modified supplemented with 20% FCS, Penicillin, Streptomycin, Fungizone, gentamicin, insulin, selenite, transferrine, and L-Glutamine (all Life Technologies Europe BV, the Netherlands). Cell line 697 was cultured in RPMI-1640 +GlutaMAX, supplemented with 10% FCS, Penicillin, Streptomycin, and Fungizone.

Ex vivo drug cytotoxicity assays

Cell-intrinsic resistance towards common chemotherapeutics was evaluated as previously described.[4] In brief, freshly isolated primary leukemic cells were incubated with a range of concentrations of prednisolone (0.076 - 250µg/mL), vincristine (0.05 – 50µg/mL), daunorubicin (0.002 – 1.2µg/mL), L-asparaginase (0.003 – 10IU/mL), 6-mercaptopurine (0.002 – 0.05µg/mL) and 6-thioguanine (0.02 – 0.5µg/mL). After four days, cell viability was evaluated by adding 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and measuring formazan-conversion with optical density measurement. LC50-values were calculated (concentration at which 50% conversion activity was measured relative to untreated control) and compared between groups using a Mann-Whitney U test. *Ex vivo* sensitivity towards trametinib was measured similarly (range 5µM to 0.6nM and four days incubation). Both primary material and patient-derived xenograft samples were used to evaluate trametinib sensitivity.

Protein isolation and western blot

Leukemic cells were lysed either using radioimmunoprecipitation buffer (Thermo Fisher Scientific) with fresh phosphatase and protease inhibitors (Sigma Aldrich) or in protein lysis buffer pH 7.4 containing 25mM Tris base, 150mM NaCl, 5mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol, 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 10mM glycerolphosphate, dithiothreitol, phenylmethylsulfonyl fluoride, Aprotinin, and Sodium-Fluoride (all Sigma Aldrich). Samples were blotted on nitrocellulose membranes using the BioRad Trans-blot Turbo system and membranes probed with anti-MEK1/2 (#4694), anti-phospho-MEK1/2 S217/221 (#9154), anti-ERK1/2 (#9107), anti-phospho-ERK1/2 T202/Y204 (#4370), and anti-alpha-tubulin (#2144) according to manufacturer's instructions (all Cell Signaling Technologies).

DNA isolation and sequencing

Genomic DNA and RNA were isolated using Trizol reagent according to manufacturer's protocol (Life Technologies), or in some cases using the DNeasy blood and tissue kit (Qiagen). Genomic DNA concentration was determined using the Qubit dsDNA Broad Range Assay Kit (Life Technologies). For TruSeq Custom Amplicon sequencing (Illumina, USA), 100-250ng genomic DNA was used to prepare sequencing libraries according to manufacturer's instructions. Successful library preparation, correct amplicon length, and concentration was assessed using the Labchip GX genomic analyzer (Caliper Life Sciences Benelux N.V., the Netherlands) with the HT DNA 12K Reagent Kit, Version 2. Samples were then pooled equimolarly sequenced on an Illumina MiSeq in paired-end reads of 250bp each. In total, 49 amplicons of mutational hotspot regions in 13 RAS pathway genes were sequenced (Supplemental Table S1). Detailed information on the design and analysis script will be provided upon request.

Sequence reads were exported in fastq format and aligned to the standard 1000 genomes human reference sequences (version b37, from the GATK resource bundle, the Broad institute, USA) using BWA version 0.7.10[5]. We applied the GATK indel realigner version 3.3-0 to remove artefacts caused by indels. Reads mapping outside targeted regions (e.g. due to false priming or poor quality) were excluded. Single nucleotide variants were called with Freebayes version 0.9.18-24[6], Varscan version 2.3.7[7], Bcftools version 1.0[8], and GATK version 3.3-0[9]. The resulting variant call format files were annotated using snpEff and snpSift version 4.1a[10] and dbNSFP version 2.7 database[11]. All variants were combined into a single table and filtered based on several criteria using an in-house developed pipeline: For each sample, variants were excluded if they were reported by only one caller, had coverage of less than 100 reads, or had less than 20 reads supporting the variant allele. Overall, variants were excluded if they did not occur at least once with a variant allele frequency (VAF) >2% or were not distributed equally between runs according to a chi-square test. Furthermore only variants were taken into account that were reported in the COSMIC V73 GRCh37 database (cancer.sanger.ac.uk)[12], lead to an amino acid change, were unlikely to be a germline variant (see below), and not a known SNP. A variant was considered to be a SNP if it had a mean population frequency of 5% or higher across the 1000Gp1 complete human population, 1000Gp1 population of European decent, and the ESP-6500 population of European decent allele frequency database. Variants were labelled as a possible germline variant if they were found in more than 10 samples with a mean and median VAF of more than 40%.

Clonal and subclonal mutations were distinguished based on VAF: assuming heterozygosity, a mutation was considered to be clonal if VAF exceeded 25% (representing 50% of the cells) and subclonal if VAF was <25%. Since a minimum number of 20 variant reads was required for variant calling, sufficient coverage was crucial to detect subclonal mutations. In the 7 most frequently mutated amplicons, coverage allowed detection of clonal mutations in on average 99% of the samples (Supplemental Tables S1 and S2). Subclonal mutations could be reliably detected up to 3% VAF in most cases and amplicons (average 87% of cases). Because detection of variants with a frequency of 1% was limited to a smaller group, the frequency of subclonal mutations is a conservative estimate. However, based on the observed frequencies we calculated that only a small number of 9 subclonal mutations might be missed

in our study (Supplemental Table S2B). In a comparison with 25 samples that were sequenced previously (Ariës et al.[13]), all variants could be identified by both platforms, indicating a low false-positive rate.

Exon 3 of NRAS was sequenced using Sanger sequencing in 253 ALL10 samples, which were part of the clinical outcome analyses. 248 yielded good quality chromatograms. Samples were analyzed by visual inspection of codons 59 to 63 as well as the R package sangerseqR in combination with the tools above to call and annotate detected variants. Primer sequences are available upon request.

Clinical characteristics and statistics

Clinical characteristics were compared using Fisher's exact test in R (version 3.2.1). We analyzed cases from the DCOG and COALL diagnosed between 1992 and 2013 with diagnostic data available (n=432). We restricted the RAS pathway mutated group to those carrying a verified activating mutation in amino acid positions 12, 13, and 146 of *NRAS* or *KRAS* or carrying a variant in *FLT3* or *PTPN11*. Event-free survival (EFS) and cumulative incidence of relapse and non-response after induction therapy (CIR) were evaluated in 244 eligible cases treated within one protocol (DCOG ALL10), and analyses were stratified for risk group. Non-eligible were cases who had resided less than 3 months in the Netherlands or who were not treated in a childhood oncology center. Since induction death precludes risk-group allocation these cases (n=5) were excluded. The COALL97/03 cohort consisted of patients treated in the consecutive COALL 06-97 and the COALL 07-03 protocols (n=131). For details see Escherich et al.[14] Treatment intensity in arms LR-R and LR-S was considered comparable to DCOG ALL10 standard risk treatment; LR-I, HR-R, and HR-S treatment is comparable to the DCOG ALL10 medium risk treatment. In the COALL97/03 study, no high risk treatment is given that is comparable to the intensity given in the DCOG ALL10 high risk arm. Mean follow-up for patients at risk was 7.7 years (±1.86 years) in ALL10 and 8.6 years (±2.63 years) for COALL97/03. CIR was estimated using a competing risks model and compared using Gray's test. EFS probabilities were estimated using the actuarial Kaplan-Meier method and compared using the log-rank test. Hazard ratios were calculated in SPSS v21 using Cox's proportional hazard model. All reported tests are two-sided, adjustments for multiple testing were deemed expendable due to low numbers of comparisons. Outcome analyses were performed in R 3.2.1, using the packages cmprsk[15] version 2.2-7, mstate version 0.2.7[16], and survival version 2.38-3[17].

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Supplemental Table S1: Median coverage per sample itemized by amplicon. Amplicons are indicated by chromosome and start/end coordinates according to GRCh37. Amplicon ID consists of gene name and a consecutive number, + or – indicate targeted strand. ULSO: Upstream Locus-Specific Oligo, DLSO: Downstream Locus-Specific Oligo

Chromo-some	Start	End	Amplicon ID	Target exon	Median read depth per sample	ULSO	DLSO
1	115258677	115259080	NRAS_1_+	2	1912	GAGGTAGAATTGCGTAACAGCTCTCT	AACTTCCGGTCTGGACGCGCAGTGC
2	39249582	39249982	SOS1_1_+	11	3405	GAGTCCTGAGACCAGAAAGTTACAAGA	CTTCAATTATATACCCGAAAAGTCTGC
2	39249854	39250256	SOS1_2_+	11	4303	GTCAGCTGAAGAGAAAAACAATTGGATG	CAACCAAAGACCTCTTTTCTTTGACTAAA
2	39250176	39250579	SOS1_3_+	11	3126	CACTAGGCAGCCTCATCTGCTCCTCTTT	CTTCCATTATAAATTCATTACAACACTGTC
2	39278250	39278665	SOS1_4_+	7	908	AACATTAGCACAATCAGACTGGAAGAA	CAAATCACAAGTGTGACTTAAAACAGG
3	12625886	12626290	RAF1_1_+	17	3037	CTCTAGAGGAAGCAGGAGACAGGG	CAAACCCACAGCGGTCCCTGGG
3	12626160	12626560	RAF1_2_+	16	3993	GCAATATAAGGTGGGAGTGTTTTGTTTTGT	AGAAGCTGCTGCTAAGGACCTTCTAG
3	12626432	12626835	RAF1_3_+	15+16	5824	AACAAAGCCCAAGAATGCTCTCATTAG	ACAAGTCCTAACCCTCTAGCTGCTTA
3	12626708	12627112	RAF1_4_+	15	8246	AAAGGGACAGCCTGGCCCCTAGATGTC	GGATATGCCTCCCCAGATCTTAGTAAG
3	12627036	12627437	RAF1_5_+	14	5165	AAACTGAATGGGTTGTTATCCTGCATTC	GAAATGTACAGAAACGCTTTAAGTTTGC
3	12629037	12629445	RAF1_6_+	13	628	TGAACTGATGGCCCAGTGAACTAATG	GACCCAGGCATCAAATTTGTCCCTAA
3	12632242	12632688	RAF1_7_+	12	1282	CAGAGGGACTGGACCGCCAGCTTTCTA	AGGTTAACTGCTATCGCTACAGTTAGG
3	12633174	12633623	RAF1_8_+	11	54	AGAAAAACCTCGTTGGAAATTAGAATAC	TTGGGGCCCTCCCTTTACTAACTGCA
3	12641157	12641607	RAF1_9_+	10	604	CTGCATGATCACAGGTCCTCTAAACAT	ATCTAAGAGGCCTGCCCACATCCCTCAT
7	140453074	140453511	BRAF_1_+	16	2800	GGAGGAAAGAAGAAATTACCAGAGGTC	CCATTACCATCTTGGACCTGGAAGAAG
11	533746	534150	HRAS_1_+	3	636	CAAGAGAATATCTGGGCCTACATTGCT	CAGTTGTCTGGATCCATTTTGTGGATG
11	534080	534480	HRAS_2_+	2	343	AGGATAGGGCTGGCTGCAGCCCCTGG	AGTACAGGTGAACCCCGTGAGGCTGG
11	119148789	119149238	CBL_1_+	8	1529	TTCTGAGTGGAATCCCTGCCACAAATCC	GAGGCACTCCGCTCAATCTTGG
11	119149169	119149569	CBL_2_+	9	597	AGCCCTGACCTTCTGATTCCTGC	GTTAACAGAAAAAGTGAAGACTTGTTTCC
12	25378499	25378910	KRAS_1_+	4	1413	GAAACCTTAGGAAGTTCAGTGCAGAGA	TAGTGACAGCCAGCCTCAGGGCAATTGT
12	25380234	25380683	KRAS_2_+	3	926	CCTGAGAACATCAGAACCTTTCTGCAT	TCTGGATGGTTTTGGGAACGTCAATAT
12	25398208	25398635	KRAS_3_+	2	2064	CCAGCAATGCACAAAGATTTCAGTGTC	AATGAGGGACCAGTACATGAGGACTG
12	112888116	112888551	PTPN11_1_+	3	2576	TAGAGCTGAGAAGCCTTCGGGGAGTAA	CCACTTTGTAGATGAATATGATCCCAC
12	112926839	112927258	PTPN11_2_+	13	1053	ATGCAACCCCTATAGGATGAGTGTAAG	GTGGGTGACAGCTCCATTTCTTCTAAA
13	28592577	28593002	FLT3_1_+	20	1381	GCAGAACAGCAGTCTGGCTATTTAGAT	TGTGGACGAATATGATCCAACAATAGA
13	28608193	28608596	FLT3_2_+	13+14	1051	CTTTTGCTCGGAATCTGCAAAAGATCT	TGTGAGGCTGCTATTTCCTACTTATTT
13	28610091	28610494	FLT3_3_+	11	172	AATGTCACACAGGAATTCTGTTTCATC	TGGTAAGAATGGAATGTGCCAAATGTT

