THE MICROBIAL EFFECT OF INHALED STEROIDS IN SEVERE COPD PATIENTS WITH ASSOCIATED BRONCHIECTASIS

[TEMPESTAS]

Statistical Analysis Plan

Version No	2.1
Date	21Jul2025
Author(s)	Cat Graham
CI Name	Dr Gourab Choudhury
CI Email address	g.choudhury@ed.ac.uk

Co-sponsors	The University of Edinburgh & Lothian Health Board ACCORD The Queen's Medical Research Institute 47 Little France Crescent Edinburgh, EH16 4TJ
Funder	GSK
Funding Reference Number	213680
Sponsor Reference	AC21046
EudraCT Number	2022-000524-38
REC Number	22/NW/0079
ISRCTN Number / ClinicalTrials.gov Reference	15449782

Signatures	
Trial Statistician: *Cat Graham*	**Date: 21Jul2025**
Chief Investigator: 	**Date:** *21/07/2025*

Document Control		
Version No	**Date**	**Summary of Revisions**
1.0		Initial Creation
2.0	27Jun2025	Review prior to final analysis
2.1	21Jul2025	Resolution of wording

Table of Contents

LIST OF ABBREVIATIONS

ACCORD	Academic and Clinical Central Office for Research & Development - Joint office for The University of Edinburgh and Lothian Health Board
AE	Adverse Event
CAT	COPD Assessment Tool
CFU	Colony forming units
CI	Confidence interval
COPD	Chronic Obstructive Pulmonary Disease
CRP	C Reactive Protein
DNA	Deoxyribose nucleic acid
ECG	Electrocardiogram
ESR	Erythrocyte sedimentation rate
FEV1	Forced Vital Capacity
FEF25/75	Forced Expiratory Flow between 25% and 75% of vital capacity
FVC	Forced expiratory volume in 1 second
GOLD	Global Initiative for Chronic Obstructive Lung disease
GSK	Glaxo SmithKline
ICAM-1	Intercellular adhesion molecule 1
ICS	Inhaled corticosteroid
IMP	Investigational Medicinal Product
LABA	Long acting beta agonist
LAMA	Long acting muscarinic antagonist
SAE	Serious Adverse Event

1. Introduction

This will be a multi-centre open label randomised control trial investigating the microbiological impact of dual bronchodilators (Umeclidinium and Vilanterol 55/22 mcg) vs. triple therapy (Fluticasone furoate, umeclidinium and vilanterol 92/55/22 mcg) over 1 year in patients with GOLD Stage D COPD and coexistent diagnoses of Bronchiectasis. The trial aims to recruit 80 participants allocated in a 1:1 ratio of study triple therapy: dual bronchodilator using a minimisation algorithm stratified on the following variables: prior ICS (yes/no) and prior long-term macrolides (yes, no). The minimisation will allocate participants to the group which minimises the treatment imbalance with a built in random component to ensure the minimisation allocation is random in a 1:1 ratio to triple therapy: dual bronchodilator.

2. Statistical Methods section from the protocol

Proposed Analyses

Results will be presented broken by treatment allocation. Where data is categorical number and percent will be presented however where data is continuous descriptive statistics including mean, standard deviation (sd), median, q1, q3, min, max will be presented. As this is a proof-of-concept study we will present descriptive statistics for markers of interest by treatment arm at each time point as well the baseline to 1-year change. No covariates are planned to be adjusted for these analyses.

Where responses are continuous for example the level of change in cfu, comparison between groups will be made using a two-sample t-test and results of this will be presented accompanied by the mean and 95% for the difference between groups. We will present the time to first exacerbation using a Kaplan-Meier survival curve stratified by treatment allocation and present this will the accompanying log-rank statistic.

Where responses are binary i.e., presence of clinically significant change in cfu, comparisons between groups will be made using a binomial test for the comparison of proportions and accompanying this we will present the difference in proportion and 95% CI for the difference.

The number of participants experiencing adverse events and serious adverse events will be reported as well as the number of events per participant. Comparison will be made between treatment arms.
As information on AE/SAE will be captured during the washout phase prior to any dosing with study drug we will present this information separately for AE/SAE occurring prior to the first dosing and AE/SAE occurring after the first dosing.

Dropouts during the study will be reported and where available a reason for dropout will be captured. The dropout rate will be compared across both treatment arms

16S and ITS data: we will summarise and visualise microbial and fungal community compositions using ordination techniques/PERMANOVA-tests, allowing for global comparisons between groups. To compare alpha diversity between samples, we will look at the richness, as well as the Shannon

diversity index. Beta diversity, which represent the between sample diversity, will be investigated using the Bray-Curtis dissimilarity. In addition, using a data-driven, unsupervised clustering approach we will discern profiles that will subsequently be linked to host phenotypes, enabling us to identify microbial structures associated with host infection susceptibility. Depending on the distribution of data, we will use either classical regression (MetagenomeSeq) or a machine learning technique (randomForest) to identify microbes driving differences between patient groups.

