Prognostic impact of the absence of biallelic deletion at the *TRG* locus for pediatric patients with T-cell acute lymphoblastic leukemia treated on the Medical Research Council UK Acute Lymphoblastic Leukemia 2003 trial

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SUPPLEMENTARY INFORMATION

Patient cohort

Diagnostic samples from previously untreated T-ALL patients enrolled on the MRC UKALL2003 trial were analyzed. The trial, registered at http://www.controlled-trials.com under the ISRCTN number 07355119, opened in October 2003 for patients aged 1-18 years. The upper age limit of the trial was increased in September 2006 to 20 years and in June 2008 to 25 years of age. Samples obtained at diagnosis from 152 of the 393 (39%) patients with T-ALL were available for analysis. Ethical approval for the trial was obtained previously from the Scottish Multi-Centre Research Ethics Committee, and samples were collected with informed consent according to the Declaration of Helsinki.

Details of the trial protocol are as published[1] and are outlined in Supplementary Figures S5-6. At trial entry, patients with National Cancer Institute (NCI) standard risk (<10 years of age and white cell count [WCC] <50x10^9/L) were assigned to regimen A while NCI high risk patients (≥10 years and/or WCC ≥50x10^9/L) received regimen B. Patients <16 years with a slow early response (≥25% blasts in the day 15 or 8 bone marrow for regimens A or B respectively) and all patients with high risk cytogenetics (*KMT2A* [*MLL*] and *TCF3-HLF* fusions, near haploidy, low hypodiploidy, and iAMP21) were assigned to regimen C. MRD was evaluated by real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements with a quantitative range of 0·01% as defined by the European MRD Study Group.[2] Patients with undetectable MRD at the end of induction (EOI, day 29) and before interim maintenance were classified as MRD low risk, as were

those who had detectable EOI MRD (<0·01%) but undetectable MRD before the start of interim maintenance. MRD low risk patients were eligible for treatment reduction randomization. Patients with EOI MRD ≥0·01% were classified as MRD high risk and were eligible for treatment intensification randomization (Figure S5).

Materials and Methods

Samples

Whole-Genome-Amplified (WGA) diagnostic genomic DNA (gDNA) from patient samples was as previously prepared.[3]

qPCR assay

The quantitative polymerase chain reaction (qPCR) assay was as previously described,[4] and evaluated the presence or absence of an amplicon within the intron between the V and J regions of the *TRG* locus at chromosome 7p14. The primers (Forward: 5'-CATCCTCACTTTCCTGCTTCTTC-3'; Reverse: 5'-CCAAGGTGAATCCCTACATGCT-3') amplified an 87 base pair (bp) amplicon 5089 bp from the 3' end of the *TRGV11* pseudoexon and 10123 bp away from the 5' end of *TRGJP1* exon. The reference gene *ANLN* lies 1.9Mb downstream of *TRG* at 7p15-14, and the primers (Forward: 5'-AAATTCTGCCCTTTGCTTGTTT-3'; Reverse: 5'-GAAAGCAACCACAGAGAATATGTAAGTAA-3') amplified an 89 bp product.

25µl PCR reactions were set up as follows: 0.5µl of each forward and reverse primer (either *TRG* or *ANLN*) was added at 0.2µM, 12.5µl 2x concentrated ready-to-use FastStart Universal SYBR Green Master reaction mix (ROX) (Roche, 2008), 2µl genomic DNA template, and 9.5µl nuclease-free water. Samples were analyzed

in triplicate for each primer pair. The reactions were run on a Mastercycler epgradient S thermocycler (Eppendorf) at 95^0C for 10mins, followed by 40 cycles of 95^0C for 15 seconds and 60^0C for 1 min for each cycle. CT (cycling time) values were obtained from the Mastercycler ep Realplex software.

Standard curves using dilutions of gDNA from the non-leukemic cell line HEK293T that does not have a rearrangement of the *TRG* locus showed that the primer pair efficiencies were very similar to each other (E=1.84 and 1.817 for *TRG* and *ANLN* respectively). The *ANLN* primer pair efficiencies were also validated using gDNA from Jurkat cells, a non-early T cell precursor cell line that does have a rearrangement of the *TRG* gene.

Mean and standard deviation (SD) between the CT values of each sample for each primer pair were calculated. If the SD between CT values of the replicates for the PCR reaction of the reference gene, *ANLN*, was >0.5, then the reactions were discarded and all the reactions for that patient sample were repeated.

TRG:ANLN fold change was calculated according to the comparative ΔΔCT method using HEK293T gDNA as the calibrator. Each run included the positive control (HEK293T gDNA) and a negative control (nuclease-free water) for each primer pair. The SD of the HEK293T CT values for each of the primer pairs showed minimal variability across the runs (n=14; *TRG*: mean CT value 23.7, range 23.4-23.9, SD 0.1; *ANLN*: mean CT value 23.8, range 23.6-23.9, SD 0.1). Therefore, the CT results of the HEK293T sample of each run was used as the calibrator value for the fold change calculations for samples on that run.

Comparison of WGA and non-WGA gDNA fold change

TRG:ANLN fold change calculated by the method above using WGA gDNA samples was validated using the same assay in the corresponding non-WGA gDNA samples available from 26 patients, which included patients with fold changes that covered the entire range (mean fold change 0.01–0.94). There was good agreement between the fold change results from the 2 types of samples (R^2=0.92; Figure S2A), with a bias of -0.023 and an agreement interval from -0.201 to 0.155, in which 95% of the differences between the 2 fold changes should lie (Figure S2B). One patient with ABD and three patients with non-ABD in the WGA samples were found to be indeterminate by qPCR assay in the corresponding non-WGA samples. However, none of the patients had their ABD status change from ABD to non-ABD, and vice-versa, when comparing the WGA and the corresponding non-WGA results.