15	38545247	38545694	SPRED1_1_+	1	529	GCAATCATTGTGCTTTTTAGAACACTGAA	GCCAAACATGAGAGACACATATTTCTCTTT
15	38591535	38591950	SPRED1_2_+	2	1906	CTTCCATCCATCCACATTATTCCCTCT	ACAGGGGCCTCACCATGGTGGATGC
15	38614381	38614826	SPRED1_3_+	3	293	CCCATCCCCCAACAAATGTGTATCAAT	GCAAAGATCAGGAATTAGCTTTTCCAT
15	38643086	38643489	SPRED1_4_+	7	3114	CATGCAATTGACATAGACTATGATGTG	ACATATATCAAATGCATCTAATGTAGCTC
15	38643358	38643759	SPRED1_5_+	7	3957	ATTTTTAATGAGGAAGGCTGCGTCTTA	TTCTGGACATTTCTATGATATGTTTTTGG
15	38643684	38644084	SPRED1_6_+	7	1655	GATTCCAGTATTCAGTTTTCTAAACCAGAC	CTTGCGATGGTTAGCCCTGGTAGCTTT
15	66727334	66727751	MAP2K1_1_+	2	434	CTGCAAATACTCTACAACCTGTAACAAAAC	GTCACACGAACAGGGATCAGAAAAATCT
15	66729037	66729486	MAP2K1_2_+	3	529	TATTGACTTGTGCTCCCCACTTTGGA	TTGCCCAGGCTGGTGTGCAACCTCCAT
17	29528006	29528443	NF1_1_+	10	1085	TTCCCTAAATTTAGAACCGAGCAGTCA	CTCCCAGTAGCCGGGACCACAGGCAT
17	29652753	29653190	NF1_2_+	37	2647	CTGAGCCAGGCAGATCTATAGAAAAAG	ACAAAAGCCTTTAAATACCCATGCAGT
17	29654472	29654917	NF1_3_+	38	4061	CATAAAATAAAATTGATTAGTGGCATCTGT	GAACAACAGAAACTACCTGCTGCCA
17	29677138	29677547	NF1_4_+	50	2071	GGTCTCATTTTAAAAGCAACAAACCCC	CGGGGTGAAGTGGAGAAGAATTAAACT
19	4099156	4099558	MAP2K2_1_+	7	179	TGAGCACTTGGGACACGTTTCTGTGTG	AACAGAGCATGTTGGGGAGAAGGAAG
19	4110438	4110845	MAP2K2_2_+	3	775	GGATCCTTTCCTTCTACACCATGAACC	AGTGGTGGGGAAGGTCCCCCTGGAAA
19	4117360	4117773	MAP2K2_3_+	2	1255	AACTATGAGAGGTGCTCTAGGTACACC	AGAGAGGTTTTATGAAAGAAAGAGGGA
X	47426580	47427029	ARAF_1_+	10	454	CTGCATGATCACAGGTCCTCTAAACAT	ATCTAAGAGGCCTGCCCACATCCCTCAT
X	47428072	47428517	ARAF_2_+	11+12	61	CTTTTGAGAGAGATACAAGGTTTCTGT	ACAGATCTGTTTTCTGCAAAATCATAA
X	47428811	47429228	ARAF_3_+	13	420	TTGTAATTCCCAGAACCCCCACAAGAG	AGTGTCAGAGGTGGGGAGGAAAAATAG
X	47429159	47429559	ARAF_4_+	14	287	AGATCCCACTGGCCACACAGGGTAC	ATCCTGACCAGGAGCCCCTTAATATCA
X	47430152	47430552	ARAF_5_+	15	220	GTCTGGACTCCTCATGTCCCACCT	AACCTAAAATTTTCTAGGATAAGGACTCCC
X	47430424	47430824	ARAF_6_+	16	527	CTCATGGTGAAACACACACACACACA	AGCTGGGGTACTGTCAGTATCAAGG
X	47430758	47431158	ARAF_7_+	16	360	GGGGCTGGACACCTTGGGTGGGTGACT	CCCAGGCCGATGAGTTGCCTGCCT

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Supplemental Table S2: Estimate of false negative cases missed due to low coverage in the 8 most frequently mutated regions. The frequency of 1-3%VAF mutations, 3-5% VAF mutations, and 5-10%VAF mutations was calculated in a set of samples with sufficient coverage (>2000rds). This frequency was used to estimate the number of cases that would be expected in the group of cases where coverage was below the required minimum. All missed cases in these three groups were summed up. WT: Wildtype for RAS pathway mutations.

Estimated number of <3%VAF mutations missed in cases with a coverage <666, based on the frequency of 1-3%VAF mutations observed in a group that did reach the coverage of >2000

amplicon	cases with enough coverage	mutation frequency VAF 1-3%	WT cases with coverage <666	Estimate missed cases
NRAS_1_+	210	10.5	20	2
KRAS_1_+	118	2.5	57	1
KRAS_2_+	10	0.0	59	0
KRAS_3_+	248	4.8	20	1
PTPN11_1_+	327	2.4	9	0
PTPN11_2_+	32	0.0	41	0
FLT3_1_+	95	4.2	24	1
FLT3_2_+	40	0.0	46	0
ΣMissed cases				6

Estimated number of 5-10%VAF mutations missed in cases with a coverage <200, based on the frequency of 5-10%VAF mutations observed in a group that did reach the coverage of >2000

amplicon	cases with enough coverage	mutation frequency VAF 5-10%	WT cases with coverage < 200	Estimate missed cases
NRAS_1_+	446	4.7	8	0
KRAS_1_+	396	0.3	28	0
KRAS_2_+	437	0.0	5	0
KRAS_3_+	448	4.5	7	0
PTPN11_1_+	454	0.4	2	0
PTPN11_2_+	427	0.0	3	0
FLT3_1_+	443	0.5	11	0
FLT3_2_+	432	0.5	16	0
Missed cases				1

Estimated number of 3-5%VAF mutations missed in cases with a coverage < 400, based on the frequency of 3-5%VAF mutations observed in a group that did reach the coverage of >2000

amplicon	cases with enough cov	mutation frequency of VAF 3-5%	WT cases with coverage <400	Estimate missed cases
NRAS_1_+	210	3.8	12	0
KRAS_1_+	118	1.7	35	1
KRAS_2_+	10	0.0	16	0
KRAS_3_+	248	2.0	10	0
PTPN11_1_+	327	0.6	6	0
PTPN11_2_+	32	0.0	18	0
FLT3_1_+	95	0.0	13	0
FLT3_2_+	40	0.0	21	0
Missed cases				2

Supplemental Table S3: Frequency and total number of clonal and subclonal RAS pathway mutations per subtype and gene. WT: Wildtype, n: number of cases, 1-10: mutations with 1 to 10% VAF, 50+: 50 to 100% VAF, all: all mutations summed up. ER: ETV6-RUNX1-rearranged; MLL: t(4;11)-rearranged, HD: high hyperdiploid, BO: B-other, TCF3: TCF3-rearranged, BA: BCR-ABL1-rearranged, BAL: BCR-ABL1-like.

