A phase I trial of ribavirin and low-dose cytarabine for the treatment of relapsed and refractory acute myeloid leukemia with elevated eIF4E

Sarit Assouline,[1] Biljana Culjkovic-Kraljacic,[2] Julie Bergeron,[3] Stephen Caplan,[1] Eftihia Cocolakis,[1] Caroline Lambert,[1] Cara J. Lau,[1] Hiba Ahmad Zahreddine,[2] Wilson H. Miller Jr,[1] and Katherine L. B. Borden[2*]*

[1]Segal Cancer Centre, Lady Davis Institute, Jewish General Hospital, McGill University; [2]Institute for Research in Immunology and Cancer & Dept. of Pathology and Cell Biology, Université de Montréal; and [3]Hôpital Maisonneuve-Rosemont, Montréal, QC, Canada

Correspondence: katherine.borden@umontreal.ca
sarit.assouline@mcgill.ca
doi:10.3324/haematol.2014.111245

Supplemental Figure 1. Cohorts of patients were treated with incrementing doses of ribavirin and two different doses of LDAC, see Table 1. Venous blood samples were collected from patients prior to start of treatment (C1D1), 24 hours (C1D2), 15 days, and 28 days (C2D1) post-start of treatment. Mean plasma levels are represented and error bars indicate standard deviations. Plasma levels of ribavirin were measured by Apredica Pharmaceutical (Watertown, MA, USA) using LC-MS methods.

Supplemental Materials and methods

Patient Selection

Patients with a diagnosis of either *de novo*, secondary AML after myelodysplastic or myeloproliferative disorder, therapy related AML of M4 or M5 French-American-British FAB subtypes subtype or high eIF4E, were eligible to participate in this study. Patients must also have been at least 18 years of age; and must have had an Eastern Cooperative Oncology Group (ECOG) performance status lower than 3; a life expectancy of at least 12 weeks; adequate hepatic and renal function (hepatic transaminase level lower than 2.5 times the institutional upper limit of normal ULN, total bilirubin level less than 1.5 times the ULN, serum creatinine level below 1.5 times the ULN). No concurrent cytoreductive chemotherapy was permitted. Patients with central nervous system (CNS) leukemia, active cardiovascular disease, intercurrent illness or medical condition precluding safe administration of the study drug and known human immunodeficiency virus infection were not permitted on study. All patients had to have reviewed and signed an appropriate informed consent approved by the institutional review boards and Health Canada in

accordance with the Declaration of Helsinki of the participating centers. ClinicalTrials.gov registry is NCT01056523.

Treatment regimen

Cytarabine was administered subcutaneously at a fixed dose of 20 mg twice a day from days 1 to day 10. Due to absorption issues, cytarabine was reduced to 10 mg twice a day from days 1 to day 10. Ribavirin (originally purchased from Zydus Pharmaceuticals, Pennington, NJ, USA and then subsequently donated by Pharmascience Inc., Montreal, Quebec, Canada) was administered continuously, twice daily with food at escalating doses.

Evaluation of response

To assess response to therapy, a bone marrow aspirate was performed prior to study start and at the end of every 28 day cycle. Clinical responses were assessed using the Cheson criteria(1). A complete remission (CR) was defined as absence of leukemic blasts from peripheral blood, fewer than 5% blasts in bone marrow, peripheral level of hemoglobin higher than 90 g/L (higher than 9 g/dL), platelet count greater than 100 × 109/L and absolute neutrophil count greater than 1×10^9/L. A designation of complete remission with incomplete blood count recovery (CRi) required that all criteria for a CR were met, but that there was either a residual neutropenia ($<1 \times 10^9$/L) or thrombocytopenia ($<100 \times 10^9$/L). Partial remission (PR) required the hematologic criteria for CR, and a 50% reduction in bone marrow blasts with a post-treatment blast count between 5 and 25%. Incomplete partial remission (PRi) required the same marrow criteria as PR but allowed for an incomplete recovery or neutrophils and platelets as for CRi. A blast response (BR)

required a greater than 2-log decrease in absolute peripheral blood blast count and/or at least a 50% decrease in bone marrow blast percentage sustained for a 28-day period in the absence of fulfilling the criteria for a CR, CRi, PR, or PRi. Progressive disease (PD) was defined as a 50% increase in the absolute number of blasts in the bone marrow relative to baseline, or an increase in the absolute peripheral blast count of at least 10×10^9/L. Stable disease (SD) was defined as failure to achieve a BR, yet not fulfilling the criteria for PD. The best response for each patient was recorded.

