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The addition of the mTORr inhibitor, Everolimus, to consolidation therapy in acute myeloid leukaemia: experience from the UK NCRI AML17 trial.

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Abstract

As part of the UK NCRI AML17 trial, adult acute myeloid leukaemia patients in remission could be randomised to receive the mTOR inhibitor everolimus, sequentially with post-induction chemotherapy. Three hundred and thirty-nine patients were randomised (2:1) to receive everolimus or not for a maximum of 84 days between chemotherapy courses. The primary endpoint was relapse free survival (RFS). At 5 years there was no difference in Relapse Free Survival (29% vs 40%; OR 1.19 (0.9-1.59) p=0.2), cumulative incidence of relapse (60% vs 54%: OR 1.12(0.82-1.52): p=0.5) or overall survival (45% vs 58%: OR 1.3 (0.94-1.81): p=0.11). The independent Data Monitoring Committee advised study termination after randomisation of 339 of the intended 600 patients due to an excess mortality in the everolimus arm without any evidence of beneficial disease control. Dose delivery of everolimus was variable, but there was no evidence of clinical benefit in patients with adequate dose delivery compared with no treatment. This study suggests that the addition of mTOR inhibition to chemotherapy provides no benefit.

Introduction

The majority (70-85%) of younger patients with acute myeloid leukaemia (AML) will enter complete morphological remission with any one of a variety of induction treatments. However nearly half will relapse. It is increasingly being recognised that a substantial proportion of those in morphological remission have residual disease as assessed by techniques of minimal/measurable residual disease assessment (flow cytometry or quantitative polymerase chain reaction[1,2]). In our previous studies we have endeavoured to define the optimum post remission chemotherapy. To date we conclude that, apart from transplantation, following two induction courses of anthracycline containing therapy, two consolidation courses of Ara-C is adequate[3]. An aim of the UK NCRI AML17 trial was also to explore a further reduction in the total number of chemotherapy courses from 4 to 3, as well as the addition of molecularly targeted treatments to consolidation therapy. Among these was the

incorporation of the inhibitor of the mammalian target of rapamycin (mTOR), everolimus.

There is plausible pre-clinical evidence both *in vitro* and *in vivo* that mTOR inhibition could be beneficial in AML. mTOR is a serine/threonine protein kinase that is predominantly modulated by PI3K-AKT-dependent mechanisms and acts as a central regulator of cellular metabolism, growth and survival[4]. Dysregulation of the mTOR pathway is closely associated with cancers including AML[5,6] and other human diseases. Part of the rationale is the evidence of constitutive activation of the PI3K-AKT pathway in 90% of AML samples and the demonstration that this activation is central to the survival of AML blasts but not of normal CD34+ cells[7]. The concept that everolimus may have the potential to eliminate leukaemia-initiating stem cells whilst sparing normal haematopoietic stem cells is also appealing. *In vivo* evidence in NOD/SCID mice has suggested that mTOR regulates a critical cell survival pathway in AML stem cells[8,9]. In an preliminary unrandomised clinical trial, the mTOR inhibitor sirolimus was administered as a single agent to 9 relapsed, refractory or poor-risk AML patients for 28 days resulting in partial responses in 4, and stable disease in a 5th patient[10]. De-phosphorylation of downstream effectors of mTOR was demonstrated. In an ongoing U.K. trial, 11 elderly patients with primary and relapsed AML have been treated with the combination of low dose Ara-C and sirolimus. Following a single 28-day course of treatment, of the 7 patients eligible for analysis, one had achieved a CR, 4 a PR, one marrow was profoundly hypocellular and one patient was a non-responder (Das Gupta, unpublished data). Patients in this trial reliably maintained trough sirolimus levels of 8-16 ng/ml, which are consistent with the published concentrations required to inhibit AML cell growth *in vitro.* The feasibility of combining mTOR inhibition (sirolimus) with intensive chemotherapy had also been assessed in AML patients in conjunction with the more intensive MEC (Mitoxantrone, Etoposide and Cytarabine) chemotherapy regimen in a phase I dose escalation study and reported in abstract form. In this study standard renal transplant doses of sirolimus were well tolerated and did not increase the non- haematological toxicity of MEC chemotherapy with a median time to neutrophil recovery of 27 days[11]. Based on this background information, the NCRI AML17 trial included the option for

eligible patients to be randomised to receive, or not, the mTOR inhibitor everolimus daily between consolidation chemotherapy courses.

Methods

The UK NCRI AML 17 trial (ISRNCTN 55675535) was a large, prospective phase 3 multi-centre trial for patients with newly-diagnosed AML or high risk myelodysplastic syndrome (MDS; >10% marrow blasts), generally under the age of 60 years, open from April 2009 to December 2014 in >130 centres in the United Kingdom, Denmark and New Zealand. It addressed several randomised questions (supplementary figure 1). Between October 2009 and October 2012, 499 adult patients who did not have acute promyelocytic leukaemia (APL), had received the first induction course and who did not have Core Binding Factor leukemia, high risk disease (defined using a multifactorial score[12]) and were not in the lestaurtinib randomisation for patients with FLT3 mutations, could be randomised between adding everolimus, or not in a 2:1 ratio, between subsequent consolidation chemotherapy courses. Treatment schedules have been set out elsewhere[13]. Allogeneic stem cell transplantation was permitted for patients with intermediate- or poor-risk disease with a recommendation of myelo-ablative conditioning for patients aged <35 years and reduced intensity conditioning for intermediate risk patients >45 years, with investigators able to choose an ablative or reduced intensity approach for patients between 35 and 45 years.

Patients eligible to enter the everolimus randomisation included 34% of all adult patients entering AML17 while the randomisation was available. Oral everolimus (10mg daily from 2 days after each chemotherapy course for up to 28 days or until 2 days before the start of the subsequent course, whichever was shorter) or not was given between each course of consolidation chemotherapy.. In patients allocated 3 courses of treatment, a final 28-day course of everolimus was given after a one-week break. In patients with side effects thought due to everolimus, subsequent doses could be reduced by 50% in daily dosing. If this did not improve tolerability, dosing could be further reduced to alternate days; if these dose reductions were not tolerated, subsequent doses would be omitted. After 65% (n=146) of the patients randomised to everolimus were assessed, the Independent Data Monitoring

Committee recommended, because of increased side effects and reduced compliance, that the starting daily dose of everolimus be reduced to 5mg with the option to increase to 10mg if well-tolerated.

Extensive Sanger sequencing (111 genes) was undertaken in 123 patients; *NPM1* status was available in 302 patients.

Patients were requested to provide a trough blood sample taken immediately prior to everolimus dosing on day 14 of each treatment course to measure the level of mTOR inhibitory activity in their plasma (PIA). Methods are summarised here (supplementary figure 2) and will be more fully reported elsewhere.

Statistical considerations:

All analyses are by intention-to-treat. Categorical endpoints were compared using Mantel-Haenszel tests, giving Peto odds ratios and confidence intervals. Continuous/scale variables were analysed by Wilcoxon rank sum tests; and time-to-event outcomes using the log-rank test, with Kaplan-Meier survival curves. Odds/hazard ratios (OR/HR) <1 indicate benefit for everolimus. All survival percentages are at 5 years unless otherwise stated.

Stratified analyses were performed with suitable tests for interaction[14] and interpreted cautiously.

It was planned to recruit 600 patients to the everolimus randomisation, giving 85% power to detect a 12.5% difference in the primary endpoint of RFS, from 50% to 62.5% (HR 0.68). Follow-up is complete until 1st March 2016 (median follow-up from diagnosis 53.5 months (range 4.3 – 76.8 months)).

The trial was conducted in accordance with the Declaration of Helsinki, sponsored by Cardiff University and approved by Wales REC3 on behalf of all UK investigators, by the Danish Medicines Agency for sites in Denmark, and by MEDSAFE for sites in New Zealand.

Results

Patient characteristics: The randomisation opened in October 2009. In 2012, the Independent Data Monitoring Committee recommended closure of the randomisation, because of an excess of early mortality in remission with everolimus and no associated evidence of relapse reduction. Between

October 2009 and October 2012, 332 of 482 eligible patients were randomised (Figure 1). Their characteristics are shown in table 1. There was no significant survival difference in outcome between eligible patients who entered the randomisation and those who did not (p=0.8), although patients with higher WBC, worse performance and secondary disease were marginally less likely to enter the randomisation. The median age was 47 years (range 16-69). The majority presented with *de novo* AML and had a WHO performance score of <2. The other protocol treatments given to patients in the everolimus randomisation are shown in table 1. In addition to standard daunorubicin/Ara-C induction additional etoposide and gemtuzumab ozogamicin was given to 43% and 45% of patients respectively in induction with no difference between arms.