Validation of fold change using a different reference gene

The fold change results that were indeterminate by the qPCR assay above (TRG:ANLN fold change 0.26-0.49) were validated using a different reference gene encoding the mitochondrial protein Cytochrome c Oxidase Assembly factor 1 (*COA1*), which lies 4MB upstream of the *TRG* locus, to abrogate the possibility that abnormalities of the *ANLN* reference gene itself may have contributed to the indeterminate fold change calculation. The *COA1* primers (Forward: 5'-GGAAAACTGGGTTGCAGGAG-3'; Reverse: 5'-AGAAGACCCAGCTTGCTTCT-3') amplified a 105bp product.

TRG and *COA1* assays were performed in triplicate using the same PCR reaction reagents, calibrator and conditions as described above and the TRG:COA1 fold

change calculated. Standard curves using dilutions of gDNA from HEK293T cells were set up using the *COA1* primers and the efficiency was comparable to that of the *TRG* primers (E=1.96 and E=1.84 for *COA1* and *TRG* respectively).

The TRG:COA1 fold change led to the same ABD status assignment as that of the TRG:ANLN fold change for 7 patients that had informative TRG:ANLN fold change results (data not shown). The TRG:COA1 fold change results for 15 patients with indeterminate TRG:ANLN did not change the ABD assignment from ABD to non-ABD or vice versa, although 3 patients had fold changes in this assay that varied from those of the TRG:ANLN assay only across the thresholds of 0.26-0.49 of the indeterminate range (Figure S2C). As a result of the inconclusive fold change results from both the TRG:ANLN and TRG:COA1 assays for these samples, these patients were assigned the ABD indeterminate status.

SNP array analysis

WGA gDNA samples were previously analyzed using the Infinium CytoSNP-850K Beadchip array (Illumina, Essex, UK).[3] Log-R intensities and B-allele frequencies for each of the 62 SNP markers across the region hg19 chr7:38288270-38385938 which includes the *TRG* locus were assessed independently by 4 individuals and the *TRG* locus deletion status was scored as no or heterozygous deletion (ABD), or homozygous deletion (non-ABD).

Statistical Analysis

Survival curves were plotted using Kaplan-Meier analysis for overall survival (time from the start of treatment until death), event-free survival (time to relapse,

secondary tumor or death, whichever came first) and relapse-free survival (time to relapse in those who achieved remission). Patients who died in remission were censored. Those who did not have an event were censored at the date last seen. Comparisons between groups were carried out using Cox regression and the log rank test. Differences between the median Day 29 MRD results in the ABD and non-ABD groups was compared using the Wilcoxon Mann-Whitney test. Statistical analysis was conducted using STATA version 14.2 (STATACORP, Texas), MRD scatter plot drawn using GraphPad Prism 6 (GraphPad Software, Inc, California).

References

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Supplementary Tables

Table S1: Comparison of the baseline characteristics, treatment allocation and response of T-ALL patients that were (n=152) or were not (n=241) included in the study.

	Patients with samples	Patients without samples	*P*
	N=152	N=241	
Baseline characteristics			
Sex, N (%)			
Male	117 (77.0)	168 (69.7)	0.12
Female	35 (23.0)	73 (30.3)	
WBC count (x10^9/L), N (%)			
<50	48 (31.6)	95 (39.4)	0.12
≥50	104 (68.4)	146 (60.6)	
Age, N (%)			
<10 years	82 (53.9)	107 (44.4)	0.07
≥10 years	70 (46.1)	134 (55.6)	
NCI risk group, N (%)			
Standard	19 (12.5)	40 (16.6)	0.27
High	133 (87.5)	201 (83.4)	
CNS disease at diagnosis, N (%)			
No	144 (94.7)	225 (93.4)	0.58
Yes	8 (5.3)	16 (6.6)	
Final Treatment Allocation and Response			
Final treatment given, N (%)			
A	14 (9.2)	30 (12.4)	0.25
B	87 (57.2)	118 (49.0)	
C	51 (33.6)	93 (38.6)	
Slow early Response, N (%)			
No	97 (63.8)	148 (61.4)	0.55[#]
Yes	37 (24.3)	55 (22.8)	
Unknown[*]	18 (11.8)	38 (15.8)	
MRD, N (%)			
Negative (<0.01% positive cells)	50 (32.9)	70 (29.0)	0.25[§]
Positive (≥0.01% positive cells)	67 (44.1)	97 (40.2)	
Indeterminate[Ψ]	35 (23.0)	74 (30.7)	

P values derived using Chi-squared tests. [*]Patients without bone marrow results at day 8 or 15 (assumed to have rapid early response for treatment escalation decision) and two patients with conflicting slow early response data and bone marrow results. [#]Excluding the unknown group: $P = 0.92$. [§]Excluding the indeterminate group: $P = 0.89$. [Ψ]Includes samples where there were either no targets for MRD assessment or the targets were not sensitive enough, and samples where MRD was either not analysed or was unevaluable due to the sample being inadequate or having missing data. WBC, white blood cell count; NCI, National Cancer Institute, CNS, central nervous system; MRD, minimal residual disease.

Table S2: Time-to-event outcomes comparing the groups with and without samples for ABD analysis.

		Events/n	HR (95% CI)	*P*	5-year rate (95% CI)
EFS					
	Without samples	59/241	1.00	0.075	76.9% (71.0 – 81.8)
	With samples	27/152	0.66 (0.42 – 1.05)		84.2% (77.4 – 89.1)
RFS					
	Without samples	37/239	1.00	0.41	84.9% (79.4 – 89.0)
	With samples	20/151	0.80 (0.46 – 1.38)		87.2% (80.7 – 91.6)
OS					
	Without samples	43/241	1.00	0.074	82.8% (77.4 – 87.0)
	With samples	18/152	0.61 (0.35 – 1.05)		89.5% (83.4 – 93.4)

P values calculated using the log rank test. EFS, Event-Free Survival; RFS, Relapse-Free Survival; OS, Overall Survival; HR, Hazard Ratio; CI, Confidence Interval.