Study design

Ribavirin dose levels were escalated following the "3+3 design" in order to determine the maximum tolerated dose (MTD). Cohorts of 3 patients were treated with incrementing doses of ribavirin, see Table 1 from MARCH/2010 and OCT/2013. Patients withdrawn prior to completing 28 days of therapy without experiencing a dose limiting toxicity (DLT) were replaced. If any grade 3 or greater haematological or non-haematological toxicity (NCI Common Toxicity Criteria for Adverse Events V3.0) considered related to study drugs that did not resolve to grade 1 or less or baseline within six weeks of the start of Cycle 1 were observed in 2 of 6 patients, the dose level would exceed the MTD. Only patients completing 28 days of therapy were considered evaluable for response unless there was evidence of clinical progression as per investigator. All patients were included in the safety assessment.

Pharmacokinetic studies

Venous blood samples were collected pre-dose (within 5 minutes prior to the morning ribavirin dose) and 1, 2, 4, 6 and 24 hours post-dose on Cycle 1 Day 1, pre-dose on Cycle 1 Day 15, pre-dose on Day 1 of every cycle, two weeks following any dose changes and a the End-of-Treatment visit. Blood was centrifuged at 1 500 rpm for 10 minutes at 4°C. The plasma was transferred into sterile, polypropylene cryovials and stored at -70°C. Plasma levels of ribavirin were measured by Apredica Pharmaceuticals (Watertown, MA, USA) using LC-MS methods. Whenever possible, specimens were divided into 2 or 3 samples and ribavirin levels analyzed in duplicate or triplicate. Pure ribavirin (Kemoprotec, Middlesborough, United Kingdom) was used as a standard. In no instance was ribavirin observed in the samples obtained prior to the start of treatment. LDAC levels were not deteremined because it is the same molecular weight as cytosine, confounding measurement efforts.

Correlative studies

Primary AML specimens and healthy volunteers: Patients were analyzed for eIF4E, ENT1, ADK, Gli1 and UGT1A mRNA and protein (whenever possible) levels. Leukemic blasts were isolated using side and forward scatter as described in (2). Briefly, white blood cells were isolated from peripheral blood or bone marrow using Ficoll Gradient. Leukemic blasts were then isolated using CD45 dim side-scatter population as was described for M4 and M5 AML previously(4). Cells were sorted on a Becton Dickson BD FACSAria flow cytometer. For comparison, normal bone marrow, or normal $CD34^+$ cells were obtained from StemCell Technologies (Vancouver, BC, Canada). Protein and RNA were isolated as described(2). Screening of FLT3 ITDs and TDKs as

well as NPM1 mutations were done from RNAs by RT-PCR using primers previously described(2,5,6).

Reverse transcription andQuantitative PCR: DNAse treated RNA samples (TurboDNase, Ambion) were reverse transcribed using Supervilo kit (Invitrogen) for primary specimens. QPCR analyses were performed using EXPRESS SYBR® GreenER™ QPCR SuperMix (Invitrogen) in AB StepOne thermal cycler using the relative standard curve method (Applied Biosystems User Bulletin #2). All conditions were described previously(2,3).

Primers list includes:

Gli1Fw(GGCTGCAGTAAAGCCTTCAGCAAT),

Gli1Rv(TGCAGCCAGGGAGCTTACATACAT),

UbcFw (ATTTGGGTCGCGGTTCTTG),

UbcRv (TGCCTTGACATTCTCGATGGT),

RPIIaFw (TGACTGCCAACACAGCCATCTACT),

RPIIaRv (GGGCCACATCAAAGTCAGGCATTT),

G6PDHFv (TGGCAAAGTCGGTTTCTCTCTGGA),

G6PDHRv (TTGGGAACATGTCTCAGACTGGCA),

AdkFwd(AGAGGCAGCGAATCGTGATCTTCA),

Adk Rv(ACCTCCAACAAATGCATCTCCAGC),

ENT1Fwd (CTCTCAGTGCCATCTTCAAC),

ENT1 Rv (CAGAAACACCAGCAGGATGG),

RPL13aFwd(TTAATTCCTCATGCGTTGCCTGCC),

RPL13aR(TTCCTTGCTCCCAGCTTCCTATGT).

