Supplementary Methods

Statistics with Supplementary Information

Statistical additional considerations

Data was extracted from trial database on 28th of March 2023. Median follow-up for censored patients in each comparison is as follows: DA vs DAC, 5 years vs 4.9 years, DA vs FLAG-Ida, 4.1 years vs 4.0 years.

Toxicity (hematologic recovery times and non-hematologic toxicity) was scored using the National Cancer Institute Common Toxicity Criteria, Version 3, and resource use data (blood product support, days on antibiotics, and hospitalization) were collected. Characteristics of the patients are summarised across the group using frequency and percentage for categorical data, and median and quartile range for quantitative data. Comparisons of patient characteristics use chi-squared, Mantel-Haenszel tests for trend, or Wilcoxon rank sum tests as appropriate.

Nonproportional hazards were not anticipated at the design of the study however since an assessment of the hazard proportional assumption showed violation, we explored alternative statistical methods to quantify the treatment effect size. Rate ratios provide an average measure of effect difference at a given time.[1,2] Rate ratios calculated with the Mantel-Haenszel method for the incidence of death at both 3yrs and 5yrs provided similar results to hazard ratios (data not shown). Restricted mean survival time (RMST) differences are an alternative validated analysis technique for time to event end points[1,3-7] applied in recent trials[8,9] when the proportional hazards assumption were violated. The analysis of RMST provides a means of comparing the overall survival experience of two treatment groups, up to a time t*, rather than focusing on a single hazard ratio. RMST for OS is reported up to 3 years based on the trial design timepoint.

For the total cohort that included patients with unknown MRD, RMST was 21.4 months (95% CI: 19.6 – 23.2) for DA and 22.8 months (95% CI: 21.3 – 24.2) for the intensification group with no significant difference in RMST 1.3 months (95% CI: -1.0 to 3.6, P=0.267) between the two groups.

In the sensitivity cohort that excluded MRD-unknown patients, the 3 year RMST was 20.6 months (95% CI: 18.5 – 22.6) for DA and 23.3 months (95% CI: 21.6 – 25.0) for the intensification group. The RMST difference between intensification versus DA was 2.7 months (95% CI: 0.1 to 5.4, P=0.045), indicating that intensification was beneficial by gain in survival at 3 years in patients with residual disease. This was also apparent by RMST analysis up to 5 years with a significant gain of 5.4 months (95% CI: 0.8 to 10.1, P = 0.022) for intensification (32.4 months, 95% CI: 9.4 – 35.4) versus DA (27.0 months, 95% CI: 23.4 – 30.6).

Original Sample Size Calculations (in trial protocol)

The sample size was calculated using the estimates from the previous trial (AML16). The finding from AML16 was that MRD following course one was highly prognostic, with MRD associated with increased relapse and worse survival. This population will be randomised 1:1:1 between DA (for two courses), FLAG-Ida, and DAC. Based upon AML16, approximately

200/1600 patients will die before the end of course 1. Of the remaining 1400 patients, about 900 will have detectable or unknown residual disease. Figures from AML16 indicate that 3-year survival in this group will be about 12.5%. With significance at P=0.025 to allow for multiple comparisons, with 400 patients in each comparison (200 per arm) with 312 events, there will be approximately 90% power to detect an absolute improvement in survival from 12.5% to 25% (hazard ratio 0.67) for each novel therapy. There will be no direct comparison of novel therapies; rather, they will be compared against control (DA).

Differences from assumptions used on standard of care arm

Although the study was designed to see an improvement in 3yr OS from 12.5% to 25% based on the results from AML16, final analysis of this AML18 randomisation showed that patients receiving DA (standard of care arm) as course 2 had a 3yr OS of 34%. This improvement in outcome compared the historical AML16 cohort could relate to several factors. 1) 48.5% of the 1015 patients alive day+30 after course 1 were not randomized (including 290 not in CR/CRi) introducing potential positive bias.

2) 36% of patients allocated daunorubicin in the course 2 randomisation had an allogeneic transplant in first remission compared to 15% overall in the AML16 trial.[12]

3) Both single dose[13,14] and fractionated gemtuzumab (GO) given with first induction have been shown to improve overall survival in older adults.[14,15] In AML16 no patients received fractionated GO and only 1 in 2 received single dose GO.

4) The Daunorubicin dose in AML18 was increased, from 50mg/m^2 in AML16 to 60mg/m^2 in AML18.

Adjusted Sample size for DAC randomisation

Between the opening of the trial in 2014 and the closure of the DAC arm in 2019, changes to the regulatory processes regarding drug supply to trials, and the associated costs, were introduced in the UK. These were beyond the control of the trial team, Sponsor and collaborating pharma company, and resulted in the arm closing earlier than planned.

As per the protocol, the estimated sample size was 400 (200 per group) with a level of significance of 2.5% and a power of 90%. Under the same assumptions, the current sample of 277 patients randomised between DA and DAC yields a power of 78% at a level of significance of 2.5% and a power of 85% at a level of significance of 5%.

Randomisation Alllocation and Comparisons

During the initial phase of the trial, patients were randomly assigned to one of three treatment groups: DA, FLAG-Ida, and DAC, with a 1:1:1 allocation ratio (1:2 randomisation period). The DA group was the control arm, while FLAG-Ida and DAC were considered as intensification treatments. However, as logistical issues caused DAC to be unavailable later in the trial, so it was removed from further randomizations. As a result, the trial changed to a 1:1 allocation between DA and FLAG-Ida.

To ensure comparisons between DA and DAC were correct and accurate, only the DA samples from the initial three-way randomization were used. This resulted in a reduced number of DA patients when analysing with the DAC and slightly different survival proportions.

Sensitivity analysis

Therapeutic questions stated in the AML18 trial protocol, included the following.

Is MRD status following course 1 of clinical value? In particular, can outcomes be improved by intensifying treatment in patients who show evidence of residual disease following course 1 of treatment?
Accordingly the SAP for the course 2 randomization included a pre-planned sensitivity analysis, excluding patients without pre-defined evidence of residual disease (as measured by the trial MRD assay) at randomization.

Multiparameter Flow Cytometry (MFC) detection of MRD

Patients' samples were sent by overnight mail to the reference laboratory. Following ammonium chloride lysis, nucleated cells of bone marrow (and /or blood at diagnosis) were labelled with antibody panel shown below for flow cytometric MRD analysis as previously described[16-18].

Tube No.	FITC	PE	PerCP	PECy7	APC	APC H7	BV 510	BV 421
1	**HLADR** L243 (BD)	**CD13** L138 (BD)	**CD34** 8G12 (BD)	**CD117** 1042D2 (BD)	**CD33** P67.6 (BD)	**CD45** 2D1 (BD)	**CD14** SJ25C1 (BD)	**CD11b** ICRF44 (BD)
2	**CD38** HB7 (BD)	**CD56** MY31 (BD)	**CD34**	**CD117**	**CD33**	**CD45**	**CD19** (Biolegend)	**CD7** M-T701 (BD)
3	**CLL1** (CLEC12A, BD)	**CD123** 7G3 (BD)	**CD34**	**CD117**	**CD19** SJ25C1 (BD)	**CD45RA** HI100 (BD)	**CD45** HI30 (BD)	**CD38** HIT2 (BD)

The conventional AML MFC-MRD assay screened for abnormal immunophenotypic expression in 2 antibody combinations (tubes 1 & 2) containing ELN recommended markers. In addition, Tube 3 was applied to detect immunophenotypic LSC-type aberrancies (from CLL1/ CD45RA/ CD123 expression on CD34+CD38-CD19- cells) with assay detection threshold of 0.02%. Cell acquisition was performed on a FACSCanto (BD Biosciences) flow cytometer. Acquisition was set for 500,000 to 1 million cells or as many cell events as possible for MRD samples. Post-acquisition analysis of the flow cytometry data was performed (blinded to clinical data) using FlowJo software (Treestar Inc). Data review for analyses included periodically updated reference control bone marrow profiles. Viability, acquisition and autofluorescence artefact and hemodilution (by CD11b / CD13 myeloid maturation profile) were assessed in acquisition generated flow cytometry standard (FCS) data files. Results from tube 3 provided additional information to tubes 1-2 for MFC-MRD but were not quantitated as LSC separately in this study.

MFC-MRD analysis

Flow cytometric MRD testing combined detection of diagnostic leukemic aberrant immunophenotypes (LAIP) and different from normal aberrant immunophenotypes (DfN) as per consensus recommendations[19,20] with any measurable level of MRD considered positive (above sensitivity threshold of 0.02-0.05% of leukocytes). An MRD negative result required

negativity in an adequate bone marrow by both DfN and LAIP analysis (prerequisite of LAIP target(s) identified at baseline).

Baseline LAIPs were selected from blast subpopulations in diagnostic samples (bone marrow and /or blood that deviated from the normal antigen profiles with sufficient detection sensitivity, usually comprised >10% of leukemic blasts and, from previous data[16,17,21] were known to be stable at follow-up (~0.02-0.05% sensitivity thresholds). Most LAIPs were defined by pre-set 'different-from normal' regions (gates) applied to CD117+ and CD34+ blasts (gated by FSC/SSC/CD45/ CD117 or CD34). This analysis approach was also applied to screen for any DfN aberrant immunophenotypes in all MRD samples including those with no baseline data. DfN pre-set gates were 'empty' for control bone marrow CD117+ or CD34+ blasts (empty defined as $<10^{-4}$ mean+SD of >20 reference bone marrows). LAIP/DfN gates that included weak CD33 as a parameter were adjusted or excluded if myeloid CD33 expression was globally low. If LAIP/DfN gates included events that might result from background (including artefact from autofluorescence), backgating was performed to check distribution of events in other marker and light scatter profiles. When there were increased myeloid blasts but no LAIPs from 'different to normal' regions, CD117+ and/or CD34+ leukemic blasts were overlayed with reference controls ('normal' CD117+ and/or CD34+ blasts) to further check for aberrant immunophenotypes. If there was an expanded myeloid blast population that was mainly or all negative for CD117 and CD34, blasts were gated by CD45/SSC or FSC/SSC then CD45intermediate and other markers (such as HLADR, CD56, CD33, CD13) followed by overlaying with reference controls to identify LAIPs for which sensitivity threshold was at least 0.05% of leukocytes. Potential LAIPs that overlapped with mature monocyte profiles (usually because of higher CD45 expression) were not reported as MRD unless these predominated in patients with clear refractory disease by flow cytometry. Our panel was insufficiently comprehensive to discriminate monocytic LAIPs for MRD sensitivity.

MRD percentages were reported as percentage of leukocytes (CD45+) expressing the identified blast LAIP with the highest frequency and/or specificity and stability[19,20]. Any level of MRD detected above the sensitivity threshold for a baseline defined LAIP was reported as MRD positive in AML18. MRD positivity was reported by different from normal approach if above sensitivity threshold (0.02%-0.05%, or >0.1% when increased background below this level from autofluorescence / viability artefact) in the pre-set different-from-normal gates. In some patients minor or major immunophenotypic changes from baseline or previous MRD sample LAIPs were detected but reported as MRD if fulfilled criteria for 'different-from-normal' approach. Samples were not reported if poor viability and/or fluorescent artefact with unacceptable background. Inadequate follow-up samples defined by <0.1% blasts and/or <100 cell events within the total blast (gated by CD45/SSC plus CD34+ and/or CD117+ gate) were also excluded from data analysis unless there was detectable MRD from a distinct aberrant cluster of at least 30-50 LAIP cell events.

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Data Supplement Figure Legends

Figure S1. AML18 Trial Schema for MRD directed randomization.