Table S3. Comparison of baseline characteristics, treatment allocation, response and molecular characterisation of patients with definitive and indeterminate qPCR results for the ABD assay.

	qPCR result N=133	Indeterminate qPCR result N=19	*P*
Baseline characteristics			
Sex, N (%)			
Male	104 (78.2)	13 (68.4)	0.38*
Female	29 (21.8)	6 (31.6)	
WBC count (x10^9 per L), N (%)			
<50	39 (29.3)	9 (47.4)	0.11
≥50	94 (70.7)	10 (52.6)	
Age, N (%)			
<10 years	72 (54.1)	10 (52.6)	0.90
≥10 years	61 (45.9)	9 (47.4)	
NCI risk group, N (%)			
Standard	14 (10.5)	5 (26.3)	0.066*
High	119 (89.5)	14 (73.7)	
CNS disease at diagnosis, N (%)			
No	125 (94.0)	19 (100.0)	0.60*
Yes	8 (6.0)	0	
Final Treatment Allocation and Response			
Final treatment given, N (%)			
A	11 (8.3)	3 (15.8)	0.53*
B	76 (57.1)	11 (57.9)	
C	46 (34.6)	5 (26.3)	
Slow early Response, N (%)			
No	85 (63.9)	12 (63.2)	>0.99*,§
Yes	32 (24.1)	5 (26.3)	
Unknown#	16 (12.0)	2 (10.5)	
MRD, N (%)			
Negative (<0.01% positive cells)	41 (30.8)	9 (47.4)	0.34*,Ψ
Positive (≥0.01% positive cells)	61 (45.9)	6 (31.6)	
Indeterminateθ	31 (23.3)	4 (21.1)	
Molecular Characterisation			
NOTCH1/FBXW7, N (%)			
Wild type	47 (35.3)	6 (31.6)	0.20*
Single Mutant	50 (37.6)	11 (57.9)	
Double Mutant	36 (27.1)	2 (10.5)	
PTEN, N (%)			
Wild type	94 (75.8)	17 (94.4)	0.12*
Mutant	30 (24.2)	1 (5.6)	
RAS, N (%)			
Wild type	120 (90.2)	17 (89.5)	>0.99
Mutant	13 (9.8)	2 (10.5)	

P values derived using Chi-squared tests unless otherwise indicated. *Fisher's exact test.
#Patients without bone marrow results at day 8 or 15 (assumed to be RER for treatment escalation) and one patient with conflicting slow early response data and bone marrow results.
§Excluding the unknown group: *P* >0.99. ΨExcluding the indeterminate group: *P* = 0.15.
θIncludes samples where there were either no targets for MRD assessment or the targets were not sensitive enough, and samples where MRD was either not analysed or was unevaluable due to the sample being inadequate or having missing data. WBC, white blood cell count; NCI, National Cancer Institute, CNS, central nervous system; MRD, minimal residual disease.

Table S4: Time-to-event outcomes comparing the groups with definitive and indeterminate qPCR results for the ABD assay.

		Events/n	HR (95% CI)	*P*	5-year rate (95% CI)
EFS					
	qPCR result	24/133	1.00	0.81	84.2% (76.8 – 89.4)
	Indeterminate qPCR	3/19	0.86 (0.26 – 2.87)		84.2% (58.7 – 94.6)
RFS					
	qPCR result	17/132	1.00	0.75	87.6% (80.6 – 92.2)
	Indeterminate qPCR	3/19	1.22 (0.36 – 4.17)		84.2% (58.7 – 94.6)
OS					
	qPCR result	16/133	1.00	0.83	89.5% (82.9 – 93.6)
	Indeterminate qPCR	2/19	0.85 (0.20 – 3.71)		89.5% (64.1 – 97.3)

P values calculated using the log rank test. EFS, Event-Free Survival; RFS, Relapse-Free Survival; OS, Overall Survival; HR, Hazard Ratio; CI, Confidence Interval.

Table S5: Time-to-event outcomes comparing the ABD and non-ABD groups.

		Events/n	HR (95% CI)	*P*	5-year rate (95% CI)
EFS					
	Non-ABD	17/110	1.00	0.09	85.4% (79.5 – 92.3)
	ABD	7/23	2.12 (0.88 – 5.12)		77.3% (55.4 – 90.3)
RFS					
	Non-ABD	13/110	1.00	0.42	88.8% (81.1 – 93.5)
	ABD	4/22	1.58 (0.52 – 4.86)		81.8% (58.5 – 92.8)
OS					
	Non-ABD	12/110	1.00	0.37	90.0% (82.7 – 94.3)
	ABD	4/23	1.67 (0.54 – 5.17)		87.0% (64.8 – 95.6)

P values calculated using the log rank test. EFS, Event-Free Survival; RFS, Relapse-Free Survival; OS, Overall Survival; HR, Hazard Ratio; CI, Confidence Interval.

Table S6: Relapse-free survival comparing the ABD and non-ABD groups treated on Regimens A/B and C.

	Events/n	HR (95% CI)	*P*	5-year rate (95% CI)
Regimen A/B				
Non-ABD	7/75	1.00	0.31	91.9% (82.8 – 96.3)
ABD	0/11	-		100%
Regimen C				
Non-ABD	6/35	1.00	0.21	82.1% (64.4 – 91.6)
ABD	4/11	2.19 (0.62 – 7.78)		63.6% (29.7 – 84.5)

P values calculated using the log rank test. HR, Hazard Ratio; CI, Confidence Interval.

Supplementary Figure Legends

Figure S1. Kaplan-Meier survival curves of the patients included or not included in the study. (A) Overall Survival, (B) Event-Free Survival, (C) Relapse-Free Survival.

Figure S2. Validation of the TRG:ANLN qPCR assay. (A) Comparison of results obtained using WGA and Non-WGA samples from 26 patients with available material. (B) Bland-Altman plot showing the differences between the TRG:ANLN WGA and non-WGA results plotted against the mean of each pair of fold changes. The shaded area shows the agreement interval in which 95% of the differences are expected to lie. (C) TRG:ANLN and TRG:COA1 fold changes for diagnostic WGA samples from 15 patients with indeterminate ABD status by TRG:ANLN fold change.