		total n tested	Wildtype		1-10%		10-20%		20-30%		30-40%		40-50%		>50%		all	
			n	% of total	n	% of total	n	% of total	n	% of total	n	% of total	n	% of total	n	% of total	n	% of total
ER	KRAS	124	107	86.3	6	4.8	3	2.4	1	0.8	5	4.0	2	1.6	0	0.0	17	13.7
	NRAS	124	110	88.7	10	8.1	0	0.0	1	0.8	2	1.6	0	0.0	1	0.8	13	10.5
	FLT3	124	119	96.0	4	3.2	0	0.0	0	0.0	0	0.0	1	0.8	0	0.0	5	4.0
	PTPN11	124	120	96.8	3	2.4	0	0.0	0	0.0	1	0.8	0	0.0	0	0.0	4	3.2
	other	124	122	98.4	2	1.6	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	1.6
t(4;11)	KRAS	15	9	60.0	3	20.0	1	6.7	0	0.0	2	13.3	0	0.0	0	0.0	6	40.0
	NRAS	15	9	60.0	2	13.3	3	20.0	1	6.7	0	0.0	0	0.0	0	0.0	6	40.0
	FLT3	15	13	86.7	1	6.7	0	0.0	1	6.7	0	0.0	0	0.0	0	0.0	2	13.3
	PTPN11	15	13	86.7	2	13.3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	13.3
	other	15	15	100.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
HD	KRAS	124	69	55.6	19	15.3	7	5.6	10	8.1	7	5.6	12	9.7	0	0.0	55	44.4
	NRAS	124	82	66.1	18	14.5	5	4.0	8	6.5	4	3.2	6	4.8	1	0.8	41	33.1
	FLT3	124	110	88.7	5	4.0	4	3.2	1	0.8	0	0.0	2	1.6	2	1.6	12	9.7
	PTPN11	124	110	88.7	4	3.2	4	3.2	1	0.8	1	0.8	4	3.2	0	0.0	14	11.3
	other	124	122	98.4	0	0.0	0	0.0	1	0.8	0	0.0	0	0.0	1	0.8	1	0.8
BO	KRAS	67	52	77.6	6	9.0	2	3.0	1	1.5	2	3.0	3	4.5	1	1.5	14	20.9
	NRAS	67	48	71.6	7	10.4	3	4.5	1	1.5	4	6.0	3	4.5	1	1.5	18	26.9
	FLT3	67	62	92.5	4	6.0	0	0.0	0	0.0	1	1.5	0	0.0	0	0.0	5	7.5
	PTPN11	67	65	97.0	2	3.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	3.0
	other	67	66	98.5	1	1.5	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.5
TCF3	KRAS	26	25	96.2	0	0.0	1	3.8	0	0.0	0	0.0	0	0.0	0	0.0	1	3.8
	NRAS	26	25	96.2	1	3.8	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	3.8
	FLT3	26	26	100.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	PTPN11	26	26	100.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	other	26	26	100.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
BA	KRAS	26	26	100.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	NRAS	26	26	100.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	FLT3	26	25	96.2	0	0.0	0	0.0	0	0.0	0	0.0	1	3.8	0	0.0	1	3.8
	PTPN11	26	26	100.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	other	26	26	100.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
BAL	KRAS	79	61	77.2	7	8.9	1	1.3	3	3.8	3	3.8	3	3.8	1	1.3	17	21.5
	NRAS	79	58	73.4	9	11.4	2	2.5	0	0.0	2	2.5	7	8.9	1	1.3	20	25.3
	FLT3	79	72	91.1	2	2.5	1	1.3	2	2.5	0	0.0	1	1.3	1	1.3	6	7.6
	PTPN11	79	72	91.1	3	3.8	0	0.0	1	1.3	0	0.0	3	3.8	0	0.0	7	8.9
	other	79	78	98.7	1	1.3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.3

Table S4: Frequencies of RAS pathway mutations among cytogenetic subtypes of BCP-ALL. Fisher's Exact test comparing the frequency of each cytogenetic subtype to the remaining BCP-ALL subtypes. ER: ETV6-RUNX1-rearranged, MLL: t(4;11)-rearranged, HD: high hyperdiploid karyotype, BO: B-other, TCF3: TCF3-rearranged, BA: BCR-ABL1-rearranged, BAL: BCR-ABL1-like gene expression profile. OR: Odds ratio. P-values < 0.05 are printed in bold. CI: confidence interval. Values for p < 0.05 are printed in bold.

	Wildtype	Mutated	Total	Frequency	Fisher's Exact p-value	OR	95% CI of OR	
ER	96	28	124	22.6%	**8.2E-08**	0.3	0.2	0.5
MLL	4	11	15	73.3%	**1.8E-02**	3.8	1.1	16.8
HD	34	90	124	72.6%	**4.7E-15**	5.7	3.5	9.3
BO	39	28	67	41.8%	0. 90	1.0	0.5	1.7
TCF3	26	0	26	0.0%	**3.6E-07**	0.0	0.0	0.2
BA	25	1	26	4.0%	**7.5E-06**	0.0	0.0	0.3
BAL	40	39	79	49.4%	0.21	1.4	0.8	2.3
total	264	197	461					

Supplemental Table S5: Frequency of common secondary aberrations in RAS pathway wildtype, clonal and subclonal mutation cases. Genomic data obtained by MLPA and array comparative genomic hybridization (aCGH) were available for a subset of patients analysed in this study. The overlap with RAS pathway mutations is shown. total: Total number of cases in the analysis and per group. Cases: Cases positive for the respective feature OR: Odds ratio. p: p-value. Values for $p < 0.05$ are printed in bold.

	Total			RAS pathway wildtype			clonal RAS pathway mutation							subclonal RAS pathway mutation						
Feature	total	freq.	cases	total	freq.	cases	total	freq.	cases	p =	OR	CI (95%)		total	freq.	cases	p =	OR	CI (95%)	
ERG deletion or break	272	3%	8	156	3%	5	70	1%	1	0.669	0.44	0.01	4.03	46	4%	2	0.659	1.37	0.13	8.73
JAK2 mutation	419	4%	15	239	4%	9	103	1%	1	0.293	0.25	0.01	1.85	77	6%	5	0.341	1.77	0.45	6.11
high CRLF2 expression	407	11%	45	231	9%	20	102	9%	9	1.000	1.02	0.39	2.45	75	21%	16	**0.006**	2.85	1.29	6.20
chromosome 21 abnormality	392	42%	164	224	32%	72	99	56%	55	**0.000**	2.63	1.58	4.42	69	54%	37	**0.002**	2.43	1.36	4.39
Morbus Down	392	4%	14	224	5%	11	99	2%	2	0.358	0.40	0.04	1.89	69	1%	1	0.306	0.29	0.01	2.03
iAMP21	390	4%	15	222	4%	8	99	1%	1	0.284	0.27	0.01	2.09	69	9%	6	0.106	2.54	0.70	8.70
dic(9;20)	390	5%	19	222	2%	5	99	11%	11	**0.002**	5.39	1.67	20.39	69	4%	3	0.399	1.97	0.30	10.42
9p deletion	390	9%	34	222	4%	9	99	21%	21	**0.000**	6.33	2.64	16.40	69	6%	4	0.515	1.45	0.32	5.42
PAX5 amplification	401	1%	6	231	0%	1	95	4%	4	**0.027**	10.03	0.98	499	75	1%	1	0.431	3.09	0.04	244
PAX5 del	401	26%	105	231	31%	71	95	27%	26	0.595	0.85	0.48	1.48	75	11%	8	**0.000**	0.27	0.11	0.60
CDKN2A deletion	404	33%	134	232	29%	68	97	46%	45	**0.003**	2.08	1.24	3.50	75	28%	21	0.884	0.94	0.50	1.72
CDKN2B deletion	403	32%	127	231	27%	63	97	44%	43	**0.003**	2.12	1.25	3.58	75	28%	21	0.883	1.04	0.55	1.91
CDKN2A and CDKN2B wildtype	403	65%	260	231	69%	159	97	52%	50	**0.004**	0.48	0.29	0.81	75	68%	51	0.887	0.96	0.53	1.77
IKZF1 deletion	400	21%	84	230	25%	57	95	19%	18	0.311	0.71	0.37	1.32	75	12%	9	**0.023**	0.41	0.17	0.91
BTG1 deletion	400	10%	38	229	14%	32	96	1%	1	**0.000**	0.07	0.00	0.40	75	7%	5	0.106	0.44	0.13	1.20
EBF1 deletion	402	7%	28	231	8%	19	96	4%	4	0.240	0.49	0.12	1.52	75	7%	5	0.807	0.80	0.22	2.32
ETV6 deletion	399	25%	101	227	30%	68	97	14%	14	**0.003**	0.40	0.19	0.76	75	25%	19	0.467	0.79	0.41	1.48
TCF3 deletion	121	26%	32	63	29%	18	37	24%	9	0.816	0.81	0.28	2.21	21	24%	5	0.782	0.78	0.19	2.69

Supplemental Table S6: Distribution of clonal and subclonal RAS pathway mutations among the treatment arms of DCOG ALL10.

SR: Standard risk treatment arm; MR: medium risk treatment arm; HR: high risk treatment arm. Values for $p < 0.05$ are printed in bold.

Within	Mutated, clonal	Mutated, subclonal
ALL10 SR	10/75 (13%)	15/75 (20%)
ALL10 MR	33/151 (22%)	46/151 (30%)
ALL10 HR	9/22 (41%)	2/22 (9%)
Fisher test, p=	**0.024**	**0.047**

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Supplemental Table S7: Clinical characteristics of BCP-ALL patients with subclonal RAS pathway mutations. WBC: White blood cell count; CNS+: non-traumatic puncture and >5 WBC/µl CSF with identifiable leukemic cells; patients with a traumatic lumbar puncture were not included; PPR: Prednisone poor responder, i.e. ≥1000 leukemic blasts/µl in peripheral blood on day 8 of induction therapy; MRD high: Minimal residual disease $\geq 10^{-3}$; d33: at the end of induction therapy (day 33); d79: at the end of consolidation therapy (day 79); [a]Fisher's Exact test p-values <0.05 are printed in bold font; [b]Odds ratios are only given if p-values in Fisher's Exact test were <0.2.

	Incidence of subclonal mutations among		**Statistics**			
Risk parameter	Risk parameter: Yes	Risk parameter: No	Fisher's p=[a]	Odds ratio[b]	95%-CI	
Age ≥10	8/75 (15%)	80/357 (30%)	**0.04**	0.43	0.17	0.97
Male	49/227 (28%)	39/205 (38%)	0.53			
High WBC (>50/nL)	10/99 (15%)	77/331 (30%)	**0.02**	0.42	0.18	0.88
Down Syndrome	1/16 (7%)	86/391 (28%)	0.12	0.18	0	1.26
CNS+	0/1 (0%)	38/248 (15%)	1			
PPR	2/17 (20%)	63/244 (32%)	0.73			
MRD high d33 ALL10	15/48 (45%)	44/191 (23%)	0.06	2.24	0.95	5.23
MRD high d79 ALL10	0/8 (0%)	60/231 (32%)	0.18	0	0	1.82

Supplemental Table S8: Baseline characteristics of the ALL10 and COALL97/03 cohorts. WBC: white blood cell count; MRD: Minimal residual disease, MRD positive: MRD ≥ 10^{-3}; PPR: poor prednisone window response. Color intensities are used to indicate the treatment intensity. Treatment intensity: COALL treatment arms LR-R and LR-S are comparable to ALL10 standard risk treatment (labeled standard); LR-I, HR-R and HR-R are comparable to ALL10 medium risk treatment (labeled medium, see method section for details).