Metagenomic sequencing: Microbial DNA will be isolated and processed as previously described. For bacterial community profiling, the V4 hypervariable region of the 16S-rRNA gene was amplified using barcoded primer pair 533F/806R.17 To profile the fungal community, we will target the ITS1 region using a two-step protocol as described by Illumina (Fungal sequencing and classification with the ITS Metagenomics Protocol) with some modifications. Amplicons will be quantified using PicoGreen (Thermo Fisher Scientific, Invitrogen, Eugene, OR, USA) and pooled in equimolar amounts. Amplicon pools of samples and controls are sequenced using the Illumina MiSeq platform (San Diego, CA, USA).

Bioinformatic processing: Bioinformatic processing of 16S reads will be performed as previously described and included quality filtering/trimming, error correction, read assembly and binning reads in OTUs of 97% similarity. Bacterial sequence variants will be annotated using the Silva database (version 119).20 Taxonomic assignment of fungal sequence variants will be annotated using the UNITE QIIME release database version 01.12.2017 and the RDP classifier in QIIME version 1.9.21

Quality control: To control for contaminating DNA we will process DNA isolation and PCR controls, and use mock communities as positive controls. Following, the decontam R-package22 will be used to identify contaminating sequence variants for 16S- and ITS-based data separately.

Quantifying biomass: We will quantify the fungal DNA concentration using Picogreen. For bacteria, we will use qPCR.

Statistical/data analysis: All analyses will be performed in SAS v9.4 or later (SAS Institute Inc., Cary, NC, USA.) or R version 3.6.3 within R studio version 1.2.5033 (Boston, MA) or a later version. We will use the packages vegan, phyloseq23, microbiome24, and ggplot225 for our microbial community structure (microbiota) analyses.

For both datasets, the relative abundances will be calculated by dividing the sequencing reads assigned to different taxa by the total number of reads per sample.

Benjamini-Hochberg (BH) adjusted P values (q values) will be used where appropriate. A P value and a q value of 0.05 will be considered significant.

All patient samples will be subjected to a similarity-based, unsupervised hierarchical clustering approach based on absolute abundances to identify community state types/clusters.

For comparisons of group differences, a one-way analysis of variance, Wilcoxon rank-sum test, Kruskal-Wallis test, or chi-square test will be used where appropriate depending on the variable(s) tested.

Group differences in diversity will be calculated using Wilcoxon tests and linear mixed-effect models. Associations between group and overall microbiota composition will be analysed using the adonis2 function (vegan package69), based on permutational multivariate analysis of variance (PERMANOVA)-tests. Differences between groups on the lowest taxonomic annotated level (OTU) will be tested using the metagenomeseq package in R studio or the machine learning method Random Forest, depending on the distribution of data in our cohort.

Please note: the section highlighted in grey above is outwith the scope of this document, this is being conducted elsewhere.

3. Overall Statistical Principles

The level of statistical significance will be defined as two-sided p=0.05 for all analyses unless otherwise stated.

3.1 Analysis populations

Participants will be included in the analysis in the groups to which they were originally assigned irrespective of treatment actually received with the exception of adverse event data which will also be presented by treatment received.

3.2 Interim Analysis

There is no planned formal interim analysis. However there is a Data Monitoring Committee who will oversee serious adverse events.

3.3 Timing of final analysis

The analysis for the main study will only be performed at study completion. It will be performed on the dataset after any 'cleaning' that may be required has been completed and the database locked.

3.4 Missing Data

The sample size has been increased to account for potential drop outs through the duration of the study. The number of participants who withdraw during the study will be presented by treatment allocation with reasons for withdrawal where available.

There are no plans to impute missing data, if required due to the amount of missing data a sensitivity analysis may be performed.

3.5 St Georges Respiratory Questionnaire for COPD patients (SGRQ-C)

Scoring of this has been conducted according to the scoring manual provided at https://www.sgul.ac.uk/research/research-operations/research-administration/st-georges-respiratory-questionnaire/docs/SGRQ-C-Manual-January-2023.pdf.

The SGRQ-C results the following:

- Symptom component: sum of weights from questions 1-7.
- Activity component: sum of weights from questions 9&12
- Impact component: sum of weights from questions 8,10,11,13,14).
- Total: sum of weights from all questions.

To convent these into scores we use the following:

$$\frac{sum\ of\ weights\ in\ that\ component}{maximum\ possible\ score\ for\ component} * 100$$

Based on the scoring manual a limited number of missing observations can be accommodated, (symptom component – maximum of 1 missing, activity component – maximum of 3 missing and impact component – maximum of 5 missing). Where missing observations are present the score is calculated

$$\frac{sum\ of\ weights\ in\ that\ component\ with\ response}{(maximum\ possible\ score\ for\ component - weight\ of\ missing\ response\ (s))} * 100$$

Scores will be presented as SGRQ-C scores.

