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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)

Initial Protocol

<Version 1.1, 3 September 2021>

Sponsor:	Institute of Tropical Medicine Nationalestraat 155 B-2000 Antwerpen - Belgium
Coordinating/Principal Investigator:	Dr. Chris Kenyon

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STATEMENT OF COMPLIANCE

By signing this protocol, the Investigator(s) acknowledge(s) and agree(s):

This protocol contains the necessary information for conducting this clinical study. The Principal Investigator will conduct this study as detailed herein and will make every reasonable effort to complete the study within the time designated. The Principal Investigator commits to carry out the study in compliance with the protocol, amendments, applicable procedures and other study-related documents provided by the Sponsor, and in compliance with the Declaration of Helsinki, Good Clinical [Laboratory] Practice (GC[L]P), the EU General Data Protection Regulation 2016/679 (GDPR), the ESF/ALLEA Code of Conduct for Research Integrity, and applicable regulatory requirements.

The protocol and all relevant study information, which is provided by the Sponsor, will be made available to the physicians, nurses and other personnel who participate in conducting this study. The Investigator will use this material for their training so that they are fully informed regarding the drugs and the conduct of the study.

The Sponsor of this study – the Institute of Tropical Medicine in Antwerp, Belgium (ITM) – can at any time have access to the source documents from which Case Report Form information may have been generated and will be permitted to perform trial-related monitoring and audits. All study material will be maintained according to regulatory requirements and until the Sponsor advises that retention is no longer necessary.

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Title, Name: Prof. Dr. Chris Kenyon Date: 03/09/2021

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Signing this document, I commit to carry out the trial in accordance with the protocol, Good Clinical Practice and applicable ethical and regulatory requirements. I also acknowledge the paragraph relevant to study confidentiality and authorize the Institute of Tropical Medicine, Antwerp, Belgium to record my data on a computerized system containing all the data pertinent to the study.

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SYNOPSIS

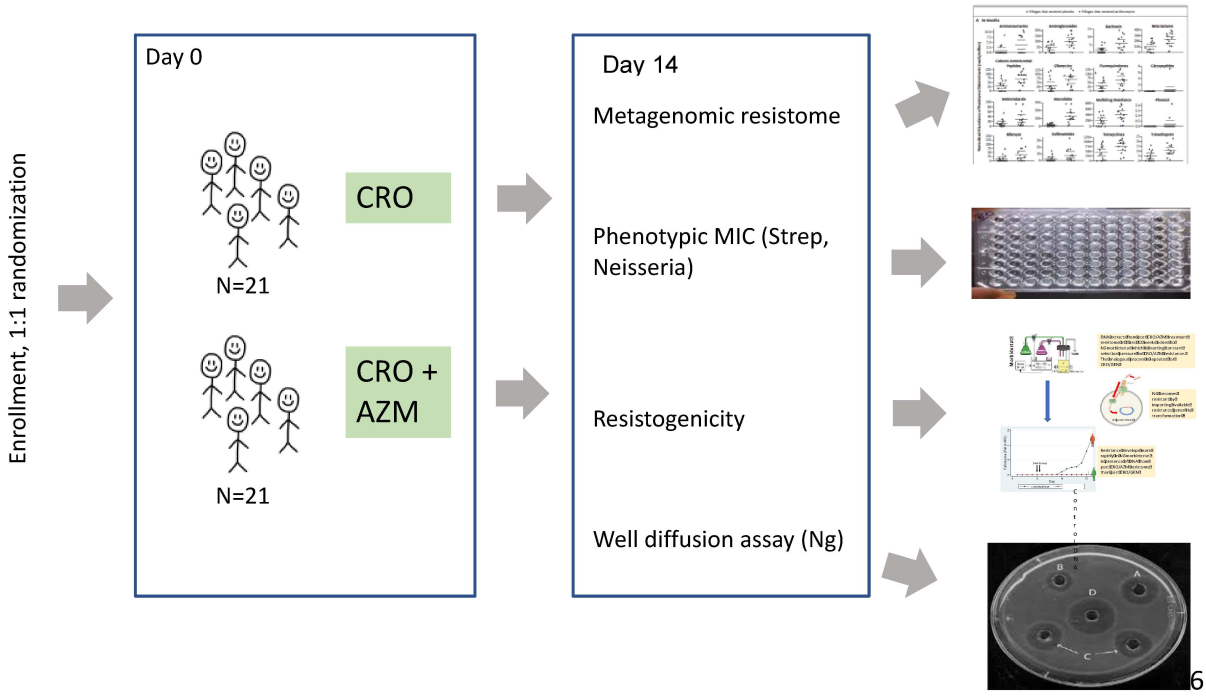
There is real possibility that *Neisseria gonorrhoeae* (Ng) may become untreatable in the near future. Understanding the reasons underpinning the emergence of this resistance could facilitate efforts to prevent pan-resistance. We hypothesize that dual therapy with ceftriaxone (CRO)/azithromycin (AZM) compared to monotherapy with CRO increases the probability of macrolide resistance emerging in Ng and other bacteria.