Figure S2. Disposition of MRD Data

Patients not in CR/CRi but with MRD unknown status post course 1 had either missing / inadequate samples (4 DA, 10 FLAG-IDA, 5 DAC) or had undetectable MRD by different-from-normal analysis but were not categorized as MRD negative due to not having a diagnostic LAIP identified (8 DA, 5 FLAG-IDA, 0 DAC). Legend. EMD, extramedullary disease.

Figure S3. % MRD by treatment arm

A. pre-randomzation. B. post course 2 in patients with MRD data at both timepoints.

*Data shown represents all patients with MRD data, including those not in CR/CRi post course-1

Figure S4. Adverse Events (intention to treat population).

A. The percentage of patients with grades 1-5 events are shown for adverse events by treatment arm.

B Causes of early deaths (up to day 60 from randomisation) by treatment arm

Figure S5. A Overall Survival by Intensification versus No Intensification (DA).

B Overall Survival by Intensification versus No Intensification (DA) excluding patients with unknown MRD status

Figure S6. A-C Overall Survival by treatment arm in known and unknown baseline MRD subgroups.

A. DA versus Intensification. B. DA versus FLAG-Ida. C. DA versus DAC

NB 1:1 intensification, DA vs FLAG-Ida after closure of DAC arm

D-E Overall Survival (Kaplan Meier) of MRD-unknown patients by treatment arm.

Figure S7 A-B. Overall Survival by treatment arm according to baseline remission status (excludes patients with unknown MRD).

A. DA versus FLAG-Ida. B. DA versus DAC

C-D Overall Survival (Kaplan Meier) by treatment arm of CR/CRi MRD+ patients

Figure S8. Subgroup analysis of overall survival for patient and disease characteristics according to treatment arm.

A. Age: DA versus FLAG-Ida, DA versus DAC

B. ECOG performance status: DA versus FLAG-Ida, DA versus DAC

C. Disease subtype: DA versus FLAG-Ida, DA versus DAC
D. Cytogenetic risk: DA versus FLAG-Ida, DA versus DAC
E. *FLT3* mutations: DA versus FLAG-Ida, DA versus DAC
F. By mutation group *TP53,* secondary type, denovo: DA versus FLAG-Ida, DA versus DAC
G. Gender: DA versus FLAG-Ida, DA versus DAC

Figure S9 Overall Survival by randomisation censored for allogeneic transplant, excluding patients with unknown MRD.

Data Supplement Table S1. Early Deaths, Recovery Times and Resource Usage by Treatment Arm

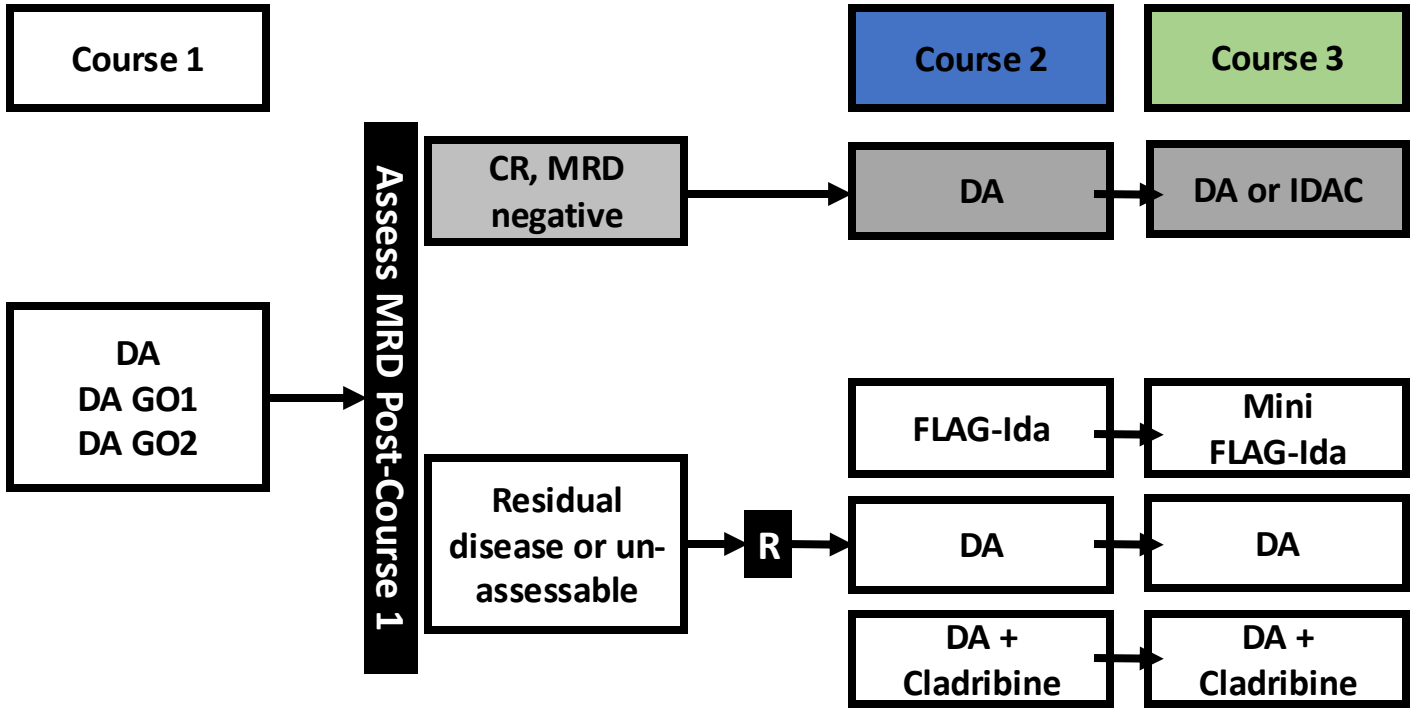
	DA	FLAG-Ida	DA-Cladribine	P value*
Early death after course 2 30 days	2 (1%)	7 (4%)	0 (0%)	0.034
60 days	7 (4%)	18 (9%)	5 (4%)	0.032
Neutrophil recovery Time from start of Course 2 Days, median [IQR]				
All patients	25 (22 – 30)	30 (24 – 37)	29 (24 – 38)	<0.001
Clinical Secondary AML	24 (22 – 32)	35 (30 – 41)	35.5 (29.5 – 41)	0.005
Genetic Secondary AML	25 (22 – 27)	26 (21 – 37)	30 (25 – 41)	0.002
Platelet recovery Time from start of Course 2 Days, median [IQR]				
All patients	26 (20 – 37)	34 (26 – 47)	33 (24 – 43)	<0.001
Clinical Secondary AML	34 (25 – 39)	34 (34 – 41)	25.5 (20 – 42)	0.602
Genetic Secondary AML	27 (22 – 39)	27 (24 – 41)	34 (24 – 43)	0.145
Resource usage				
Units of blood	5 (3 – 7)	8 (5 – 13)	7 (5 – 10)	<0.001
Units of platelets	4 (2 – 7)	8 (4.5 – 14.5)	7 (4 – 11)	<0.001
Days of IV antibiotics	6 (0 – 11)	13 (8 – 21)	11.5 (6 – 18)	<0.001
Days of oral antibiotics	7 (0 – 18)	9 (0 – 22)	11 (0 – 23)	0.090
Nights in hospital	24 (12 – 30)	32 (23 – 43)	29 (21 – 39)	<0.001
Transplantation				
Time to allograft in CR1 from start of course 2. median (IQR) days *	116.5 (83 – 164)	122.5 (92 – 150)	110.5 (80 – 135)	0.6136
Number of allografts within 120 days post C2	38 (54%%)	29 (46%)	33 (57%)	0.508

Data Supplement Table S2 Patients with unknown MRD: Demographics and Clinical Characteristics

	Overall	**DA**	**FLAG-Ida**	**DA -Cladribine**
	N = 131	**N = 47**	**N = 51**	**N = 33**
Age median (range)	67 (58 – 79)	67 (58 – 79)	67 (58 – 77)	67 (60 – 76)
Age ≥ 65yrs	92 (70%)	31 (66%)	38 (74%)	23 (69%)
Age ≥ 70yrs	39 (30%)	16 (34%)	12 (24%)	11 (33%)
Male	75 (57%)	27 (58%)	28 (55%)	20 (61%)
WBC x 10^9 / L median (range)	3.9 (0.5 – 198.2)	6.6 (0.6 – 198.2)	3.7 (0.6 – 101)	6.3 (0.5 – 118.6)
<10	89 (68%)	35 (75%)	35 (69%)	19 (58%)
≥ 50	14 (11%)	3 (6%)	5 (10%)	6 (18%)
Diagnosis				
Clinical De Novo AML	93 (71%)	33 (70%)	37 (73%)	23 (70%)
Clinical Secondary AML	17 (13%)	5 (11%)	4 (10%)	7 (21%)
High Risk MDS	21 (16%)	9 (19%)	9 (18%)	3 (9%)
Performance ID (ECOG)				
0	66 (50%)	22 (47%)	26 (51%)	18 (55%)
1	54 (41%)	23 (49%)	21 (41%)	10 (30%)
2	11 (8%)	2 (4%)	4 (8%)	5 (15%)
Course 1 treatment				
DA	47 (36%)	19 (40%)	19(37%)	9 (27%)
DA GO1	36 (28%)	10 (21%)	16 (31%)	10 (30%)
DA GO2	48 (37%)	18 (38%)	16 (31%)	14 (42%)
Small Molecule from Course 2				
Long quizartinib	23 (18%)	12(26%)	5(10%)	6(18%)
Short quizartinib	14 (11%)	6(13%)	5(10%)	3(9%)
No quizartinib	94 (72%)	29 (62%)	41 (80%)	24 (73%)
Genetic risk				
Cytogenetic (Grimwade2010)				
Favourable	1(1%)	1(1%)	0(0%)	0(0%)
Intermediate	100(77%)	37(79%)	40(80%)	23(72%)
Adverse	17(13%)	6(13%)	7(14%)	4(13%)
Failed	11(9%)	3(6%)	3(6%)	5(16%)
Not reported	2	0	1	1

TP53+	6 (5%)	1 (2%)	3 (6%)	2 (6%)
ELN 2017				
Favourable	21 (27%)	9 (32%)	5 (19%)	7 (29%)
Intermediate	26 (33%)	9 (32%)	9 (33%)	9 (33%)
Adverse	30 (38%)	9 (32%)	12 (44%)	9 (38%)
Unknown	2 (3%)	1 (4%)	1 (4%)	0 (0%)
Not Reported	59	19	24	9
***FLT3* mutations**	14 (11%)	4 (9%)	6 (12%)	4 (12%)
***NPM1* mutations**	25 (19%)	10 (25%)	8 (18%)	7 (23%)
MDS-related mutations	35 (44%)	14 (50%)	11 (41%)	10 (42%)

Figure S1. AML18 Trial Schema



Abbreviations: DA – Daunorubicin/AraC; GO – Gemtuzumab Ozogamicin; MRD – measurable residual disease; R – randomisation; IDAC – intermediate-dose cytarabine; DAC – Daunorubicin/Ara-C/cladribine; FLAG-Ida – fludarabine, cytarabine, G-CSF, idarubicin

Course 2: FLAG-Ida (for patients aged 60-69 years)

- Fludarabine 30mg/m2 daily IV, days 2-6 (5 doses)
- Cytosine Arabinoside 1g/m2 daily IV, days 2-6 (5 doses)
- G-CSF 263 µg (1 vial) s.c. daily days 1-6 (6 doses)
- Idarubicin 8mg/m2, days 4-6 (3 doses)

