# RAndomized Clinical Trial Of NAfamostat Mesylate, A Potent Transmembrane Protease Serine 2 (TMPRSS2) Inhibitor, in patients with Covid-19 pneumonia: Safety

## *SUPPLEMENTAL DATA*

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**Running title**: Nafamostat in Covid-19 patients

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**Protocol of RACONA Study**

**Study design and aims**

The RACONA is a randomized, double-blind, placebo-controlled on top of best standard of care, phase 2 trial, with group-sequential parallel-arms. The primary aim was to assess the clinical efficacy of nafamostat mesylate in hospitalized patients with Covid-19 infection, with the efficacy defined as at least a 2-point improvement in a clinically validated seven-category ordinal scale (categories ranging from 1 to 7, with higher categories indicating a worse condition; Table S1). The secondary objective was to evaluate the effects of the drug on the clinical safety and the impact on inflammation biomarkers. The trial, registered at clinicaltrial.gov in April 2020 (Identifier: NCT04352400), received approval from Ethic Committee of INMI Lazzaro Spallanzani (IRCCS) and Italian Medicines Agency (AIFA).

**Patients**

Patients with age 18-85 years who were hospitalized because of Covid-19 infection, confirmed by a positive polymerase-chain-reaction (PCR) test and imaging, were eligible for enrollment. Inclusion and exclusion criteria are reported in Table S2. Briefly, inclusion criteria entail written signed informed consent form; body temperature >37.3°C, or history of fever in the two weeks before hospital admission, or use of fever-killing medications, and abnormal oxygenation (any of the following: *i)* oxygen saturation ≤95% on room air; *ii)* PaO2/FiO2 ratio ≤300 mmHg; *iii)* need of oxygen supplementation; respiratory rate (RR) ≥18 beats/min. Patients were excluded if *i)* pregnant or lactating females; *ii)* unwilling or not able to perform the study; *iii)* eGFR < 30 ml/min/m2 (CKD EPI formula); *iv)* liver disease (Child Pugh score ≥ 10); *v)* participation to a clinical trial with an investigational product; *vi)* history of anaphylactic shock; v*ii)* unstable hemodynamics; *viii)* active bleeding; *x)* malignancy; *xi)* chronic interstitial infiltration that can mislead diagnosis of covid-19 disease at imaging; *xii)* hyperkalemia.

**Treatments**

The study comprised 2 treatment groups: active group (nafamostat mesylate, 0.10 mg/kg/h i.v. dissolved in 5% dextrose); control group (placebo, sterile 5% dextrose i.v.). Nafamostat was kindly provided by Kyoso Mirai Pharma, Japan.

Nafamostat, an ester conjugate of p-guanidinobenzoic acid (GBA) and 6-amidino-2-naphthol, inhibits the activity of TMPRSS2 with an IC50 of between 5 and 55 nM [1,2]. The half-maximal inhibitory concentration (IC50) of nafamostat in preventing infection of alveolar epithelial cells by SARS-CoV-2 is in the range of 5-10 nM.6 The steady-state plasma concentrations of nafamostat when infused to patients with DIC at 0.1 mg/kg/h or 0.2 mg/kg/h is reported to be between 14 and 130 ng/mL, which exceeds the IC50 for TMPRSS2 inhibition.

With a half-life of 8 minutes[3] nafamostat mesylate is rapidly eliminated from the blood [4], being metabolized into inactive compounds by arylesterases and carboxylesterase 2 in the blood and liver [5].

Hence, nafamostat administration was planned as a continuous i.v. infusion over 24 hours for seven days at a dose of 0.10 mg/Kg/h. Decrease (down to 0.05 mg/Kg/h) or increase (up to 0.20 mg/kg) of the dose was allowed, depending on adverse effects, e.g. mild to moderate hyperkalemia, or persistence of fever or dyspnea. Detectable levels of the metabolite support a rapid breakdown in vivo.

Eligible patients, as defined above, were randomly allocated (1:1) to the active or control groups at baseline (day 1). To enforce control over bias, an algorithm was specifically created for this study. The algorithm uses a permuted block randomization sequence with stratification [6] Strata were defined by the cross-combination of use of oxygen therapy (nasal duct, mask, etc.) and ongoing treatment with inhibitors of the renin-angiotensin-aldosterone system, as these drugs were suggested to affect outcomes of covid-19 patients.[7–9] Investigators and patients were blinded to the treatment administered.

