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Prognostic impact of the absence of biallelic deletion at the *TRG* locus for pediatric patients with T-cell acute lymphoblastic leukemia treated on the MRC UKALL2003 trial

Nadine Farah[1], Amy A Kirkwood[2], Sunniyat Rahman[1], Theresa Leon[1], Sarah Jenkinson[1], Rosemary E Gale[1], Katharine Patrick[3], Jeremy Hancock[4], Sujith Samarasinghe[5], David C Linch[1], Anthony V Moorman[6], Nicholas Goulden[5], Ajay Vora[3,5] and Marc R Mansour[1]

[1]Department of Haematology, UCL Cancer Institute, London, UK

[2]Cancer Research UK & UCL Cancer Trials Centre, UCL, London, UK

[3]Department of Haematology, Sheffield Children's Hospital, Sheffield, UK

[4]Bristol Genetics Laboratory, Southmead Hospital, Bristol, UK

[5]Department of Haematology, Great Ormond Street Hospital, London, UK

[6]Leukaemia Research Cytogenetics Group, Northern Institute for Cancer Research, Newcastle, UK

Running head: Prognostic Impact of ABD in T-ALL in UKALL2003

Contact information for correspondence:

Dr. Marc R Mansour, UCL Cancer Institute, 72 Huntley St, London WC1E 6DD, UK

m.mansour@ucl.ac.uk

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Risk-stratification and treatment intensification according to minimal residual disease (MRD) analysis has improved outcomes of patients with acute lymphoblastic leukemia (ALL).[1,2] However, a significant proportion of patients with T-cell ALL (T-ALL) still experience early relapse or refractory disease. Robust prognostic markers able to identify high-risk patients at diagnosis have proved challenging, and risk-adapted management for T-ALL remains an unmet need. The work presented here shows that the molecular marker, absence of biallelic deletion at the *TRG* locus, does not have a poor prognostic impact on the outcome of pediatric and adolescent patients with T-ALL treated on the risk-directed protocol of the MRC UKALL2003 trial.

Early T-cell Precursor (ETP) T-ALL identified by immunophenotyping was previously reported to confer a poor prognosis in pediatric, adolescent and adult patients.[3,4] However, immunophenotyping was found to underestimate the number of patients with an ETP gene-expression signature,[5] and inter-laboratory diagnosis for this immunophenotype is not standardized. Moreover, gene-expression profiling is not widely utilized for clinical use. Therefore, an alternative molecular method based on the V-J recombination status of the T-cell receptor gamma (*TRG)* gene has been described.[6] Given that *TRG* recombination occurs early during T-cell development,[7] cells that have not undergone biallelic deletion at the *TRG* locus have been termed 'ABD' (Absence of Biallelic Deletion), with the majority of such ABD T-ALL cases having an ETP-ALL gene-expression signature.[6] Consistent with the original reports of ETP-ALL,[3] ABD was reported to be associated with inferior survival,[6] although the patients studied were not treated on MRD risk-directed protocols. Thus we investigated whether ABD status adds further prognostic information for pediatric and

adolescent patients with T-ALL treated on the MRC UKALL2003 trial that used MRD risk to direct treatment intensity.[1,8]

Whole genome amplified (WGA) DNA was available from diagnostic samples of 152 of 393 (39%) T-ALL patients treated on this trial.[9] Full details of the patient cohort, trial protocols, methods and statistical analyses are described in the Supplementary Information. The trial is registered at http://www.controlled-trials.com under ISRCTN number 07355119. Ethical approval for the trial was obtained previously from the Scottish Multi-Centre Research Ethics Committee on 25/02/2003, ref: 02/10/052, and samples were collected with informed consent according to the Declaration of Helsinki.

Baseline characteristics and survival outcomes of these 152 patients were not significantly different from the 241 patients not included in the study (Tables S1-2, Figure S1).

The *TRG* quantitative polymerase chain reaction (qPCR) assay determined fold change using the comparative ΔΔCT method with *ANLN* as the reference gene (TRG:ANLN), as previously described.[6] There was good agreement between results from WGA material and their corresponding non-WGA sample where available (r^2=0.92) (Figure S2A-B). Patients were assigned to the ABD group if the TRG:ANLN fold change was ≥0.5 and the diagnostic blast count ≥50% (to exclude lack of deletion due to contamination with non-leukemic cells), non-ABD if ≤0.25, and indeterminate if between these values (Figure 1A). Indeterminate results were

confirmed using *COA1* as an alternative reference gene (Figure S2C). *TRG* locus deletions were also determined using data from Illumina CytoSNP-850K arrays.[9]

By qPCR, 23 of 152 samples (15%) were classified as ABD, 110 (72%) as non-ABD, and 19 (13%) were indeterminate (16 with fold change 0.26-0.49; 3 with fold change >0.5 but blast count <50%) (Figure 1A). Baseline characteristics and survival of the 19 patients with indeterminate status were similar to the other 133 patients (Tables S3-4, Figure S3). Baseline characteristics of the 133 patients according to ABD status are outlined in Table 1. Of these, 118 also had SNP array results at the *TRG* locus that were concordant with the qPCR findings (Figure 1 B-C), including 22 ABD patients; array data was uninterpretable or not available for the remaining 15 cases.

However, there was no statistically significant difference in overall survival (OS) between the ABD and non-ABD groups (5-year OS, 87% vs 90% respectively; hazard ratio (HR) 1.67, 95% confidence intervals 0.54–5.17; *P*=0.37) (Figure 2A; Table S5). There was also no significant difference in the relapse-free survival (RFS) (82% vs 89%; HR 1.58, 0.52–4.86; *P*=0.42) (Figure 2B; Table S5). Although there was a trend towards an inferior event-free survival (EFS) in the ABD group, this did not reach statistical significance (78% vs 85%; HR 2.12, 0.88–5.12; *P*=0.09) (Figure 2C; Table S5), and was impacted by 7 non-relapse events in the ABD group (1 infection-related death at induction, 4 deaths from causes other than ALL and 2 second malignancies).

We have previously reported that patients treated on this trial with T-ALL and *NOTCH1*/*FBXW7* double-mutant status had excellent OS (100%).[10] However, the comparable survival of the ABD and non-ABD groups in our cohort could not be

explained by this molecular subtype, since only 2 of the 23 ABD patients (9%) had *NOTCH1*/*FBXW7* double-mutant status.

There was, however, a significant association between MRD levels and ABD status. Only 9% of the ABD patients were MRD-negative at day 29 compared with 35% of the non-ABD patients (P=0.01, Fisher's exact test). Also, patients with ABD status and MRD results had higher median MRD levels compared with those with non-ABD status (2.519% vs 0.0173% positive cells; P=0.03) (Figure 2D), suggesting that ABD status may highlight a high-risk group that is already identified by high MRD levels.