Both CRO/AZM and CRO are highly efficacious therapies for Ng. Whilst no randomized controlled trials (RCTs) have directly compared these two treatments, the results from other RCTs and observational studies have established very high cure rates for both regimens. The main reasons for why different national treatment guidelines recommend either CRO/AZM or CRO relates primarily to preventing the emergence of antimicrobial resistance (AMR) in Ng. Remarkably the authors of guidelines advocating both AZM/CRO and CRO claim that their first-choice recommended therapy results in a lower risk of the emergence of resistance than the alternative. In part these differences relate to the fact that no RCTs have ever been done to compare the effects of these treatments on the probability of AMR emerging in Ng.

Two important conclusions emerge from these observations. Firstly, there is equipoise in the evidence for the clinical efficacy of CRO and CRO/AZM. Secondly, there is a need for studies which compare the effects of CRO and CRO/AZM on the probability of gonococcal AMR emergence.

In this RCT, we will recruit 42 men attending the STI (Sexually transmitted infection) and HIV clinic at the Institute of Tropical Medicine, with a diagnosis of Ng and randomize them 1:1 to receive either CRO or CRO/AZM. They will be followed-up for a test of cure visit at day 14 post-treatment where urine, oropharyngeal and anorectal samples will be taken to test for cure and monitor treatment effects on the microbiome and resistome (Figure 1). The primary outcome will be evaluating the difference in the abundance of resistance conferring genes in the rectal microbiome in the two arms, 14 days after the receipt of therapy.

Figure 1. *Overview of entire ResistAZM study.*
Legend: Ng - Neisseria gonorrhoeae; MIC - Minimum Inhibitory Concentration; CRO - ceftriaxone; AZM - azithromycin.



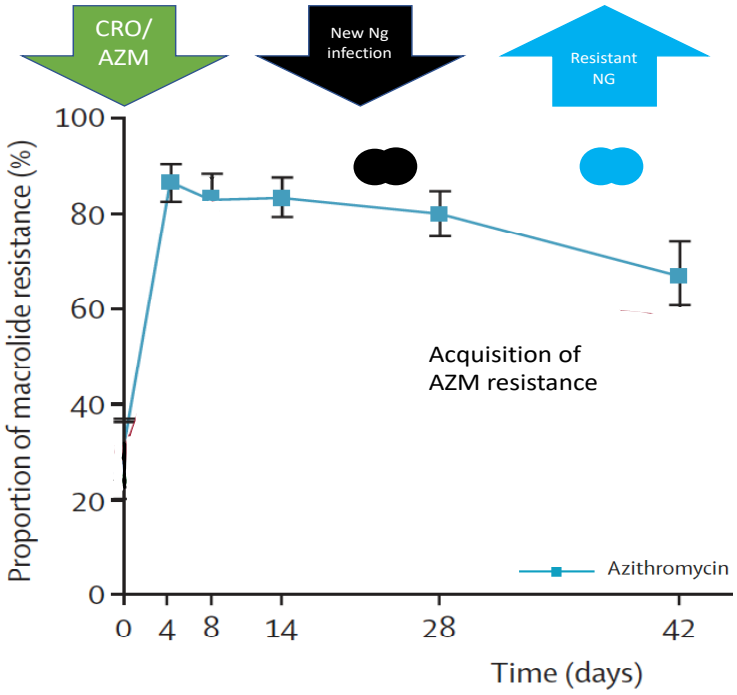
HYPOTHESIS	CRO/AZM is more resistogenic than CRO
DESIGN	Unblinded, single center, randomized controlled trial
STUDY SITE & POPULATION	Subjects will be recruited from the HIV/STI Clinic at the Institute of Tropical Medicine Antwerp. A maximum of 42 will be recruited (21 in each arm)
DURATION	Each participant will be enrolled for 15 days. Total study duration will be 8 months
OBJECTIVES AND ENDPOINTS	***Primary Objective:*** Assess if there is a difference in the ratio of macrolide resistance determinants in the anorectal microbiome of the CRO/AZM group to those in the CRO group at 14 days post-treatment. ***Primary Endpoint*:** The mean macrolide resistance determinants in the day 14 anorectal samples in the CRO/AZM group over the corresponding mean quantity in the CRO group
INCLUSION & EXCLUSION CRITERIA	• ***Inclusion criteria:*** 1. Able and willing to provide written informed consent 2. Male sex at birth 3. At least 18 years old 4. Confirmed diagnosis of urethritis, proctitis or pharyngitis Ng – symptomatic or asymptomatic • ***Exclusion criteria:*** 1. Use of any macrolide antibiotics in previous 6 months 2. Known contra-indications or allergy to ceftriaxone, azithromycin or lidocaine 3. Indication for any other antibiotic treatment at the time of enrollment
SCREENING, RECRUITMENT & RANDOMIZATION	Subjects (identified from the HIV/STI clinic) will be screened at ITM. If eligible, they will be enrolled and randomized to group 1 (CRO) or group 2 (CRO/AZM)
STUDY DRUG	Group 1: Single dose of Rocephine® (= ceftriaxone 1g + lidocaine 35mg) intramuscular injection Group 2: Single dose of Rocephine® (= ceftriaxone 1g + lidocaine 35mg) intramuscular injection + single dose of azithromycin 2g orally
FOLLOW-UP	Single follow-up visit at day 14
SAFETY	Safety of the intervention will be evaluated by recording (Serious) Adverse Events. Data will be monitored by means of a sponsor regulated pharmacovigilance system
STATISTICAL METHODS	The primary analysis of assessing a difference between the mean macrolide resistance determinants in anorectal microbiome between the two groups will be done using permutation test.