Figure S3. Kaplan-Meier survival curves of the patients with definitive and indeterminate qPCR results. (A) Overall Survival, (B) Event-Free Survival, (C) Relapse-Free Survival.

Figure S4. Kaplan-Meier survival curves grouped according to ABD status for patients on different treatment regimens. Relapse-Free Survival for patients on (A) Regimen C, (B) Regimen A or B, and (C) those with positive MRD regardless of treatment regimen.

Figure S5. Schematic diagram of the UKALL2003 trial protocol. (A) Treatment intensity decision points, (B) Randomization arms of the trial based on MRD

analysis. Slow Early Response defined as ≥25% blasts in the Day 15 bone marrow for Regimen A and Day 8 marrow for Regimen B; High Risk Cytogenetics includes *KMT2A* [*MLL*] and *TCF3-HLF* fusions, near haploidy, low hypodiploidy, and iAMP21; MRD positive defined as ≥0.01% positive cells and MRD negative as <0.01% positive cells; MRD indeterminate includes samples with no targets for MRD assessment or targets were present but not sensitive enough, and samples where MRD was either not analysed or was not evaluable due to the sample being inadequate or having missing data. Abbreviations: ALL, Acute Lymphoblastic Leukemia; MRD, Minimal Residual Disease; WCC, White Cell Count.

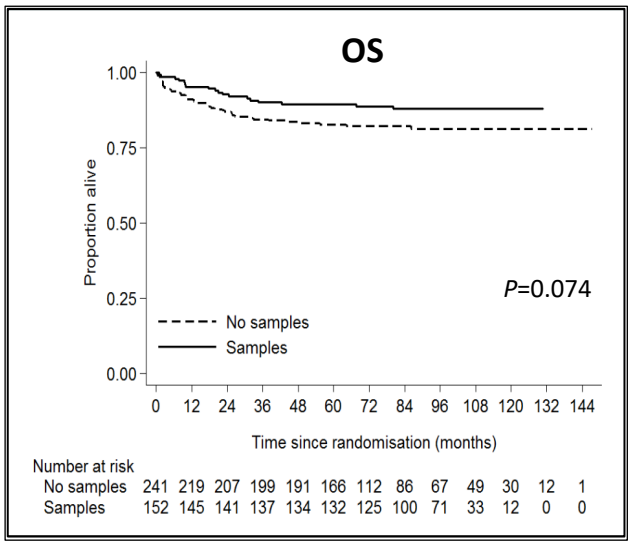
Figure S6. Schematic diagram of the UKALL2003 treatment regimens A, B and C. Regimen A: 3 drug induction with vincristine, dexamethasone, asparaginase followed by consolidation with daily mercaptopurine and central nervous system (CNS)-directed therapy with weekly intrathecal methotrexate. Interim Maintenance (IM): daily mercaptopurine, weekly methotrexate, monthly vincristine and corticosteroid pulses; Delayed Intensification (DI): asparaginase, vincristine, dexamethasone, doxorubicin, cyclophosphamide and cytarabine; Continuing Therapy: oral mercaptopurine and methotrexate, monthly vincristine, corticosteroid pulses, and intrathecal methotrexate every 3 months. Regimen B: 4 drug induction: daunorubicin in addition to dugs used in regimen A induction. Consolidation phase as in Regimen A with the addition of BFM, Berlin Frankfurt Munster (4 weeks of cyclophosphamide and cytarabine). Regimen C: Augmented consolidation by addition of 4 doses of vincristine and 2 doses of pegylated asparaginase during BFM consolidation. Capizzi maintenance as interim maintenance consisted of escalating doses of intravenous methotrexate without

folinic acid rescue, and vincristine and pegylated asparaginase. Abbreviations: MRD, minimal residual disease; CNS, central nervous system; IM, interim maintenance; DI, delayed intensification; BFM, Berlin Frankfurt Munster.

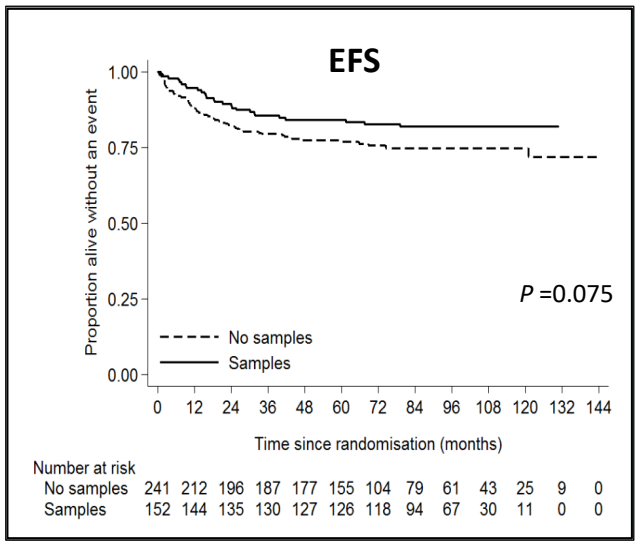
Further treatment regimen details available at Vora et al[5] and O'Connor et al.[6]

Figure S1

A



B



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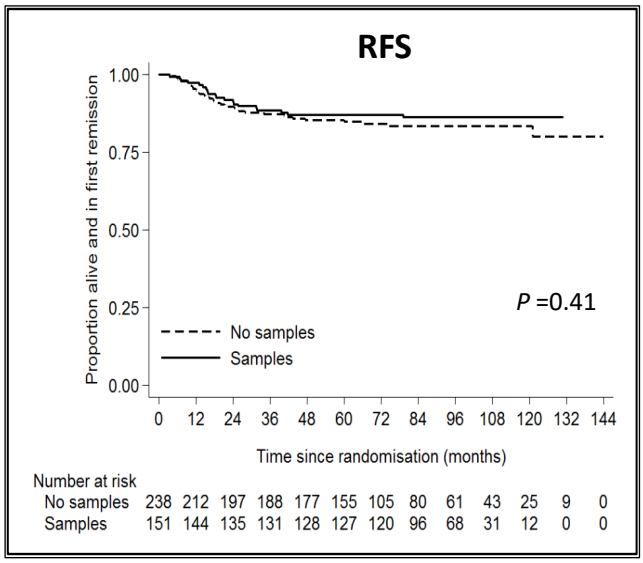