After screening (day -1), the patients were treated with nafamostat mesylate or placebo starting from day 1 until day 7 as specified below. Follow-up visits were done at a daily basis for the first seven days, and then on day 10, 14 and 28 to asses safety (Table S3). Vital signs, including body temperature, heart rate, respiratory rate, systolic and diastolic blood pressure (BP), oxygen saturation, were reported daily. Clinical examination included assessment of SOFA, PSI and CURB65 score and registration of adverse events, if any. Chest X-ray or CT were performed at the screening, and days 7 and 28 after the treatment (Table S3).

Blood pressure was monitored noninvasively with automated devices throughout the infusion. Since hyperkalemia was occasionally found after nafamostat treatment, serum K+ levels were measured after the first 6 hours of infusion and daily during the 7 days of drug administration (Table S4).

**Data collection**

The anonymized data were entered and stored securely in an ad hoc created web-based collection data form (Zucchetti SpA, Lodi, I), and stored securely in a server protected with firewalls and passwords.

**Concomitant treatments**

Patients were allowed to continue their treatment for pre-existing conditions, including ACE-inhibitors, angiotensin II AT1 receptor blockers (ARB). Mineralocorticoid receptor antagonists (MRA), which act via eNaC, were also allowed, but utmost attention was given to detect the onset of hyperkalemia that, if occurring, would lead to their immediate withdrawal and use of polystyrene sulfonate. Antiplatelet drugs, if needed, and ongoing treatments with antiviral and anti-inflammatory drugs, if ongoing, were allowed.

**Outcome measures**

The primary efficacy outcome was defined as the time-to-clinical improvement, defined as the time from randomization to an improvement of two points (from the status at randomization) on a seven category ordinal scale. The outcome followed the recommendations of the WHO R&D Blueprint expert group [10].

The secondary efficacy outcomes included 1) the rate of responders, defined as patient showing improvement of at least two points in seven-category ordinal scale at day 7, 10 ,14, 21 and 28; 2) the proportion of patients who progressed to critical illness/death; 3) change in pO2/FiO2 ratio over time; 4) change Sequential Organ Failure Assessment score (SOFA score) over time; 5) improvement at imaging, as assessed at chest CT, 6) duration of hospitalization (days) in survivors, 7) proportion of patients who require mechanical ventilation and duration of mechanical ventilation (Table S5).

The safety outcomes entailed adverse events (fatal and non-fatal), premature discontinuation of treatment due to adverse events, proportion of patients who develop arrhythmia, or myocardial infarction, or other cardiovascular disease not present at the baseline, hyperkalemia defined as S-K+ > 5.0 mmol/L, or hyponatremia defined as Na+< 130.0 mmol/L, hemorrhages (Table 1).

The safety biochemical outcomes included evaluation of changes over time of all investigated parameters (renal and liver function, blood cells, coagulation, and inflammation)(Tables S5 and S6).

**Setting.** University Hospital of Padova.

**Sample size.** The study was designed as an event-driven group-sequential parallel-arm trials [11], with two interim analyses, with non-binding formal control for futility. The primary objective of the study was to detect a statistically significant difference in time to 2-point improvement of the seven-category ordinal scale for the nafamostat arm relative to the control arm, using the classical Lachin design [12]. As the divergence between cases following a non-fatal vs a fatal clinical course started to occur on average after 7-10 days, it was anticipated that the median time for starting detecting a difference between the nafamostat group and the placebo group fall in between 7 to 10 days, with a hazard ratio between the treatment arms of 0.70 [13] We estimated that 200 events are needed at the final analysis, assuming a censoring time at 30 days, an overall study duration of 6 months and a uniform overall accrual time of 4 months, with a symmetric two-sided alpha level of 0.05 and a power

of 0.80. This number of events corresponds to an expected total sample size of 256 patients. Futility analyses performed at the time of interim analysis (Table S7). Computations have been performed using the R System [14, 15]and the gsDesign libraries [13].