1. INTRODUCTION

1.1 Background and Rationale

Neisseria gonorrhoeae (Ng) has developed resistance to all antimicrobials used to treat it. As a result, there are fears that it may become untreatable in the near future [1-3]. Although Ng has been shown to use the full repertoire of possible resistance mechanisms [4], it is particularly adept at taking up genetic material from its environment via a well-developed system of transformation [5, 6]. Phylogenetic analyses have, for example, provided strong evidence that Ng acquired the cefixime-resistance-inducing PBP2A gene from commensal pharyngeal Neisseria [7]. The same has been established with macrolide resistance [8-11]. A range of studies have concluded that excessive macrolide consumption first results in macrolide resistance in commensal Neisseria and that this resistance is then transferred to Ng via transformation [8-11]. An important implication of Ng's ability to take up resistance mechanisms from other bacteria is that it can acquire resistance by passing through environments that have been enriched with relevant resistance genes (Figure 2) [12].

Figure 2. *A schematic illustration of how the AZM (azithromycin) component of ceftriaxone /azithromycin (CRO/AZM) dual therapy could result in AZM resistance in Ng (Neisseria gonorrhoeae). AZM given at day 0 results in a prolonged increase in the proportion of commensal oral Neisseria/Streptococci that have macrolide resistance (blue squares). A new Ng infection during this period of elevated resistance could acquire the resistance conferring determinants from these resident commensals via horizontal gene transfer.*



The importance of evaluating the effect of antibiotics on the resistome

Importantly this means that AMR surveillance needs to monitor the prevalence of AMR determinants in commensal Neisseria and other bacteria and not just pathogens such as Ng. The commensal Neisseria can be viewed as a canary-in-the-mine type of early warning system [13]. In particular when evaluating the risks and benefits of various antimicrobial options, we need to evaluate their effects

on the commensal resistome. The primary objective of this study is to compare the effects of ceftriaxone (CRO) vs. ceftriaxone/azithromycin (CRO/AZM) on the resistome.

Switch in treatment guidelines and absence of clinical evidence of superiority of CRO/AZM vs CRO in the treatment of N. gonorrhoeae

There are currently two main schools of thought as to the optimal therapy of Ng: monotherapy with CRO and dual therapy with CRO/AZM (henceforth termed dual therapy). Dual therapy rose to prominence in multiple countries around 2012 [14, 15]. Around this time both the European IUSTI and the United States CDC Ng treatment guidelines changed to advocating dual therapy [14, 15]. The rationale was not based on any clinical trials or observational studies, but on expert opinion that dual therapy would be predicted to retard the emergence of gonococcal AMR. Of note the combination of AZM and CRO has not been shown to exhibit any synergy in vitro or in vivo [16, 17]. No RCTs have been performed to directly compare the two treatment options (Box 1 – page 12) [18]. Over the past 2 years a number of prominent groups have reevaluated the evidence for the two treatments options and changed their recommendations. In the case of the United States and the United Kingdom, the national guidelines have recently been changed to explicitly recommend monotherapy with CRO as the preferred therapy for Ng [19]. The European IUSTI Ng treatment guidelines were also revised in 2020 to include mono-therapy as an alternative treatment strategy (Box 1). The guidelines in the Netherlands continue to recommend CRO monotherapy [20].

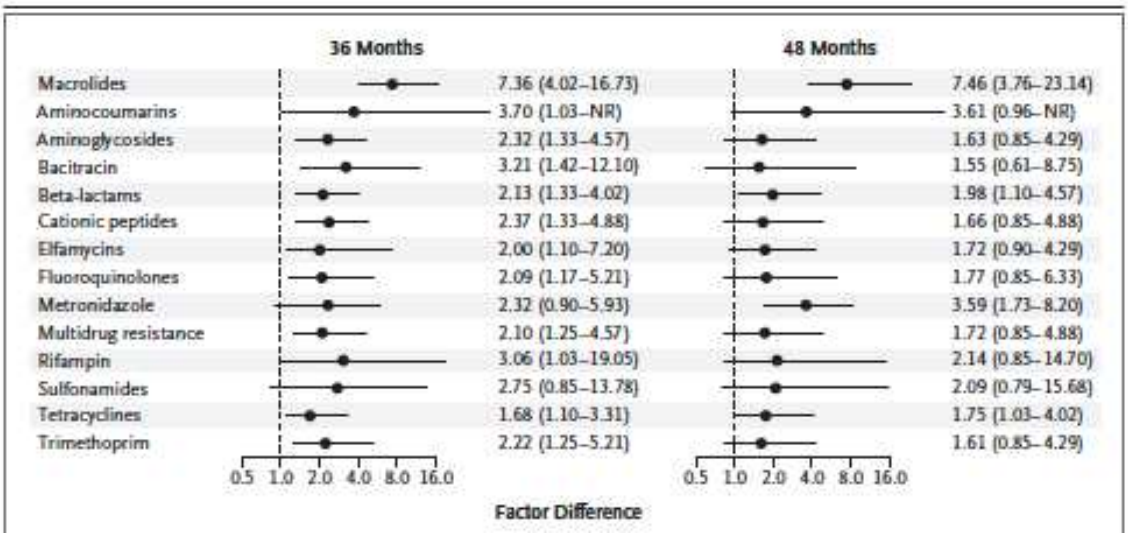
Five lines of evidence contributed to this shift:

1. **Increasing AZM resistance.** In Belgium the percentage of Ng isolates with reduced susceptibility to AZM (MIC – Minimum Inhibitory Concentration > 1mg/L) has increased from 0.2 to 16% over the past 8 years – the period that dual therapy has been used [21]. An increase in AZM resistance has been noted in multiple countries in Europe [22]. In the USA there has been an increase in AZM reduced susceptibility isolates from 0.6% in 2013 to 4.6% in 2018 [19]. The prevalence of Ng AZM resistance is so high in a number of populations around the world that certain authors have argued that its use can no longer be justified [15, 24, 25]. Confirmed cases of treatment failure following dual therapy have reinforced this perspective [26].
2. **Macrolide resistance and stewardship.** AZM is classified by the WHO AWARE typology as a second line antimicrobial whose use should be avoided where ever possible so as to prevent the emergence of AMR and preserve its clinical utility [27, 28]. The use of dual therapy for Ng has played an important role in elevating macrolide consumption levels in contemporary HIV Pre-Exposure Prophylaxis (PrEP) populations to 7-fold higher than thresholds for the induction of macrolide resistance in a range of bacteria including *Treponema pallidum, Mycoplasma genitalium, Streptococcus pneumoniae* and Ng [21, 29, 30]. We have confirmed this high macrolide consumption both in Belgium and other countries and shown that treatment for Ng plays an important role in driving this high consumption [21, 31]. In Belgium we have gone one step further and ascertained that the prevalence of high level resistance to AZM in commensal Neisseria species in Men who have Sex with Men (MSM) attending the ITM STI clinic is considerably higher than that ever recorded anywhere in the world [32]. We have hypothesized that a likely driver of this resistance is high levels of macrolide consumption including those used as dual therapy for Ng [32].

These concerns have been reinforced by studies that have demonstrated the long-term adverse effects of macrolides on the resistome. Studies have shown that the quantity of ermB genes (conferring macrolide resistance) in the colonic microbiome for example increase 3 to 5 orders of

magnitude for 2 to 4 years after a single course of macrolides [33-35]. These changes in the resistome are mediated by increases in macrolide-resistant enterococci [36], streptococci [37] and staphylococci [36, 38]. A more recent study, MORDOR, found that 6-monthly mass administration of AZM to children in Niger, resulted in a large increase in the abundance of genes in the gastro-intestinal tract conferring resistance to macrolides and a range of other classes of antimicrobials (Figure 3) [39]. This study also revealed a significant increase in the prevalence of macrolide resistance in S. pneumoniae in communities receiving mass AZM [40]. Other studies have found that a single course of AZM can lead to elevated AZM MICs in oropharyngeal streptococci and that this effect can persist for 6 months [37].

Figure 3. *Results from the MORDOR study showing the difference in abundance of antibiotic-resistance determinants in the azithromycin-treated group as compared with the group that received placebo, with associated 95% confidence intervals. The abundance of macrolide and other antibiotic classes resistance determinants was ascertained from rectal swabs from recipients at 36 months and 48 months (From [39]).*



3. **Pharmacokinetic considerations of dual vs. monotherapy.** Although human studies have not been performed to evaluate the length of time that CRO needs to be above the MIC to eradicate Ng at each relevant anatomical site, Monte Carlo models have revealed that this time should be above 20 hours to eradicate urogenital Ng [41]. Various lines of evidence including those from a mouse model of Ng suggest that a CRO dose of 500-1000mg given IM will provide a time above the MIC of 24 hours which is sufficient to eradicate Ng [42]. These considerations suggest that monotherapy with CRO dosed at 500mg to 1g IM would be sufficient to consistently eradicate Ng.

4. **Epidemiology of Ng in populations using monotherapy.** Certain countries such as the Netherlands never advocated the use of dual AZM/CRO therapy in their national guidelines. There is no evidence of either a higher treatment failure rate or a decrease in cephalosporin and other antimicrobial class susceptibility in these populations [18].