Moreover, more than 3 times as many ABD patients had an MRD indeterminate status compared to the non-ABD group (57% vs 16%; P<0.001, Mantel-Haenszel test) (Table 1). This was mainly due to the fact that a higher proportion of ABD patients lacked MRD targets at diagnosis than the non-ABD patients (38% vs 1% respectively; P<0.001, Fisher's exact test). MRD was evaluated by real-time quantitative PCR analysis of the T-cell receptor gene rearrangement, and failure to detect a gene rearrangement target as an MRD marker in the ABD group is consistent with the same underlying biology as that of the ABD status, where developmental arrest occurs prior to VJ recombination. To address whether ABD status might be a useful alternative prognostic marker in this MRD indeterminate group, we analyzed outcome in this subgroup of patients. Our results show that in the MRD indeterminate cases who were eligible for RFS analysis, only 3 of 18 non-ABD (17%) and 1 of 12 ABD (8.3%) relapsed. Notably, MRD indeterminate status itself directed treatment intensity as these patients were not eligible on the trial for randomization to reduction of chemotherapy intensity. Thus, numbers are too small to make firm conclusions on the

additional prognostic significance of ABD in this subgroup, which would need to be addressed in a larger prospective trial.

Our data is in marked contrast to the results from another study where the ABD subgroup, identified using the same *TRG* qPCR assay, was associated with a dismal outcome (5-year OS: 25% vs 72% in the ABD and non-ABD groups respectively).[6] However, these patients were not treated using MRD-directed therapy, suggesting that differences in treatment protocols may impact on prognosis of this subgroup. Within the ABD group of our cohort, 11 patients (48%) received the more intensive chemotherapy arm, Regimen C, including all the 8 MRD-positive patients, although none of the ABD patients proceeded to an allogeneic stem cell transplant in first remission. Notably, there was no statistical difference in the RFS of ABD and non-ABD patients treated on Regimen C (P=0.21) (Figure S6A, Table S6). The RFS for ABD patients treated on Regimens A or B was 100%, although it should be noted that none of them were MRD-positive (Figure S6B, Table S6). There was a trend towards an increased risk of relapse in MRD-positive ABD patients when compared to MRD-positive non-ABD patients (HR 3.22, 0.83–12.52; P=0.07) (Figure S6C), which might relate to higher median MRD levels.[11]

The comparable survival of the ABD and non-ABD groups treated on the UKALL2003 trial is consistent with the outcome reported for patients on this trial according to their ETP status by immunophenotyping[12]. Moreover, the comparable survival between these two groups is also consistent with the outcome reported for ETP and non-ETP patients from other pediatric MRD risk-directed studies.[13,14] In addition to this, our findings are similar to those recently reported for ABD status in

adult T-ALL patients treated using response-based risk stratification and therapy intensification, including allogeneic stem cell transplantation.[15]

In conclusion, our data indicate that in pediatric/adolescent T-ALL, ABD status does not add further prognostic information nor justify treatment escalation beyond what can already be inferred by MRD analysis using a risk-adapted protocol.

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Table 1: Baseline characteristics, treatment allocation, response and molecular characterization of the ABD and non-ABD groups.

	Non-ABD N=110	ABD N=23	*P*
Baseline characteristics			
Sex, N (%)			
Male	88 (80.0)	16 (69.6)	0.27
Female	22 (20.0)	7 (30.4)	
WBC count (x10^9 per L), N (%)			
<50	24 (21.8)	15 (65.2)	<0.001
≥50	86 (78.2)	8 (34.8)	
Age, N (%)			
<10 years	64 (58.2)	8 (34.8)	0.041
≥10 years	46 (41.8)	15 (65.2)	
NCI risk group, N (%)			
Standard	10 (9.1)	4 (17.4)	0.26*
High	100 (90.9)	19 (82.6)	
CNS disease at diagnosis, N (%)			
No	102 (92.7)	23 (100.0)	0.35*
Yes	8 (7.3)	0	
Treatment Allocation and Response			
Final treatment given, N (%)			
A	8 (7.3)	3 (13.0)	0.12*
B	67 (60.9)	9 (39.1)	
C	35 (31.8)	11 (47.8)	
Slow early Response, N (%)			
No	74 (67.3)	11 (47.8)	0.14§
Yes	25 (22.7)	7 (30.4)	
Unknown#	11 (10.0)	5 (21.7)	
MRD, N (%)			
Negative (<0.01% positive cells)	39 (35.5)	2 (8.7)	<0.001Ψ
Positive (≥0.01% positive cells)	53 (48.2)	8 (34.8)	
Indeterminate	18 (16.4)	13 (56.5)	
No targets	*1*	*8*	
Targets not sensitive enough	*9*	*2*	
Not evaluableθ	*6*	*2*	
OtherΩ	*2*	*1*	
Molecular Characteristics			
NOTCH1/FBXW7, N (%)			
Wild type	33 (30.0)	14 (60.9)	0.011
Single Mutant	43 (39.1)	7 (30.4)	
Double Mutant	34 (30.9)	2 (8.7)	
PTEN, N (%)			
Wild type	77 (74.8)	17 (81.0)	0.55
Mutant	26 (25.2)	4 (19.1)	
RAS, N (%)			
Wild type	100 (90.9)	20 (87.0)	0.70*
Mutant	10 (9.1)	3 (13.0)	