Course 2: Mini FLAG-Ida (for patients aged 70+ years)

- Fludarabine 25mg/m2 daily IV, days 2-5 (4 doses)
- Cytosine Arabinoside 1g/m2 daily IV, days 2-5 (4 doses)
- G-CSF 263 µg (1 vial) s.c. daily, days 1-6 (6 doses)
- Idarubicin 8mg/m2, days 3-5 (3 doses)

Course 2: DA 3+8

- Daunorubicin 50mg/m2 daily by IV infusion, days 1, 3, 5 (3 doses)
- Cytosine Arabinoside 100mg/m2 12-hourly IV, days 1-8 (16 doses)

Course 2: DAC

- Daunorubicin 50mg/m2 daily by IV infusion, days 1, 3, 5 (3 doses)
- Cytosine Arabinoside 100mg/m2 12-hourly IV, days 1-8 (16 doses)
- Cladribine 5mg/m2 daily, days 1-5 by s.c. injection (max 10mg/dose)

Course 3: Mini FLAG-Ida (for all patients)

- As above

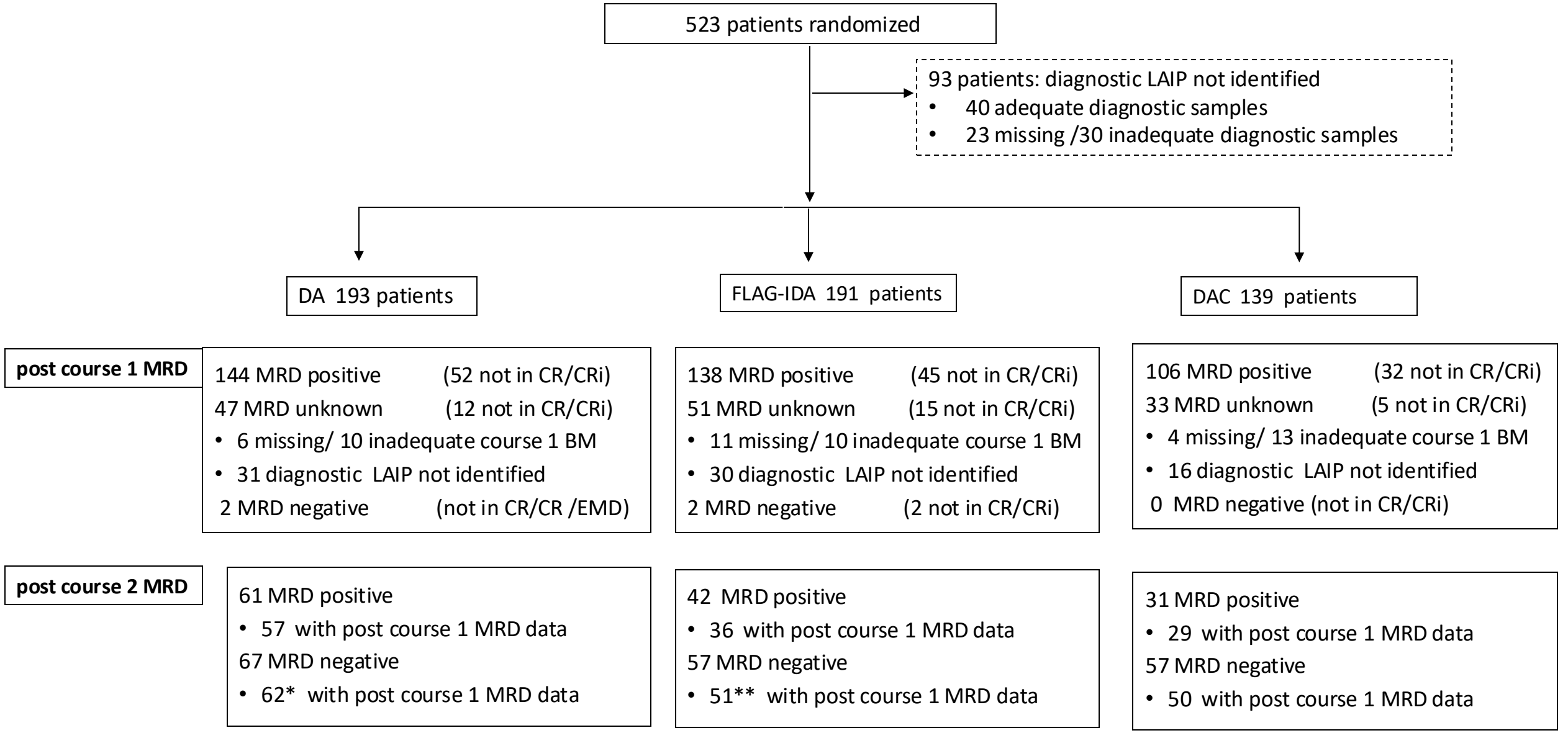
Course 3: DA 2+5

- Daunorubicin 50mg/m2 daily by IV infusion, days 1, 3 (2 doses)
- Cytosine Arabinoside 100mg/m2 12-hourly IV, days 1-5 (10 doses)

Course 3: DAC

- As above

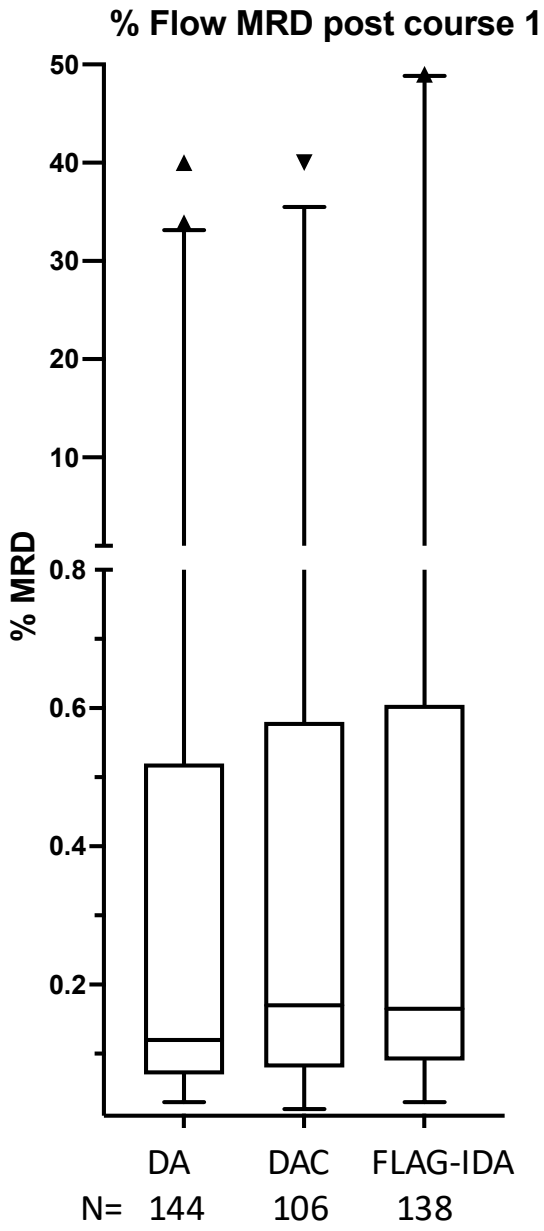
Figure S2. Disposition of MRD data



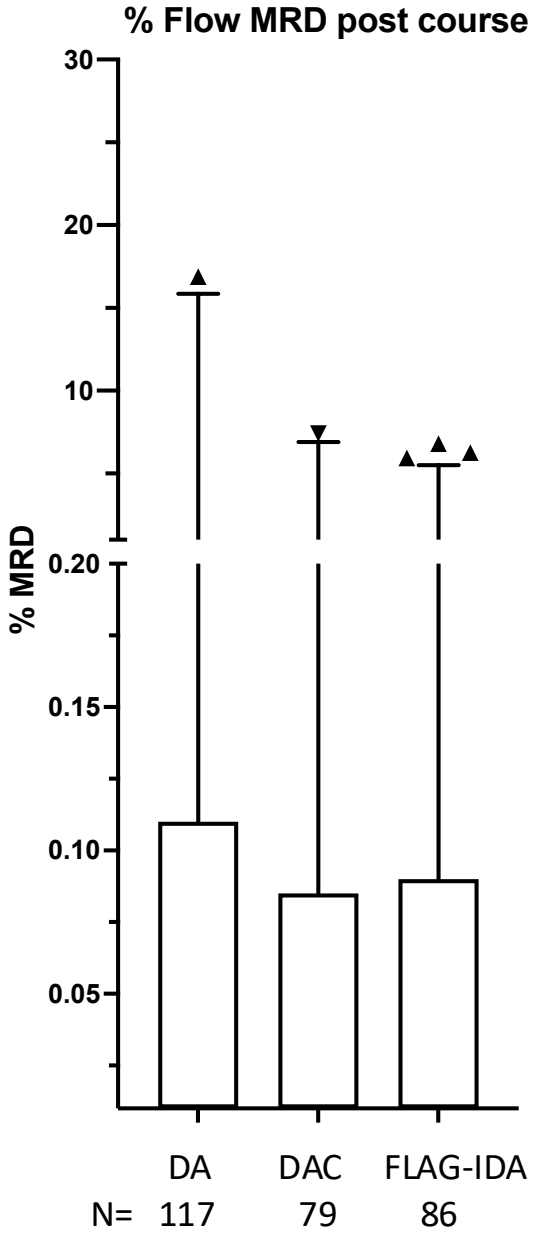
* Includes 2 not in CR/CRi but MRD negative. ** Includes 1 not in CR/CRi but MRD negative

Figure S3. % MRD by treatment arm

A. pre randomization



B. post course 2 in patients with MRD data at both timepoints



% MRD assessable after course 2 of all post course 1 MRD+ patients*		
DA	DAC	FLAG-Ida
81.3% 117/144	74.5% 79/106	62.3% 86/138

% MRD Conversion to MRD negative after course 2*		
DA	DAC	FLAG-Ida
51.3%	63.3%	58.1%