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**Table S1. Seven-category ordinal scale**

| Score | Descriptor |
|---|---|
| 1 | Not hospitalized with resumption of normal activities |
| 2 | Not hospitalized, but unable to resume normal activities |
| 3 | Hospitalized, not requiring supplemental oxygen |
| 4 | Hospitalized, requiring supplemental oxygen |
| 5 | Hospitalized, requiring nasal high-flow oxygen therapy, noninvasive mechanical |
| 6 | Hospitalized, requiring ECMO, invasive mechanical ventilation, or both |
| 7 | Death |

**Table S2. Inclusion and exclusion criteria**

| **Inclusion criteria** |
| --- |
| Written signed informed consent form;<br>Body temperature >37.3℃, or history of fever in the two weeks before hospital admission, or use of fever-killing medications,<br>and<br>abnormal oxygenation (any of the following: i) oxygen saturation ≤95% on room air; ii) PaO2/FiO2 ratio ≤300 mmHg; iii) need of oxygen supplementation;<br>Respiratory rate (RR) ≥18 beats/min |
| **Exclusion criteria** |
| Pregnant or lactating females;<br>Unwillingness or not ability to perform the study;<br>eGFR < 30 ml/min/m2 (CKD EPI formula);<br>Liver disease (Child Pugh score ≥ 10);<br>Participation to a clinical trial with an investigational product;<br>History of anaphylactic shock;<br>Unstable hemodynamics;<br>Active bleeding;<br>Malignancy;<br>Chronic interstitial infiltration that can mislead diagnosis of covid-19 disease at imaging;<br>Hyperkalemia. |

**Table S3. Flow-chart of the study**

| Task | Enrolment | Randomization | Treatment | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Days | | | | | | | | | | |
| | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 | 21 | 28 |
| Informed consent | X | | | | | | | | | | | | |
| Inclusion/exclusion criteria | X | | | | | | | | | | | | |
| Demographics[a] | X | | | | | | | | | | | | |
| Clinical history | X | | | | | | | | | | | | |
| Randomization | | X | | | | | | | | | | | |
| Seven-category ordinal scale | X | X | X | X | X | X | X | X | X | X | X | X | X |
| SARS-CoV-2 nucleic acid detection | X | | | | | | | | X | | | | X |
| Vital signs & clinical examination | X[b] | X[b] | X[b] | X[b] | X[b] | X[b] | X[b] | X[b] | X[b] | X[b] | X[b] | X[b] | X[b] |
| Lab tests | X[c] | X[c] | X[c] | X[c] | X[c] | X[c] | X[c] | X[c] | X[c] | X[c] | X[c] | X[c] | X[c] |
| ECG | X | | | X | | | | | X | | X | | X |
| Chest X-ray | X | | | | | | | | X | | | | X |
| SOFA score | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Pneumonia Severity Index (PSI) | X | X | X | | X | | X | | X | X | X | X | X |
| CURB65 score | X | X | X | | X | | X | | X | | X | X | X |
| Adverse events | X | X | X | X | X | X | X | X | X | X | X | X | X |

a: Demographics as in WHO Global COVID-19 Clinical Platform.

b: Measurement of temperature, heart rate, respiratory rate, systolic and diastolic blood pressure, oxygen saturation.

c: see Table Laboratory tests.

**Table S4. Laboratory tests**

| Test | Enrolment | Randomization | Treatment | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Days | | | | | | | | | | | | |
| | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 | 14 | 21 | 28 |
| Angiotensin peptides | X | | | | | | | | X | | | | X |
| Direct renin concentration and plasma aldosterone concentration | X | | | | | | | | X | | | | X |
| Angiotensin-converting enzyme (ACE) | X | | | | | | | | X | | | | X |
| (ACE2 | X | | | | | | | | X | | | | X |
| Interleukin 6 (IL-6), | X | | | X | | X | | | X | | | | X |
| Soluble tumor necrosis factor receptor type II (sTNFrII) | X | | | X | | X | | | X | | | | X |
| Plasminogen activator inhibitor type-1 (PAI-1) | X | | | X | | X | | | X | | | | X |
| Tumor necrosis factor-α (TNF-α) | X | | | X | | X | | | X | | | | X |
| Soluble receptor for advanced glycation end products (sRAGE) and Surfactant Protein-D | X | | | X | | X | | | X | | | | X |
| Endothelin-1 | X | | | X | | X | | | X | | | | X |
| Lymphocyte and neutrophil counts | X | X | X | X | X | X | X | X | X | X | X | X | X |
| SARS-Cov2 IgM/IgG*** | X | X | | | | X | | | X | | X | X | X |
| Haemoglobin and red blood cells | X | X | | | | | | X | X | X | X | X | X |
| Platelet counts | X | X | X | X | X | X | X | X | X | X | X | X | X |
| PT-INR, aPTT, fibrinogen, D-Dimer and fibrinogen | X | X | X | X | X | X | X | X | X | X | X | X | X |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| degradation products (FDP) | | | | | | | | | | | | | |
| Serum ferritin | X | | | X | | X | | | X | | | | X |
| Lactate dehydrogenase | X | | | X | | X | | | X | | | | X |
| C-reactive protein and procalcitonin | X | | | X | | X | | | X | | | | X |
| S-Urea, creatinine, eGFR | X | X | X | X | X | X | X | X | X | X | X | X | X |
| S-Na+, S-K+, | X | X | X | X | X | X | X | X | X | X | X | X | X |
| U-Na+,US-K+, | X | X | X | X | X | X | X | X | X | X | X | X | X |
| AST ALT | X | X | X | X | X | X | X | X | X | X | X | X | X |
| High-sensitivity cardiac troponin* | X | | | X | | X | | | X | | | | X |
| Viral RNA titer | X | | | X | | | | | | | | | |
| Pregnancy Test** | X | | | | | | | | | | | | |
| Urinalysis | X | | | X | | X | | | X | | | | X |