ALL10						COALL97/03					
	n	%	Median	25th	75th		n	%	Median	25th	75th
Total	244	100%				Total	131	100%			
Treatment intensity											
Standard	22	9%				Stanard	31	24%			
Medium	148	61%				Medium	100	76%			
High	74	30%						-			
Risk characteristics											
Age			5	3	8	Age			4	2	9
WBC			11	4.925	32.15	WBC			24.1	9.47	55
MRD positive d33	48/239	20%				MRD positive d29	20/61	33%			
MRD positive d79	8/239	3%				MRD positived79	N/A				
PPR	15	6%				PPR	N/A				
Down S.	8	3%				Down S.	3/110	3%			
Events	32	13%				Events	29	22%			
Relapse	28	11%				Relapse	26	20%			

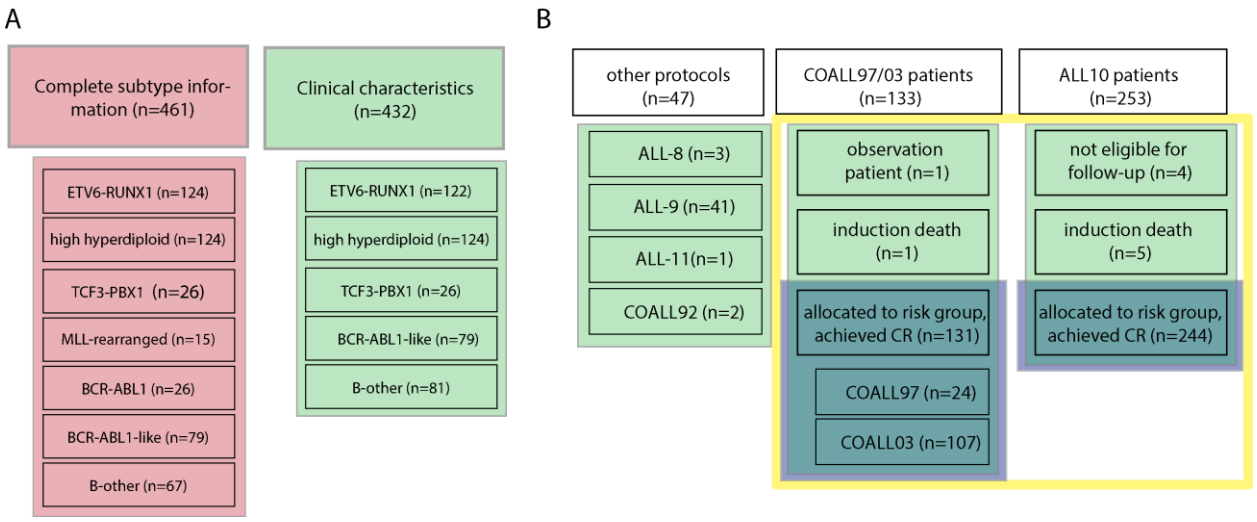
Supplemental Table S9: Univariate and multivariate analysis of Cox's proportional hazard ratio in Standard-risk treated DCOG ALL10 cases. WBC: White blood cell count; Clonal mutation: VAF≥25%; subclonal VAF<25%; 95% CI: 95% confidence interval. Values for $p < 0.05$ are printed in bold.

	Univariate		Multivariate	
Variable	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Age (≥10 years)	0.05 (0 - >1000)	0.66	0 (0-∞)	0.99
WBC (≥ 50/nL)	1.1 (0.1-9.2)	0.91	0.7 (0.08 – 5.9)	0.73
RAS pathway status		0.11		0.074
Clonal	4.6 (1.0 – 21)	**0.047**	5.4 (1.2 - 25)	**0.032**
subclonal	0.9 (0.1 – 7.8)	0.9	0.9 (0.1 – 7.7)	0.9

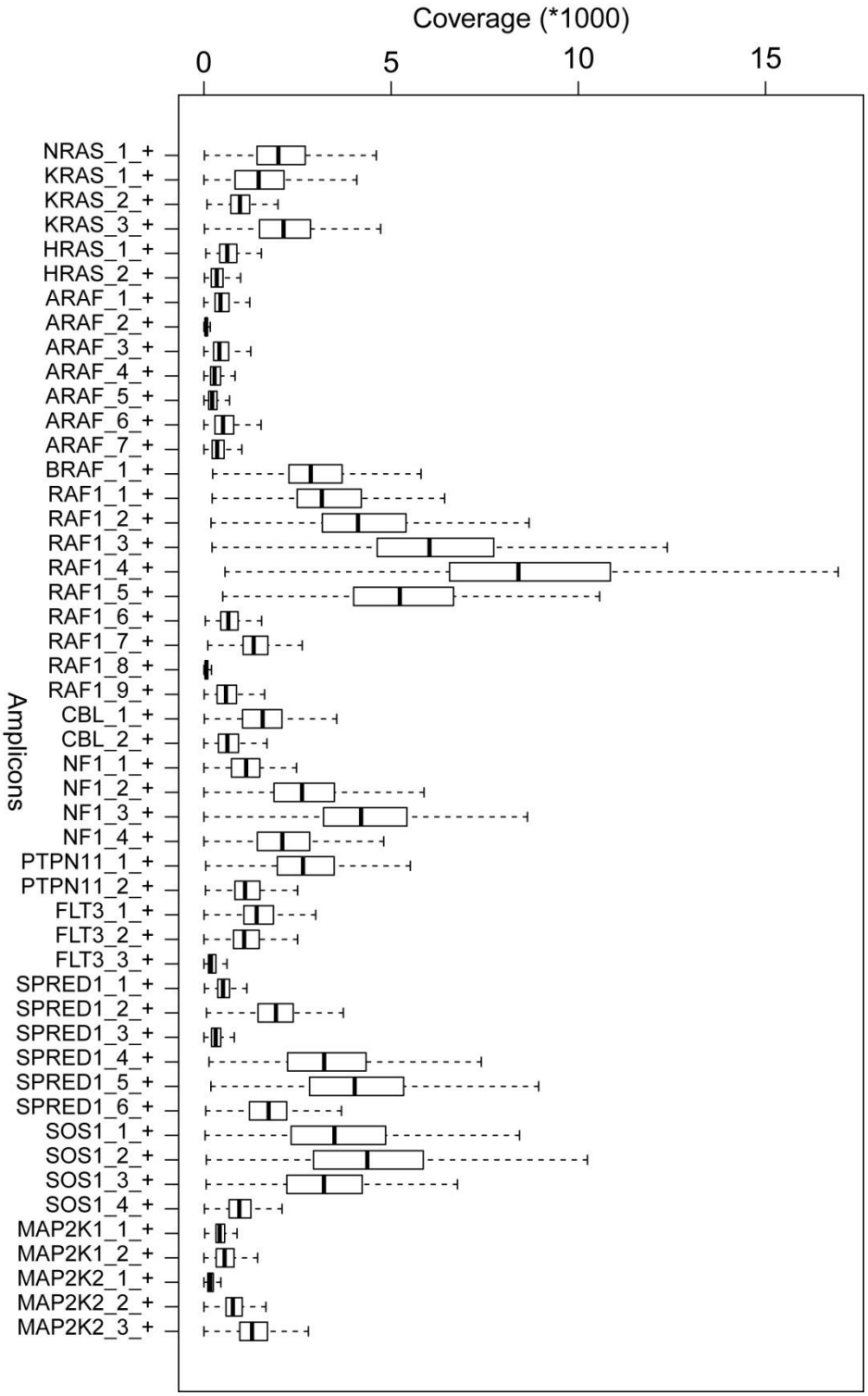
Supplemental Table S10: Median ex vivo LC50-values per RAS pathway mutations. The allocated group is determined by the largest clone. LC50-values are given in IU/mL for L-asparaginase and in µg/mL for all other chemotherapeutic agents. p-value is determined with a Mann-Whitney U test. Significant p-values are depicted in red. wt: wildtype, Combined: all RAS pathway mutations taken together. PRED: Prednisolone, VCR: Vincristine, ASP: L-asparaginase, DNR: Daunorubicine, 6TG: 6-Thioguanine, 6MP: 6-Mercaptopurine. Values containing ≥ or ≤ represent the highest or lowest concentration in the range, which was used as IC50 if limits were exceeded.