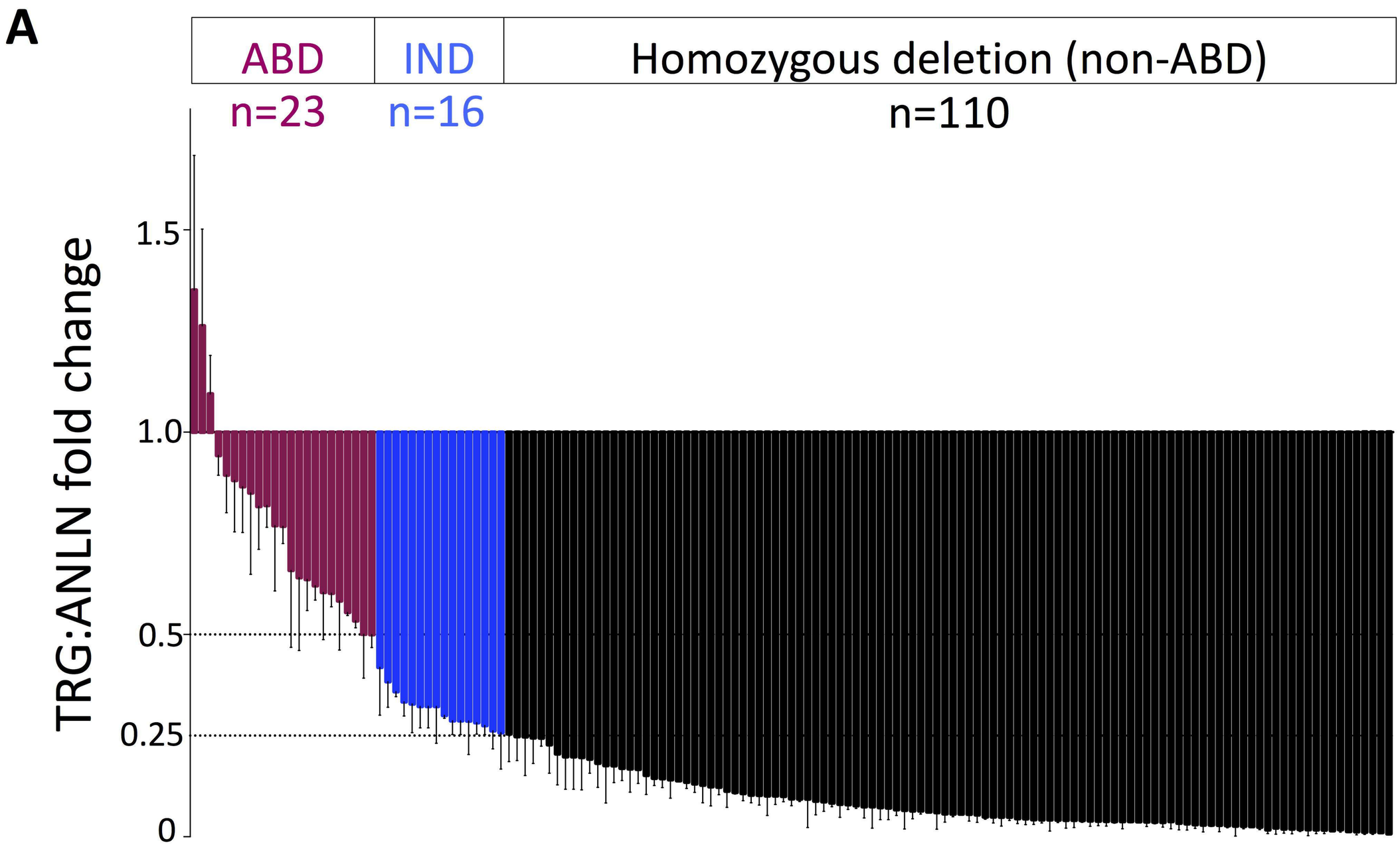
P values derived using Chi-squared tests unless otherwise indicated. *Fisher's exact test. #Patients without bone marrow results at day 8 or 15 (assumed to be RER for treatment escalation) and one patient with conflicting slow early response data and bone marrow results. §Excluding the unknown group: $P = 0.26$. ΨExcluding the indeterminate group: $P = 0.31$*.θ Induction death (n=1), Inadequate diagnostic day 29 sample (n=7). ΩNot analysed (n=1), reason missing (n=2). WBC, white blood cell count; NCI, National Cancer Institute; CNS, central nervous system; MRD, minimal residual disease.

Figure Legends

Figure 1. qPCR and SNP array analysis at the *TRG* locus. (A) ABD status by qPCR TRG:ANLN fold change of WGA diagnostic samples from 149 patients. Three patients with an indeterminate (IND) result are not shown as their blast counts were <50%. (B) Representative Log-R ratio plot at the *TRG* locus (GRCh37/hg19 chr7:37868112-38678273) from CytoSNP-850K arrays for an ABD and (C) a non-ABD patient. Location of the *V-J* region amplified in the qPCR assay is shown.

Figure 2. Outcome and MRD-positivity in patients grouped according to their ABD status. Kaplan-Meier curves for (A) OS and (B) RFS (C) EFS (D) Scatter-plot showing percentage of MRD-positive cells at day 29. Patients with ≥0.01% positive cells were classified as MRD-positive. Red line: median level.

Figure 1


A
ABD
IND
Homozygous deletion (non-ABD)
n=23
n=16
n=110
TRG:ANLN fold change
1.5
1.0
0.5
0.25
0