Figure S4 A. Toxicities

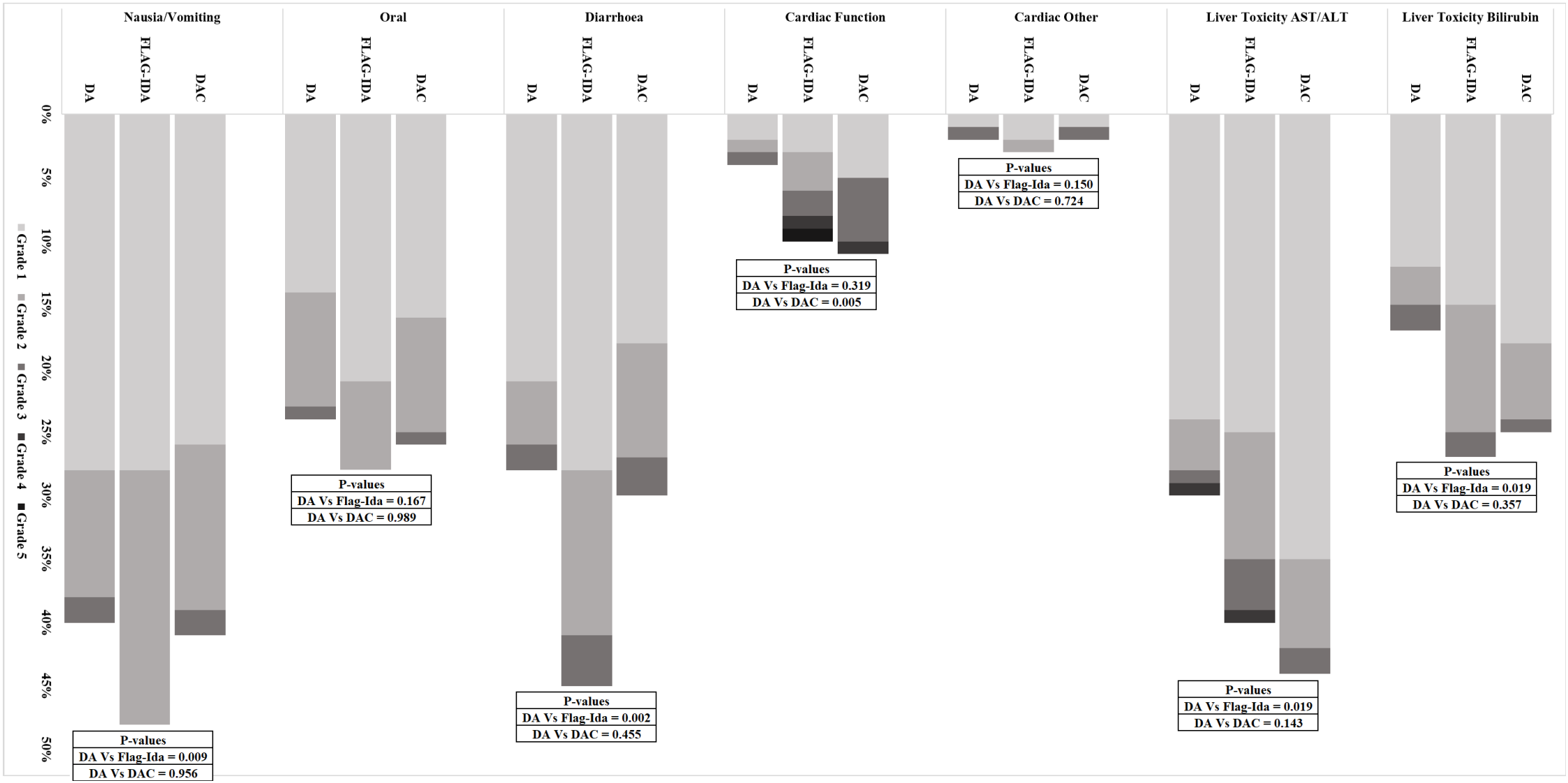
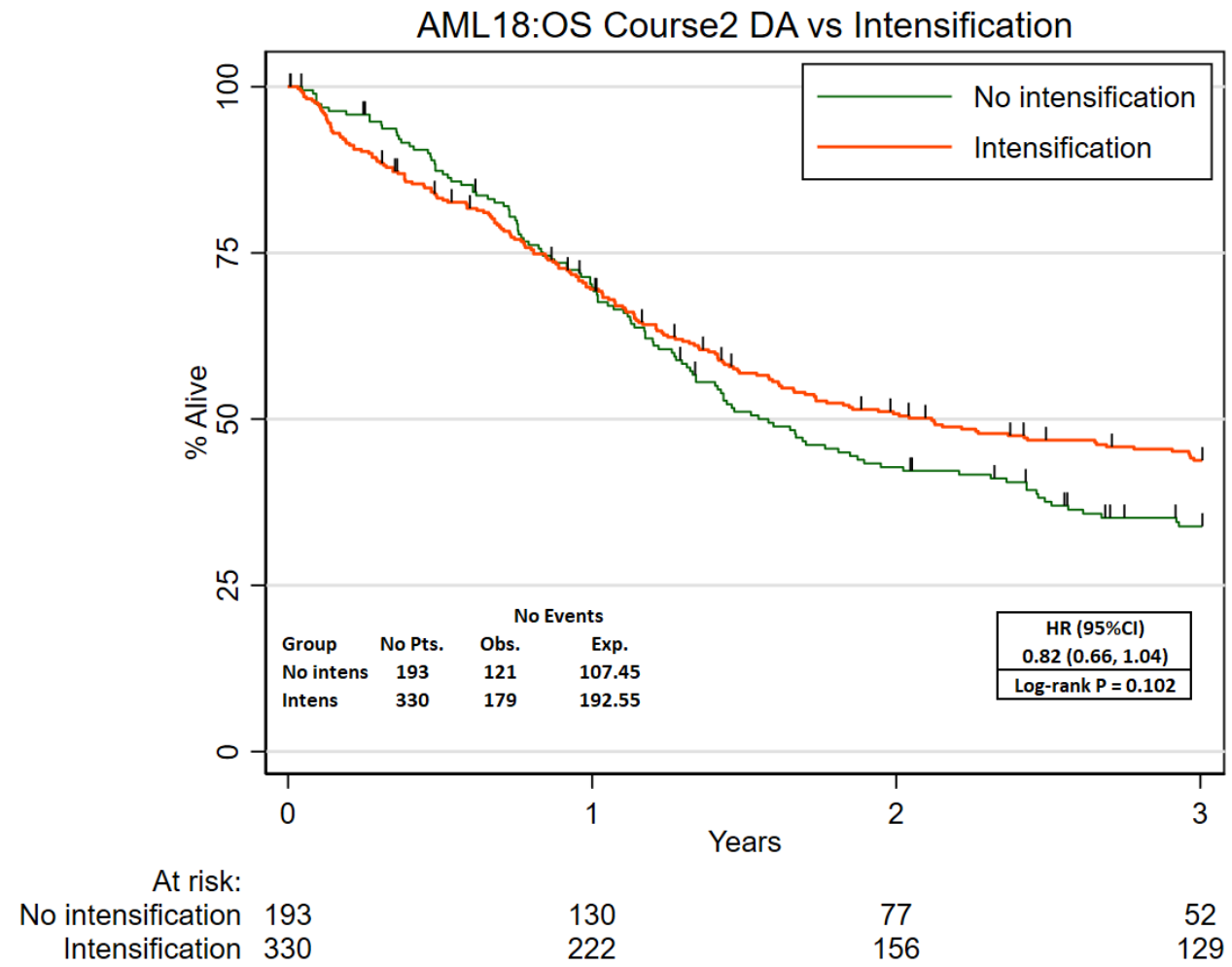


Figure S4 B. Causes of Early Deaths

Cause of Death	DA	FLAG-Ida	DAC	Total
Infection	3	5	2	**10**
Resistant disease 1st remission failure	1	4	1	**6**
Other /Multiple Causes	3	9	2	**12**
Total	**7**	**18**	**5**	**30**

Figure S5 Overall Survival. DA vs Intensification (All Patients)



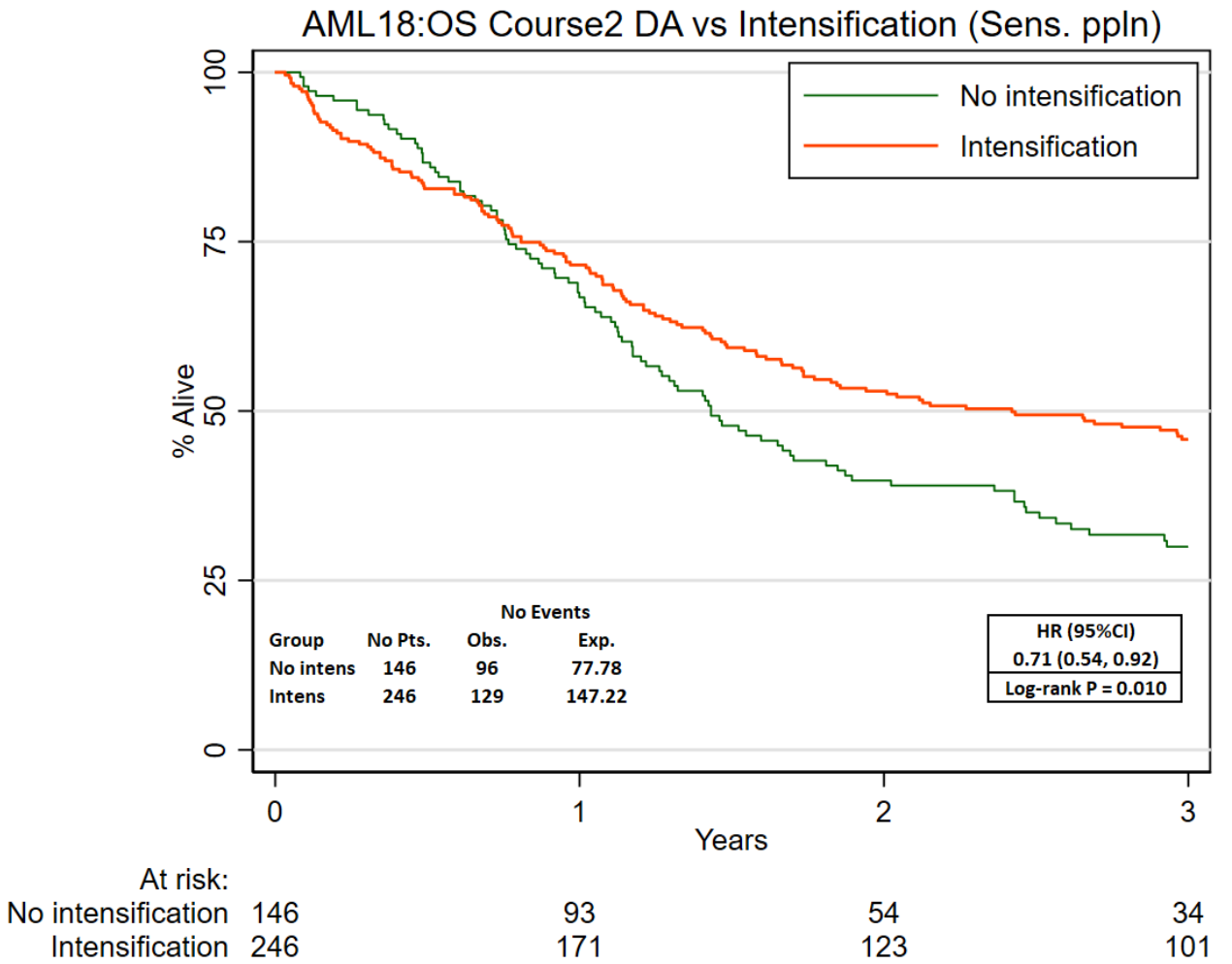
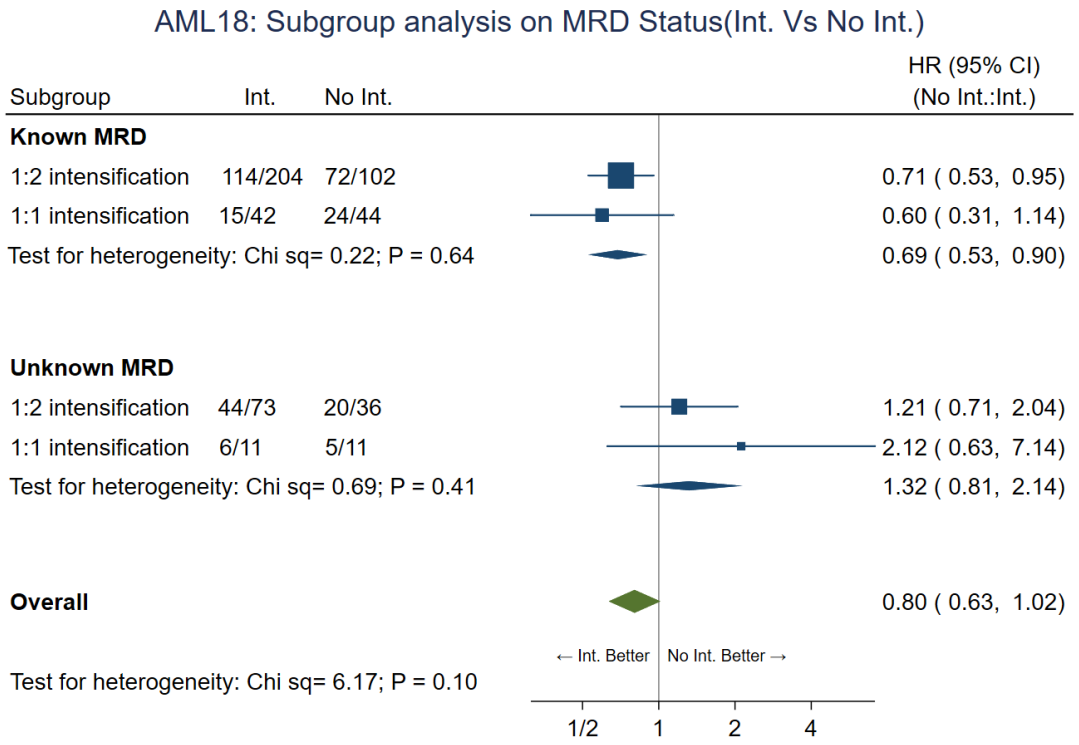
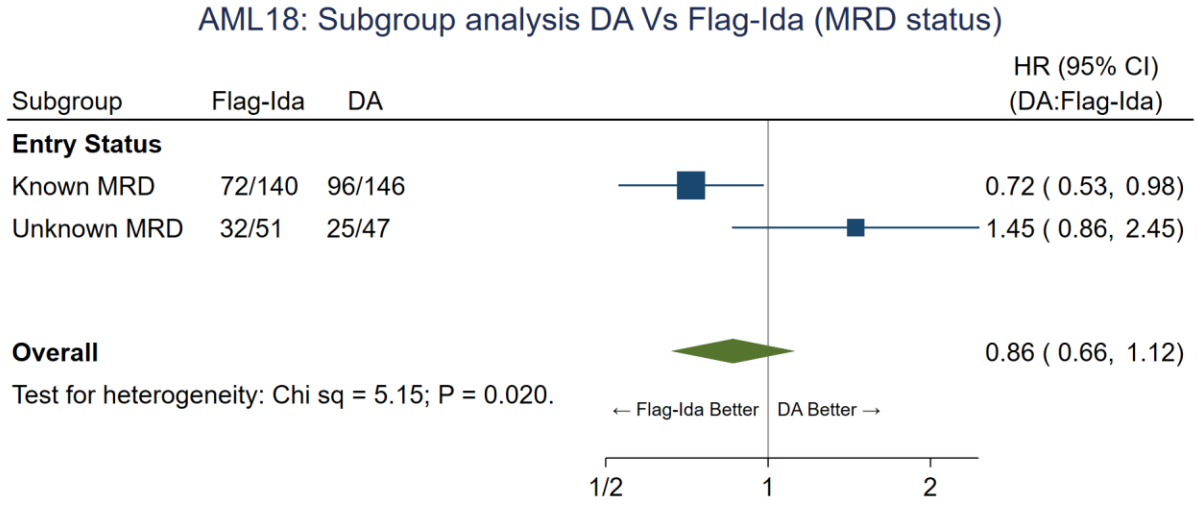