* At any time if patient symptomatic for chest pain or other symptoms suggesting myocardial ischemia

**If positive, do not enroll the patient. *** These variables will be assessed also after discharge at 3, 6, 12 and 24 months.

**Table S5. Secondary Outcomes**

| **Efficacy Outcomes** |
|---|
| • Rate of responders<br>*Responder is defined as patient showing improvement of at least two points in seven-category ordinal scale at day 7, 10 ,14, 21 and 28*<br>• Proportion of patients who progressed to critical illness, defined as need of mechanical ventilation, or death<br>• Change in pO2/FiO2 ratio over time<br>• Change Sequential organ failure assessment score (SOFA score) over time<br>• Improvement at imaging, as assessed at the chest CT<br>• Duration of hospitalization (days) in survivors<br>• Proportion of patients who require mechanical ventilation and its duration |
| **Safety Outcomes** |
| • Adverse events (fatal and non-fatal) occurring during treatment<br>• Proportion of premature discontinuation of treatment due to adverse events<br>• Proportion of patients who develop cardiovascular disease not present at the baseline (*e.g. arrhythmia, myocardial infarction*)<br>• Hyperkalemia defined as S-$K^+$ $\geq$ 5.0 mmol/L<br>• Hyponatremia defined as $Na^+$< 130.0 mmol/L<br>• Hemorrhages |
| **Safety Biochemical Outcomes** |
| Changes in biomarkers over time<br>• Coagulation (platelet count, PT-INR, aPTT, fibrinogen, D-Dimer and fibrinogen)<br>• Infection/inflammation/tissue damage (C-reactive protein, procalcitonin, S-ferritin, AST, ALT)<br>• Renal function (urea, creatinine, eGFR [calculated as CKD-Epi],S-$Na^+$ and $K^+$) |

**Table S6. Secondary biomarker endpoints**

| |
|---|
| **Coagulation and disseminated intravascular coagulation (DIC)**<br>PT-INR, aPTT, fibrinogen, D-Dimer and fibrinogen degradation products (FDP), factor XII, X, VII and II activity, Plasminogen activator inhibitor type-1 (PAI-1) |
| **Renin-angiotensin-aldosterone system**<br>Circulating plasma levels of angiotensin peptides (angiotensin 1-8, angiotensin 1-7, angiotensin 1-5), and angiotensin-converting enzyme-1 (ACE-1) and angiotensin-converting enzyme 2 (ACE-2) activity, Direct renin concentration, Plasma aldosterone concentration |
| **Infection/inflammation/tissue damage**<br>Measurement of viral RNA titer over time and area under-the-curve (AUC)<br>Interleukin 6 (IL-6), Soluble tumor necrosis factor receptor type II (sTNFrII), Tumor necrosis factor-α (TNF-α), Soluble receptor for advanced glycation end products (sRAGE) and Surfactant Protein-D (markers of lung injury),<br>AST, ALT, High-sensitivity cardiac troponin I, C-reactive protein and procalcitonin, serum ferritin, lactate dehydrogenase |
| **Endothelial dysfunction**<br>C-terminal fragment of endothelin-1, Von Willebrand factor (vWF); |
| **Blood cells and immunity**<br>Lymphocyte and neutrophil counts,<br>Hemoglobin, red blood cells and platelet count,<br>IgM and IgG for Sars-Cov-2 antigens will be assessed during the in-hospital phase and afterward up to 24 months in order to determine the effect of nafamostat on acquired immunity |
| **Renal function**<br>Urea, creatinine, eGFR (CKD-Epi),<br>Serum levels of $Na^+$ and $K^+$<br>24-h urinary excretion of $Na^+$ and $K^+$, and 24h urine albumin excretion (albumin/creatinine, mg/g) |