3.6 COPD Assessment Tool (CAT)

The COPD assessment tool (CAT) consists of 8 questions each scored from 0 – I am very happy to 5 – I am very sad. The assessment results in a total score which is determined but summing the responses to all the questions, this generates a score ranging from 0 to 40. The higher the score the greater the impact of the disease.

If one or two components are missing the total score can still be generated with the missing observation be set to the average of the non-missing scores[1].

If more than two responses are missing then the CAT cannot be calculated and will be left as a missing value.

The redcap data base has a field for total score 'cat_total_score' however this should not be used as this field is the sum of responses to the 8 questions and does not account for any missing observations. The total score has been calculated to take missing observations into account.

3.7 Spirometry

The way spirometry data has been captured changed over the course of the trial. Initially there was space to record up to 3 readings for each of the spirometry markers however this was changed to capture only the maximum value.

For the purpose of the analysis described here we will use only the maximum value captured at the post-bronchodilation point.

4. List of Analyses

4.1 Recruitment

A standard accrual plot showing the cumulative total over time with a planned recruitment line shown to illustrate the projected target. In addition a plot showing the number of participants recruited each quarter.

4.2 Attendance/Compliance

For each visit the number and percent of participants will be presented and broken down by treatment.

Study drug compliance will be assessed and recorded by trial personnel by checking the dose indicators on the medication returned at the one month, six month and twelve-month (end of study).

Absolute compliance is calculated as: number of doses dispensed - number of doses returned

Percentage compliance is calculated as:

$$\frac{(number\ of\ doses\ dispensed - number\ of\ doses\ returned)}{(final\ visit\ date\# - dispensing\ visit\ date)} * 100$$

where a person has terminated the trial early, the timeframe over which the drugs should have been taken will be determined up to the point at which they withdrew from the trial so the change of status date will be used in place of the final visit date.

A 50% threshold for compliance will apply for the duration of the study.

4.3 Baseline Data

Presentation of baseline characteristics of those participating in the trial will be split by treatment allocation for the following: demographic variables (age, sex, ethnicity, socioeconomic status), employment history (currently employed, history of occupational dust exposure), lifestyle (smoking history, alcohol history), physical examination, relevant medical/surgical history, vital signs, pregnancy test/contraception, exacerbations, ECG, spirometry, concomitant medications, SGRQ-C, COPD Assessment, blood tests, sputum sampling, modified MRC breathlessness score.

A table will be presented separately from the baseline table showing the distribution of participants according the variables used in the minimisation: prior ICS (yes/no) and prior long-term macrolides (yes, no). These variables are all expressed in a binary form and will be presented as number of participants and percentage of each treatment allocation.

Where variables are categorical (including binary) numbers and percentages will be presented. Where variables are continuous the following information will be presented: number, number missing, mean, standard deviation, minimum, lower quartile, median, upper quartile and maximum.

4.4 Primary Outcome

A comparison between treatment arms for baseline to 1-year change (expressed in log units) in colony forming units per ml will be analysed using a two-sample t-test. The change will be calculated as:

$$\log(\ CFU\ per\ mL\ at\ 12\ months) - \log(\ CFU\ per\ mL\ at\ baseline)$$

Descriptive statistics will be presented by treatment arm at each time point in addition to the baseline to 1-year change, and the pattern of change over time shall be presented graphically.

4.5 Secondary Outcomes

4.5.1 Sputum

Table showing the number and percent of participants with a of clinically significant change in CFU at 12 months compared to baseline split by treatment allocation. Where a clinically significant change in CFU is defined as a difference of 1 log (CFU /ml) . Comparisons between treatment allocation will be made using a binomial test for the comparison of proportions and accompanying this we will present the difference in proportion and 95% CI for the difference.

For each of the following markers we will perform a comparison between treatment arms for baseline to 1-year change will be analysed using a two-sample t-test. Descriptive statistics will be presented by treatment arm at each time point in addition to the baseline to 1-year change, and the pattern of change over time shall be presented graphically.

- Sputum microbial diversity – ***although stipulated as an endpoint in the protocol this information is not available and will not be presented in the statistical analysis.***
- Microbial biomass expressed as quantified by 16S rDNA pg/ml

4.5.2 Sputum Colour

Sputum colour is assessed by a standardised colour chart- patients are graded as mucoid (clear or grey phlegm, muco-purulent (light yellow or green) and purulent (dark yellow or green). We will present the number (%) of participants in each group who have had an exacerbation with a change in colour. We will also provide descriptive statistics for the proportion of exacerbations per person where a colour change is present.