Overall, 132/332 (40%) of patients received a transplant (everolimus 39%, control 42%, p=0.6), with a minority of these (34/132) being allografts in first remission (20 vs 14; p=0.3). There was no evidence of differences in transplantation rates or types of transplants between the arms (any SCT 39% vs 42%; p=0.6; allograft 31% vs 34% p-0.6; allograft in CR1 9% vs 13%, p=0.3; Table 1).

Extensive Sanger sequencing (111 genes) was undertaken in 123 patients, and the gene panel and distribution is shown in supplementary figure 3. In addition *NPM1* status using previously published methods, was available in 302 patients.

Treatment compliance: Of the 220 patients allocated to receive everolimus, 16 never started therapy. Approximately 25% of patients did not receive 14 days of everolimus; about half completed the first 28 day course. At the time of the second course of everolimus (course 3 of chemotherapy), 35% of patients with second everolimus course information did not receive drug (figure 2). Reasons were given for about two thirds of patients (39/61) – 11 patients had not completed the previous course; 11 patients chose to discontinue (often because of toxicity in the previous course), in 3 cases the DMC had recommended closure and stopping treatment; in 5 cases patients did not reach the starting point for everolimus therapy on protocol; in 2 cases

the clinician decided, and in 4 other cases, everolimus was not given due to a variety of toxicities.

Toxicity: The recorded toxicities are shown in figure 3. There was more haematological toxicity in the everolimus arm which was most obvious after the first everolimus course, with median time to platelet recovery to >100 x 10^9/L being 9 days longer (39 vs 29 days; p= 0.006), which was reflected in a significantly greater requirement for platelet support.(table 3). The kinetics of neutrophil recovery was unaffected by everolimus, but there was significantly more use of antibiotics and a longer hospital stay with the first course of everolimus, as well as increased oral toxicity (course 1) and higher alanine transaminase levels (course 2).

Cumulative Risk of Relapse and Death in Remission: The overall outcomes are shown in table 2. The cumulative incidence of relapse at 5 years (figure 4a) did not differ significantly between arms (60% vs 54%, HR 1.12 (0.82-1.52), p=0.5). There was a significant excess of deaths in remission in the everolimus arm in the first 6 months following randomisation (8% vs 1%, HR 3.57 (1.36-9.42), p=0.009), with no significant differences thereafter leading to a non-significant excess of overall mortality with everolimus (11% vs 6%, HR 1.75 (0.83-3.70), p=0.14, Figure 4b). The causes of death in remission were: in the first 6 months 17 vs 1 (infection 9 vs 1; infection+haemorrhage 3 vs 0; haemorrhage/CVA 3 vs 0; cardiac 1 vs 0; multiple 1 vs 0); beyond 6 months 6 vs 6 (infection 1 vs 1; cardiac 1 vs 0; hepatic 1 vs 0; second cancer 1 vs 0; GVHD 0 vs 1; multiple 0 vs 2; unknown/other 2 vs 2).

Relapse Free and Overall Survival: Both relapse free and overall survival were non-significantly inferior in the everolimus arm, (figures 4c and d),reflecting the adverse hazard ratios for both relapse and death in remission, and no evidence of differences in salvage between arms after relapse (RFS: 29% vs 40%, HR 1.19 (0.90-1.59), p= 0.2; OS: 45% vs 58%, HR 1.30 (0.94-1.81), p=0.11). A sensitivity analysis, censoring patients at stem cell transplant showed results which were consistent with the overall analysis (table 2).

Exploratory Analyses: The correlation with PIA did not show a convincing pattern. Using this assay, even patients whose samples showed deep and sustained inhibition, did not have an associated reduction in relapse (supplementary figure 2). There was no relationship between the level of inhibition and toxicity or excess mortality. Prior induction chemotherapy, age, gender, WBC, and minimal residual disease status after course one, all had no impact on outcomes (Supplementary Figure 4A). In addition no relationship between other treatment modalities given and response was found, and no gene mutation, including the 110 genes assayed by Sanger sequencing in 123 patients showed a differential response (Supplementary Figure 5). Because of concerns about compliance with everolimus treatment, RFS was compared between patients with satisfactory drug delivery (defined as at least 14 days of treatment per course), those with inadequate drug delivery (less than 14 days treatment per course) and those allocated to no treatment. Although inadequate drug delivery (n=85) had a worse RFS (29%) there was no difference in RFS between patients with satisfactory drug delivery (n=63) at 41% and no everolimus (n=99) at 40%(supplementary figure 4).

Discussion

In this trial there was no benefit of the addition of the mTOR inhibitor everolimus to post-induction chemotherapy, despite the pre-clinical *in vitro* and *in vivo* rationale for its use. The main observed explanation appears to be excess toxicity, which was primarily gastrointestinal (mucositis and diarrhoea) and biochemical evidence for liver toxicity at the dose chosen. Infection was a major issue in the first 6 months of treatment with 12 vs 1 deaths attributed to infection. This did not appear to be the result of prolonged neutropenia but may be attributable to the immunosuppressive effects of everolimus when given with chemotherapy, which reflects what has been seen with its use in solid tumours[15]. This in turn contributed to sub-optimal drug delivery for many patients. The chosen schedule of 10mg daily was not feasible in this setting, but drug delivery improved when a 5mg daily dose was introduced. Other studies in leukaemia have used equivalent schedules[16,17] or a loading dose (12mg) followed by 4mg/day for 7 days per cycle[11] or lower doses in

combination with low dose Ara-C[18]. However even when the subgroup of good compliers was compared separately, there was no evidence of improved disease control.

We had hoped that the development of an assay to quantitate PIA would provide insight to response or toxicity, but unlike the experience of PIA in the setting of *FLT3* inhibitor[19,20], this was not found to be consistent. In a phase 2 study in relapsed AML treated with clofarabine and temsirolomus, correlation of response to dephosphorylation of pS6RP (S6 ribosomal protein) was demonstrated.[21] However the target cells were the patient's own blasts, which were not available in the current study and it was unclear if the clinical outcome was superior to that which clofarabine alone could achieve.

Finally the mTOR inhibitors tested to date have been inhibitors of the TORC1 pathway. This may be by-passed by the TORC2 pathway which is insensitive to this class of mTOR inhibitors, but may be sensitive to agents which have dual inhibition.

Author Contributions:

AKB was co-Chief Investigator; devised the study; wrote the manuscript. EDG co-ordinated the study and wrote the manuscript. AK, LK, LP, PC, REC were highest recruiting investigators. SK and MS developed the PIA assay. RKH undertook the Statistical and data supervision and analysed the data. NHR was co-Chief Investigator. All authors reviewed the manuscript.

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Table 1: Patient Characteristics

Characteristic	**Everolimus** (n=220)	**Control** (n=112)
Age:		
16-29	33 (15%)	16 (14%)
30-39	36 (16%)	17 (15%)
40-49	58 (26%)	31 (28%)
50-59	73 (33%)	37 (33%)
60+	20 (9%)	11 (10%)
Median	48	46
Range	16-69	17-66
Sex:		
Female	117 (53%)	70 (63%)
Male	103 (47%)	42 (37%)
Diagnosis:		
De Novo	203 (92%)	103 (92%)
Secondary	5 (2%)	3 (3%)
MDS	12 (5%)	6 (5%)
WHO PS:		
0	178	88
1	37	19
2	4	3
3	1	2
4		
WBC:		
0-9.9	138 (63%)	65 (58%)
10-49.9	61 (28%)	34 (30%)
50-99.9	15 (7%)	9 (8%)
100+	6 (3%)	4 (4%)
Median	5.8	5.5
Range	0.4-177.7	0.5-249.0
Cytogenetics		
Intermediate	194 (88%)	106 (95%)
Unknown	26 (12%)	6 (5%)
FLT3 ITD		
WT	199 (96%)	101 (99%)
Mutant	8 (4%)	1 (1%)
Unknown	13	10
NPM1c		
WT	132 (65%)	61 (61%)
Mutant	70 (35%)	39 (39%)
Unknown	18	12
FLT3 TKD		
WT	204 (99%)	100 (98%)
Mutant	3 (1%)	2 (2%)
Unknown	13	10

Induction chemotherapy ADE (not randomised)	13 (6%)	7 (6%)
ADE	29 (13%)	14 (13%)
ADE+GO3	26 (12%)	14 (13%)
ADE+GO6	26 (12%)	14 (13%)
DA+GO3	22 (10%)	11 (10%)
DA+GO6	26 (12%)	12 (11%)
DA 90mg	37 (17%)	19 (17%)
DA 60mg	41 (19%)	21 (19%)
Post Course 1 Risk Score Good risk Standard Risk	 27 (13%) 193 (87%)	 11 (10%) 101 (90%)
MRD status post course 1 (CR only) CR, MRD -ve CR, MRD +ve No MRD data/no CR	 43 (20%) 63 (29%) 114 (52%)	 24 (21%) 24 (21%) 64 (57%)
Transplanted Any allograft Any transplant in CR1 Allograft in CR1	85 (39%) 69 (31%) 24 (11%) 20 (9%)	47 (42%) 38 (34%) 16 (14%) 14 (13%)