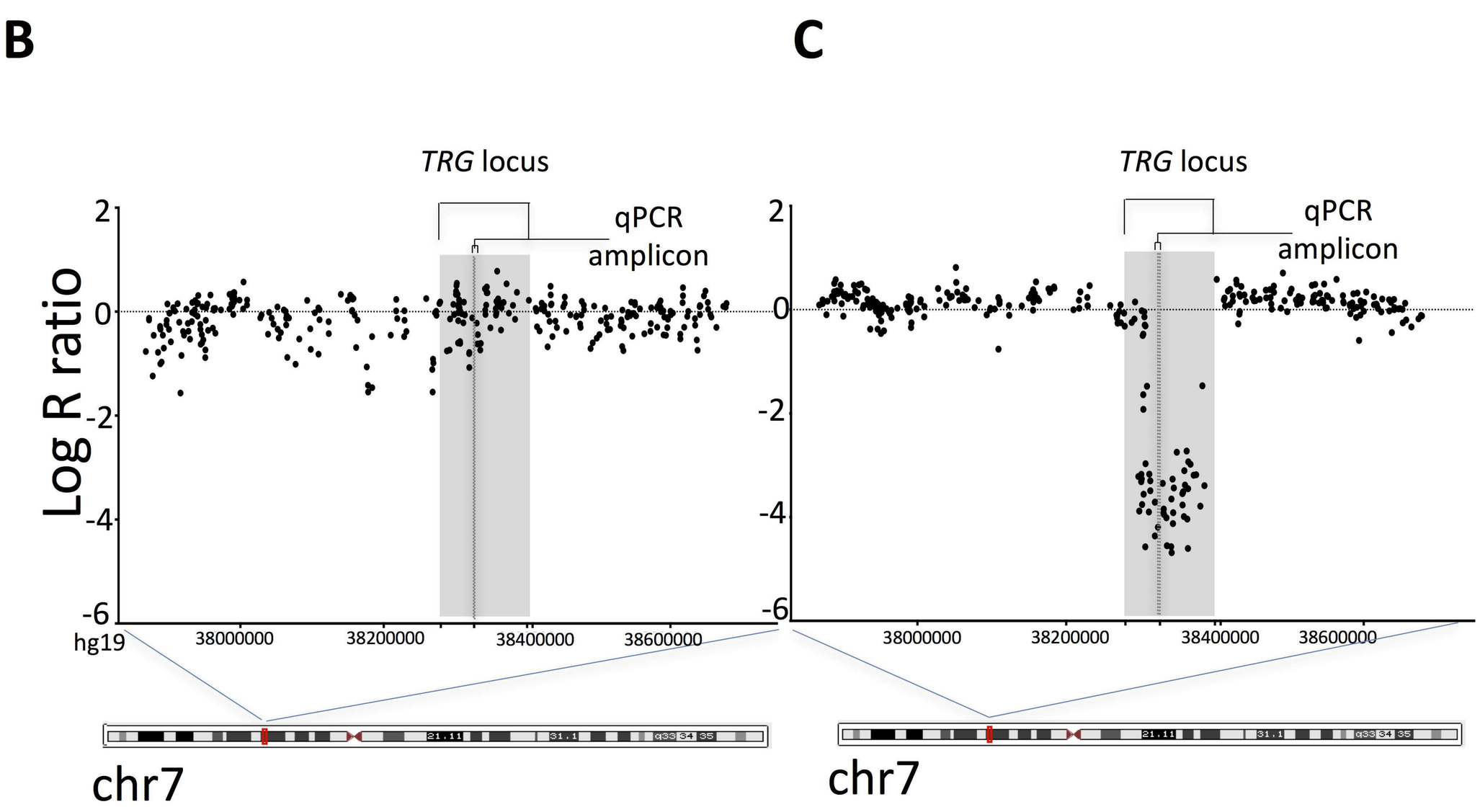
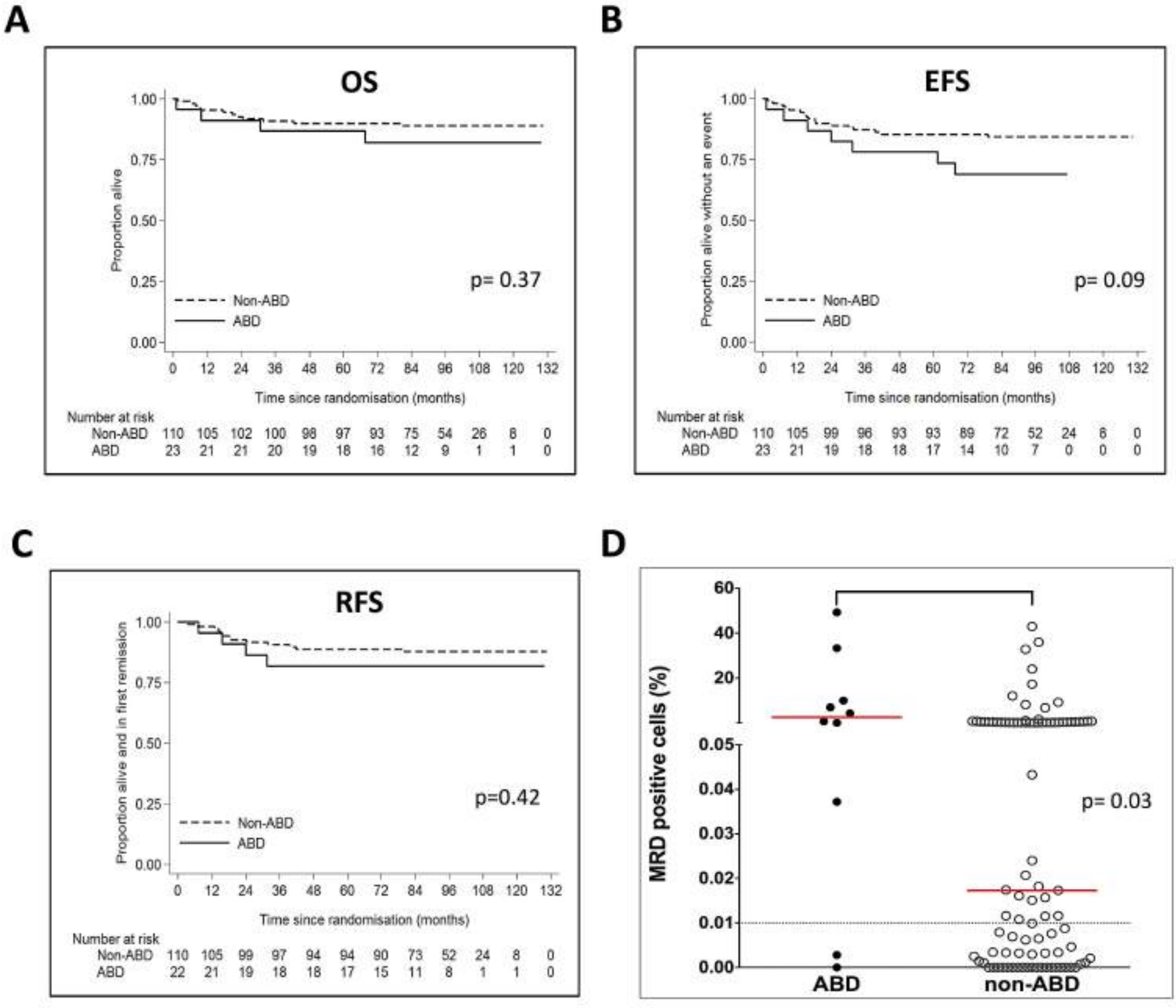

B
C
TRG locus
qPCR amplicon
Log R ratio
2
0
-2
-4
-6
hg19
38000000
38200000
38400000
38600000
chr7

Figure 2


A
OS
Proportion alive
1.00
0.75
0.50
0.25
0.00
p= 0.37
Non-ABD
ABD
0 12 24 36 48 60 72 84 96 108 120 132
Time since randomisation (months)
Number at risk
Non-ABD 110 105 102 100 98 97 93 75 54 26 8 0
ABD 23 21 21 20 19 18 16 12 9 1 1 0
B
EFS
Proportion alive without an event
1.00
0.75
0.50
0.25
0.00
p= 0.09
Non-ABD
ABD
0 12 24 36 48 60 72 84 96 108 120 132
Time since randomisation (months)
Number at risk
Non-ABD 110 105 99 96 93 93 89 72 52 24 8 0
ABD 23 21 19 18 18 17 14 10 7 0 0 0
C
RFS
Proportion alive and in first remission
1.00
0.75
0.50
0.25
0.00
p=0.42
Non-ABD
ABD
0 12 24 36 48 60 72 84 96 108 120 132
Time since randomisation (months)
Number at risk
Non-ABD 110 105 99 97 94 94 90 73 52 24 8 0
ABD 22 21 19 18 18 17 15 11 8 1 1 0
D
MRD positive cells (%)
60
40
20
0.05
0.04
0.03
0.02
0.01
0.00
p= 0.03
ABD
non-ABD

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 Figure S4-5. 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 S4).

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).

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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. 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.