Western Blot Analysis: Western analysis was performed as described previously(2,3).

Immunofluorescence and laser-scanning confocal microscopy: Immunostaining was carried out as described (2). Briefly, upon methanol fixation (10 minutes at -20^0C), cells were blocked for 1h, and incubated with eIF4E-FITC antibody (BD) overnight at 4^0C,washed 4 times with 1xPBS (pH 7.4) and mounted in Vectashield with DAPI (Vector laboratories). For Gli1 or UGT1A staining cells were incubated with 1^0 antibodies (1:500 dilution)overnight at 4^0C,followed by 3 washes in blocking solution. Cells were then incubated with 2^0 donkey anti-rabbit IgG-Texas Red antibody (Jackson Immunolaboratories, diluted 1:100 in blocking solution), washed 4 times with 1xPBS (pH 7.4) and mounted in Vectashield with DAPI. Analysis was carried out using a laser-scanning confocal microscope (LSM510 META; Carl Zeiss, Inc.), exciting 405 and 488nm or 543nm with a 100x objective, 2x digital zoom (where indicated), and numerical aperture of 1.4. Channels were detected separately, with no cross talk observed. Confocal micrographs represent single sections through the plane of the cell. Images were obtained from LSM510 software version 3.2 (Carl Zeiss, Inc) and displayed using Adobe Photoshop CS2 (Adobe).

References

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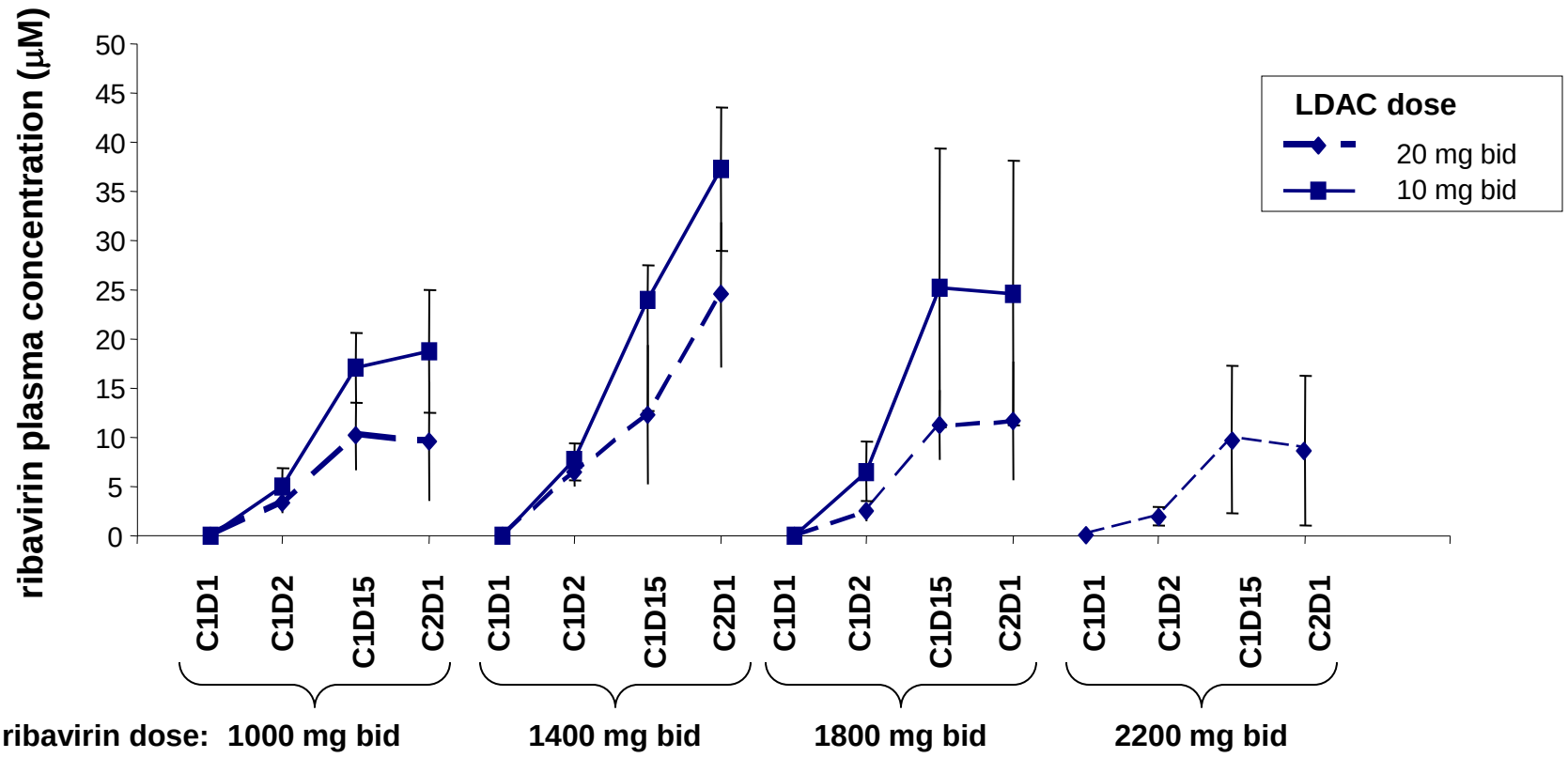
Supplemental Figure 1. LDAC 20 mg bid dose decreases ribavirin plasma levels