5. **Importance of horizontal versus vertical transmission of AMR.** The rationale for using multiple agents to prevent the emergence of AMR is likely to be stronger in infections where

horizontal gene transfer (HGT) does not play a prominent role such as *Mycobacterium tuberculosis* (MTB) and *Plasmodium falciparum* [31, 43, 44]. In the case of MTB, using rifampicin in combination with isoniazid has been shown to reduce the probability of resistance emerging to either agent. Combination therapy will not result in isoniazid resistance via selection of resistance in commensal Mycobacteria and subsequent transmission of this resistance to MTB via HGT [44]. This is because HGT occurs at an extremely low frequency in MTB [44].

There is however increasing evidence that this indirect collateral pathway plays an important role in the emergence of AMR in Ng [7, 8, 31, 43]. Using AZM in dual therapy may thus select for AMR in Ng via first selection of macrolide resistance in commensal Neisseria or other bacteria (Figure 2) [8, 9]. As noted already a range of studies have confirmed that macrolide resistance in Ng has emerged via this pathway. Wadsworth et al., for example have established that the acquisition of sections of the *mtrCDE* gene by Ng from commensal Neisseria has played a crucial role in the genesis of macrolide resistance in Ng [8, 9].

We have found evidence that macrolide resistance determinants may be transferred from oral streptococci to commensal Neisseria and play an important role in macrolide resistance. In a whole genome analysis of the determinants of high-level AZM resistance in circulating Neisseria subflava in MSM attending the ITM STI clinic, for example we found clear evidence that 9 out of 11 isolates had taken up the *msrD* gene from oral streptococci and that this in turn was the major determinant of high level AZM resistance (MICs > 256 mg/L; unpublished data).

These five types of evidence have been instrumental in effecting the change to CRO monotherapy in the USA and UK treatment guidelines and the European IUSTI guidelines newly classifying CRO monotherapy as alternative therapy. The fact that there is this still discrepancy in treatment guidelines, taken in conjunction with the absence of RCTs comparing these different treatment options could be reasonably interpreted as reflecting equipoise in the choice between mono- and dual-therapy. In this setting, there is a crucial need to generate better evidence to inform guidelines. Conducting large RCTs to compare the clinical efficacy of mono- versus dual-therapy would be costly. As an alternative/complementary strategy, in this study we will conduct a small RCT with as primary outcome the difference in the abundance of resistance conferring genes in the rectal microbiome in the two arms 14 days after the receipt of therapy.

The specific hypothesis we will be testing is that dual therapy increases the probability of the acquisition of macrolide resistance by Ng indirectly via its effect on commensal organisms. This is illustrated in Figure 2. The Y axis represents the proportion of oral Streptococci/Neisseria that are macrolide resistant following the receipt of AZM/CRO. The figure is based on an RCT by Malhotra-Kumar et al., who administered 3 days of AZM or placebo to healthy volunteers [37]. The study found a large increase in the proportion of oral streptococci that exhibited macrolide resistance between days 4 to 28. In a small study conducted in MSM attending the STI clinic at ITM, we have found that the receipt of CRO/AZM for Ng is associated with an increase in the proportion of all Neisseria isolates that have AZM resistance 14 days following treatment [32]. This was in a very small study without a control group. Based on this work, and other lines of evidence, we hypothesize that the receipt of CRO/AZM will result in a greater increase in macrolide resistance in commensal Neisseria and other bacteria in both the pharyngeal and rectal microbiomes than CRO. Furthermore, we hypothesize that this effect will persist for at least 14 days and that incoming Ng could acquire AZM-resistance-conferring-determinants from these commensals.

Box 1. Summary of systematic review of efficacy of different therapies for eradication of pharyngeal Ng. Largely because of pharmokinetic considerations pharyngeal Ng has been consistently found to be the most difficult form to eradicate [45]. For this reason, we provide a summary of a systematic review and meta-analysis of all RCTs conducted to evaluate treatment efficacy for pharyngeal Ng [45]. Nine studies were included studying 19 treatment regimens. Three studies used CRO/AZM (red box in figure below) and three CRO monotherapy (green box; various doses). All the 6 studies except one had a small sample-size below 100. The authors found no evidence of a difference in treatment efficacy between CRO/AZM and CRO.