Figure S2

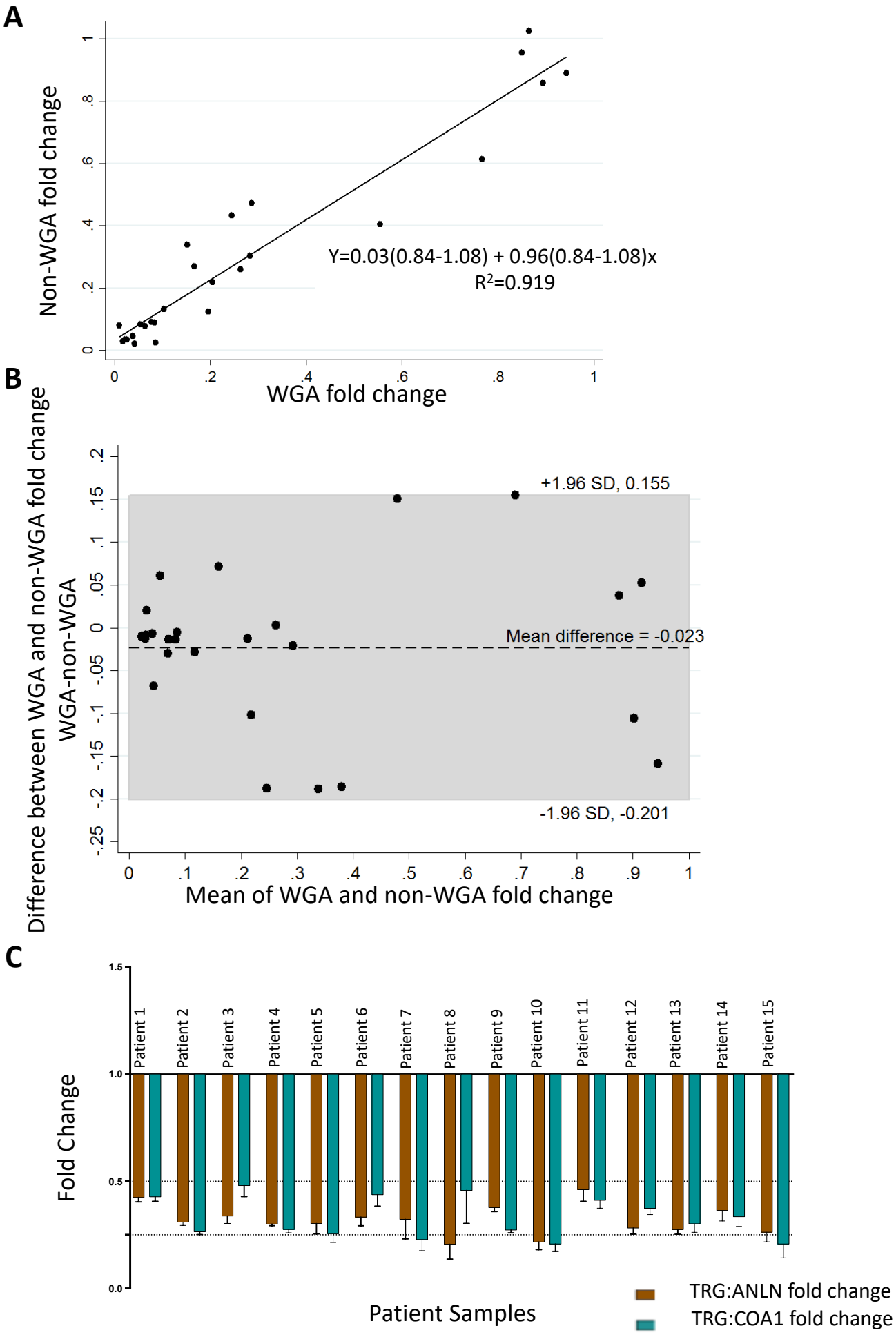
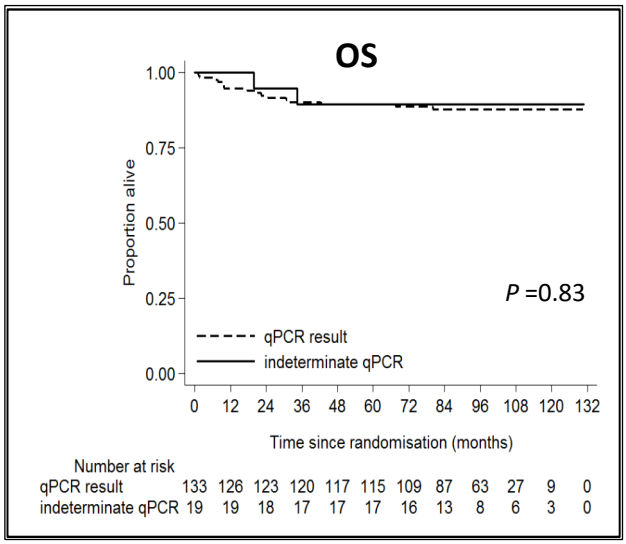
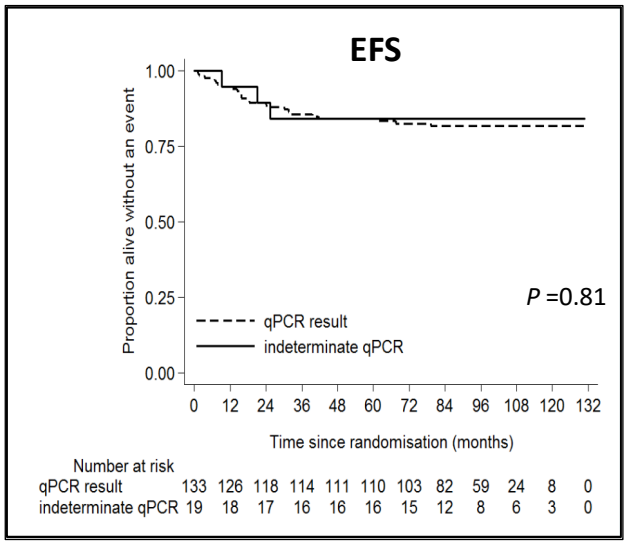