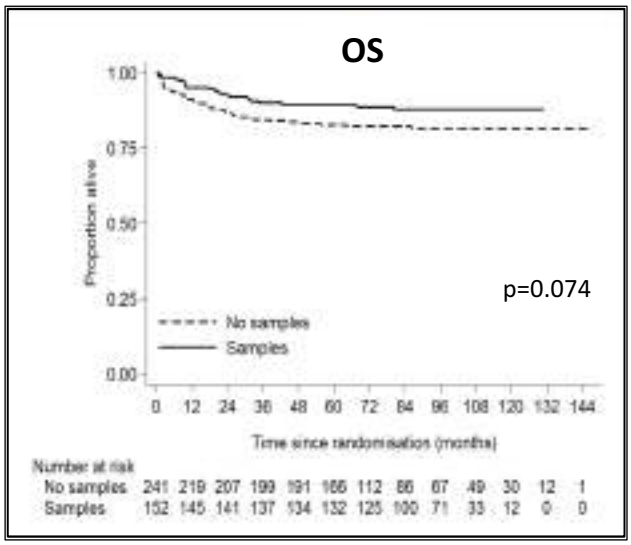
Figure S5. Schematic diagram of the UKALL2003 treatment regimens A, B and C. Regimen A: 3 drug induction with vincristine, dexamethasone, asparaginase followed by consolidation with daily mercaptopurine and central nervous system (CNS)-directed therapy with weekly intrathecal methotrexate. Interim Maintenance (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]

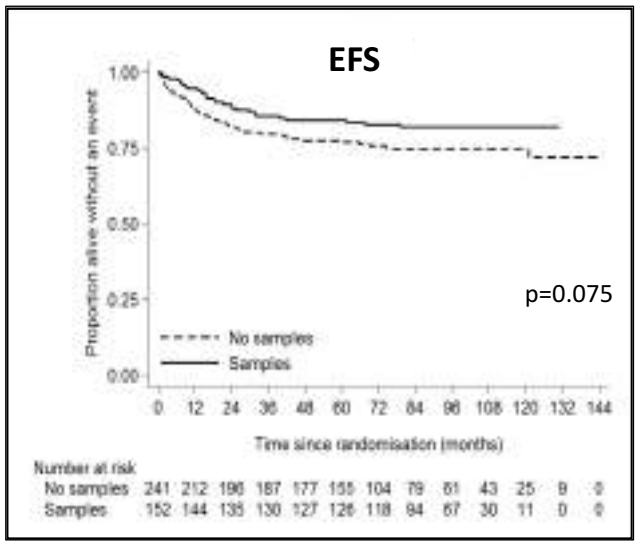
Figure S6. Kaplan-Meier survival curves grouped according to ABD status for patients on different treatment regimens. Relapse-Free Survival for patients on (A) Regimen C, (B) Regimen A or B, and (C) those with positive MRD regardless of treatment regimen.