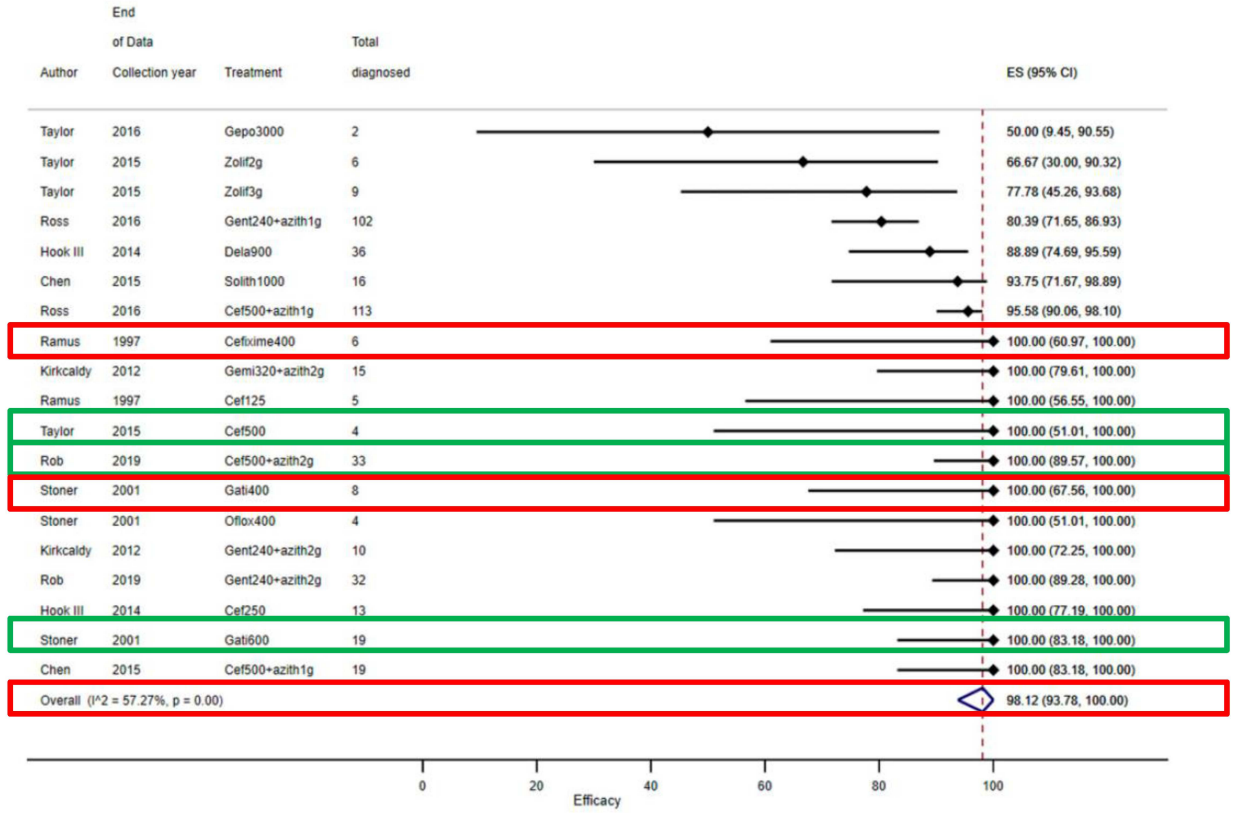


Figure 4 Overall treatment efficacy (lowest to highest efficacy). ES, effect size measured as treatment efficacy, defined as the percentage with microbial cure at follow-up. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Using an oropharyngeal rinse to characterize the microbiome, resistome of the oropharynx

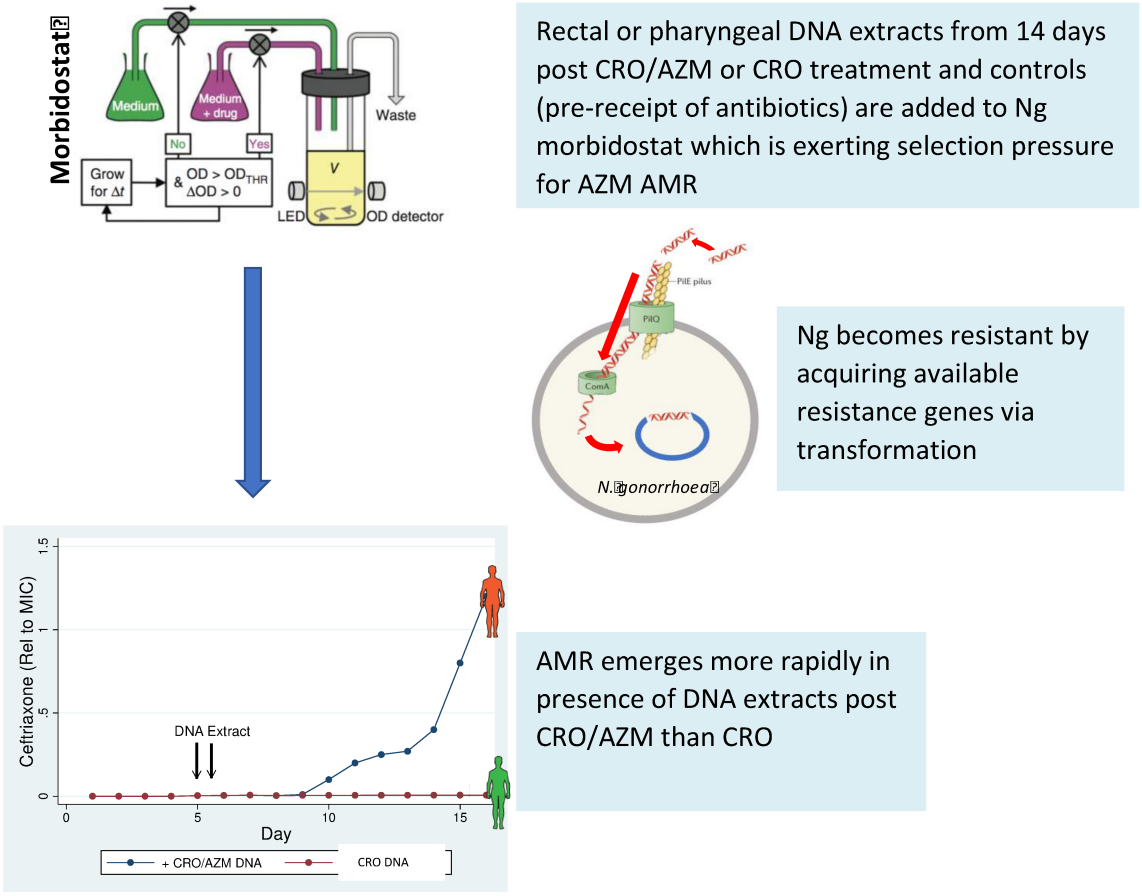
Our research team as well as others have been trying to optimize a protocol to accurately characterize the microbiome and resistome of the oropharynx. Initially we collected up to 3 swabs to do this [46-48]. This protocol had numerous problems including being laborious, unpopular with participants and subject to interindividual sampling errors. More recently we have had good results using a oropharyngeal rinse specimen (henceforth termed an oral rinse).