A
Non-WGA fold change
WGA fold change
Y=0.03(0.84-1.08) + 0.96(0.84-1.08)x
R²=0.919
B
Difference between WGA and non-WGA fold change
WGA-non-WGA
Mean of WGA and non-WGA fold change
+1.96 SD, 0.155
Mean difference = -0.023
-1.96 SD, -0.201
C
Fold Change
Patient Samples
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Patient 8
Patient 9
Patient 10
Patient 11
Patient 12
Patient 13
Patient 14
Patient 15
TRG:ANLN fold change
TRG:COA1 fold change

Figure S3

A



B



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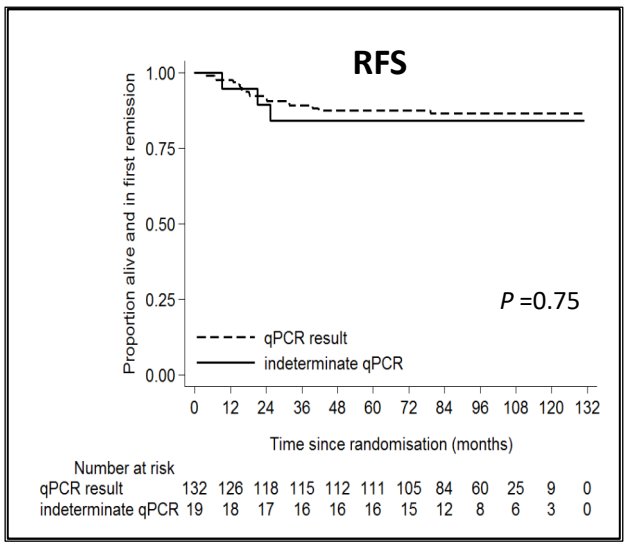


