Statistical Analysis Plan

An open-label randomized controlled trial comparing the effect of ceftriaxone plus azithromycin versus ceftriaxone for the treatment of Neisseria gonorrhoeae on the resistome

ResistAZM trial

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1. Introduction

This Statistical Analysis Plan (SAP) provides a detailed and comprehensive description of the main, pre-planned analyses for the study. "An open label randomized controlled trial comparing the effect of ceftriaxone plus azithromycin versus ceftriaxone for the treatment of *Neisseria gonorrhoeae* (Ng) on the resistome". The purpose of this study is to examine whether dual therapy with ceftriaxone (CRO)/azithromycin (AZM) compared to monotherapy with CRO increases the probability of macrolide resistance emerging in Ng and other bacteria. The study conduct is described in the Protocol (EudraCT 2021-003616-10).

These planned analyses will be performed by the statistician(s) at the Clinical Trials Unit of the Institute of Tropical Medicine (Antwerp) in collaboration with the research consortium. The analysis results will be described in a statistical analysis report to be used as the basis of the primary research publications. This document describes statistical methods for the primary and secondary objectives of the study as defined by the protocol. Additional analyses may be performed and are not covered in the current analysis plan.

This analysis plan will be finalized and approved before database lock. Major changes in the statistical methodology used for the main and pre-planned analyses from this SAP will require detailed description and justification in the statistical analysis report. The final analysis datasets, programs, and outputs are archived following good clinical practice guidelines (ICH E9).

2. Study design and objectives

2.1. Study design

This is an open-label, single-center, randomized controlled trial in men who have sex with men (MSM) attending the ITM HIV/STI clinic. A total of 42 subjects will be recruited and randomized (1:1) to either a dual-therapy regime CRO/AZM (n = 21) or to a monotherapy regime CRO (n = 21). MSM attending the HIV/STI Outpatient Clinic of the ITM with a diagnosis of symptomatic or asymptomatic urethritis, proctitis, or pharyngitis Ng will be invited for a screening visit by a member of the site research team. During the screening visit, inclusion and exclusion criteria will be evaluated. All MSM with a lab-confirmed diagnosis (NAAT or positive by Gram/methylene blue stain) who have signed the informed consent will be randomized via a computer-generated schema to either dual-therapy (CRO/AZM) or monotherapy (AZM). In both arms, participants will be followed up in an identical fashion and will be asked to give the same number of samples.

2.2. Study objectives and endpoints

The primary hypothesis of this study is that AZM/CRO therapy for Ng, results in an increase in the abundance of macrolide resistance determinants compared to CRO therapy.

Primary objective:

Assess if there is a difference in the mean macrolide resistance determinants in the anorectal microbiome of the CRO/AZM group compared to those in the CRO group at 14 days post-treatment.

Secondary objectives:

1) Assess if there is a difference in the mean **non-macrolide** resistance determinants in the **anorectal** microbiome of the CRO/AZM group compared to those in the CRO group at 14 days post-treatment.

2) Assess if there is a difference in the mean macrolide and **non-macrolide** resistance determinants in the oro**pharyngeal** microbiome of the CRO/AZM group to those in the CRO group at 14 days post-treatment.

3) Assess if there is a difference in the proportion of oro**pharyngeal** *Neisseria* spp. that are macrolide resistant in the CRO/AZM group compared to the CRO group at day 0 and day 14 and the CRO/AZM group at day 0.

4) Assess if the DNA extracts from the **oropharyngeal** *Neisseria* cultures obtained from the CRO/AZM group on day 14 result in a more rapid acquisition in AZM resistance than those from the CRO group.

5) Assess if the **oropharyngeal** commensals *Neisseria* (Gram-negative) and *Streptococci* (Gram-positive) obtained from the CRO/AZM group at day 14 have less of an inhibitory effect on the growth of Ng than those obtained from the CRO group.