**Table S7. Details of the group-sequential design adopted for the study.**

| Analysis | N events | Z (Efficacy) | Z (Futility) | Nominal P | Alpha spent | HR at bound for Efficacy | HR at bound for Futility | P(cross) if HR=1 for Efficacy or Futility | P(cross) if HR=0.7 for Efficacy |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 67 (33%) | 2.96 | -2.96 | 0.0015 | 0.0015 | 0.4833 | 2,069 | 0.0015 | 0.0660 |
| **2** | 133 (67%) | 2.09 | -2.09 | 0.0181 | 0.0172 | 0.6952 | 1.438 | 0.0187 | 0.4879 |
| **3** | 200 (100% | 1.71 | -1.71 | 0.0437 | 0.0313 | 0.7848 | 1.274 | 0.0500 | 0.8000 |
| **Total** | | | | | 0.0500 | | | | |

P(Cross) is the probability of crossing the bound at or before the given analysis under the assumed hazard ratio (HR). P(cross) if HR=0.7 for Futility is <0.001 at each interim.

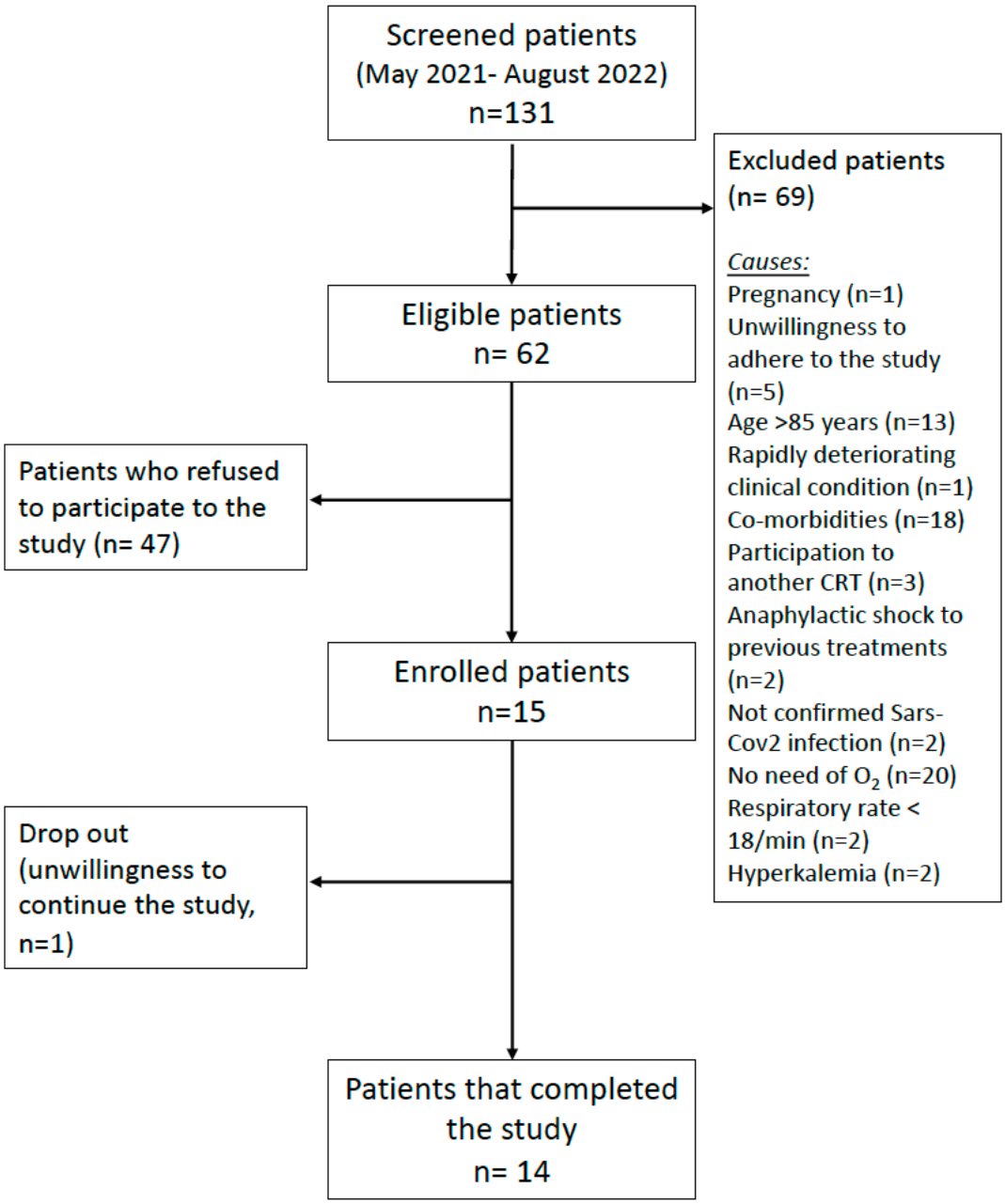


**Figure S1.** Flow chart of the RACONA Study. After screening patients who were hospitalized because of Covid-19 infection, participation to the RACONA study was offered to those who met inclusion criteria in absence of all causes that precluded their participation (see list of exclusion criteria). One of the 15 enrolled patients, one withdrew the consent from the study and, therefore, the analysis was performed in 14 patients.

**ANNEX S1: CONSORT ChecklistCONSORT 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) | 1 |
| **Introduction** | | | |
| Background and objectives | 2a | Scientific background and explanation of rationale | 1-2 |
| | 2b | Specific objectives or hypotheses | 2-3 |
| **Methods** | | | |
| Trial design | 3a | Description of trial design (such as parallel, factorial) including allocation ratio | 3 & Supplemental data |
| | 3b | Important changes to methods after trial commencement (such as eligibility criteria), with reasons | N.A. |
| Participants | 4a | Eligibility criteria for participants | 5 & Supplemental data |
| | 4b | Settings and locations where the data were collected | Supplemental data |
| Interventions | 5 | The interventions for each group with sufficient details to allow replication, including how and when they were actually administered | 3 & Supplemental data |
| Outcomes | 6a | Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed | 2-3 & Supplemental data |
| | 6b | Any changes to trial outcomes after the trial commenced, with reasons | N.A. |
| Sample size | 7a | How sample size was determined | Supplemental data |
| | 7b | When applicable, explanation of any interim analyses and stopping guidelines | 4; 10; Supplemental data |