4.5.3 Sputum composition

Microbial community composition – ***this will be conducted by the laboratory and will not form part of the statistical analysis.***

Fungal microbiome – ***although stipulated as an endpoint in the protocol this information is not available and will not be presented in the statistical analysis.***

4.5.4 Spirometry

For each of the following markers we will perform a comparison between treatment arms for baseline to 1-year change will be analysed using a two-sample t-test. Descriptive statistics will be presented by treatment arm at each time point in addition to the baseline to 1-year change, and the pattern of change over time shall be presented graphically.

- FEV_1
- FVC
- FEV_1 / FVC ratio
- Mid-expiratory flows FEF25/75

4.5.5 Quality of life

For each of the following markers we will perform a comparison between treatment arms for baseline to 1-year change will be analysed using a two-sample t-test. Descriptive statistics will be presented by treatment arm at each time point in addition to the baseline to 1-year change, and the pattern of change over time shall be presented graphically.

- SGRQ-C total score
- SGRQ-C symptom score
- SGRQ-C activity score
- SGRQ-C impact score
- CAT total score

4.5.6 Sputum inflammation

For each of the following markers we will perform a comparison between treatment arms for baseline to 1-year change will be analysed using a two-sample t-test. Descriptive statistics will be presented by treatment arm at each time point in addition to the baseline to 1-year change, and the pattern of change over time shall be presented graphically.

- sputum myeloperoxidase
- free elastase activity

4.5.7 Serum inflammation

For each of the following markers we will perform a comparison between treatment arms for baseline to 1-year change will be analysed using a two-sample t-test. Descriptive statistics will be presented by treatment arm at each time point in addition to the baseline to 1-year change, and the pattern of change over time shall be presented graphically.

- white cell count
- eosinophil
- neutrophil
- monocyte
- lymphocyte
- erythrocyte sedimentation rate (ESR)
- C Reactive Protein (CRP)
- intercellular adhesion molecule 1 (ICAM-1)

4.5.8 Exacerbations

Table showing the number and percent of participants with radiologically confirmed pneumonia split by treatment allocation.

Descriptive analysis split by treatment allocation, of:

- number of exacerbations requiring treatment with antibiotics and or steroids
- number of exacerbations requiring treatment with antibiotics and or steroids that also includes hospitalisation

A Kaplan-Meier survival curve stratified by treatment allocation will be presented along with the accompanying log-rank statistic for the time to first moderate or severe exacerbation where this is defined as an exacerbation requiring treatment with antibiotics and or steroids.

4.5.9 Mortality rate

Table showing number and percentage of participants who died during the follow-up period split by treatment allocation.

A Kaplan-Meier survival curve stratified by treatment allocation will be presented along with the accompanying log-rank statistic for the time from baseline to death or censor (this will either be the date of 12 month visit or date of withdrawal if withdrawn prior to completion).

4.5.10 Safety Endpoints

Table showing number and percent of participants dropping out from trial (IMP only or all aspects of the trial) due to worsening COPD symptoms split by treatment allocation. Comparisons between treatment allocation will be made using a binomial test for the comparison of proportions and accompanying this we will present the difference in proportion and 95% CI for the difference.

Table showing number and percent of participants with respiratory failure split by treatment allocation. Comparisons between treatment allocation will be made using a binomial test for the comparison of proportions and accompanying this we will present the difference in proportion and 95% CI for the difference.

Exacerbations resulting in hospitalisation are previously covered in 4.5.8 and will not be covered further in this section.

4.6 Adverse Events/Serious Adverse Events

The number and percent of participants experiencing at least one AE/SAE will be presented. The number of AE/SAEs experienced by each person will be presented by treatment allocation along with descriptive statistics of the number of AEs/SAEs experienced by each participant (mean, standard deviation, minimum, 25th centile, median, 75th centile and maximum) presented by treatment.

A line listing for all SAEs will be presented by treatment allocation.

4.7 Withdrawal

The number and percent of participants withdrawing will be presented by treatment allocation, along with reason for withdrawal if available.

4.8 Protocol violations/deviations, compliance

This information will come from the sponsors violations & deviations reporting system. A line listing will be provided for violations and separately for deviations.

4.9 Unblinding

As an open label study participants and treating team are aware of treatment allocation so reporting of emergency unblinding in this instance is not applicable. Those carrying out the microbiology work are blind to the treatment allocation.

5. Validation and QC

The primary outcome will be independently validated by a second statistician or if that is not possible the primary outcome will be recoded independently of the original programming.

6. References

1 Miravitlles M, Molina J, Quintano JA, Campuzano A, Pérez J, Roncero C; DEPREPOC study investigators. Depressive status explains a significant amount of the variance in COPD assessment test (CAT) scores. Int J Chron Obstruct Pulmon Dis. 2018 Mar 6;13:823-831. doi: 10.2147/COPD.S154791. PMID: 29563782; PMCID: PMC5846753.