	Clonal						Subclonal					
	n =	Median LC50	Min. (µg/mL)	Max. (µg/mL)	Fold change	p-Value	n =	Median LC50	Min. (µg/mL)	Max. (µg/mL)	Fold change	p-Value
PRED wildtype	111	0.10	≤0.0076	≥250	.	.	111	0.10	≤0.0076	≥250	.	.
PRED combined	49	0.32	≤0.0076	≥250	3.2	0.02	34	0.17	≤0.0076	≥250	1.7	0.34
PRED *KRAS* G13	8	>250	0.280	≥250	>2000	0.00	7	>250	0.06	≥250	>2000	0.01
PRED *KRAS* G12	15	0.25	0.025	≥250	2.5	0.19	6	0.35	0.04	≥250	3.4	0.37
PRED *NRAS* G13	6	1.47	0.041	≥250	14.4	0.18	0	NA			.	.
PRED *NRAS* G12	11	0.23	≤0.0076	154.89	2.2	0.37	13	0.06	0.01	≥250	0.6	0.98
PRED *FLT3*	3	0.10	0.028	14.84	1	1	5	0.55	0.16	≥250	5.4	0.06
PRED *PTPN11*	4	0.10	≤0.0076	≥250	1	0.66	2	159.24	68.48	≥250	1565.8	0.06
PRED *KRAS* other	2	0.03	0.015	0.05	0.3	0.22	1	0.36	0.36	0.36	3.5	0.54
VCR wildtype	110	0.49	≤0.049	44.68	.	.	110	0.49	≤0.049	44.68	.	.
VCR combined	49	0.73	≤0.049	≥50	1.5	0.15	35	0.50	≤0.049	>50	1	0.91
VCR *KRAS* G13	8	7.09	0.397	43.95	14.6	0.01	7	0.67	0.12	>50	1.4	0.33
VCR *KRAS* G12	15	0.77	≤0.049	≥50	1.6	0.21	6	0.51	≤0.049	2.26	1	0.77
VCR *NRAS* G13	6	1.44	0.191	36.50	3	0.07	0	NA			.	.
VCR *NRAS* G12	11	0.09	≤0.049	3.03	0.2	0.06	14	0.45	≤0.049	2.66	0.9	0.86
VCR *FLT3*	3	0.05	≤0.049	2.03	0.1	0.21	5	0.41	≤0.049	1.65	0.8	0.95
VCR *PTPN11*	4	2.48	0.488	43.38	5.1	0.04	2	3.41	0.10	6.72	7	0.94
VCR *KRAS* other	2	0.32	0.157	0.48	0.7	0.50	1	1.51	1.51	1.51	3.1	0.37
ASP wildtype	109	0.22	≤0.003	≥10	.	.	109	0.22	≤0.003	≥10	.	.
ASP combined	49	0.04	≤0.003	≥10	0.2	0.18	35	0.07	≤0.003	≥10	0.3	0.48
ASP *KRAS* G13	8	0.06	≤0.003	1.58	0.3	0.55	7	0.13	≤0.003	≥10	0.6	0.86
ASP *KRAS* G12	15	0.03	≤0.003	≥10	0.1	0.56	6	0.05	≤0.003	1.68	0.2	0.51
ASP *NRAS* G13	6	0.87	0.009	1.98	3.9	0.52	0	NA			.	.
ASP *NRAS* G12	11	0.02	≤0.003	1.58	0.1	0.21	14	0.01	≤0.003	≥10	0.1	0.09
ASP *FLT3*	3	0.10	≤0.003	0.13	0.4	0.36	5	0.03	0.01	0.27	0.1	0.33
ASP *PTPN11*	4	0.56	≤0.003	1.31	2.5	0.72	2	0.01	0.01	0.02	0.1	0.23
ASP *KRAS* other	2	0.01	0.010	0.01	0.1	0.14	1	0.01	0.01	0.01	0.1	0.31
DNR wildtype	109	0.05	≤0.002	0.32	.	.	109	0.05	≤0.002	0.32	.	.
DNR combined	48	0.03	≤0.002	1.13	0.5	0.64	34	0.05	≤0.002	1.18	1	0.79
DNR *KRAS* G13	8	0.09	0.018	0.25	1.7	0.15	7	0.03	0.02	0.42	0.6	0.66
DNR *KRAS* G12	15	0.03	≤0.002	1.13	0.6	0.87	6	0.05	0.01	0.15	0.9	0.82
DNR *NRAS* G13	6	0.03	0.020	0.10	0.5	0.36	0	NA			.	.
DNR *NRAS* G12	11	0.02	0.007	0.12	0.4	0.14	14	0.04	≤0.002	0.22	0.8	0.67
DNR *FLT3*	3	0.02	0.006	0.27	0.4	0.59	5	0.05	0.02	0.08	1	0.84
DNR *PTPN11*	3	0.19	≤0.002	0.23	3.7	0.44	1	1.18	1.18	1.18	22.9	0.09
DNR *KRAS* other	2	0.02	0.022	0.02	0.4	0.23	1	0.12	0.12	0.12	2.4	0.21
6TG wildtype	83	0.00	≤0.002	0.02	.	.	83	0.00	≤0.002	0.02	.	.
6TG combined	37	0.00	≤0.002	≥0.05	1.1	0.89	25	0.00	≤0.002	0.02	0.9	0.73
6TG *KRAS* G13	5	0.00	≤0.002	0.02	1.4	0.31	4	0.00	0.00	0.02	0.8	0.58
6TG *KRAS* G12	9	0.00	≤0.002	≥0.05	1.3	0.40	5	0.00	0.00	0.00	0.8	0.08
6TG *NRAS* G13	5	0.01	≤0.002	0.01	1.8	0.80	0	NA			.	.
6TG *NRAS* G12	11	0.00	≤0.002	0.01	0.7	0.25	12	0.00	≤0.002	0.01	0.9	0.45
6TG *FLT3*	3	0.01	≤0.002	0.01	1.5	0.85	2	0.01	0.01	0.01	2.8	0.03
6TG *PTPN11*	2	0.00	0.003	0.00	1	0.81	1	0.01	0.01	0.01	2.3	0.25
6TG *KRAS* other	2	0.00	≤0.002	0.00	0.6	0.08	1	0.00	≤0.002	0.00	0.4	0.11
6MP wildtype	82	0.09	≤0.016	≥0.5	.	.	82	0.09	≤0.016	≥0.5	.	.
6MP combined	32	0.06	≤0.016	≥0.5	0.7	0.07	25	0.08	≤0.016	≥0.5	0.9	0.39
6MP *KRAS* G13	4	0.14	0.044	≥0.5	1.6	0.43	4	0.04	0.02	0.09	0.4	0.07
6MP *KRAS* G12	8	0.05	≤0.016	≥0.5	0.5	0.15	5	0.10	0.02	0.10	1.1	0.41
6MP *NRAS* G13	5	0.07	≤0.016	≥0.5	0.8	0.70	0	NA				.
6MP *NRAS* G12	10	0.09	≤0.016	0.36	1	0.57	12	0.03	≤0.016	0.27	0.3	0.01
6MP *FLT3*	1	0.04	0.038	0.04	0.4	0.22	2	0.38	0.27	≥0.5	4.3	0.05
6MP *PTPN11*	2	0.02	≤0.016	0.03	0.2	0.03	1	0.12	0.12	0.12	1.4	0.52
6MP *KRAS* other	2	0.05	0.034	0.06	0.5	0.15	1	0.02	0.02	0.02	0.2	0.14

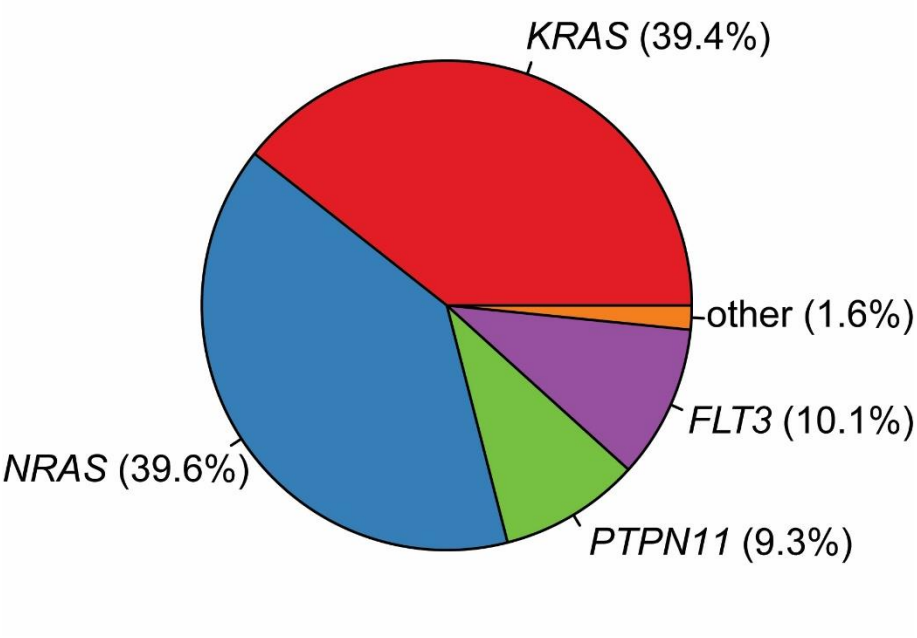
Supplemental Figure S1: Diagram with detailed information about the patient cohorts. A Subtype distribution among patients with complete subtype information, used to determine RAS pathway mutation frequency among genetic subtypes of BCP-ALL (red) and subtype distribution among cases used to compare clinical characteristics in wildtype or RAS pathway mutated patients (green). **B** Treatment protocols of cases used for analysis of clinical characteristics (green) and those used for analysis of event-free survival and cumulative incidence of relapse (blue). Cases with *ex vivo* drug sensitivity (yellow frame) data were treated in the COALL97/03 and DCOG ALL10 cohorts, but only a subset of these cases had data available (n=211). Observation patient: not eligible for follow-up, only baseline characteristics analyzed.



Supplemental Figure S2: Amplicon coverage across samples. Coverage was calculated from the number of reads mapped for each sample. Amplicons are identified by their amplicon ID (see also Supplemental Table S1). Boxes represent first and third quartile, whiskers represent outer two quartiles (max. 1.5-times the inner quartile range), and bar represents group median.



233 **Supplemental Figure S3: Relative frequency of *NRAS*, *KRAS*, *PTPN11*, *FLT3* and other RAS pathway mutations.**