Figure S1

A



B



C

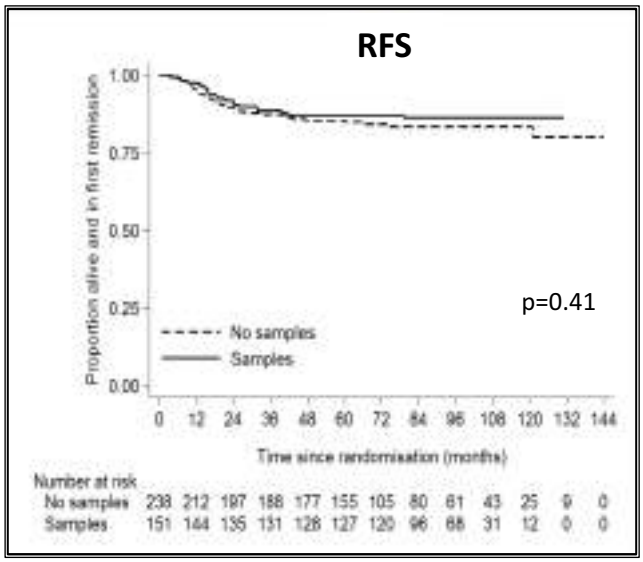


Figure S2

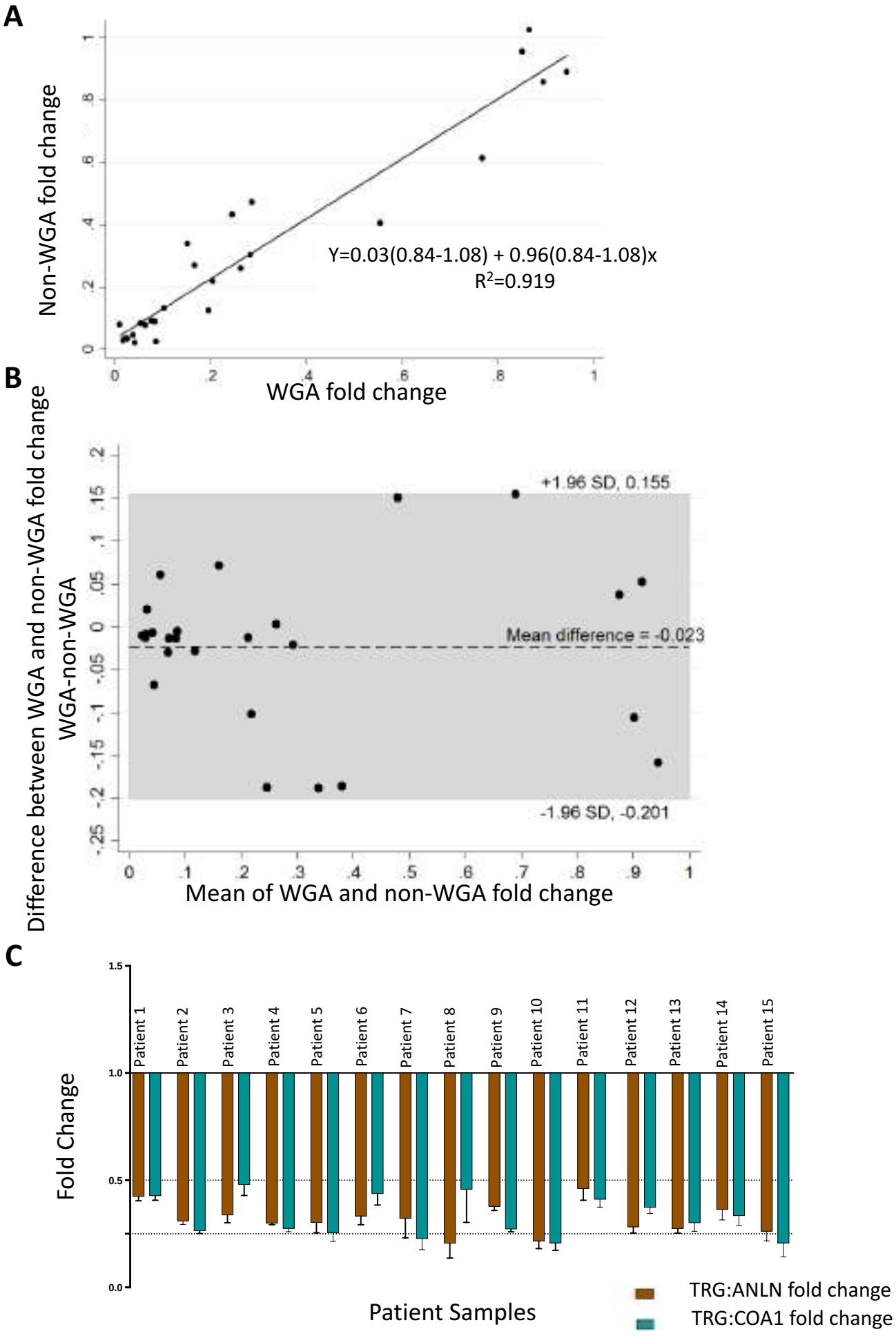
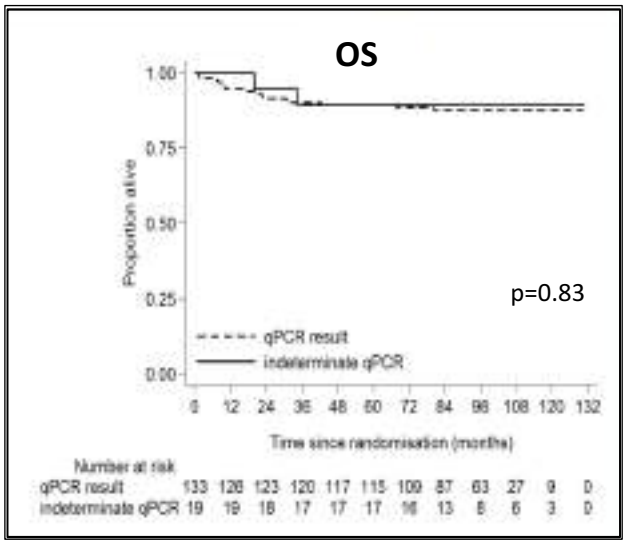
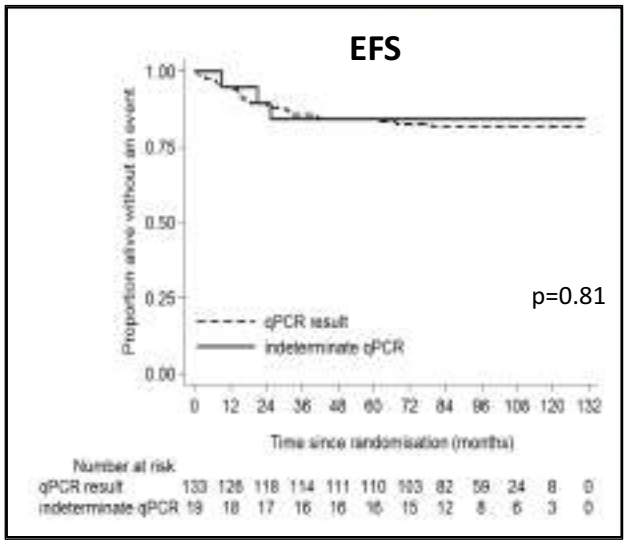

A
Non-WGA fold change
WGA fold change
Y=0.03(0.84-1.08) + 0.96(0.84-1.08)x
R2=0.919
B
Difference between WGA and non-WGA fold change
WGA-non-WGA
+1.96 SD, 0.155
Mean difference = -0.023
-1.96 SD, -0.201
Mean of WGA and non-WGA fold change
C
Fold Change
Patient 1
Patient 2
Patient 3
Patient 4
Patient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

Figure S3

A



B



C

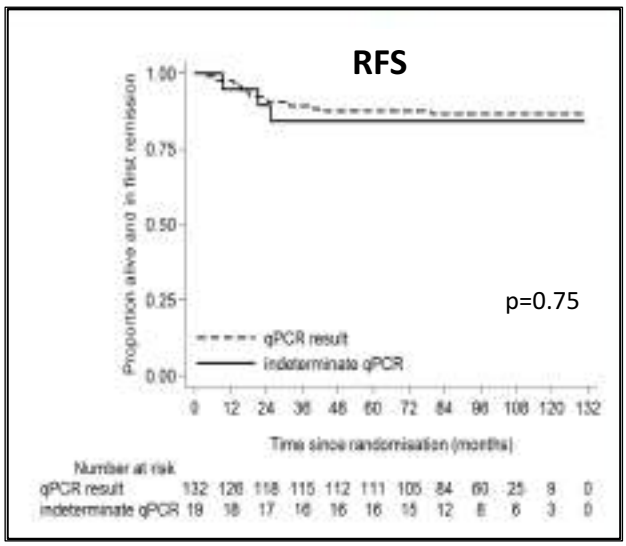
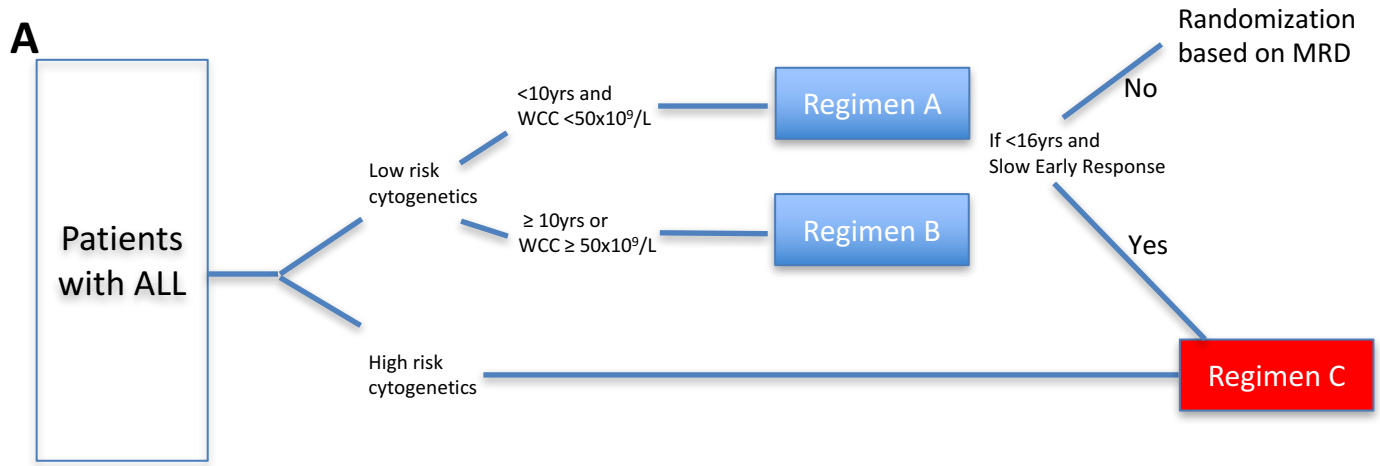