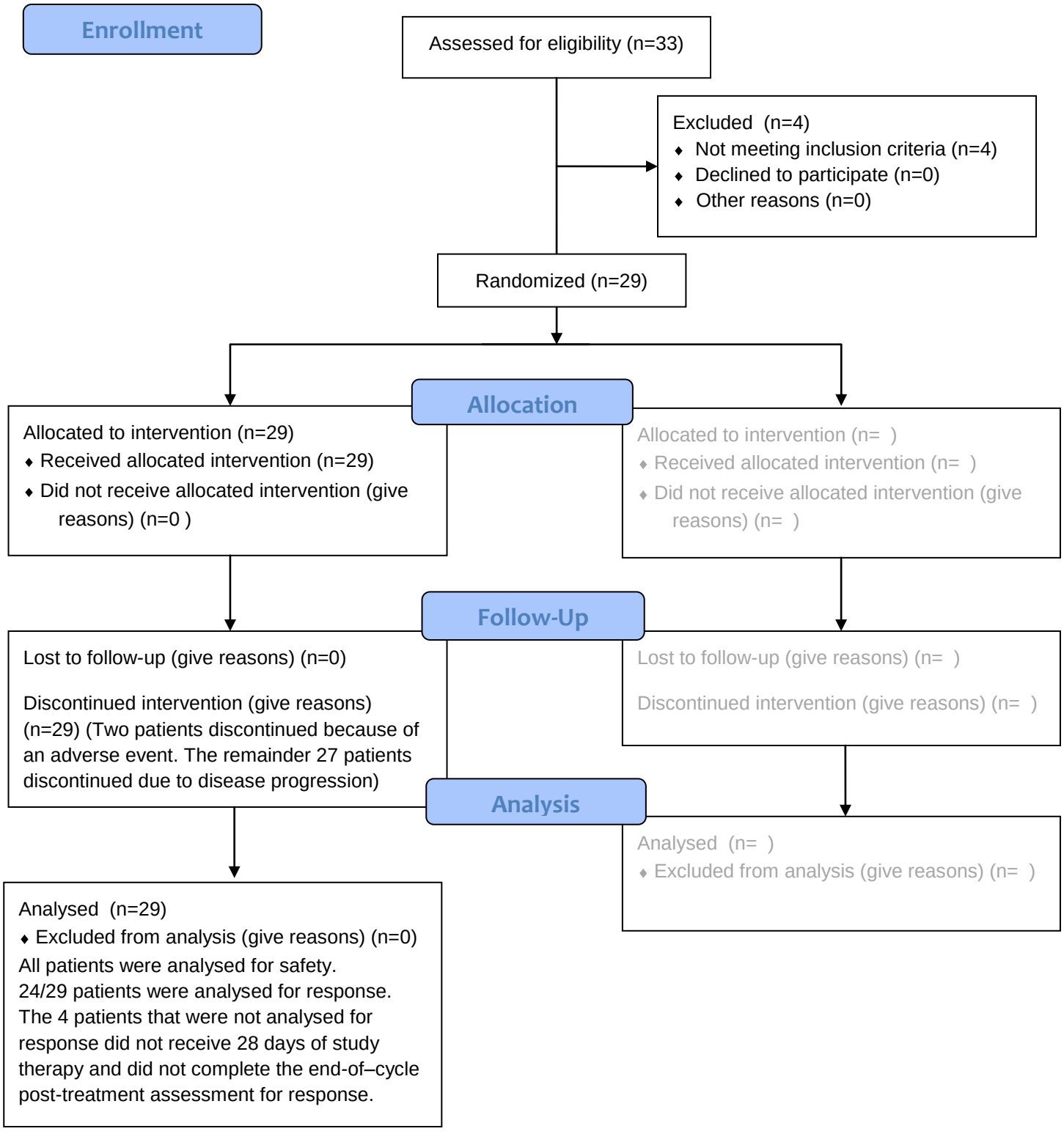


Supplemental Table 1

Patient / Response	eIF4E molecular response		Gli1 levels EOT or relapse / BT	Adk levels EOT / BT	ENT1 levels EOT / BT
	Levels of BMR or EOT / BT	Relocalization			
1. CR	0.4	+	x4.5	no change	no change
2. PD	n/a	n/a	n/a	n/a	n/a
3. SD	0.6	+/-	no change	no change	no change
4. PD	no change	no change	no change	no change	0.46
5. SD	n/a	no change	n/a (BT levels 4x higher than Normal controls)	n/a	n/a (BT levels 2x lower than Normal controls)
6. BR	0.7	+	x3.5	no change	0.5
7. PD	no change	no change	no change (BT levels 3x higher than Normal controls)	no change	no change
8. SD	0.4	no change	x1.8	no change	no change
9. PD	n/a	n/a	n/a	n/a	n/a
10. SD	0.5	+/-	x1.5	no change	0.3
11. N/A	n/a	n/a	n/a (BT levels 7.5x higher than Normal samples)	n/a (lower protein levels BT than Normal samples)	n/a
12. SD	no change	+/-	x2	no change	no change
14. PD	no change	no change	x6	no change	no change
15. N/A	n/a	n/a	n/a (BT levels 5x higher than Normal samples)	n/a (BT levels 2x lower than Normal controls)	n/a
16. PR	0.3	+	no change	no change	no change
17. N/A	n/a	n/a	n/a (Levels 3x higher than Normal samples at EOT)	n/a	n/a
18. PD	n/a	no change	n/a (BT levels 6x higher than Normal samples)	n/a (BT levels 2x lower than Normal controls)	n/a
19. CR	0.4	+	x20	0.25	no change
20. SD	no change	+	x3	no change	0.5
21. PD	no change	no change	x7 (BT levels 6x higher than Normal samples)	no change (BT levels 2x lower than Normal controls)	0.3
22. SD	0.4	+	x2.5	no change	no change
23. BR	0.3	+	x3	no change	no change
24. SD	no change	no change	X2	no change	no change
25. SD	no change	no change	x4	0.5	no change
27. N/A	n/a	n/a	n/a (High Gli1 protein levels)	n/a	n/a
28. SD	n/a	no change	n/a (BT levels 3x higher than Normal samples)	n/a	n/a
29. SD	no change	+/-	x12	0.2	0.5

CR, complete remission; PR, partial remission; BR, blast response; SD, stable disease; PD, progressive disease; N/A, not assessable; BMR, best molecular response; EOT, end of treatment; BT, before treatment;

CONSORT 2010 Flow Diagram



CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	n/a
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	1
	2b	Specific objectives or hypotheses	2-3
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	Suppl. p. 3
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	n/a
Participants	4a	Eligibility criteria for participants	Suppl. p. 1-2
	4b	Settings and locations where the data were collected	
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	Table 1, supp. p.2
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	supp. p.2
	6b	Any changes to trial outcomes after the trial commenced, with reasons	n/a
Sample size	7a	How sample size was determined	supp. p.3
	7b	When applicable, explanation of any interim analyses and stopping guidelines	n/a
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	n/a
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	n/a
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	n/a
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	n/a
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	n/a

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	n/a
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	n/a
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	n/a
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	p. 2
	13b	For each group, losses and exclusions after randomisation, together with reasons	supp. p10
Recruitment	14a	Dates defining the periods of recruitment and follow-up	supp. p.3
	14b	Why the trial ended or was stopped	p. 3
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 2
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	p. 2-4
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	p. 2-4
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	n/a
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	all exploratory
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	p. 3
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	p.4, 6
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	p. 5-6
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	p. 2-6
Other information			supp. p.2
Registration	23	Registration number and name of trial registry	
Protocol	24	Where the full trial protocol can be accessed, if available	not available
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	p. 6-7

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.