Figure S6 A-C Overall Survival by treatment arm in known and unknown baseline MRD subgroups.

A. DA vs Intensification



B. DA vs FLAG-Ida



C. DA vs DAC

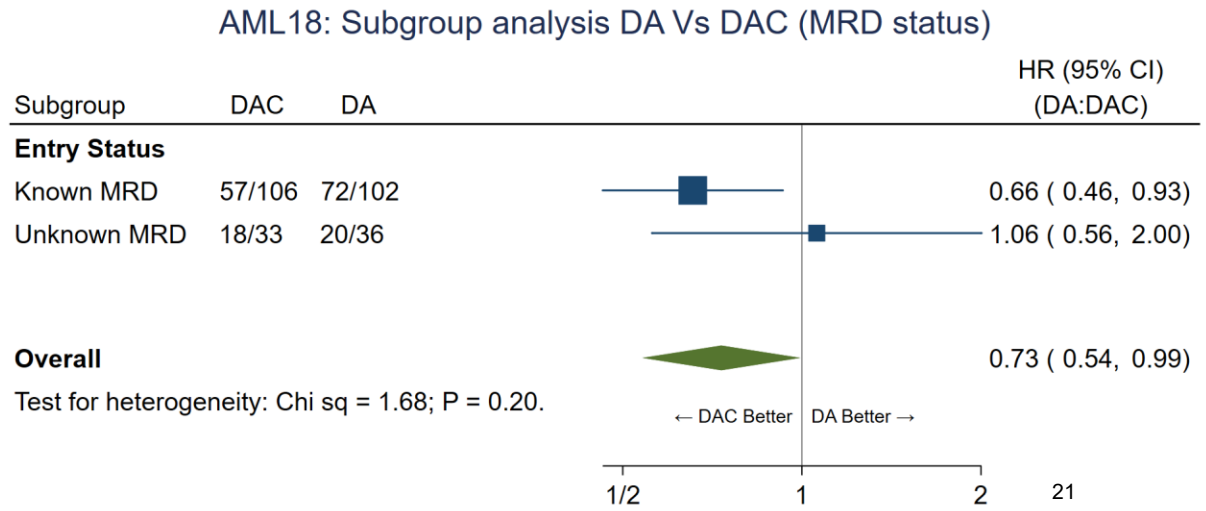
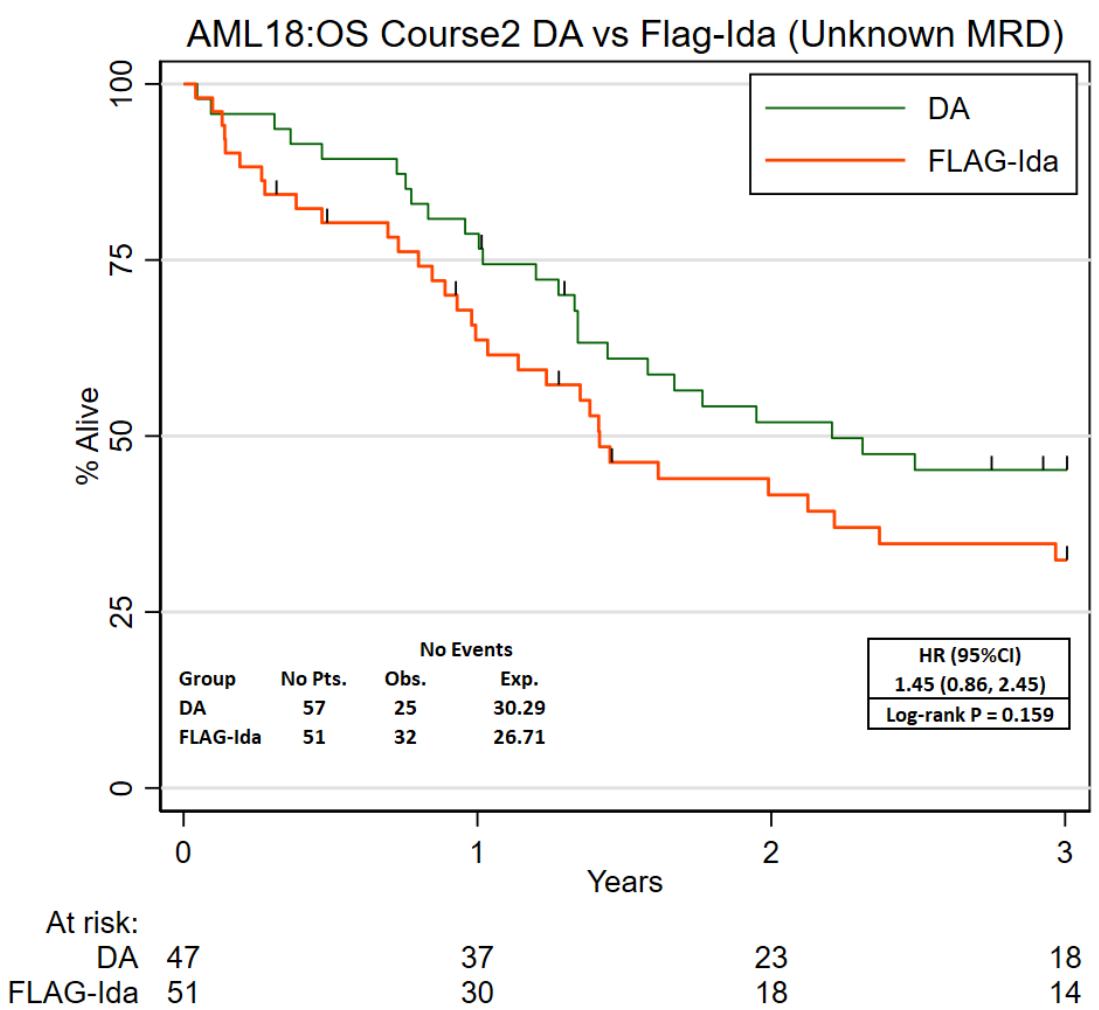


Figure S6 D-E Overall Survival (Kaplan Meier) of MRD-unknown patients by treatment arm