Figure S4


A
Patients with ALL
Low risk cytogenetics
<10yrs and WCC <50x10⁹/L
Regimen A
≥ 10yrs or WCC ≥ 50x10⁹/L
Regimen B
High risk cytogenetics
Regimen C
If <16yrs and Slow Early Response
No
Randomization based on MRD
Yes

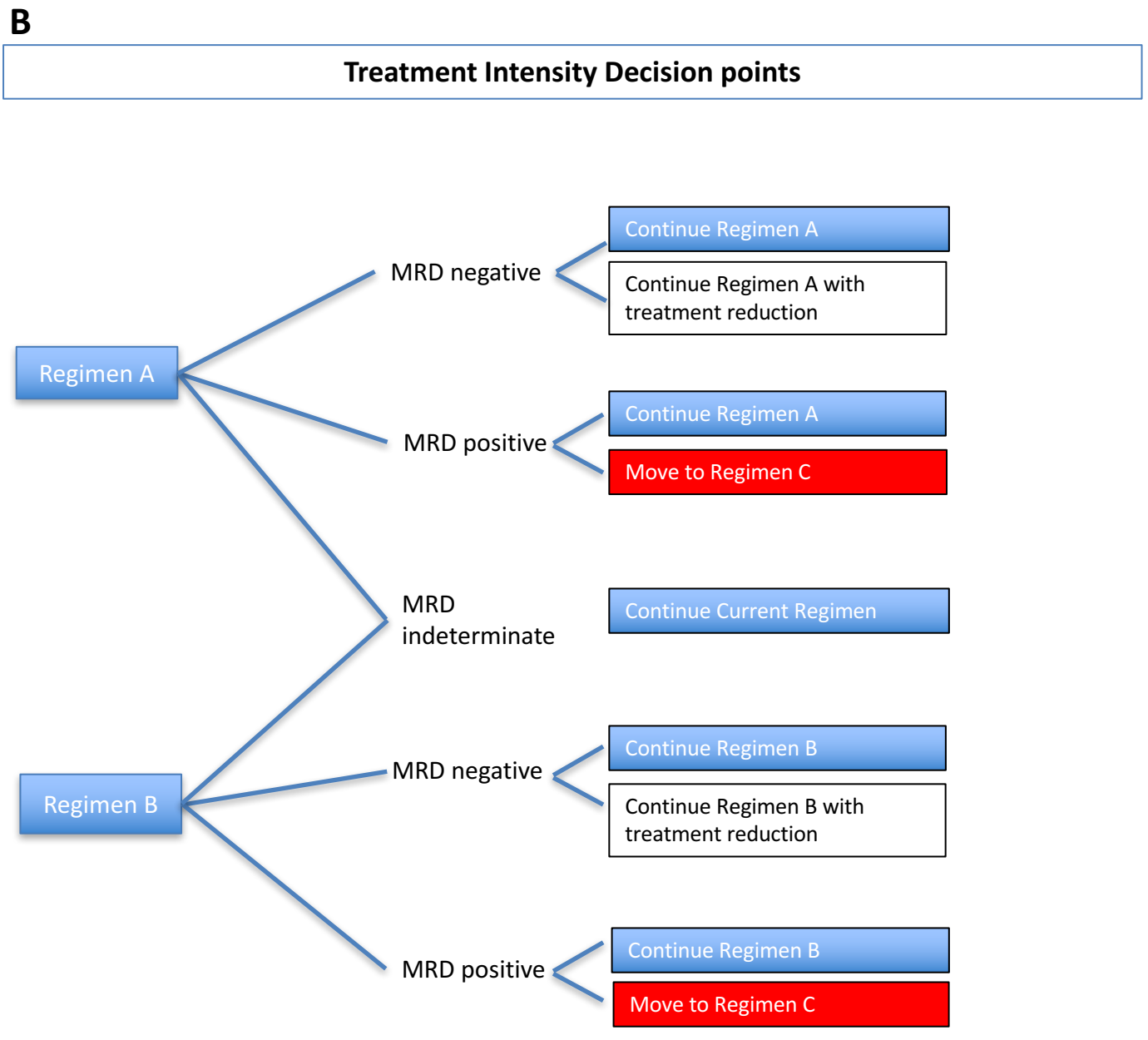

B
Treatment Intensity Decision points
Regimen A
MRD negative
Continue Regimen A
Continue Regimen A with treatment reduction
MRD positive
Continue Regimen A
Move to Regimen C
MRD indeterminate
Continue Current Regimen
Regimen B
MRD negative
Continue Regimen B
Continue Regimen B with treatment reduction
MRD positive
Continue Regimen B
Move to Regimen C
Randomization based on MRD