Secondary objective 2 will not be analyzed because it was decided not to perform oropharyngeal microbiome analyses among study participants. Secondary objectives 3-5 will also not be a part of the main study analysis and will be part of a lab sub-study.

Exploratory objective
Assess if there is a difference in the mean macrolide and non-macrolide resistance determinants in the anorectal microbiome of the CRO/AZM group compared to those in the CRO groups at 14 days post-treatment, adjusting for the respective values at day 0. Additional microbiome analyses, including alpha and beta diversity, will be performed by the bioinformatician and not by the study statistician.

3. Definitions and analysis strategy

Resistance determinants: The number of genes conferring resistance to specific antibiotic classes will be calculated during the microbiome analysis and the results will be provided to the study statistician as a separate database. The database will contain unique information such as subject id, visit in order to be able to combine with the main study database.

Macrolide resistance determinants: The number of genes that confer resistance to any of the antibiotics of the macrolide-lincosamide-streptogramin (MLS) category

Non-macrolide resistance determinants: The number of genes conferring resistance to different classes of antibiotics (one column per category). The categories included are:

- Aminocoumarins
- Aminoglycosides
- Bacitracin
- Betalactams
- Cationic antimicrobial peptides
- Elfamycins
- Fluoroquinolones
- Metronidazole
- Rifampin
- Sulfonamides
- Tetracyclines
- Trimethoprim

Resistance in any combination of the above groups will be coded as multi-drug resistance

Total number of reads: The total number of sequencing reads per sample. This information will be used as a denominator and as an offset in the analyses. A value total reads of 0 would indicate an incorrectly taken swab or a lab failure to amplify the collected DNA. Samples with insufficient sequencing depth will be excluded from the analysis. The threshold sequencing depth will be set based on the sequencing data from all day 14 anorectal samples, before unblinding.

Normalized resistance determinants: The number of (non-)macrolide resistance reads will be divided by the total number of bacterial reads to calculate the normalized resistance determinants. This quantity could be multiplied by 10^6 to present results in normalized determinants per million reads.

4. Description of the study population

4.1. Patient accounting

Details of participants who are randomized, those who withdraw from the study after randomization and those who are lost to follow-up will be summarized in a CONSORT flow diagram, together with the reason of exclusion or discontinuation. The number (%) of participants attending scheduled follow-up visits will be reported.

4.2. Description of the study population

Participants in each treatment group will be described with respect to baseline characteristics. Data collected during the study visits, such as STI cases, number of sex partners, mouthwash use, smoking etc, will also be described. The description will be in terms of medians and quartiles for continuous characteristics and using counts and

percentages for categorical characteristics. The clinical importance of any imbalance will be noted, though statistical tests of significance will not be undertaken.

5. Description of patient populations and outcomes

5.1. Patient populations

We will analyse the primary outcome both using Intention-to-Treat and Per-Protocol approaches, with the Intention-to-Treat approach being the primary one. In the per-protocol analysis, only participants who receive intervention as planned and follow the protocol as planned are included. For the safety analysis, all patients are included in the intervention group they actually received (all-patients-treated approach).

5.1.1. Intention to treat (ITT) analysis

In the Intention-to-Treat analysis, all randomized participants who gave at least a sample on day 14 will be analysed according to their randomized allocation, even in case they receive another intervention, show protocol violations prior to or during the study, or are lost to follow-up.

5.1.2. Per protocol (PP) analysis

In the per-protocol analysis, only participants who receive intervention as planned and follow the protocol as planned are included.
In Table 1 the protocol violations are classified as minor and major where minor violations can be included in the PP analysis population and major violations are excluded.