D. DA vs FLAG-Ida



E. DA vs DAC

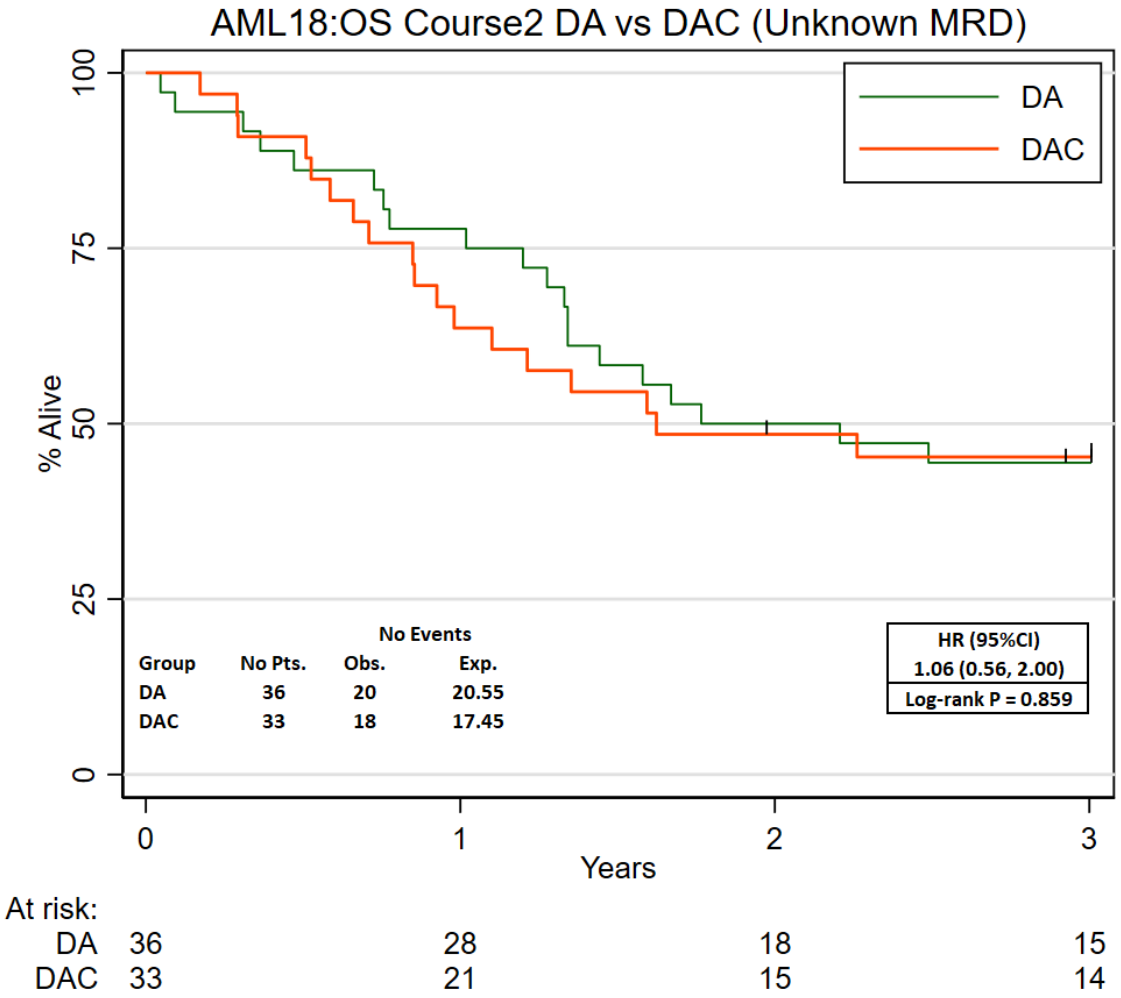
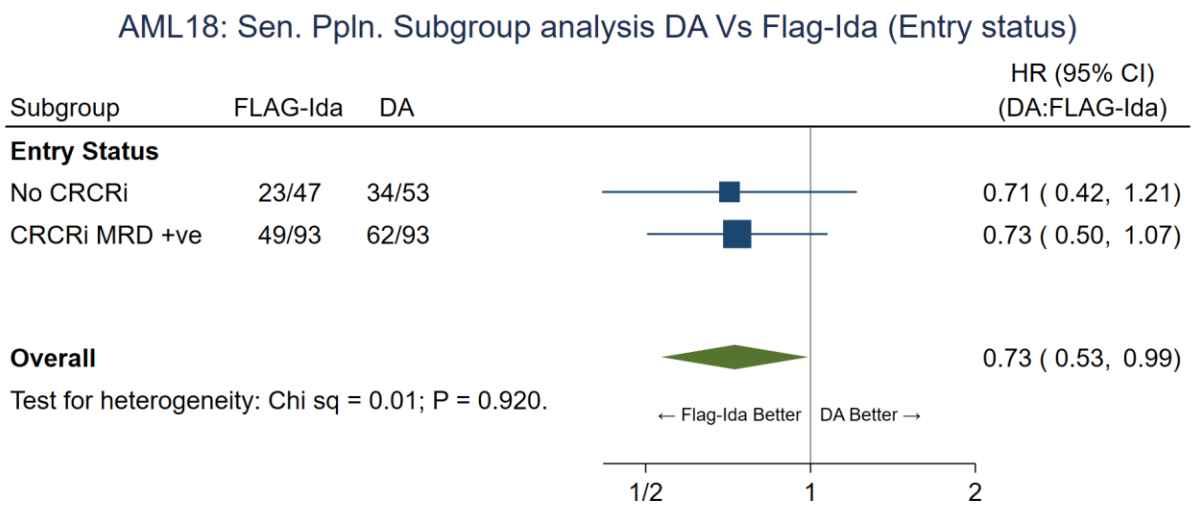


Figure S7 A. & B. Overall Survival by treatment arm according to baseline remission status

A. FLAG-Ida vs DA



B. DAC vs DA

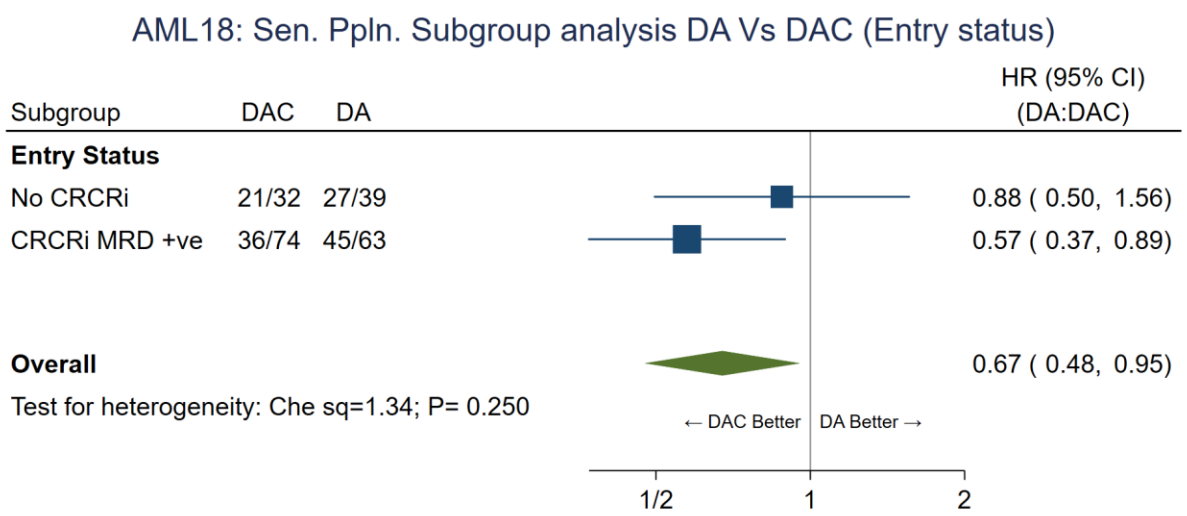
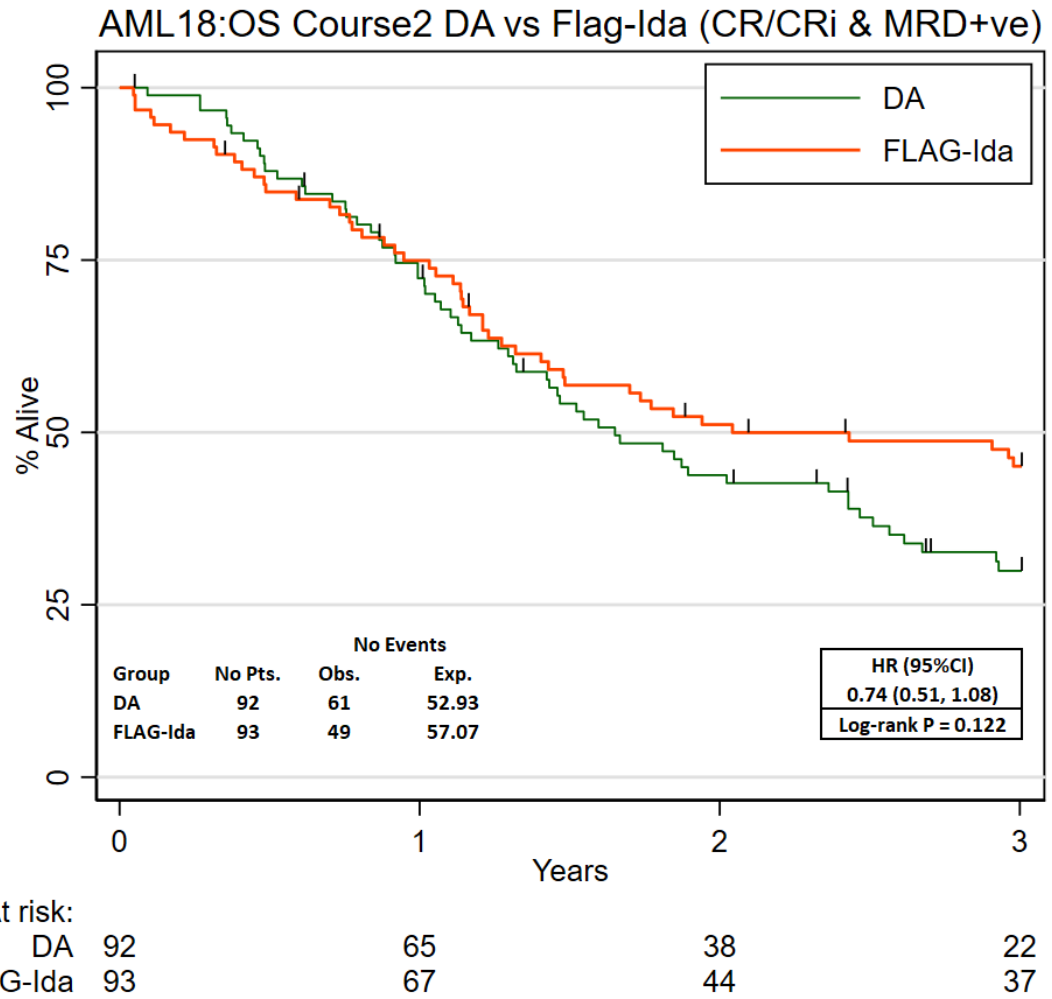


Figure S7 C. & D. Overall Survival (Kaplan Meier) by treatment arm of CR/CRi MRD+ patients