Table 2: Clinical Outcomes by Treatment Arm

	Everolimus	**Control**	**OR/HR & CI**	**P-value**
CR/CRi	99%	99%	1.02 (0.09-11.2)	1.0
MRD positivity post course 2 (CR only)	63%	65%	1.07 (0.50-2.33)	0.9
30-day mortality	1%	1%	1.48 (0.19-11.7)	0.7
60-day mortality	4%	1%	2.77 (0.70-11.0)	0.15
5 year OS	45%	58%	1.30 (0.94-1.81)	0.11
5 year RFS	29%	40%	1.19 (0.90-1.59)	0.2
5 year cumulative incidence of relapse	60%	54%	1.12 (0.82-1.52)	0.5
6 month death in CR	8%	1%	3.57 (1.36-9.42)	0.009
5 yr cumulative incidence of death in CR	11%	6%	1.75 (0.83-3.70)	0.14
5 yr survival post relapse	19%	30%	1.17 (0.81-1.70)	0.4
5 yr OS censored at SCT	57%	66%	1.34 (0.87-2.06)	0.18

Table 3: Recovery and Supportive Care in Everolimus Randomisation

Type of Care	Course	Randomisation		p-value
		Everolimus	Control	
Neutrophil recovery (median	2	28	29	0.4
From start of course	3	29	27	0.08
Platelet recovery (median	2	38	29	0.006
From start of course	3	42	36	0.10
Blood (mean units)	2	4.6	5.0	0.08
	3	6.3	6.1	0.5
Platelets (mean units)	2	5.1	3.7	0.009
	3	6.4	5.5	0.4
Antibiotics (mean days)	2	10.2	7.7	0.002
	3	12.5	10.8	0.14
Hospitalisation (mean days)	2	25.2	22.3	0.02
	3	24.8	23.5	0.3
Hospitalisation (median days)	2	25.5	23	
	3	25	24.5	

† Logrank test. * Wilcoxon test

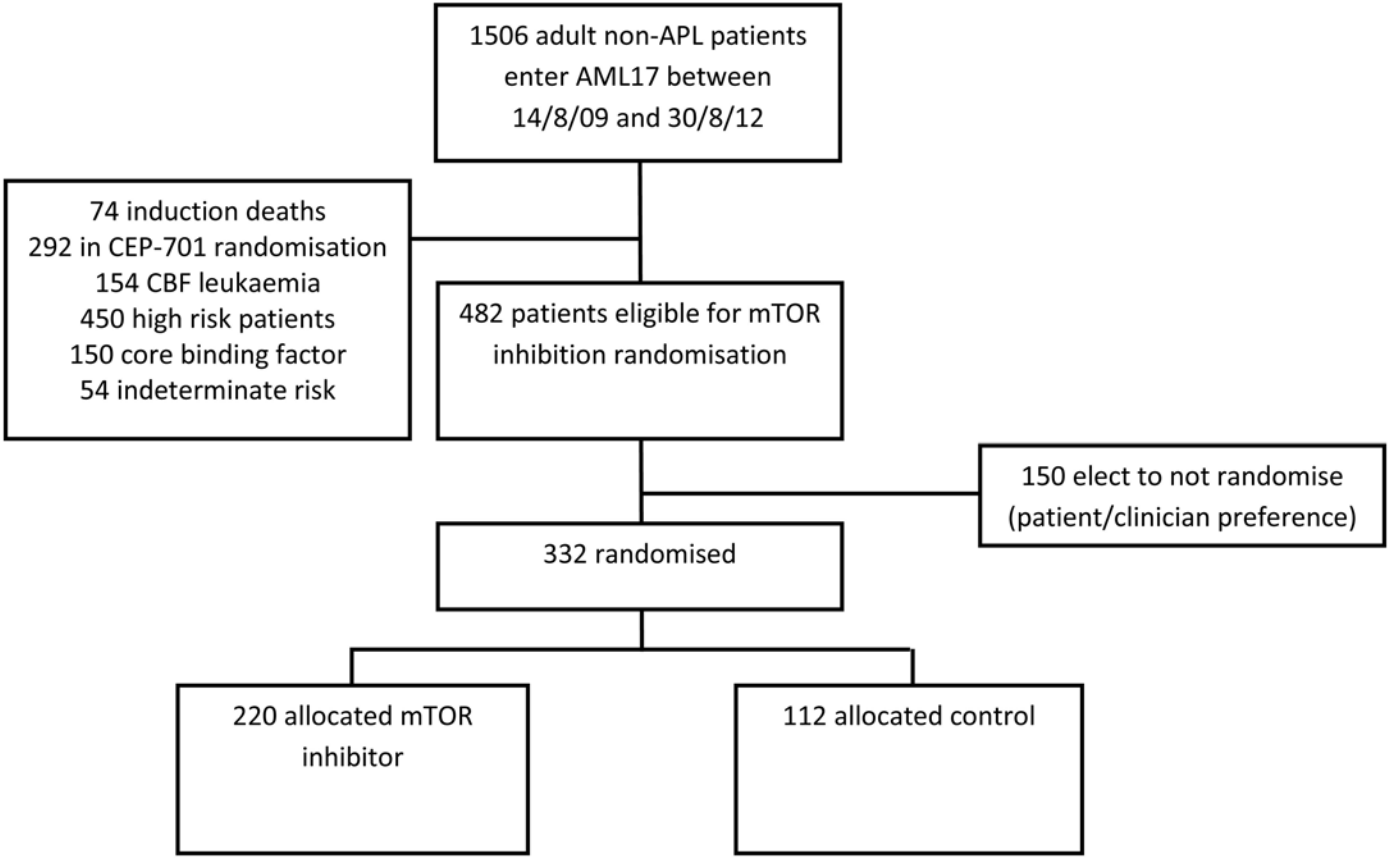
Figure Legends:

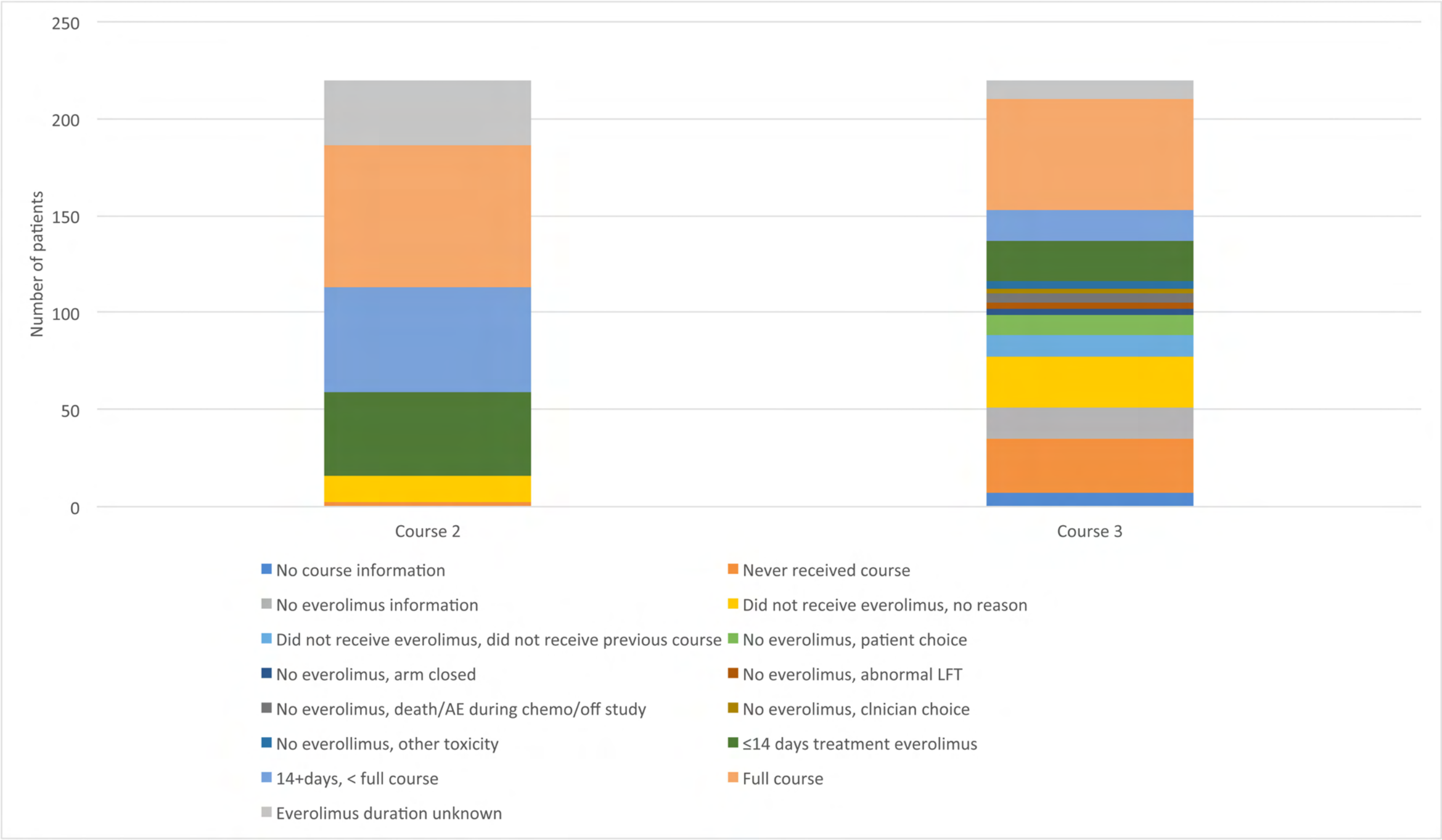
Figure 1: CONSORT diagram

Figure 2: Compliance with treatment

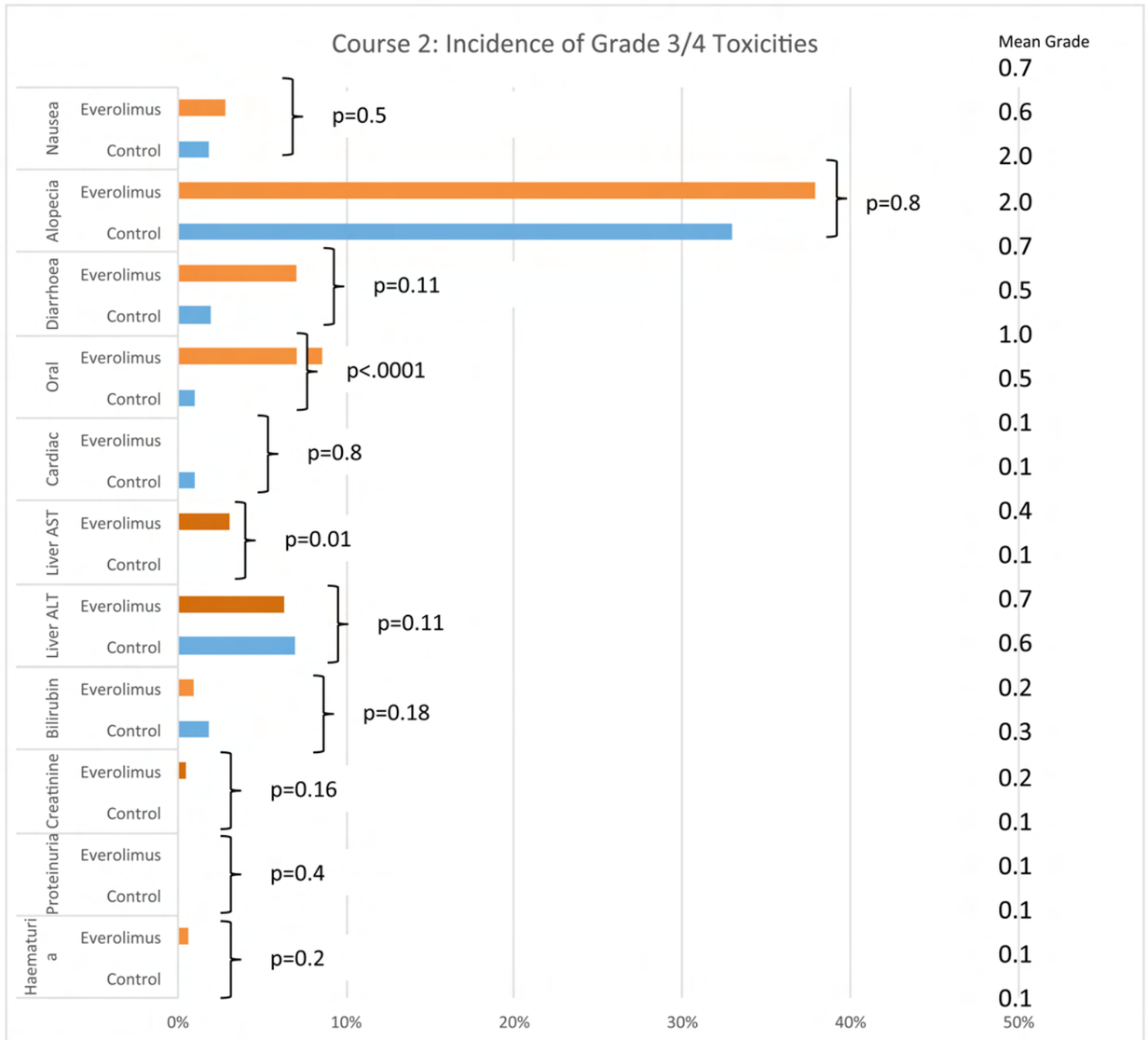
Figure 3 Toxicity associated with treatment in courses 2 and 3. A) Course 2; B) Course 3 of treatment

Figure 4: Relapse, Death in Remission, Relapse Free Survival and Overall Survival within the Everolimus Randomisation: A) Cumulative Incidence of Relapse; B) Cumulative Incidence of Death in Remission; C) Relapse Free Survival; D) Overall Survival

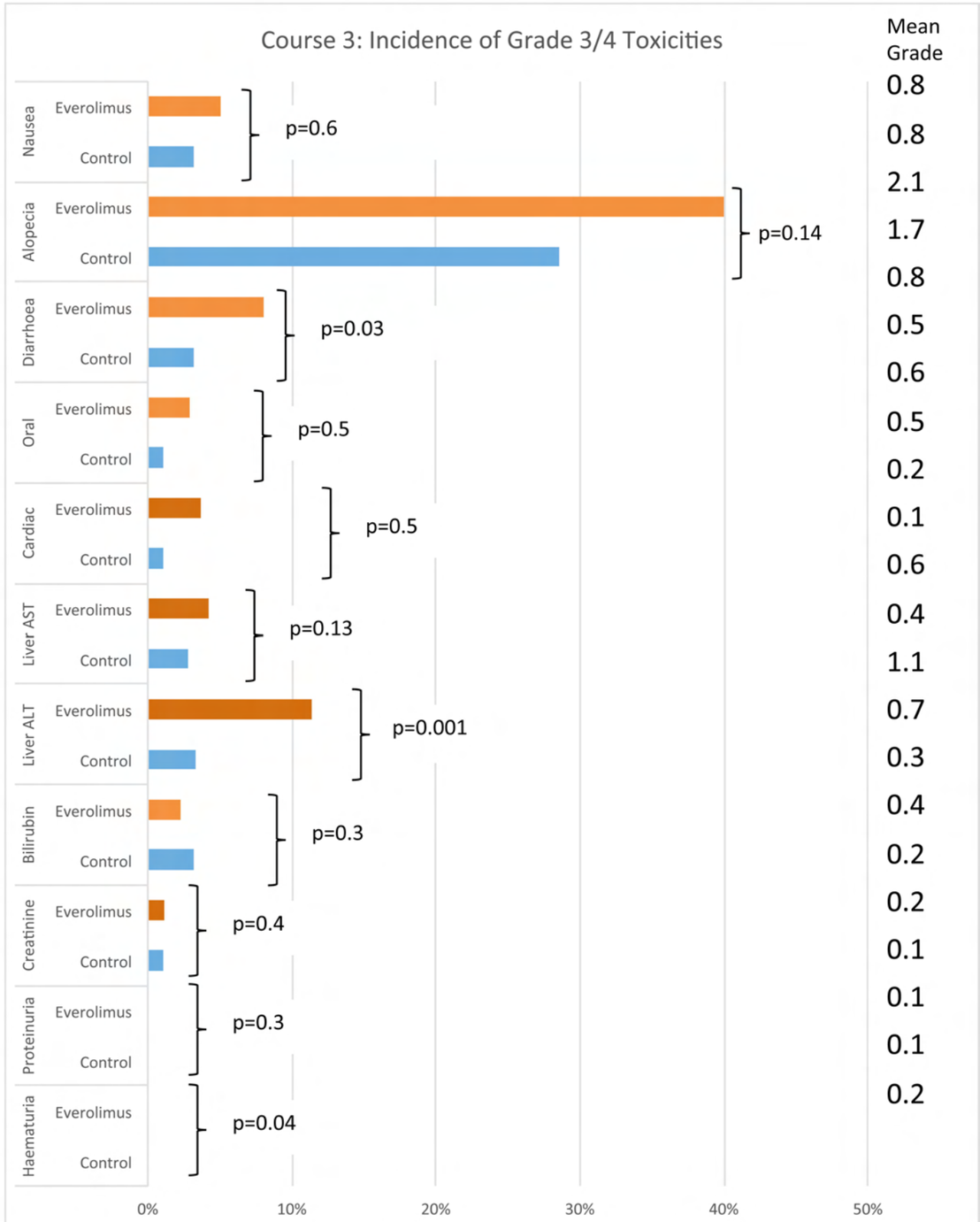

1506 adult non-APL patients enter AML17 between 14/8/09 and 30/8/12
74 induction deaths
292 in CEP-701 randomisation
154 CBF leukaemia
450 high risk patients
150 core binding factor
54 indeterminate risk
482 patients eligible for mTOR inhibition randomisation
150 elect to not randomise (patient/clinician preference)
332 randomised
220 allocated mTOR inhibitor
112 allocated control