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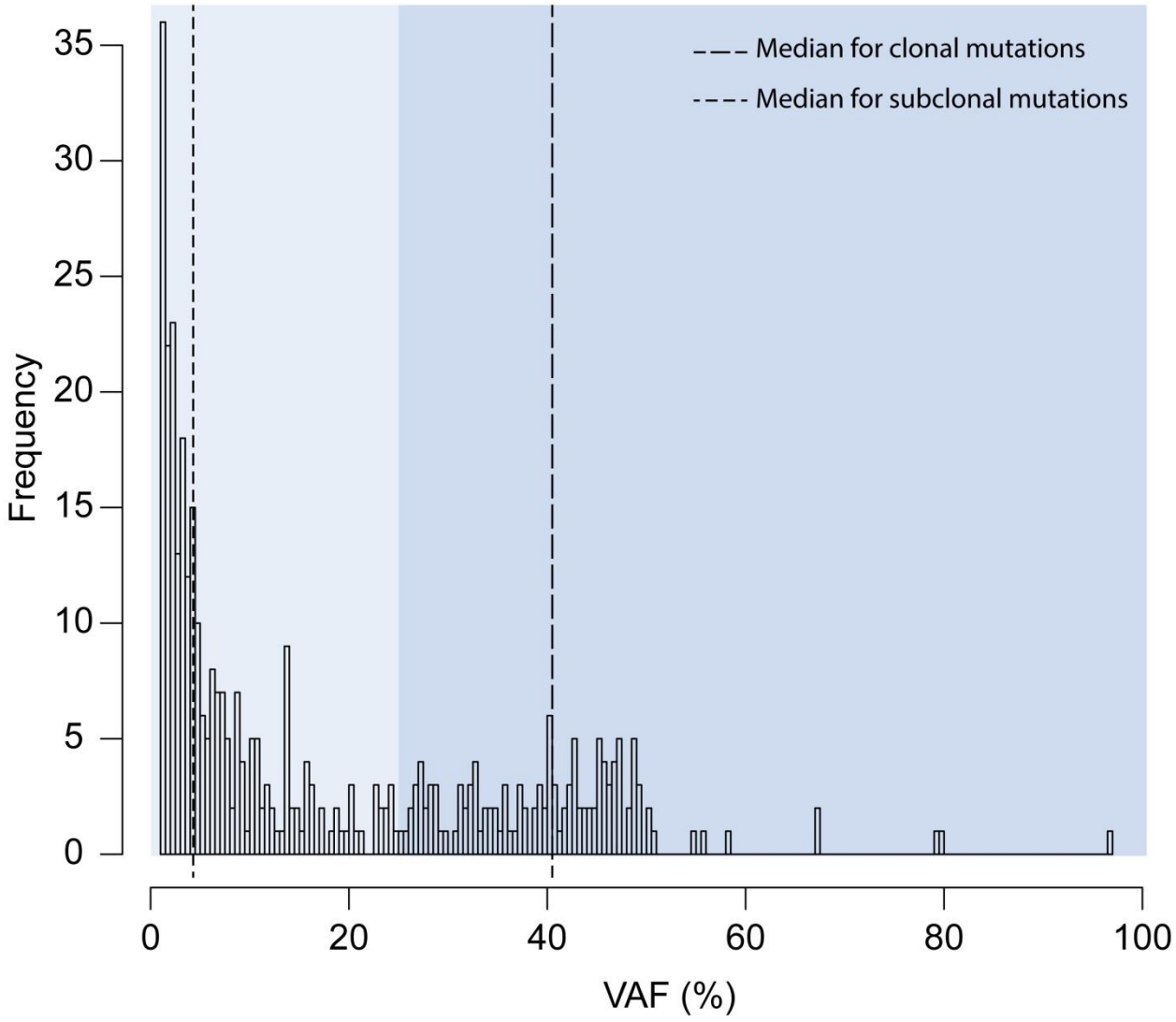
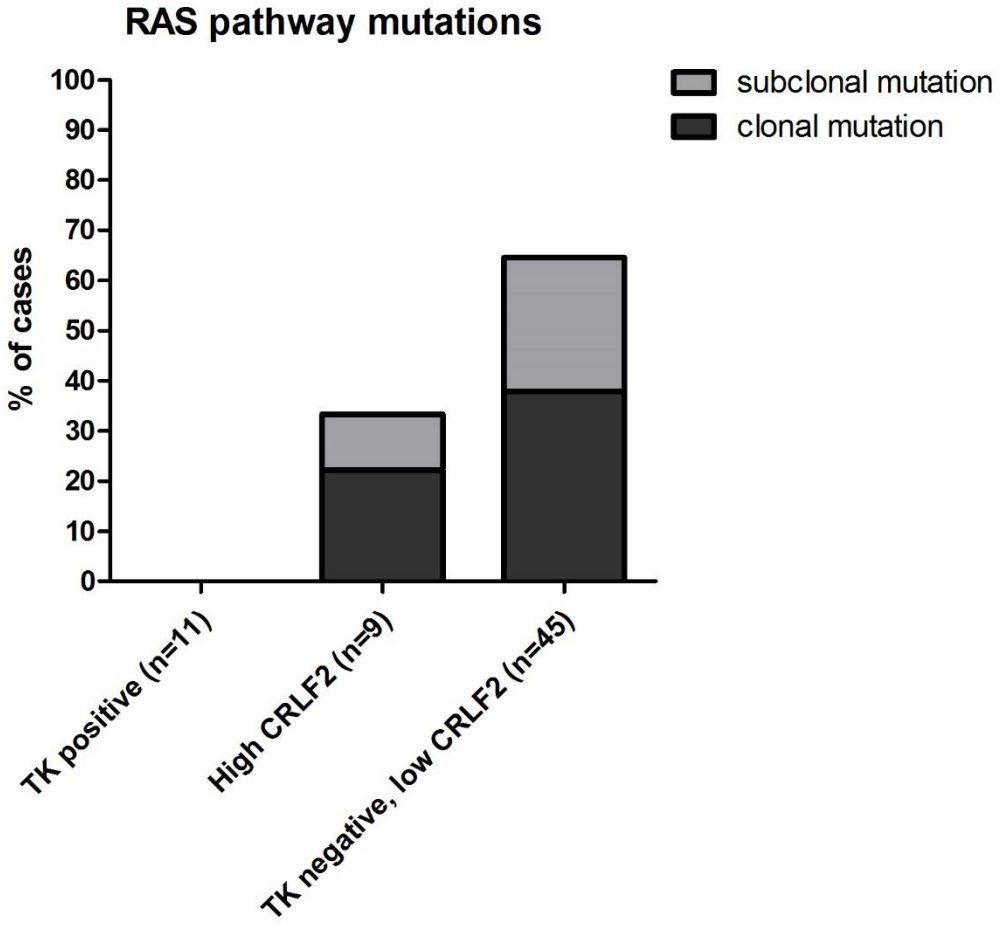
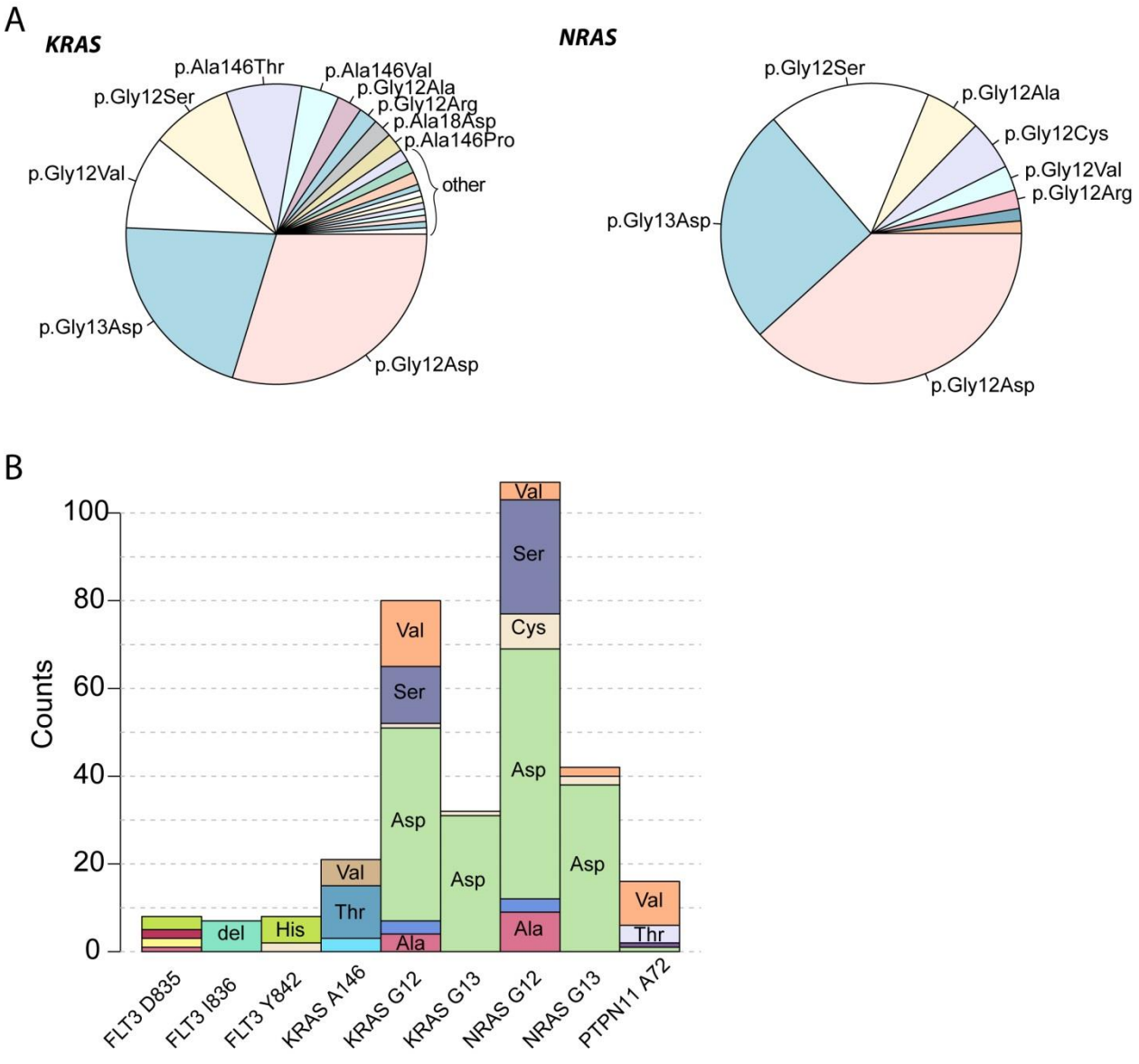
Supplemental Figure S4: Histogram of observed variant allele-frequencies. Histogram of the VAF of all mutations in RAS pathway genes identified in our dataset. Dashed lines depict median VAF of all clonal or subclonal mutations. Background shading represents subclonal (light) and (dark) clonal mutations.



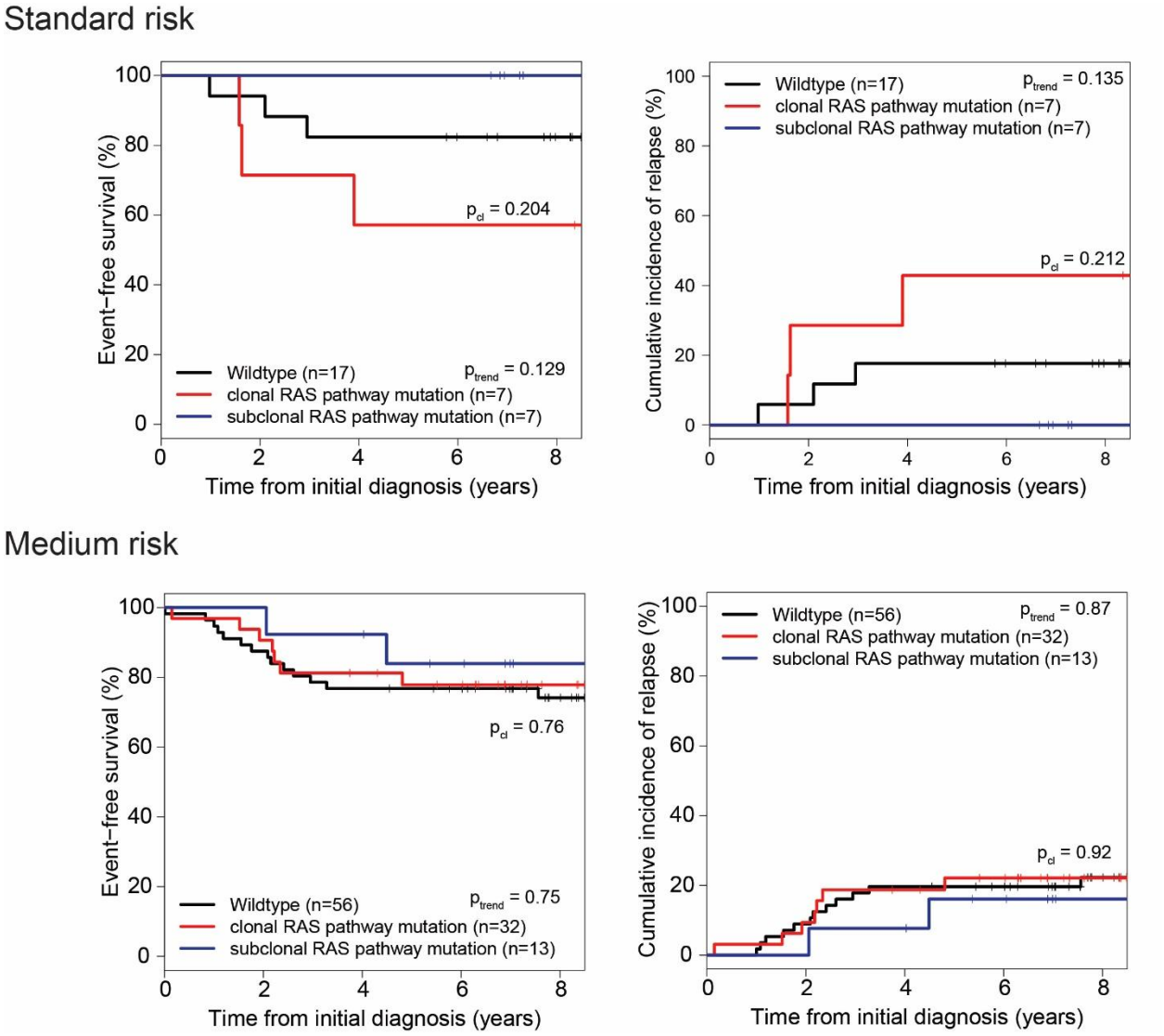
Supplemental Figure S5: Frequency of clonal and subclonal RAS pathway mutations among tyrosine kinase (TK) fusion positive *BCR-ABL1*-like cases, *CRLF2*-high *BCR-ABL1*-like cases, and TK fusion negative *BCR-ABL1*-like cases with low *CRLF2* expression. TK fusions were identified as described by Boer et al. (manuscript submitted) and included the following fusions: EBF1-PDGFRB (3 cases), PAX5-JAK2 (3), BCR-JAK2 (1), FOXP1-ABL1 (1), SSBP-CSFR (1), TERF2-JAK2 (1), and ZMIZ1-ABL1 (1). High *CRLF2* expression was, as previously, defined as the 90th percentile of the entire BCP-ALL cohort.