C. FLAG-Ida vs DA



D. DAC vs DA

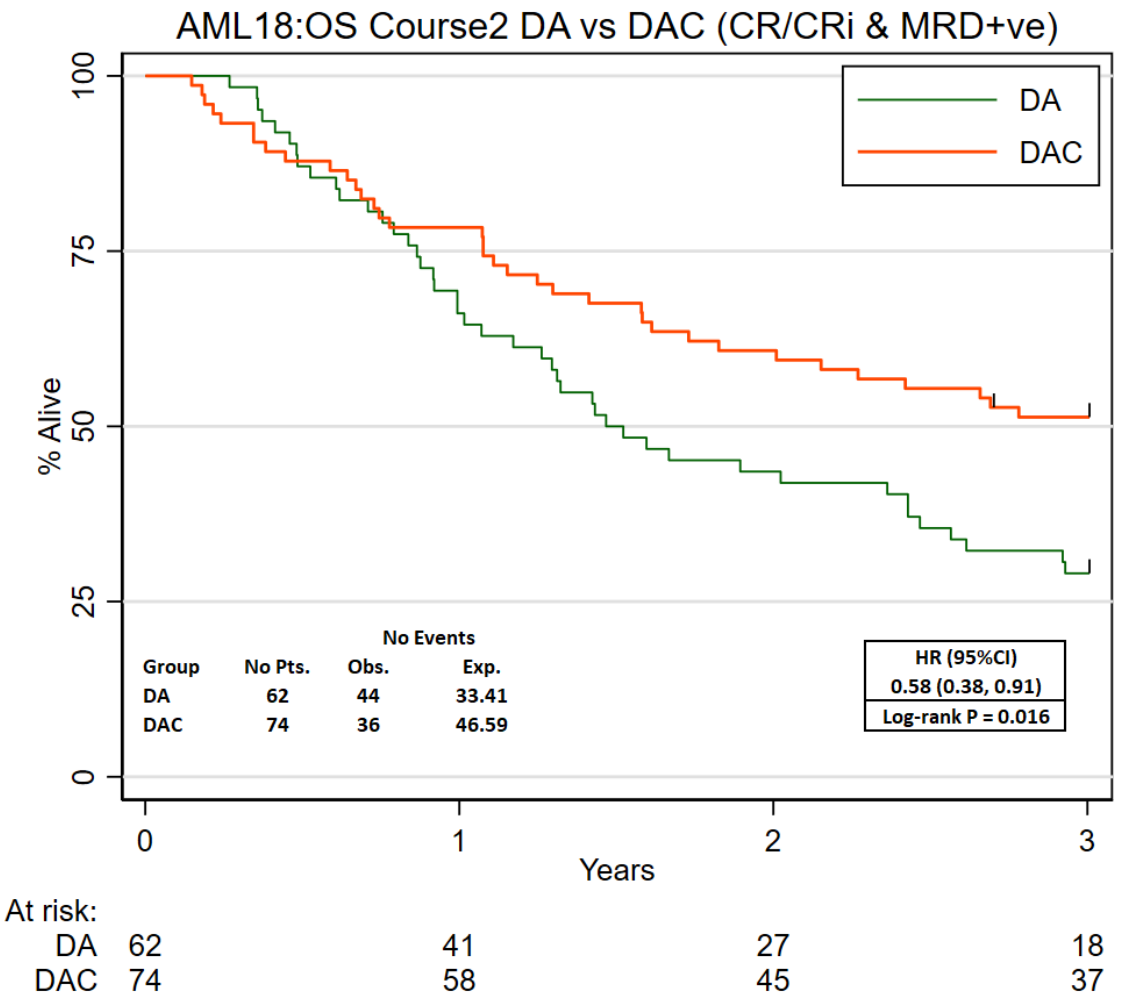
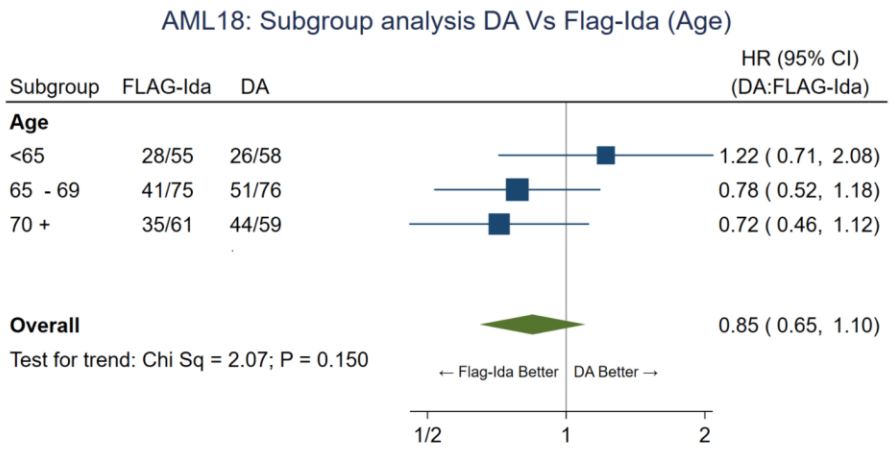


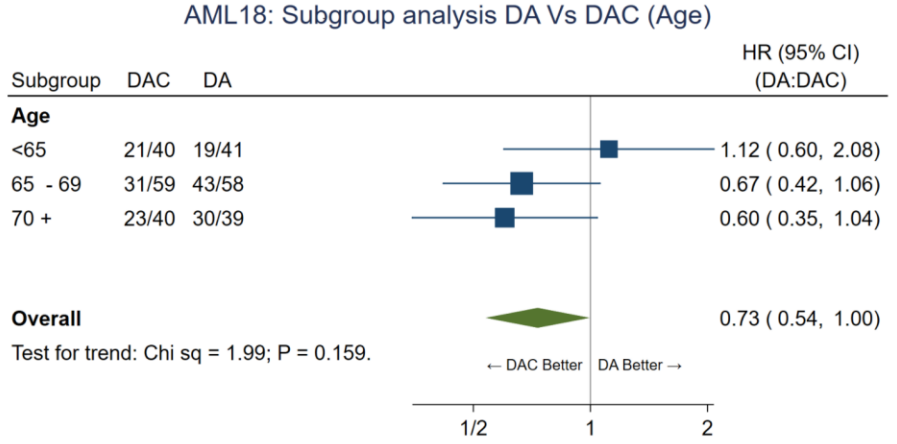
Figure S8. Subgroup analysis of overall survival for patient and disease characteristics according to treatment arm.

FLAG-Ida vs DA

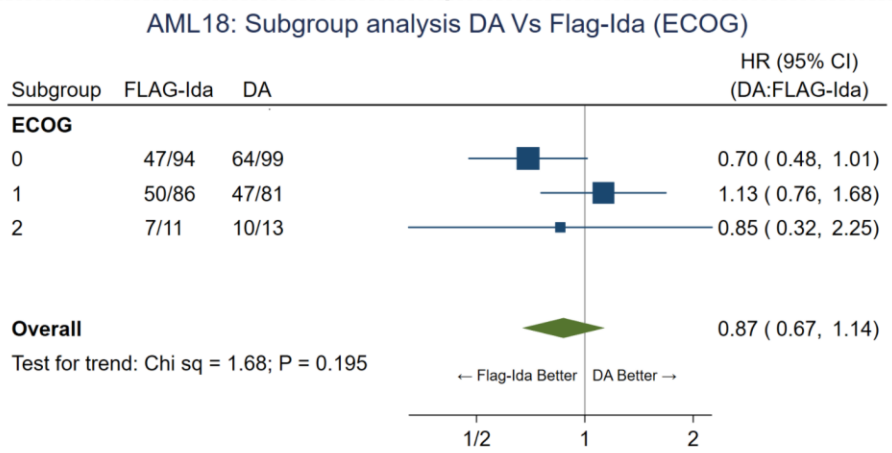
DAC vs DA

A. Age





B. ECOG Performance status



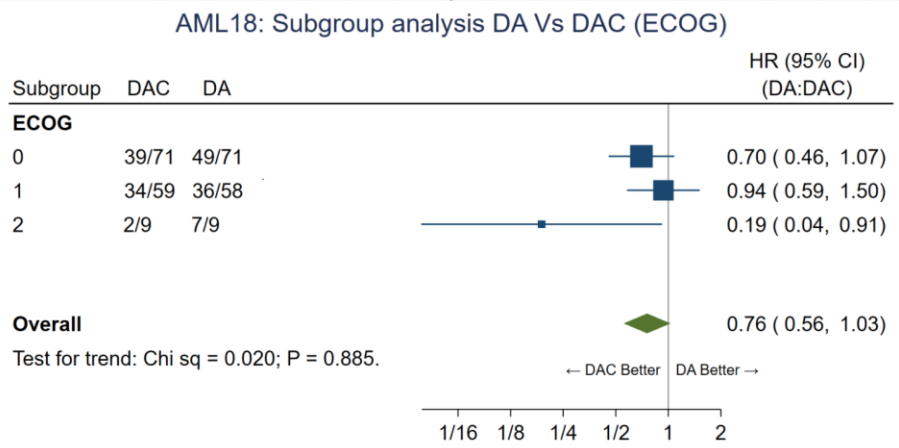
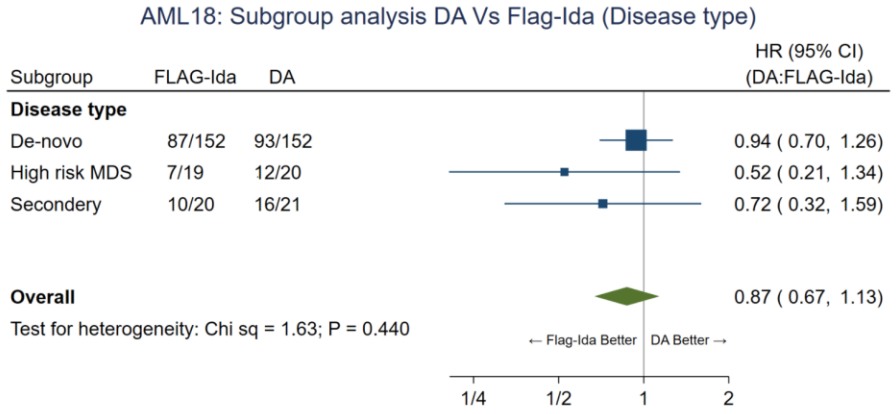


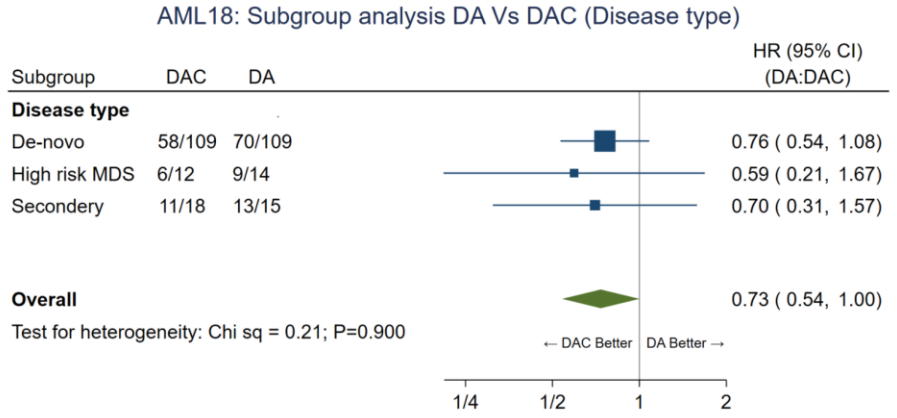
Figure S8. Subgroup analysis of overall survival for patient and disease characteristics according to treatment arm.

C. Clinical Disease subtype

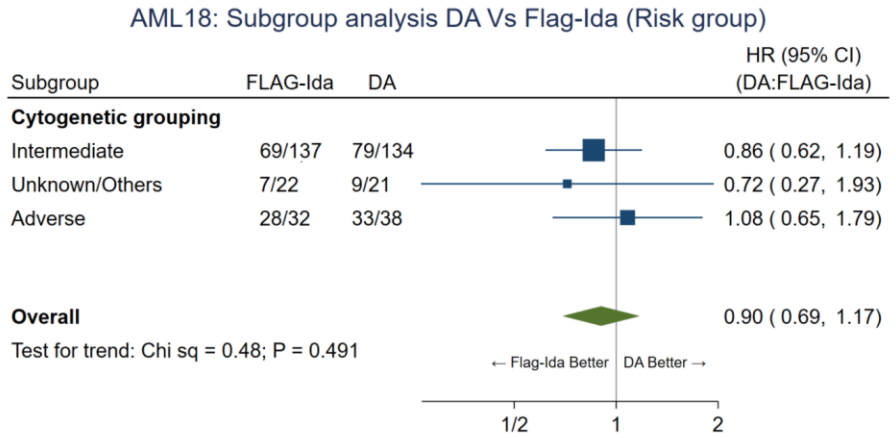
FLAG-Ida vs DA



DAC vs DA



D. Cytogenetic risk group



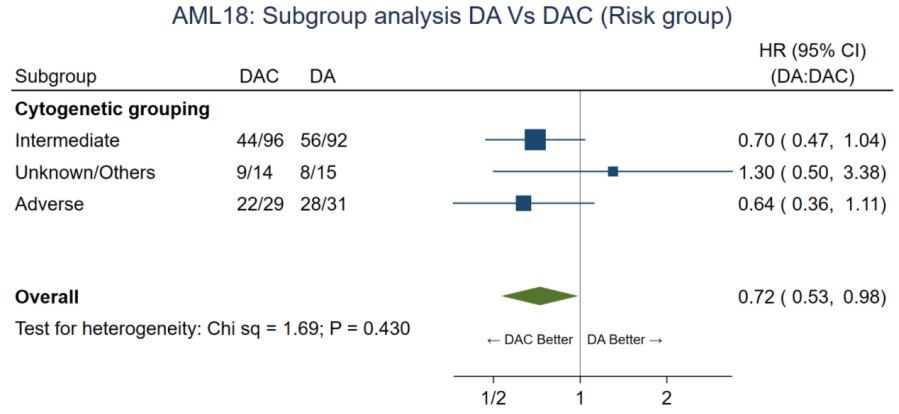
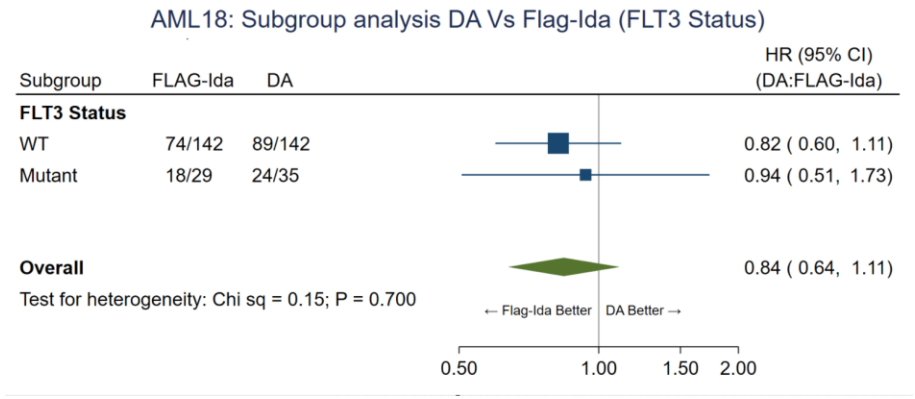