250
200
150
100
50
0
Number of patients
Course 2
Course 3
No course information
Never received course
No everolimus information
Did not receive everolimus, no reason
Did not receive everolimus, did not receive previous course
No everolimus, patient choice
No everolimus, arm closed
No everolimus, abnormal LFT
No everolimus, death/AE during chemo/off study
No everolimus, clnician choice
No everollimus, other toxicity
≤14 days treatment everolimus
14+days, < full course
Full course
Everolimus duration unknown

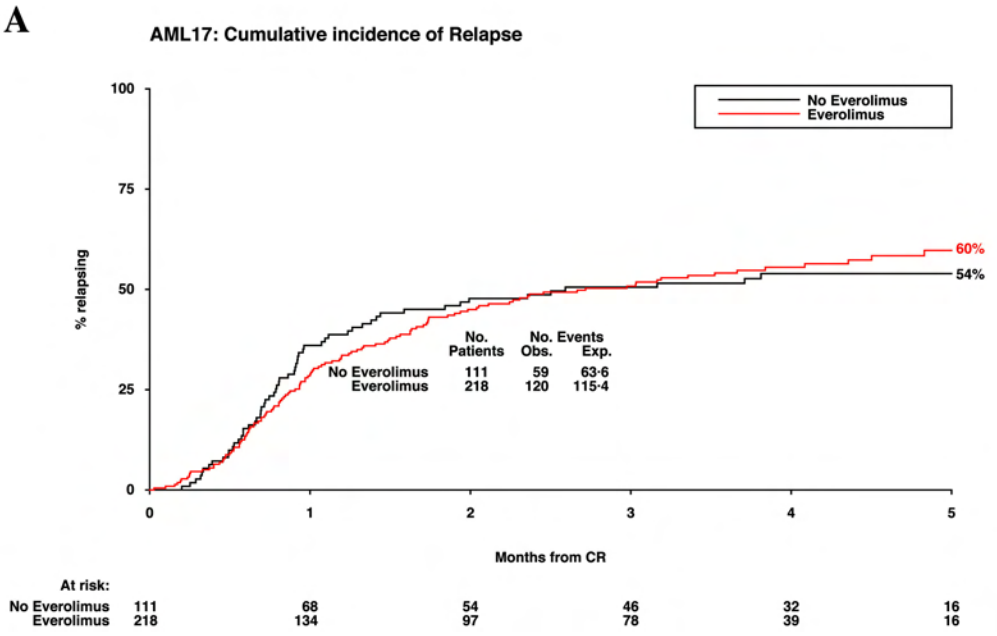
A


Course 2: Incidence of Grade 3/4 Toxicities
Mean Grade
Nausea
Alopecia
Diarrhoea
Oral
Cardiac
Liver AST
Liver ALT
Bilirubin
Creatinine
Proteinuria
Haematuria
Everolimus
Control
p=0.5
p=0.8
p=0.11
p<.0001
p=0.8
p=0.01
p=0.11
p=0.18
p=0.16
p=0.4
p=0.2
0.7
0.6
2.0
2.0
0.7
0.5
1.0
0.5
0.1
0.1
0.4
0.1
0.7
0.6
0.2
0.3
0.2
0.1
0.1
0.1
0.1
0%
10%
20%
30%
40%
50%

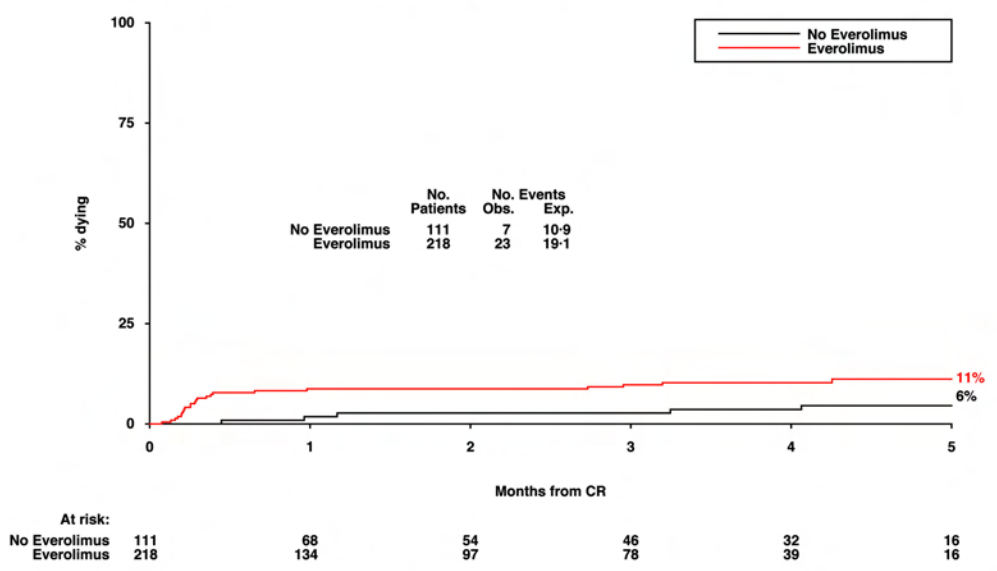
B


Course 3: Incidence of Grade 3/4 Toxicities
Mean Grade
Nausea
Alopecia
Diarrhoea
Oral
Cardiac
Liver AST
Liver ALT
Bilirubin
Creatinine
Proteinuria
Haematuria
Everolimus
Control
p=0.6
p=0.14
p=0.03
p=0.5
p=0.5
p=0.13
p=0.001
p=0.3
p=0.4
p=0.3
p=0.04
0.8
0.8
2.1
1.7
0.8
0.5
0.6
0.5
0.2
0.1
0.6
0.4
1.1
0.7
0.3
0.4
0.2
0.2
0.1
0.1
0.1
0.2
0%
10%
20%
30%
40%
50%

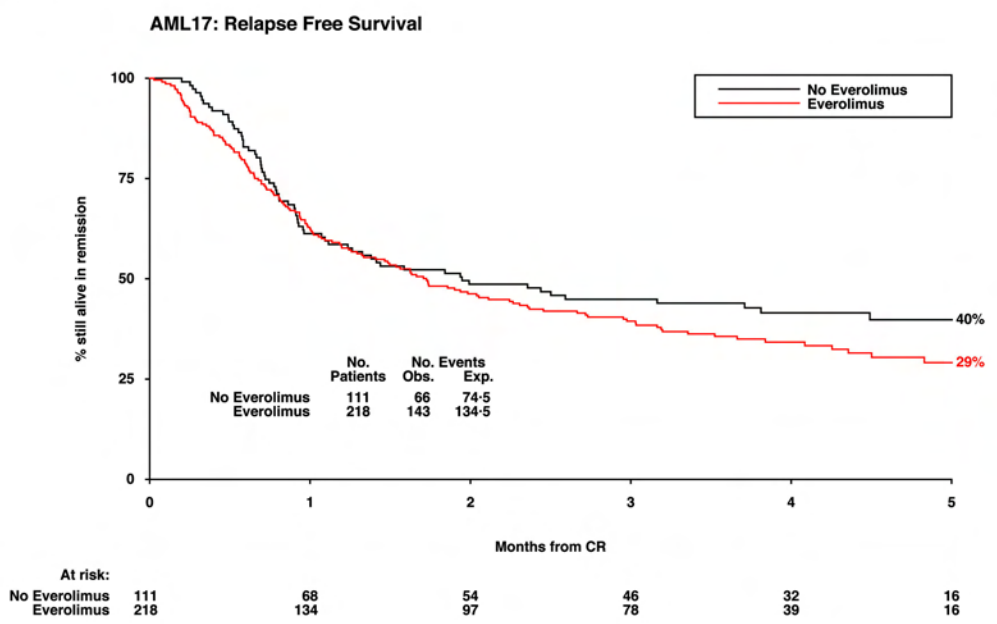
A


AML17: Cumulative incidence of Relapse
No Everolimus
Everolimus
% relapsing
100
75
50
25
0
60%
54%
No. Patients
No. Events Obs.
No. Events Exp.
No Everolimus 111 59 63·6
Everolimus 218 120 115·4
0 1 2 3 4 5
Months from CR
At risk:
No Everolimus 111 68 54 46 32 16
Everolimus 218 134 97 78 39 16