Table 1: The protocol violations classified as minor or major violation

Protocol Violation	Major/Minor Violation
Inclusion criteria	
1. Able and willing to provide informed consent	Major
2. Male sex at birth	Major
3. At least 18 years old	Major
4. Confirmed diagnosis of urethritis, proctitis or pharyngitis Ng – symptomatic or asymptomatic (Diagnosis of Ng will be by a positive NAAT performed according to the ITMs current laboratory protocols or for patients with urethritis a positive gram/methylene blue stain)	Major
Exclusion criteria	
5. Use of any macrolide antibiotics (azithromycin, clarithromycin, erythromycin, roxithromycin, spiramycin) in the previous 6 months	Major
6. Known contra-indications or allergy to ceftriaxone, azithromycin or lidocaine	Major

7. Presence of any other condition, including other STIs that will (likely) require the administration of another antibiotic at the time of enrolment, as assessed by the treating physician	Major
Treatment violations	
1. Not following the randomized intervention	Major
Follow-up violations	
1. Visits performed outside the specified time window (day 13-15)	Major
Sampling violations	
1. Did not provide any samples	Major

5.2. Outcomes

Primary outcome

During the bioinformatic analyses, each identified antibiotic-resistance determinant will be categorized at the class level using a read-based classification tool. The primary outcome will be the ratio of mean macrolide resistance determinants in the day 14 anorectal samples between the two treatment groups. This ratio will be calculated by dividing the mean normalized read count of macrolide-resistance determinants categorized at the class level in the CRO/AZM group by the corresponding mean quantity in the CRO group.

Secondary outcomes

The ratio of mean normalized read count of resistance determinants for each non-macrolide antibiotic class in the day 14 anorectal samples between the two treatment groups

Exploratory outcomes

The mean (normalized) resistance determinants per treatment arm on days 0 and 14.

Safety outcome

The number of participants with (severe) adverse events in each treatment group. *Since the IMPs are already marketed and widely used, a predefined list of AE (as listed below) will be reviewed at every study visit and reported in the source documents. These events will also be recorded in the eCRF (electronic Case Report Form):*

- *Diarrhea (CRO, AZM)*
- *Skin rash (CRO)*
- *Headache (CRO, AZM)*
- *Dizziness (CRO, AZM)*
- *Nausea or vomiting (CRO, AZM)*
- *Abdominal pain (AZM)*
- *Pruritus (CRO)*
- *Pain at injection site (CRO)*

6. Statistical Methods

6.1. Primary Efficacy Objective

The primary analysis of assessing the ratio between the mean normalized read count of macrolide resistance determinants in anorectal microbiome between the two groups will be done using permutation test. The p-value of the test will determine if there is a statistically significant difference between the two means at a 5% significance level. The estimated ratio of the two arms is presented with a 95% confidence interval. As an alternative analysis, we will use (zero-inflated) negative binomial or Poisson models with the number of resistant determinants as the outcome, the allocation arm as the only effect and the logarithm of the number of bacterial load as the offset term.

6.2. Secondary Efficacy Objectives

The secondary objectives regarding mean comparisons will be done similarly to the primary analysis. Alternatively, a t-test in the log-transformed means of the two groups will be performed as a comparison to the permutation test. A 95% CI for the ratio of the two groups will be calculated using permutation testing.

6.3. Exploratory Efficacy Objectives

Analysis of covariance will be used to compare the results from day 14 adjusting for baseline results between the two treatment arms. Since the sample size is small, the individual difference scores will be calculated by subtracting the normalized resistance determinants of baseline from the ones of day 14. The two groups will, then, be compared using a two sample t-test.

6.4. Safety Objectives

The patient count with adverse events in the two groups will be compared using Fisher's exact test.

6.5. Other aspects

a. Subgroup analyses

No a priori subgroup analyses are defined.

b. Multiplicity

As this is a study with a single primary efficacy endpoint. For the non-macrolide comparisons, the significance level of the p-values will be adjusted using the Benjamini-Hochberg procedure.

c. Interim analyses

No interim analyses are planned.

d. Missing data

We estimate that 5% of the day 14 visits will be missed. These individuals will be dropped from the analyses, but the amount of missing data in each characteristic will be described.