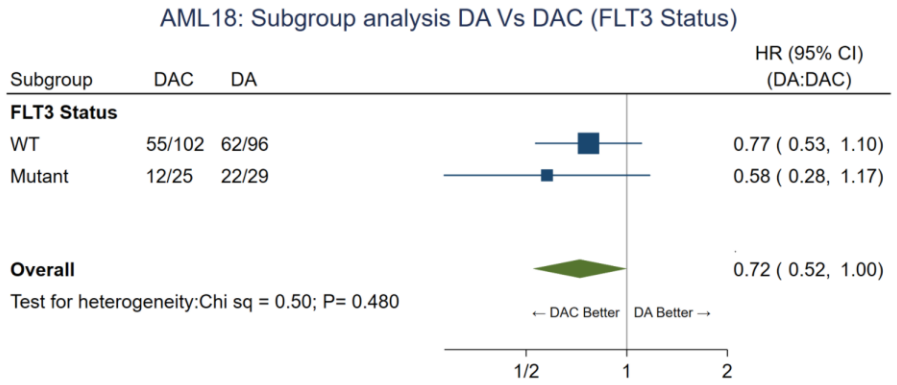
Figure S8. Subgroup analysis of overall survival for patient and disease characteristics according to treatment arm.

DA vs FLAG-Ida

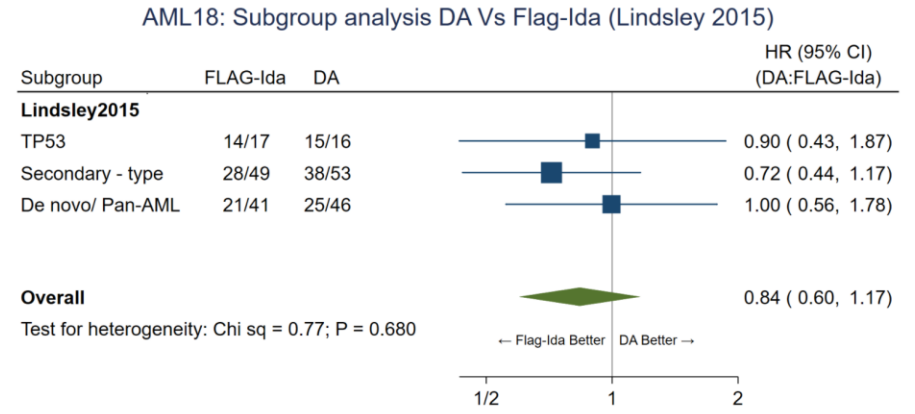
DA vs DAC

E. FLT3 mutations





F. By mutation group *TP53,* secondary type, denovo



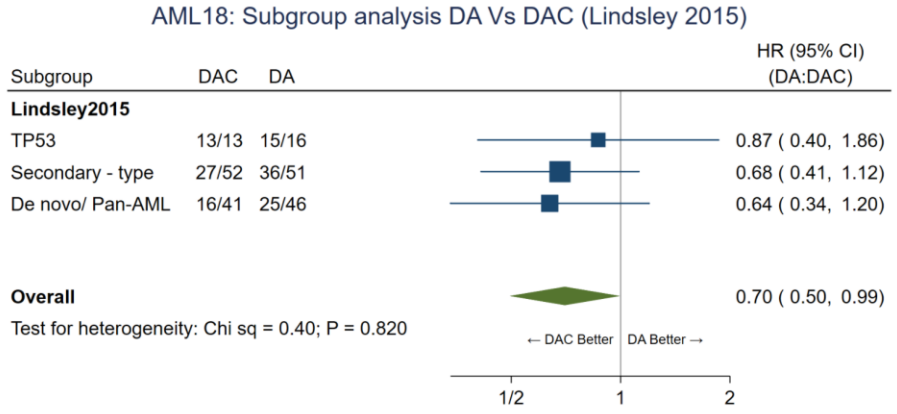
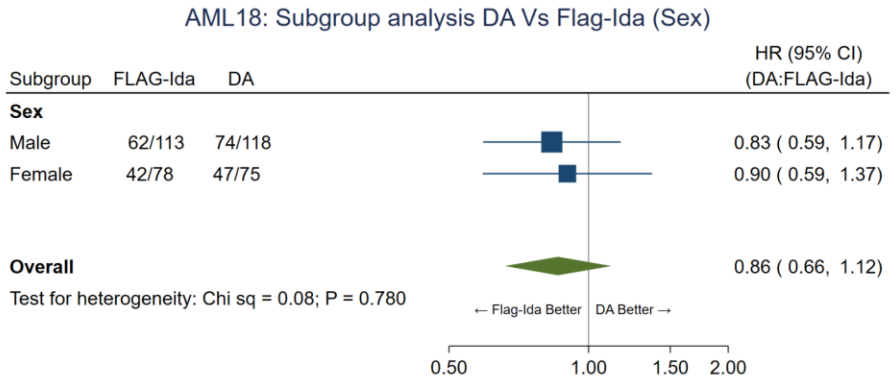


Figure S8. Subgroup analysis of overall survival for patient and disease characteristics according to treatment arm.

FLAG-Ida vs DA

DAC vs DAC

G. Gender



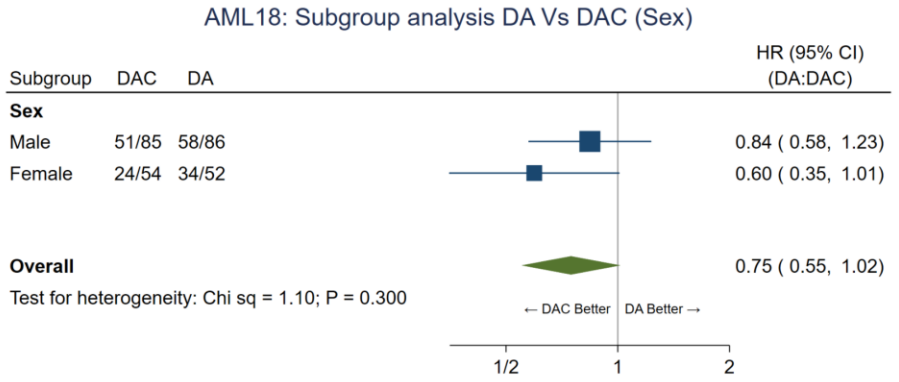
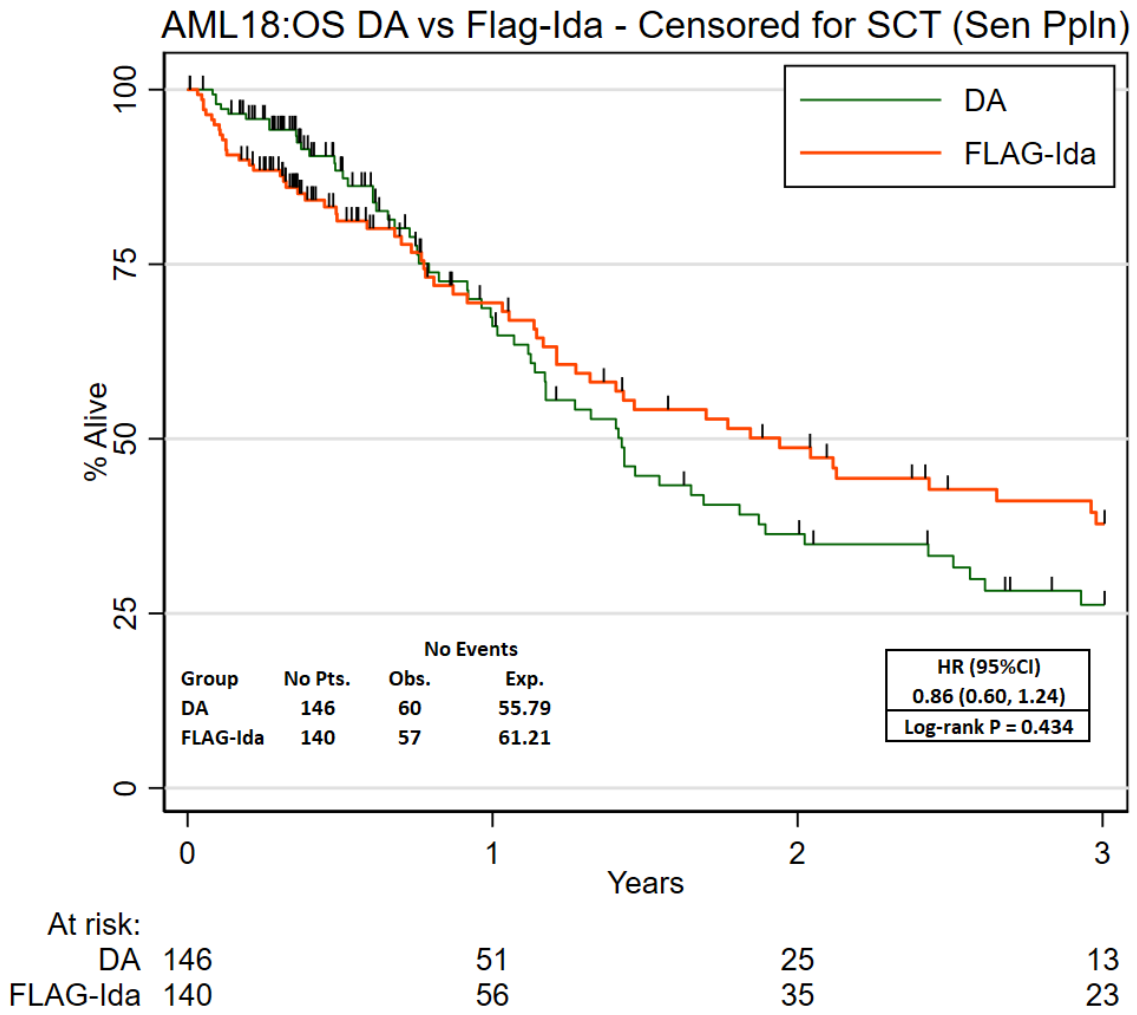


Figure S9. Overall Survival by randomisation censored for allogeneic transplant excluding patients with unknown MRD status

A. DA vs FLAG-Ida



B. DA vs DAC