Figure S4

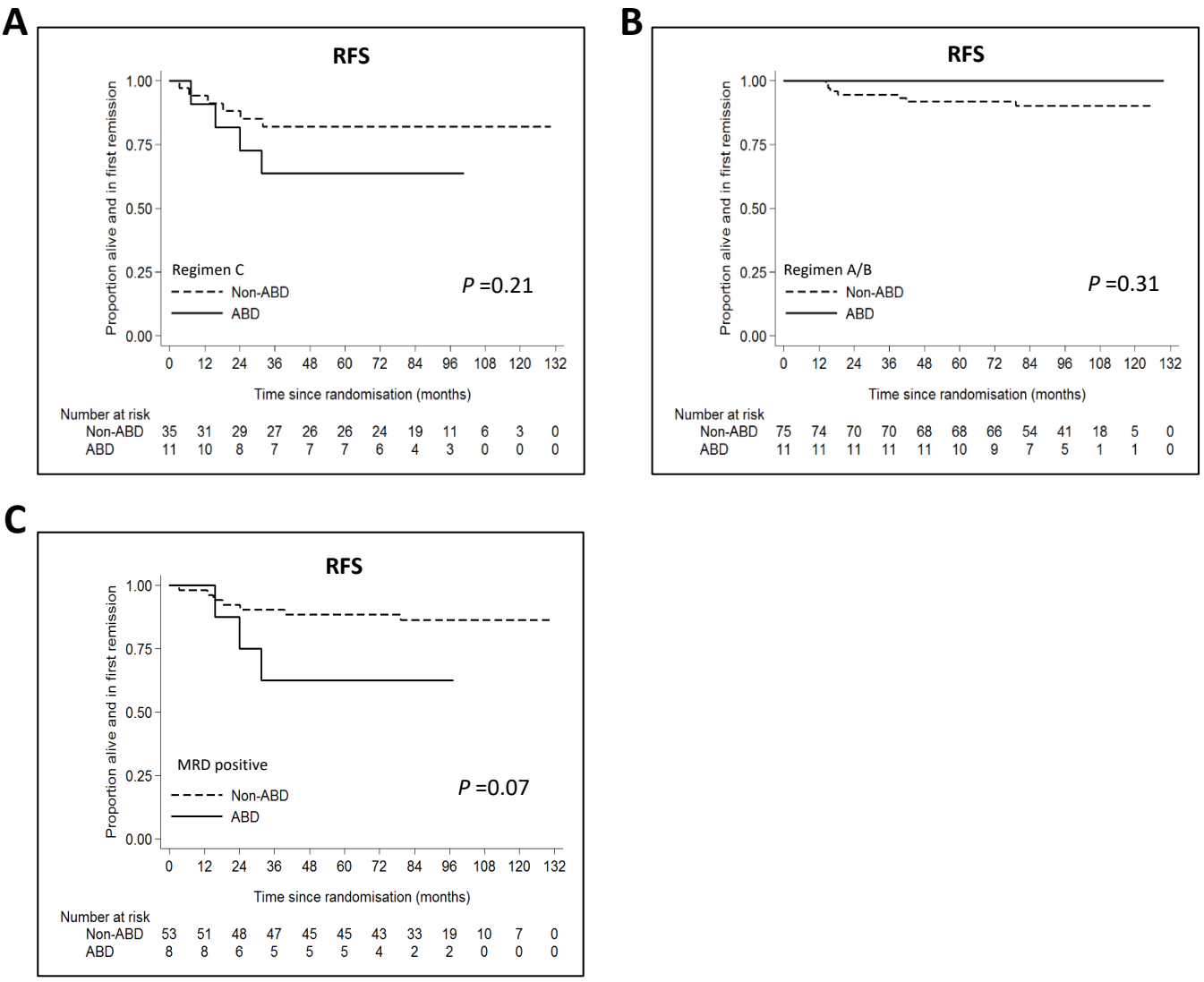
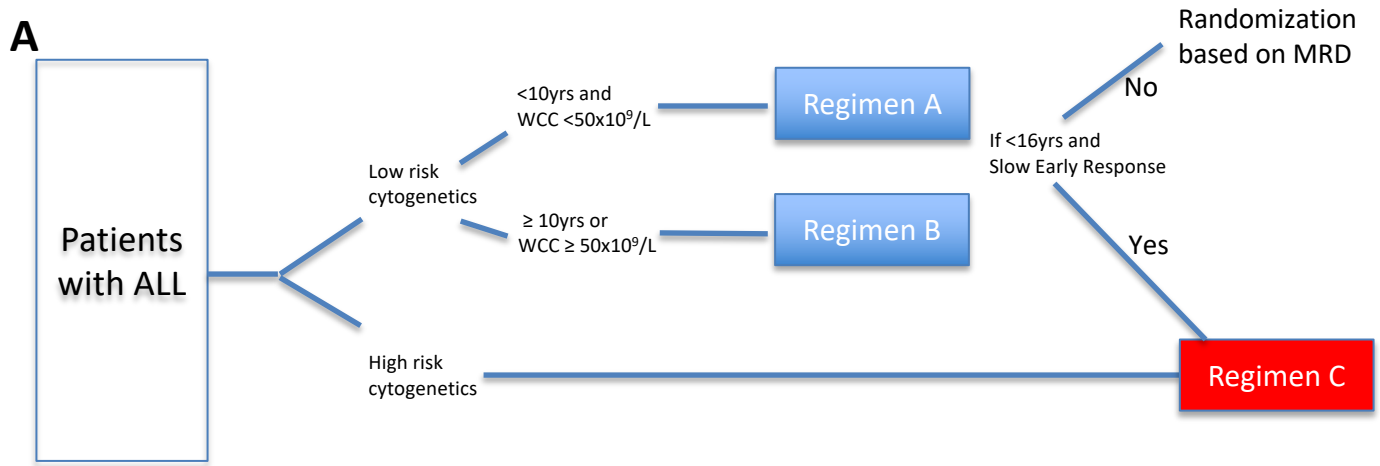
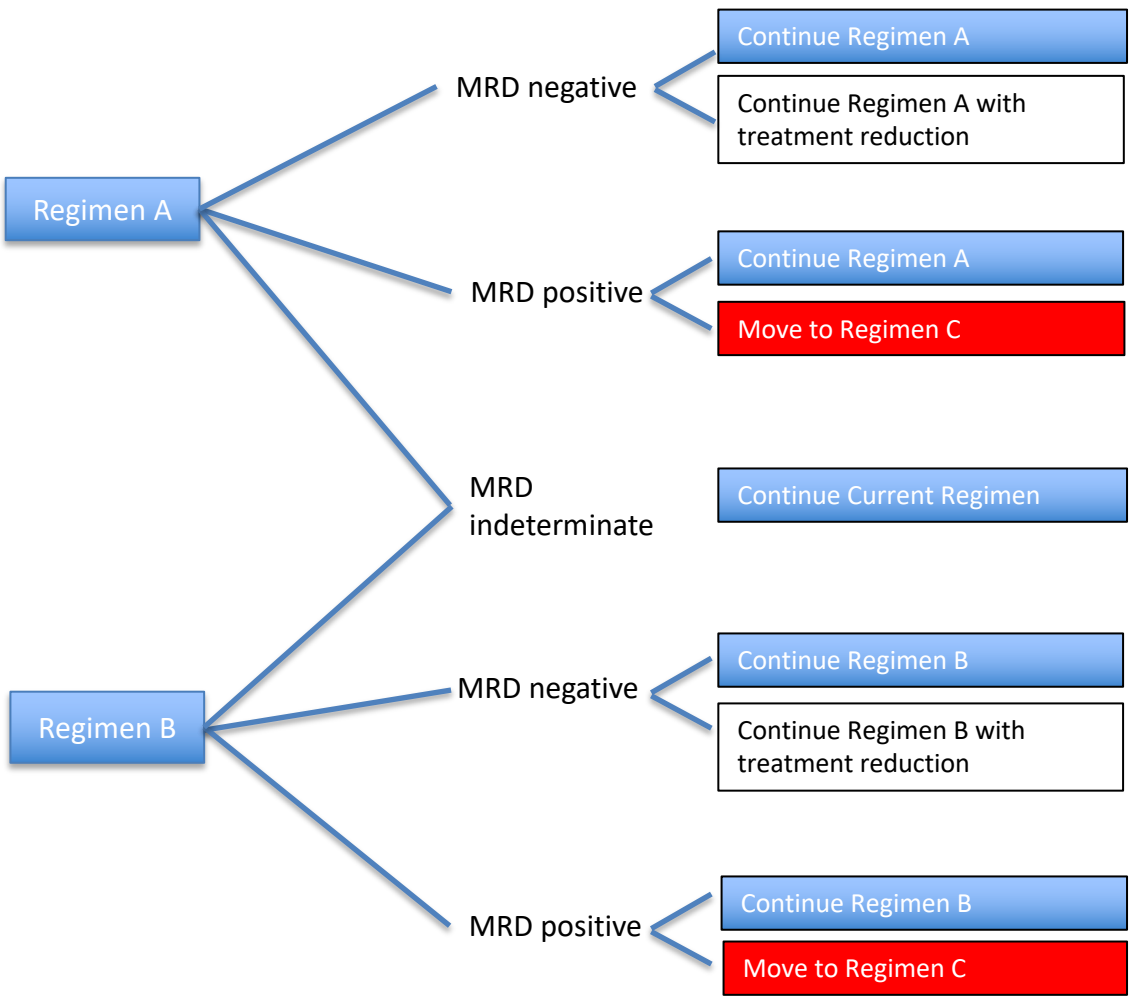