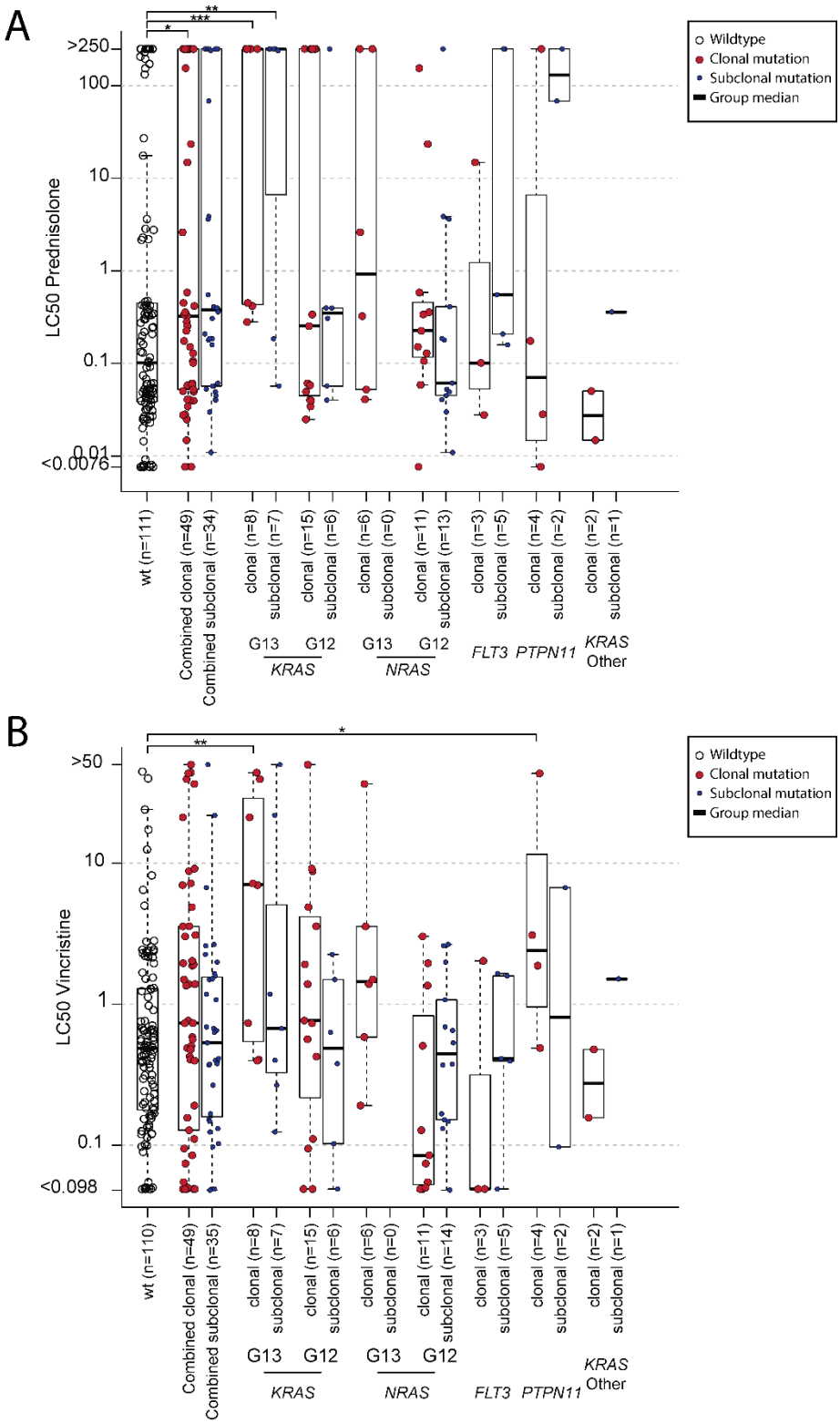
Supplemental Figure S6: Overview of amino acid changes in RAS mutated cases. A Relative frequency of different *KRAS* and *NRAS* variants observed in pediatric BCP-ALL. **B** Histogram of amino acid substitutions in *KRAS, NRAS, FLT3* and *PTPN11* variants.



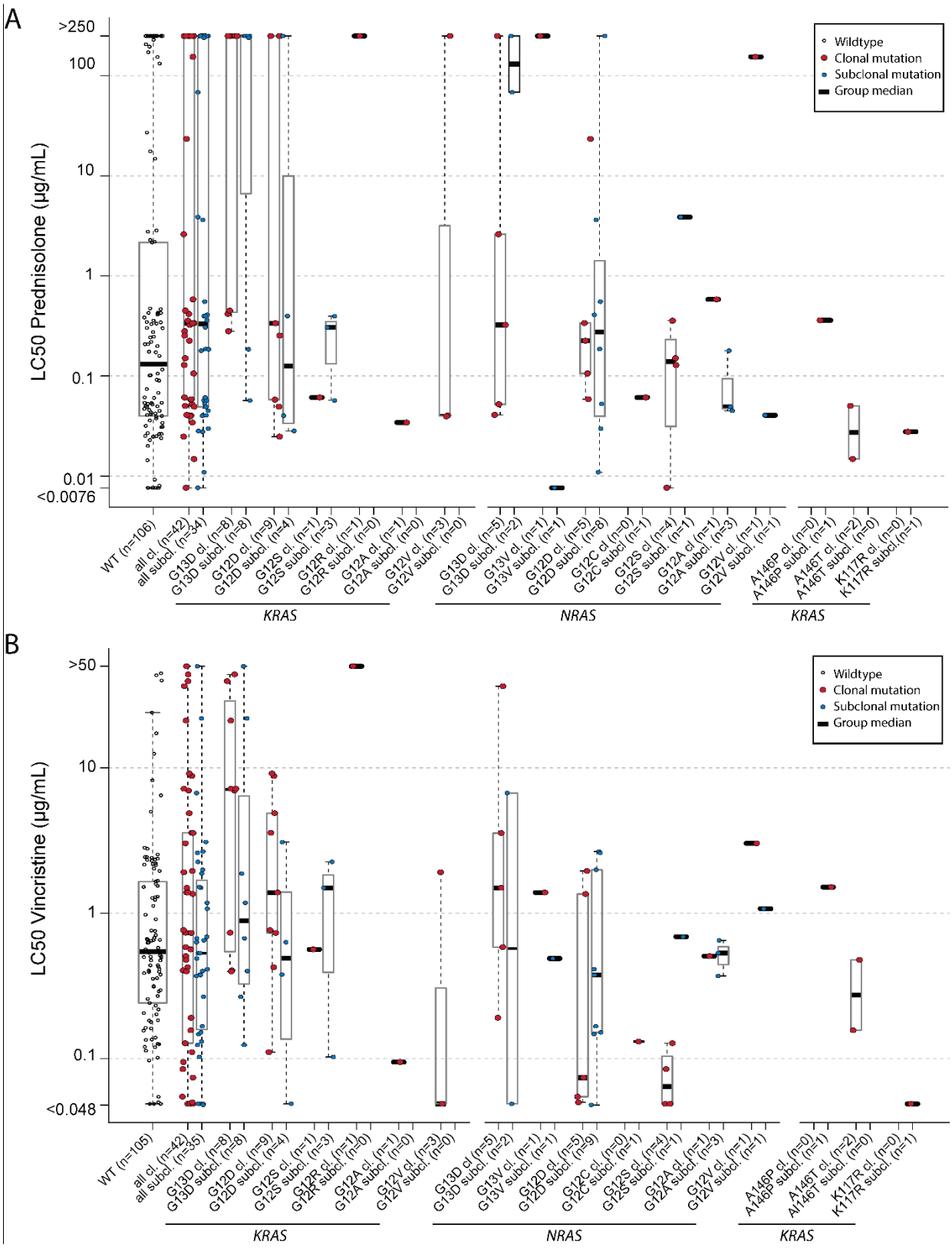
Supplemental Figure S7: Event-free survival and cumulative incidence of relapse in the COALL97/03 cohort. p_{trend} = p-value for the analysis comparing wildtype, subclonal, and clonal mutation cases; p_{cl} = p-value for the analysis comparing wildtype to cases with clonal RAS pathway mutations.