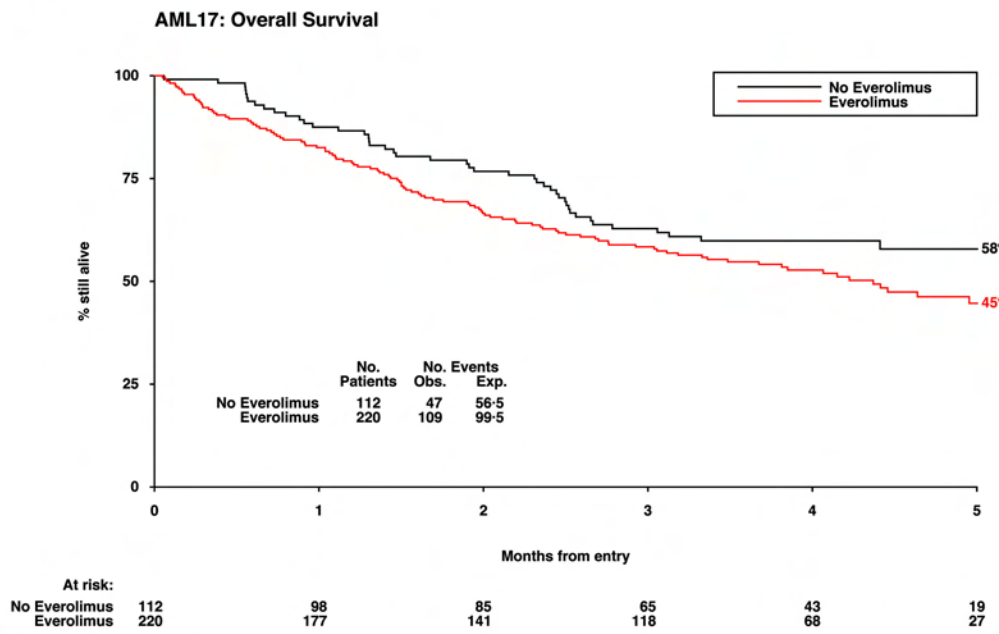
B


AML17: Cumulative incidence of death in remission
No Everolimus
Everolimus
% dying
100
75
50
25
0
11%
6%
No. Patients
No. Events Obs.
No. Events Exp.
No Everolimus 111 7 10·9
Everolimus 218 23 19·1
0 1 2 3 4 5
Months from CR
At risk:
No Everolimus 111 68 54 46 32 16
Everolimus 218 134 97 78 39 16

C

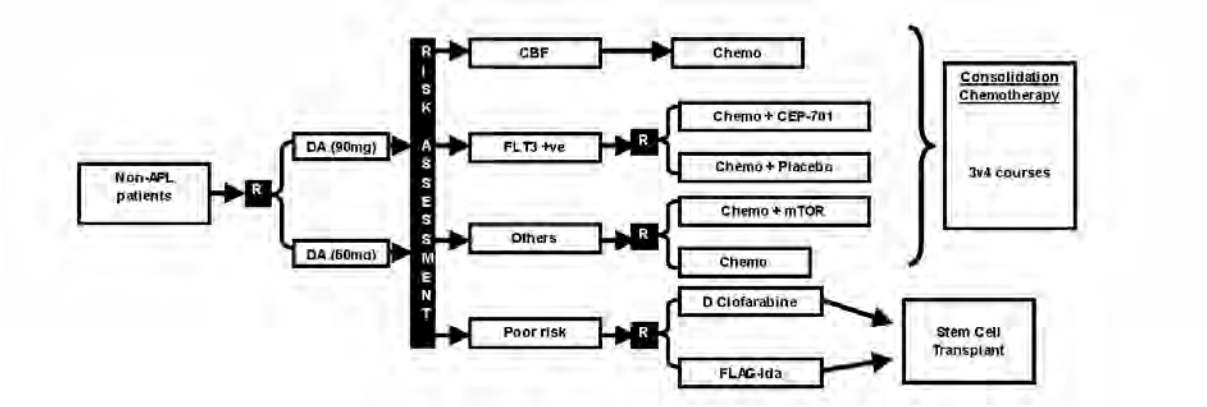

AML17: Relapse Free Survival
No Everolimus
Everolimus
% still alive in remission
100
75
50
25
0
40%
29%
No. Patients
No. Events Obs.
No. Events Exp.
No Everolimus 111 66 74·5
Everolimus 218 143 134·5
0 1 2 3 4 5
Months from CR
At risk:
No Everolimus 111 68 54 46 32 16
Everolimus 218 134 97 78 39 16

D


AML17: Overall Survival
No Everolimus
Everolimus
% still alive
100
75
50
25
0
58%
45%
No. Patients
No. Events Obs.
No. Events Exp.
No Everolimus 112 47 56·5
Everolimus 220 109 99·5
0 1 2 3 4 5
Months from entry
At risk:
No Everolimus 112 98 85 65 43 19
Everolimus 220 177 141 118 68 27