This methodology was developed and validated by Caselli et al., who found that an oral rinse specimen provided an effective way to characterize the oropharyngeal microbiome [49]. An oral rinse is also less invasive/uncomfortable than taking a posterior pharyngeal/tonsillar swab [49]. The oral rinse specimen was particularly good at characterizing the oral Neisseria species, which is the main focus of our research. The oral rinse technique is easy and quick to perform. It involves the individual rinsing their mouth with 15ml of sterile phosphate buffered saline (PBS) for 60 seconds and then spitting this out into a container.

Morbidostat: an alternative method to assess if CRO/AZM could be more resistogenic than CRO

One of the secondary objectives of the study will be to use the morbidostat to test if dual therapy is more likely to transfer macrolide resistance to Ng than monotherapy. The morbidostat is an in-vitro culture system that is able to place Ng under varying antimicrobial selection pressures [50]. We have previously used it as a way to map out the molecular pathways to high level macrolide and chlorhexidine resistance [50, 51]. In this experiment we will compare the time to the emergence of high-level macrolide resistance between the two treatment arms in Ng cultured in the morbidostat following regular infusions of DNA extracts of commensal Neisseria obtained from the day 14 swabs of participants (Figure. 3). The hypothesis we will be testing is that the commensal organisms obtained from the dual therapy arm are more likely to contain DNA that confers resistance to macrolides and this DNA can be taken up by Ng and accelerate the acquisition of AZM resistance.

Figure 5. *Graphical representation of how the Ng (N. gonorrhoea) morbidostat is used to assess the resistogenicity of CRO/AZM (ceftriaxone/azithromycin) versus CRO.*
Legend: AMR - antimicrobial resistance; MIC - Minimum Inhibitory Concentration.



Evaluating the effect of CRO vs. CRO/AZM on colonization resistance

Colonization resistance is the mechanism whereby a microbiota protects itself against incursion by new and often harmful microorganisms. Most of the work on the topic has been done in the gastrointestinal microbiome [52]. We recently completed a placebo-controlled randomized controlled trial to assess if a mouthwash (Listerine Cool Mint – LCM) could reduce the incidence of bacterial STIs in general and oropharyngeal Ng in particular (PReGo Study)[46]. To our surprise, we found that the use of LCM was associated with a statistically significant increase in the incidence of oropharyngeal Ng [46]. To explain this finding, we hypothesized that the use of LCM altered the oropharyngeal colonization resistance by reducing the prevalence/abundance of commensal species that inhibit the acquisition of Ng. A number of in vitro and in vivo experiments have established that a range of commensal bacterial species including commensal Neisseria can inhibit the growth/colonization by Ng [53-56]. If broad spectrum antimicrobials such as CRO/AZM were to diminish the prevalence/abundance of these inhibitory commensals to a greater extent than CRO then this could place these individuals at increased risk of reinfection with Ng and other pathogens. Various lines of evidence indicate that CRO/AZM has a larger negative effect on the human microbiome than CRO monotherapy [57, 58]. To test this hypothesis, we will use agar well diffusion assays to compare the inhibitory zone produced by oropharyngeal microbiota after receipt of either CRO/AZM or CRO.

2. STUDY OBJECTIVES

2.1 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.