| | | | |
|---|---|---|---|
| Randomisation: | | | Supplemental data |
| Sequence generation | 8a | Method used to generate the random allocation sequence | |
| | 8b | Type of randomisation; details of any restriction (such as blocking and block size) | Supplemental data |
| 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 | Supplemental data |
| Implementation | 10 | Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions | Supplemental data |
| Blinding | 11a | If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how | Supplemental data |
| | 11b | If relevant, description of the similarity of interventions | 3 & Supplemental data |
| Statistical methods | 12a | Statistical methods used to compare groups for primary and secondary outcomes | 3 & Supplemental data |
| | 12b | Methods for additional analyses, such as subgroup analyses and adjusted analyses | 3 & Supplemental data |
| **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 | Supplemental data |
| | 13b | For each group, losses and exclusions after randomisation, together with reasons | Supplemental data |
| Recruitment | 14a | Dates defining the periods of recruitment and follow-up | 4 |
| | 14b | Why the trial ended or was stopped | 4,10 |
| Baseline data | 15 | A table showing baseline demographic and clinical characteristics for each group | 4 |
| Numbers analysed | 16 | For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups | Supplemental data |
| 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) | 5-9 |

| | | | |
|---|---|---|---|
| | 17b | For binary outcomes, presentation of both absolute and relative effect sizes is recommended | 9 |
| Ancillary analyses | 18 | Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory | 9 |
| Harms | 19 | All important harms or unintended effects in each group (for specific guidance see CONSORT for harms) | 7 |
| **Discussion15** | | | |
| Limitations | 20 | Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses | 15 |
| Generalisability | 21 | Generalisability (external validity, applicability) of the trial findings | 17 |
| Interpretation | 22 | Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence | 9-11 |
| **Other information** | | | 11 |
| Registration | 23 | Registration number and name of trial registry | |
| Protocol | 24 | Where the full trial protocol can be accessed, if available | Supplemental data; www.clinicaltrial.gov |
| Funding | 25 | Sources of funding and other support (such as supply of drugs), role of funders | 11 |

*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.