Figure S5

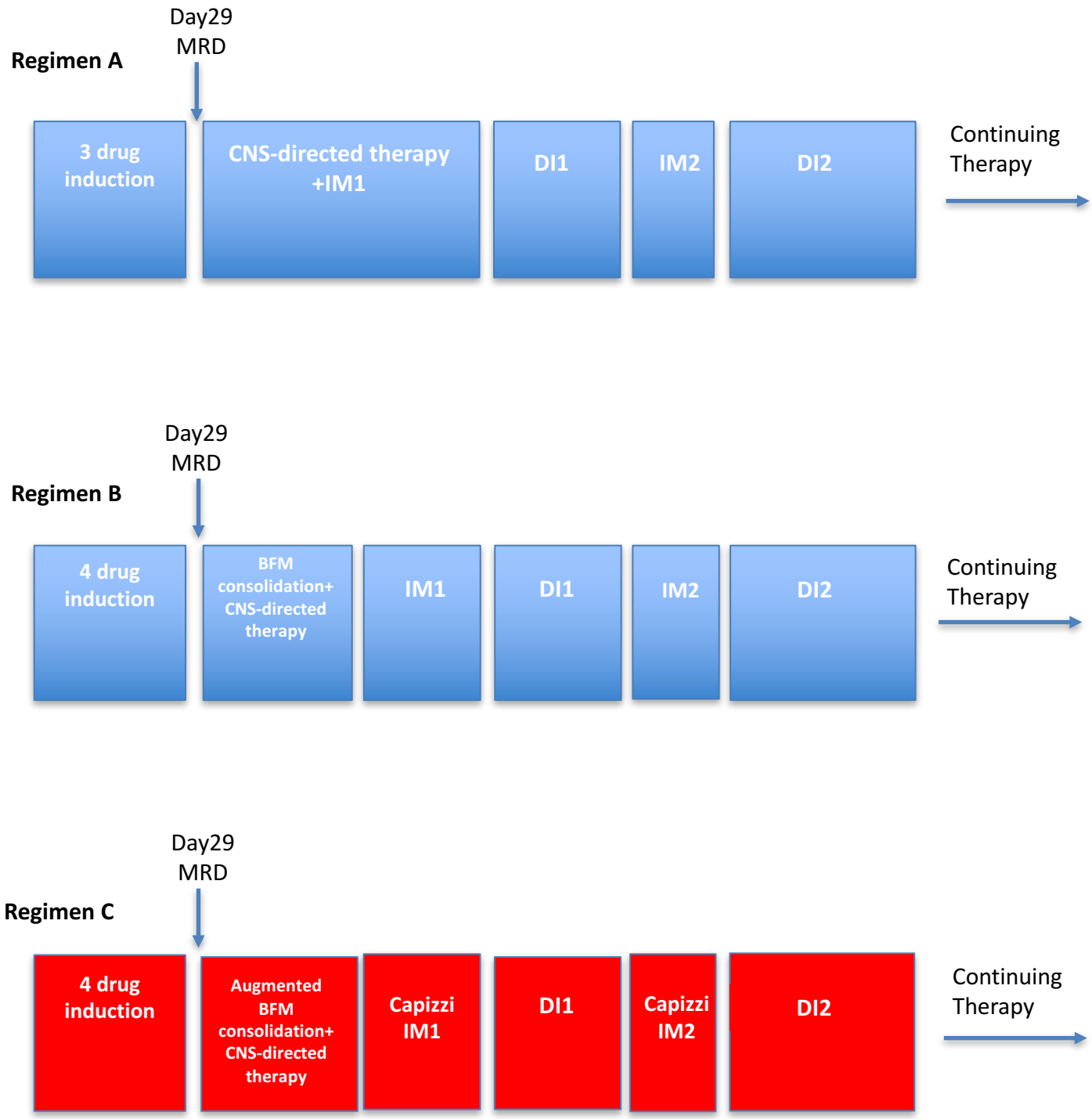
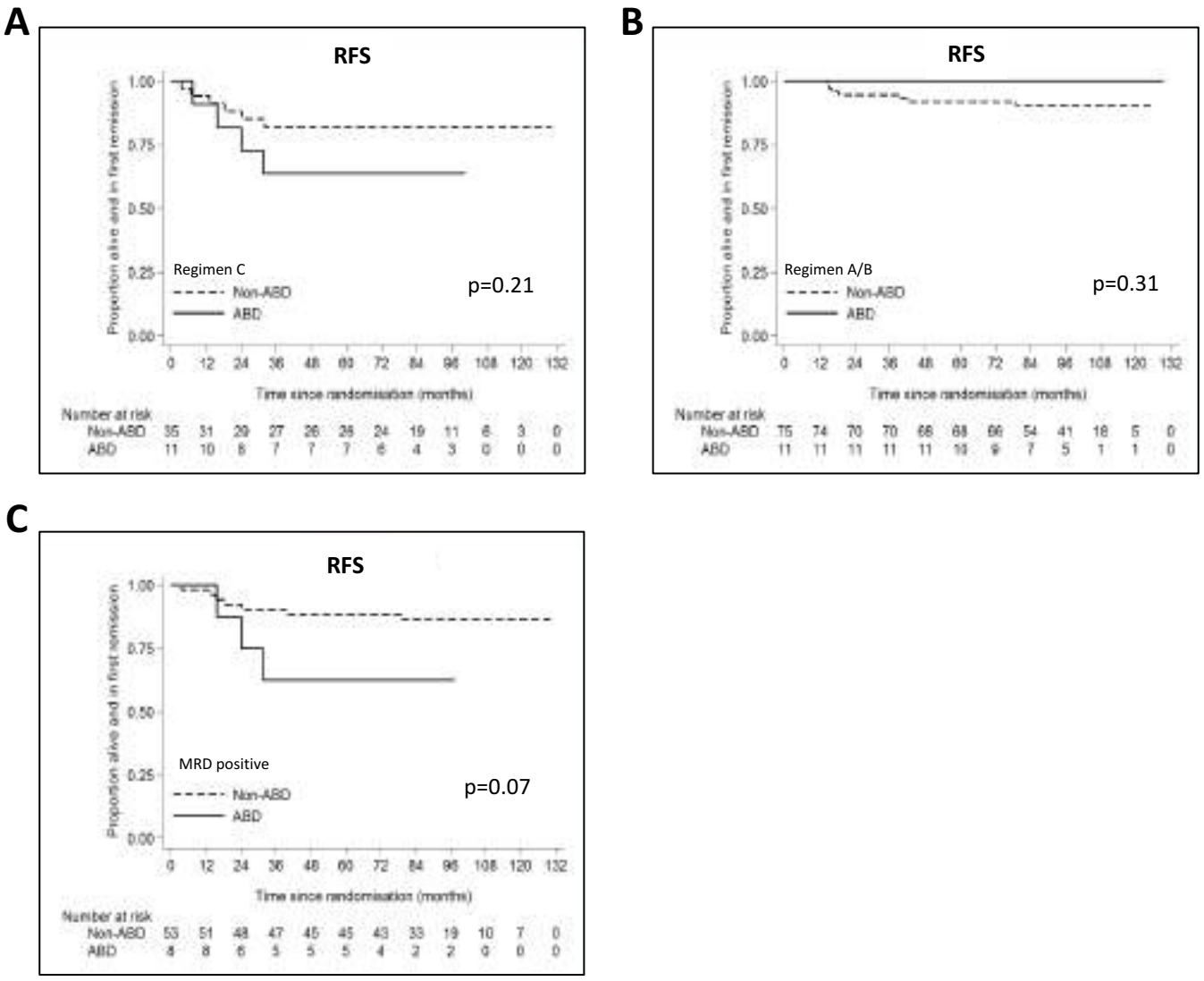

Regimen A
Day29
MRD
3 drug induction
CNS-directed therapy +IM1
DI1
IM2
DI2
Continuing Therapy
Regimen B
Day29
MRD
4 drug induction
BFM consolidation+ CNS-directed therapy
IM1
DI1
IM2
DI2
Continuing Therapy
Regimen C
Day29
MRD
4 drug induction
Augmented BFM consolidation+ CNS-directed therapy
Capizzi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

Figure S6


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 66 66 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