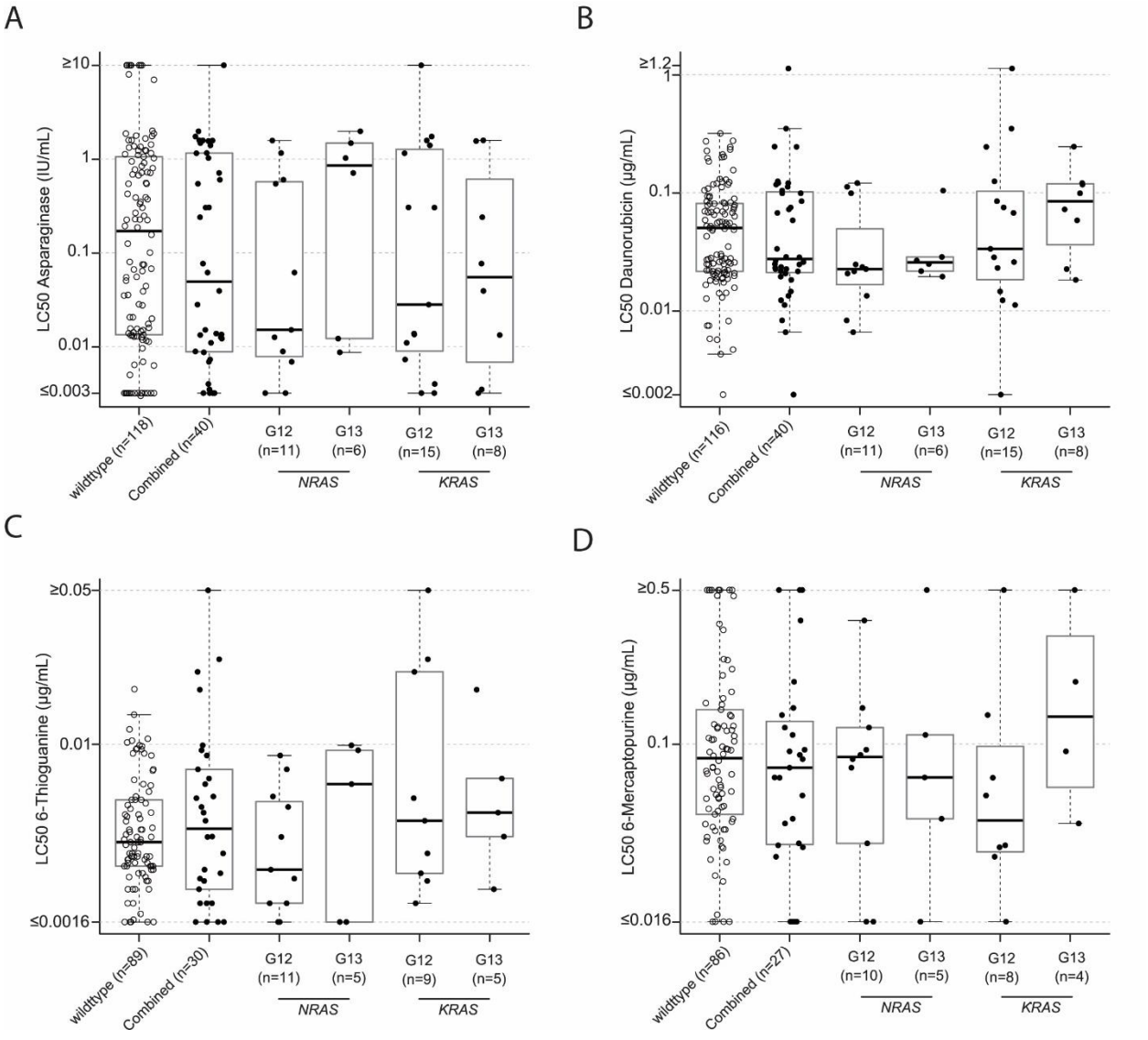
Supplemental Figure S8: *Ex vivo* sensitivity to prednisolone and vincristine in BCP-ALL cases carrying subclonal or clonal RAS pathway mutations including *FLT3* and *PTPN11*. Sensitivity of primary patient cells towards prednisolone and vincristine, distinguished by RAS mutation status. LC50-values of cases with clonal and subclonal mutations are depicted. "All": All cases with a mutation in *NRAS*, *KRAS*, *PTPN11*, or *FLT3*. In cases with multiple mutations, the mutation with the highest VAF (largest clone) was used to classify cases.



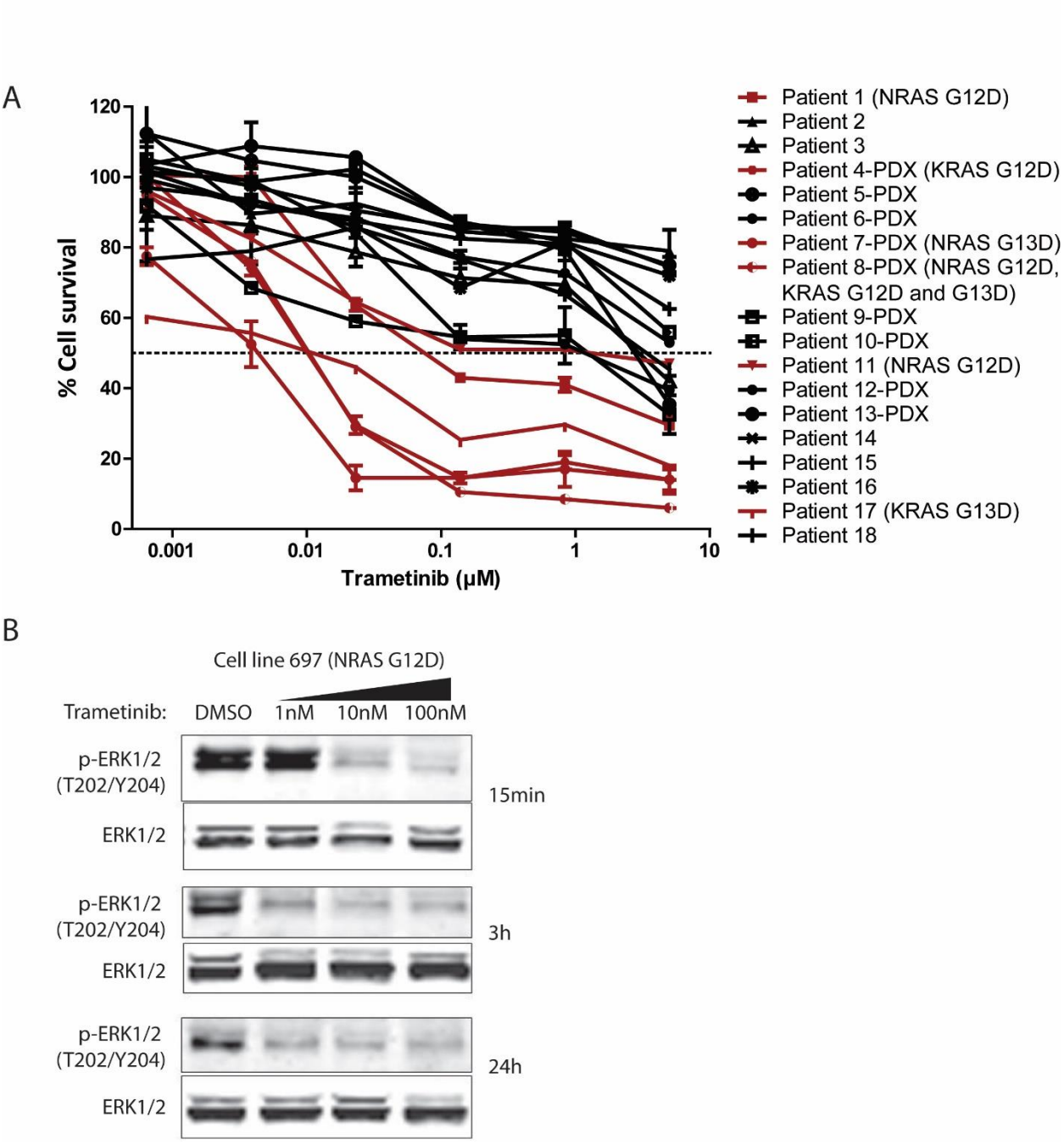
Supplemental Figure S9: *Ex vivo* chemotherapeutic sensitivity of RAS mutated BCP-ALL cases distinguished by the amino acid variant in the *NRAS* or *KRAS* gene. In cases with multiple mutations, the mutation with the highest VAF (largest clone) was used to classify cases. **A** Prednisolone sensitivity. **B** Vincristine sensitivity.



Supplemental Figure S10: RAS pathway mutations and *ex vivo* cytotoxicity of chemotherapeutic agents. *Ex vivo* sensitivity of 211 primary patient samples towards (A) L-asparaginase, (B) daunorubicin, (C) 6-thioguanine, and (D) 6-mercaptopurinol, distinguished by RAS mutation status. Only clonally mutated cases are depicted. Only *KRAS* and *NRAS* mutated groups are shown due to low recurrence of other mutations (see also supplement). Combined: All cases with a clonal mutation in *NRAS*, *KRAS*, *PTPN11*, *FLT3*. Groups were compared by Mann-Whitney U test, * p<0.05, ** p<0.01. LC50-values were evaluated by MTT assays as reported previously.



Supplemental Figure S11: Efficacy of the MEK-inhibitor trametinib in RAS pathway mutant BCP-ALL. A *Ex vivo* sensitivity of primary BCP-ALL cases towards trametinib. Red lines represent RAS pathway mutated cases, black lines represent RAS pathway wildtype cases. **B** Western blot of phosphorylated ERK1/2 to confirm effective MEK-inhibition by a range of trametinib concentrations.



References

1. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D*, et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *J Clin Oncol* 2003 Sep 1; **21**(17)**:** 3262-3268.

2. Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JG, Peters ST*, et al.* A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. *Lancet Oncol* 2009 Feb; **10**(2)**:** 125-134.

3. van der Veer A, Waanders E, Pieters R, Willemse ME, Van Reijmersdal SV, Russell LJ*, et al.* Independent prognostic value of BCR-ABL1-like signature and IKZF1 deletion, but not high CRLF2 expression, in children with B-cell precursor ALL. *Blood* 2013 Oct 10; **122**(15)**:** 2622-2629.

4. Kaspers GJ, Veerman AJ, Pieters R, Van Zantwijk CH, Smets LA, Van Wering ER*, et al.* In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. *Blood* 1997 Oct 1; **90**(7)**:** 2723-2729.

5. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv preprint arXiv:13033997* 2013.

6. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. *arXiv preprint arXiv:12073907* 2012.

7. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L*, et al.* VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* 2012 Mar; **22**(3)**:** 568-576.

8. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics* 2011 Nov 1; **27**(21)**:** 2987-2993.

9. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C*, et al.* A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature genetics* 2011 May; **43**(5)**:** 491-498.

10. Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L*, et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly* 2012 Apr-Jun; **6**(2)**:** 80-92.

11. Liu X, Wu C, Li C, Boerwinkle E. dbNSFP v3.0: A One-Stop Database of Functional Predictions and Annotations for Human Nonsynonymous and Splice-Site SNVs. *Hum Mutat* 2016 Mar; **37**(3)**:** 235-241.

12. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H*, et al.* COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res* 2015 Jan; **43**(Database issue)**:** D805-811.

13. Aries IM, van den Dungen RE, Koudijs MJ, Cuppen E, Voest E, Molenaar JJ*, et al.* Towards personalized therapy in pediatric acute lymphoblastic leukemia: RAS mutations and prednisolone resistance. *Haematologica* 2015 Apr; **100**(4)**:** e132-136.

14. Escherich G, Troger A, Gobel U, Graubner U, Pekrun A, Jorch N*, et al.* The long-term impact of in vitro drug sensitivity on risk stratification and treatment outcome in acute lymphoblastic leukemia of childhood (CoALL 06-97). *Haematologica* 2011 Jun; **96**(6)**:** 854-862.

15. Gray RJ. cmprsk: Subdistribution Analysis of Competing Risks. 2013; **R package version 2.2-7** http://CRAN.R-project.org/package=cmprsk.

16. de Wreede LC, Fiocco M, Putter H. mstate: An R Package for the Analysis of Competing Risks and Multi-State Models. *J Stat Softw* 2011 Jan; **38**(7)**:** 1-30.

17. Therneau T. A Package for Survival Analysis in S. 2012; **R Package version 2.38-3**.