A
RFS
Proportion alive and in first remission
1.00
0.75
0.50
0.25
0.00
Regimen C
Non-ABD
ABD
P =0.21
0 12 24 36 48 60 72 84 96 108 120 132
Time since randomisation (months)
Number at risk
Non-ABD 35 31 29 27 26 26 24 19 11 6 3 0
ABD 11 10 8 7 7 7 6 4 3 0 0 0
B
RFS
Proportion alive and in first remission
1.00
0.75
0.50
0.25
0.00
Regimen A/B
Non-ABD
ABD
P =0.31
0 12 24 36 48 60 72 84 96 108 120 132
Time since randomisation (months)
Number at risk
Non-ABD 75 74 70 70 68 68 66 54 41 18 5 0
ABD 11 11 11 11 11 10 9 7 5 1 1 0
C
RFS
Proportion alive and in first remission
1.00
0.75
0.50
0.25
0.00
MRD positive
Non-ABD
ABD
P =0.07
0 12 24 36 48 60 72 84 96 108 120 132
Time since randomisation (months)
Number at risk
Non-ABD 53 51 48 47 45 45 43 33 19 10 7 0
ABD 8 8 6 5 5 5 4 2 2 0 0 0

Figure S5



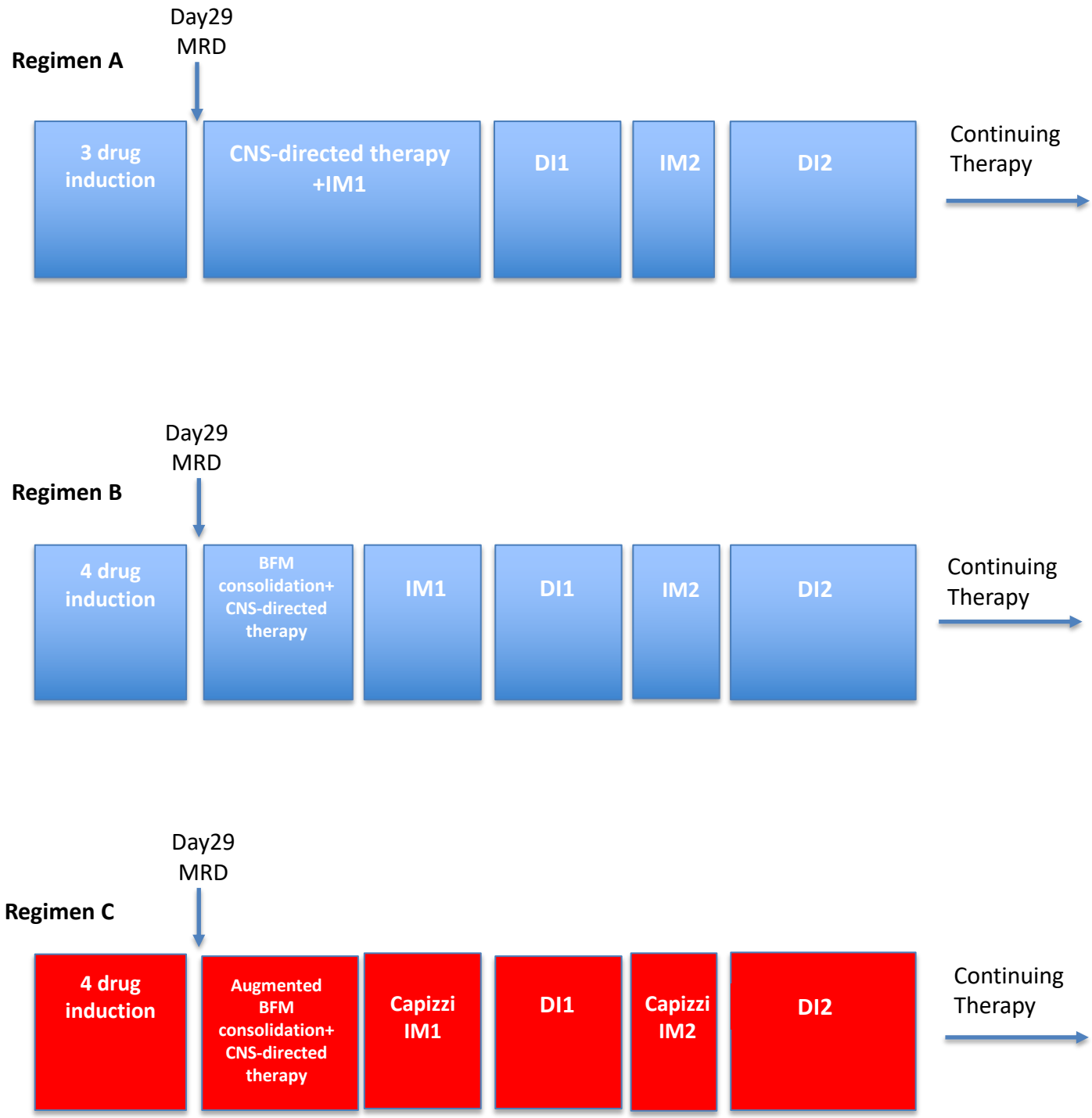
B

Treatment Intensity Decision points



Randomization based on MRD

Figure S6


Regimen A
Day29
MRD
3 drug induction
CNS-directed therapy +IM1
DI1
IM2
DI2
Continuing Therapy
Regimen B
Day29
MRD
4 drug induction
BFM consolidation+ CNS-directed therapy
IM1
DI1
IM2
DI2
Continuing Therapy
Regimen C
Day29
MRD
4 drug induction
Augmented BFM consolidation+ CNS-directed therapy
Capizzi IM1
DI1
Capizzi IM2
DI2
Continuing Therapy