Supplemental Figure 1: Randomisations Addressed in AML17

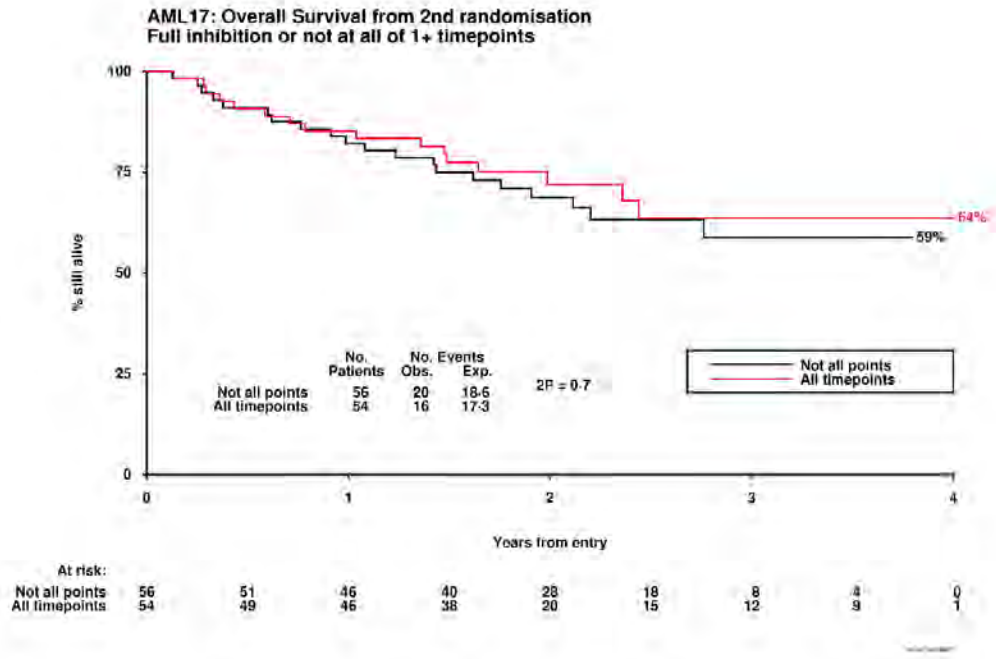
Protocol Version 7



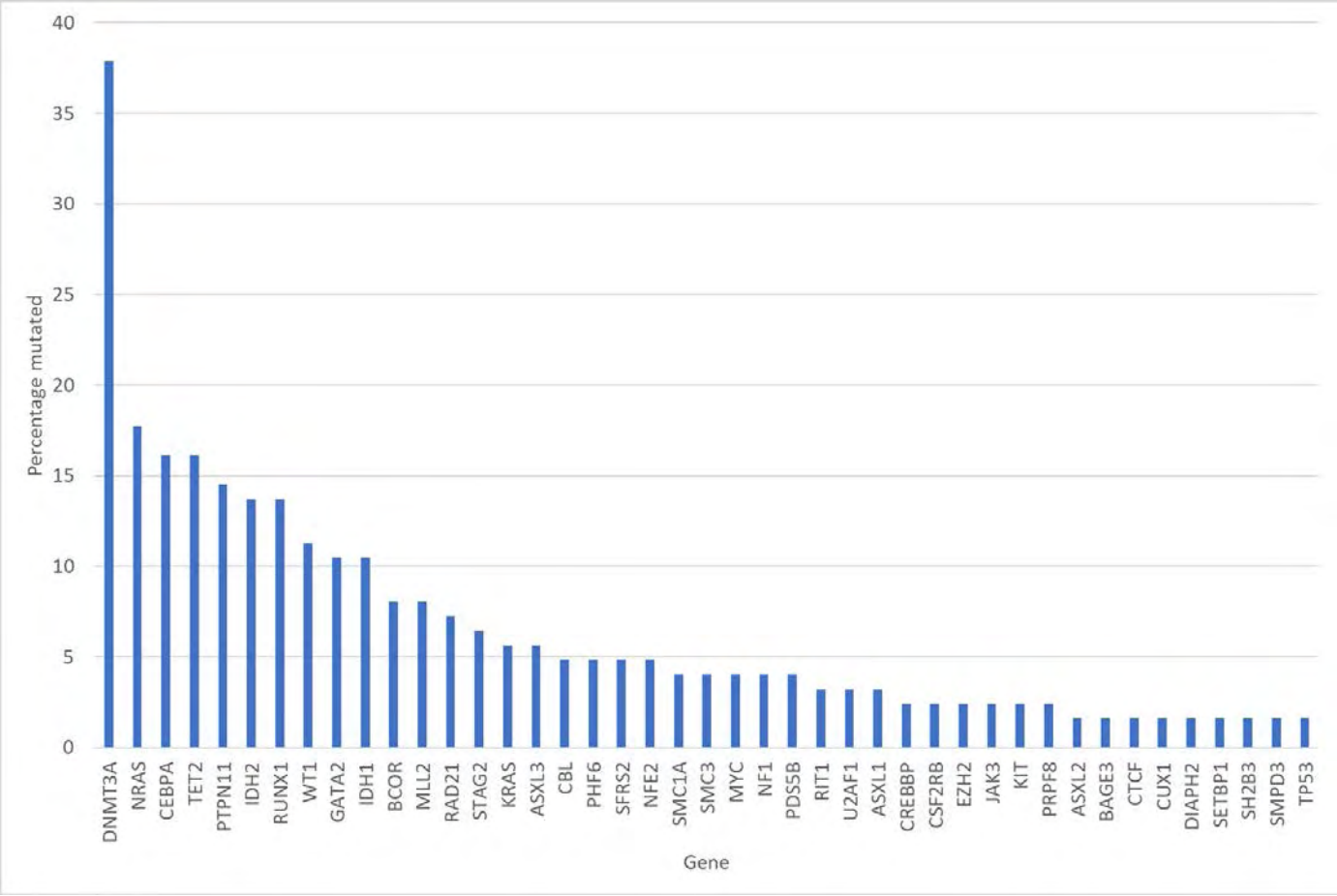
Gemtuzumab Ozogamicin 3mg/m^2 vs 6mg/m^2 (day 1 course 1)
ADE vs DA
DA (Daunorubicin 90mg/m^2) vs DA (Daunorubicin 60mg/m^2)
Lestaurtinib vs not (FLT3 mutant)
Everolimus vs not (courses 2-4)
3 vs 4 courses in total (if not high risk)
FLAG-Ida vs Daunorubicin/Clofarabine

Supplementary Figure 2: Plasma Inhibitory activity (PIA) Measurement.

The protocol requested, with patient agreement, the collection of blood samples pre-dose on day 14 of each course of everolimus treatment. To assess mTOR inhibitory activity, 400µl patient plasma was incubated in triplicate with $5x10^5$ HEL cells for 1h at 37°C in a humidified incubator with 5%CO_2. The approach was similar to those reported for other inhibitory assays.[18] A standard curve of phospho-S6 ribosomal protein (pS6-RP) PIA versus everolimus concentration was generated by spiking healthy volunteer plasma with everolimus that produce clinically-relevant concentrations ranging from 1 to 200ng/ml. In this context an estimate of plasma inhibition of phospho-S6 ribsomal protein (p S6-RP) in response to patient plasma was measured in cell lysates by immunoblotting and ELISA. The results were expressed as a percentage reduction of pS6-RP inhibitory activity compared to the maximum inhibition achieved by a 200ng/ml everolimus concentration which was run in parallel along with a no drug control.



Supplementary Figure 3: Resolution of Sanger data with AML17 mTOR randomisation (n=124)

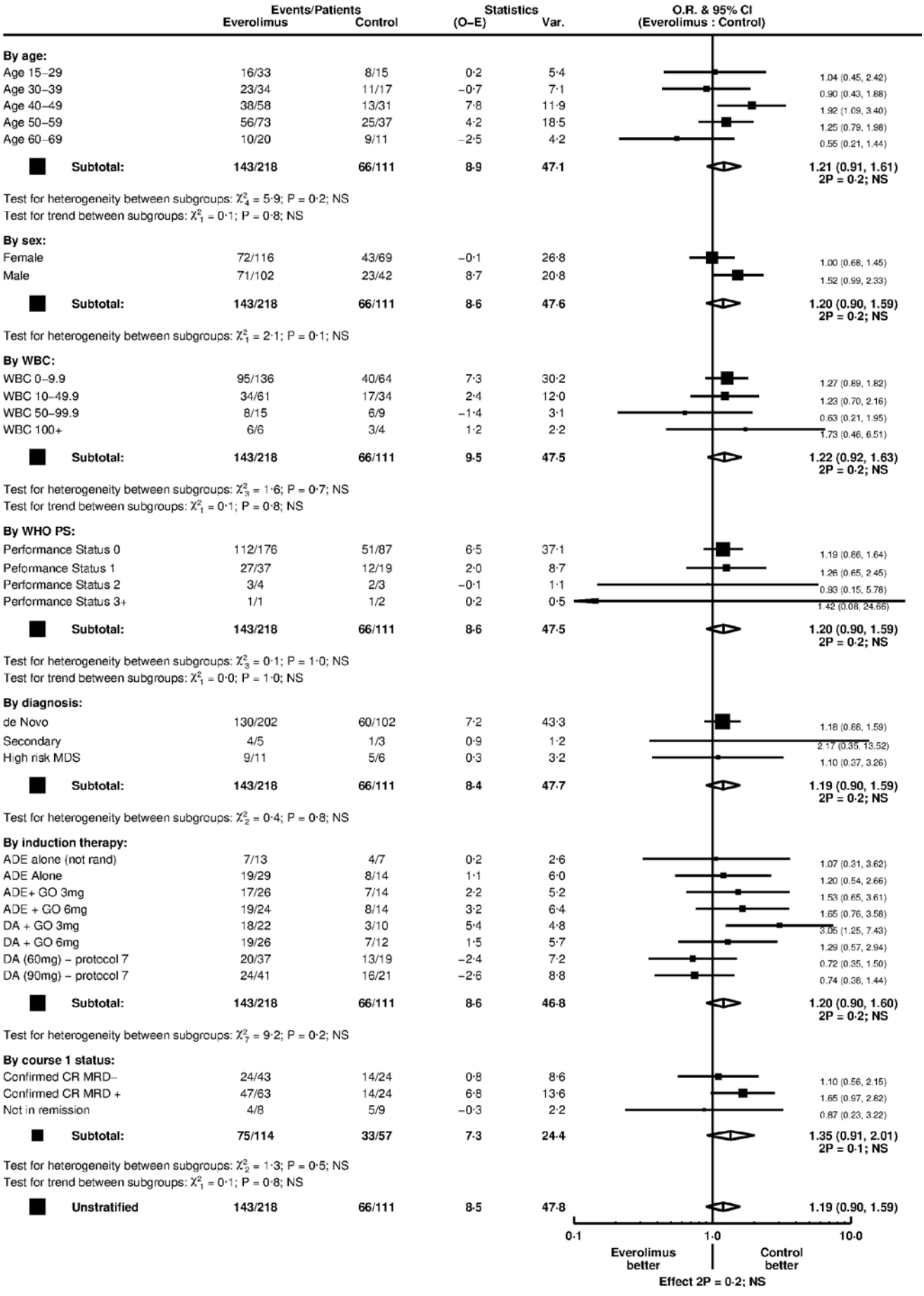


Mutations in the following genes were found in only one patient and are not shown in the graph:

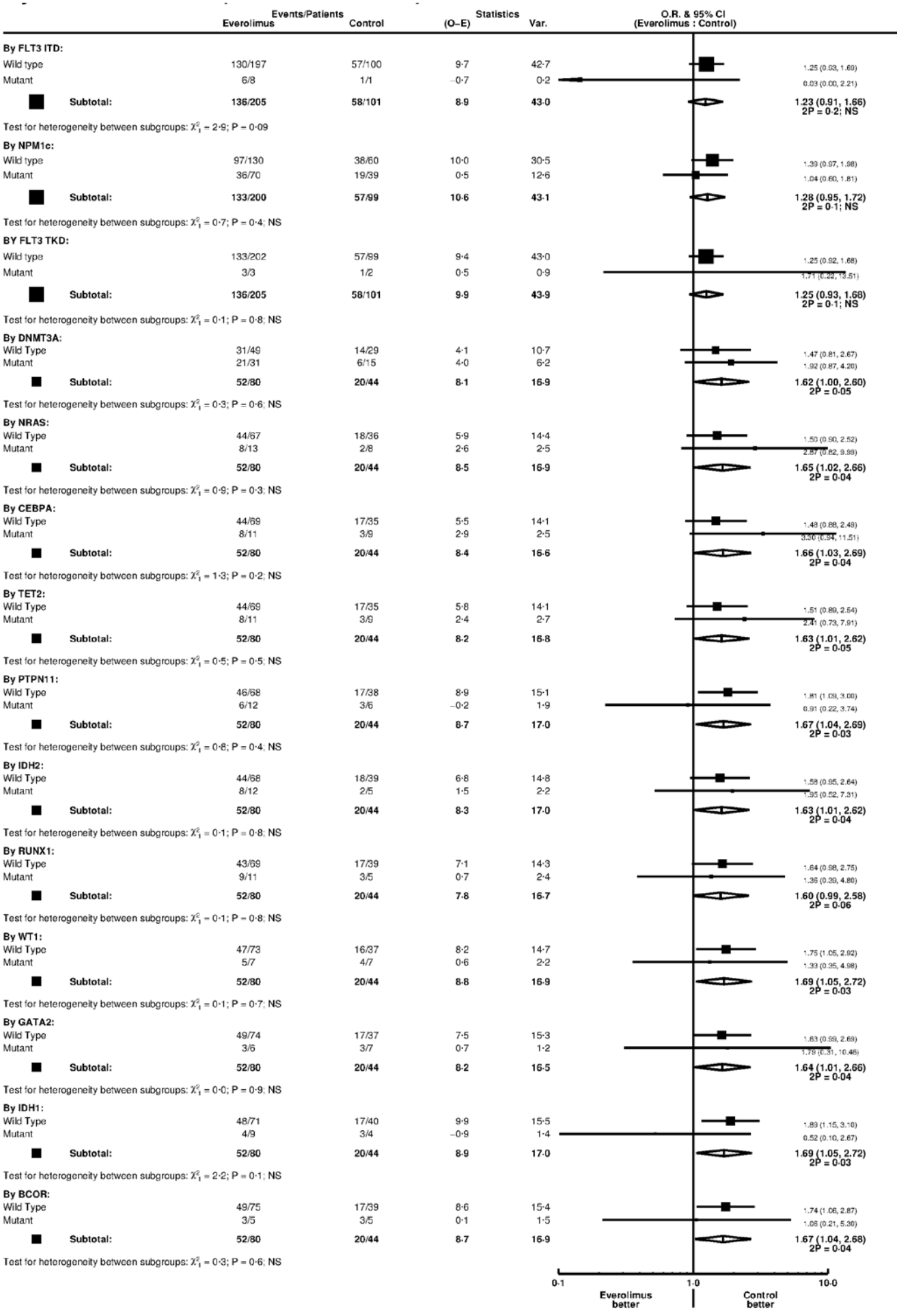
ATRX	**ERCC2**	**KIAA1267**	**PTEN**	**SMG1**
CBFB	**FBXW7**	**LUC7L2**	**PTPRF**	**STAG1**
CBLB	**GATA1**	**MED12**	**SF1**	**U2AF2**
CBLC	**KDM6A**	**MYH11**	**SF3B1**	**ZRSR2**
CSF3R				

Supplementary Figure 4: Stratified analysis of Relapse Free Survival. A) Demographics; B) Mutation status (minimum 10 mutant patients with RFS data)

A)



B)


Events/Patients
Everolimus
Control
Statistics
(O–E)
Var.
O.R. & 95% CI
(Everolimus : Control)
By FLT3 ITD:
Wild type 130/197 57/100 9·7 42·7 1.25 (0.93, 1.69)
Mutant 6/8 1/1 −0·7 0·2 0.03 (0.00, 2.21)
Subtotal: 136/205 58/101 8·9 43·0 1.23 (0.91, 1.66) 2P = 0·2; NS
Test for heterogeneity between subgroups: χ^2_1 = 2·9; P = 0·09
By NPM1c:
Wild type 97/130 38/60 10·0 30·5 1.39 (0.97, 1.98)
Mutant 36/70 19/39 0·5 12·6 1.04 (0.60, 1.81)
Subtotal: 133/200 57/99 10·6 43·1 1.28 (0.95, 1.72) 2P = 0·1; NS
Test for heterogeneity between subgroups: χ^2_1 = 0·7; P = 0·4; NS
BY FLT3 TKD:
Wild type 133/202 57/99 9·4 43·0 1.25 (0.92, 1.68)
Mutant 3/3 1/2 0·5 0·9 1.71 (0.22, 13.51)
Subtotal: 136/205 58/101 9·9 43·9 1.25 (0.93, 1.68) 2P = 0·1; NS
Test for heterogeneity between subgroups: χ^2_1 = 0·1; P = 0·8; NS
By DNMT3A:
Wild Type 31/49 14/29 4·1 10·7 1.47 (0.81, 2.67)
Mutant 21/31 6/15 4·0 6·2 1.92 (0.87, 4.20)
Subtotal: 52/80 20/44 8·1 16·9 1.62 (1.00, 2.60) 2P = 0·05
Test for heterogeneity between subgroups: χ^2_1 = 0·3; P = 0·6; NS
By NRAS:
Wild Type 44/67 18/36 5·9 14·4 1.50 (0.90, 2.52)
Mutant 8/13 2/8 2·6 2·5 2.87 (0.82, 9.99)
Subtotal: 52/80 20/44 8·5 16·9 1.65 (1.02, 2.66) 2P = 0·04
Test for heterogeneity between subgroups: χ^2_1 = 0·9; P = 0·3; NS
By CEBPA:
Wild Type 44/69 17/35 5·5 14·1 1.48 (0.88, 2.49)
Mutant 8/11 3/9 2·9 2·5 3.30 (0.94, 11.51)
Subtotal: 52/80 20/44 8·4 16·6 1.66 (1.03, 2.69) 2P = 0·04
Test for heterogeneity between subgroups: χ^2_1 = 1·3; P = 0·2; NS
By TET2:
Wild Type 44/69 17/35 5·8 14·1 1.51 (0.89, 2.54)
Mutant 8/11 3/9 2·4 2·7 2.41 (0.73, 7.91)
Subtotal: 52/80 20/44 8·2 16·8 1.63 (1.01, 2.62) 2P = 0·05
Test for heterogeneity between subgroups: χ^2_1 = 0·5; P = 0·5; NS
By PTPN11:
Wild Type 46/68 17/38 8·9 15·1 1.81 (1.09, 3.00)
Mutant 6/12 3/6 −0·2 1·9 0.91 (0.22, 3.74)
Subtotal: 52/80 20/44 8·7 17·0 1.67 (1.04, 2.69) 2P = 0·03
Test for heterogeneity between subgroups: χ^2_1 = 0·8; P = 0·4; NS
By IDH2:
Wild Type 44/68 18/39 6·8 14·8 1.58 (0.95, 2.64)
Mutant 8/12 2/5 1·5 2·2 1.95 (0.52, 7.31)
Subtotal: 52/80 20/44 8·3 17·0 1.63 (1.01, 2.62) 2P = 0·04
Test for heterogeneity between subgroups: χ^2_1 = 0·1; P = 0·8; NS
By RUNX1:
Wild Type 43/69 17/39 7·1 14·3 1.64 (0.98, 2.75)
Mutant 9/11 3/5 0·7 2·4 1.36 (0.39, 4.80)
Subtotal: 52/80 20/44 7·8 16·7 1.60 (0.99, 2.58) 2P = 0·06
Test for heterogeneity between subgroups: χ^2_1 = 0·1; P = 0·8; NS
By WT1:
Wild Type 47/73 16/37 8·2 14·7 1.75 (1.05, 2.92)
Mutant 5/7 4/7 0·6 2·2 1.33 (0.35, 4.98)
Subtotal: 52/80 20/44 8·8 16·9 1.69 (1.05, 2.72) 2P = 0·03
Test for heterogeneity between subgroups: χ^2_1 = 0·1; P = 0·7; NS
By GATA2:
Wild Type 49/74 17/37 7·5 15·3 1.63 (0.99, 2.69)
Mutant 3/6 3/7 0·7 1·2 1.79 (0.31, 10.46)
Subtotal: 52/80 20/44 8·2 16·5 1.64 (1.01, 2.66) 2P = 0·04
Test for heterogeneity between subgroups: χ^2_1 = 0·0; P = 0·9; NS
By IDH1:
Wild Type 48/71 17/40 9·9 15·5 1.89 (1.15, 3.10)
Mutant 4/9 3/4 −0·9 1·4 0.52 (0.10, 2.67)
Subtotal: 52/80 20/44 8·9 17·0 1.69 (1.05, 2.72) 2P = 0·03
Test for heterogeneity between subgroups: χ^2_1 = 2·2; P = 0·1; NS
By BCOR:
Wild Type 49/75 17/39 8·6 15·4 1.74 (1.06, 2.87)
Mutant 3/5 3/5 0·1 1·5 1.06 (0.21, 5.30)
Subtotal: 52/80 20/44 8·7 16·9 1.67 (1.04, 2.68) 2P = 0·04
Test for heterogeneity between subgroups: χ^2_1 = 0·3; P = 0·6; NS
0·1
1·0
10·0
Everolimus better
Control better

***Supplementary Figure 5: Relapse Free Survival Related to Treatment Compliance*.**