2.2 Secondary objectives

- Assess if there is a difference in the mean **non-macrolide**[1] resistance determinants in the **anorectal** microbiome of the CRO/AZM group compared to those in the CRO group at 14 days post-treatment.
- Assess if there is a difference in the mean macrolide and **non-macrolide**[1] resistance determinants in the oro**pharyngeal** microbiome of the CRO/AZM group to those in the CRO group at 14 days post-treatment.
- Assess if there is a difference in the proportion of oro**pharyngeal** Neisseria 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
- Assess if the DNA extracts from the **oropharyngeal** Neisseria cultures obtained from the CRO/AZM group at day 14 result in a more rapid acquisition in AZM resistance than those from the CRO group
- Assess if the **oropharyngeal** commensal Neisseria and commensal Streptococci 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

3. STUDY DESIGN

3.1 General study design

This is an open label, single center, randomized controlled trial in MSM attending the ITM HIV/STI clinic. A total of 42 subjects will be recruited and randomized (1:1) to one of the following groups:

1) CRO/AZM (n= 21)
2) CRO (n= 21)

Visit 1 Day 0

Diagnosis of Ng will be by a positive Nucleic Acid Amplification Test (NAAT) performed according to ITM's current laboratory protocols or for patients with urethritis, a positive gram/methylene blue stain or culture result. Details of Ng infection will be collected (site of diagnosis, symptomatic, methods of confirmation and date).

After the informed consent procedure, urine - oral rinse and anorectal swab samples will be taken, for Ng culture (of the site where Ng was detected), for microbiome/resistome profiling, and culturing oropharyngeal streptococcal and Neisseria species (Table 1).

Further STI screening will be in accordance with current local best practice and in consultation with

[1] *aminoglycosides, bacitracin, beta-lactams, cationic peptides, elfamycins, fluoroquinolones, metronidazole, rifampin, sulphonamides, tetracyclines, trimethoprim*

the patient. Generally, for MSM, this includes a test for syphilis, HIV and hepatitis C unless these were carried out recently. All diagnosed STIs will be managed according to local best practice.

Participants will be randomized 1:1 to either an intramuscular injection of CRO 1g or an intramuscular injection of CRO 1g plus a 2g oral dose of AZM.

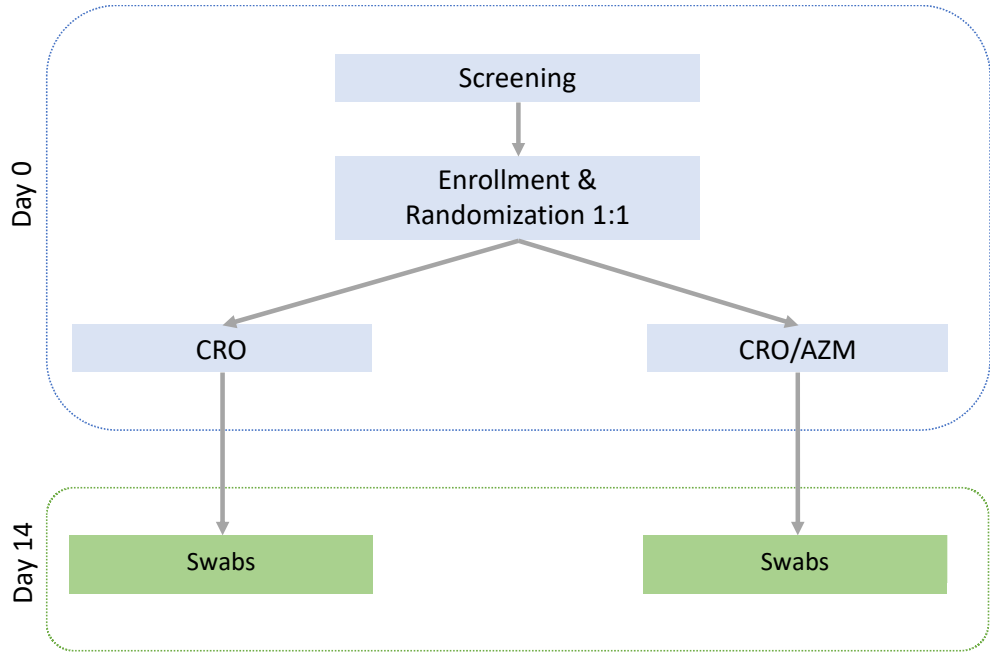
As it is recommended by IUSTI (International Union Against Sexually Transmitted Infections) Europe gonorrhoea treatment guidelines, participants will be asked to return to clinic 14 days after their treatment for a follow-up visit [14][59, 60]. Other aspects of routine STI care such as partner tracing will be carried out in accordance with current best practice guidance [14].

Visit 2 Day 14

During this visit, swabs from the previously infected sites will be taken for NAAT and culture to assess clearance of Ng in line with current guidelines [14, 59]. Oral rinse and anorectal swab will be taken for microbiome/resistome profiling and DNA extracts for transformation studies. If it is not possible for the participant to return at day 14, then they will be asked to return on day 13 or 15.

In addition, participants will be asked to return to the clinic at any point if they develop symptoms or signs of gonococcal infection. If any results reveal that the participant has an ongoing or new Ng infection further specimens will be taken for culture, sensitivity and phylogenetic analyses. The participants will also be advised to return for retesting for STIs at 12 weeks in accordance with current best practice guidelines [14]. This visit will not be included as a study visit.

Figure 6. *Study flowchart*
Legend: CRO: ceftriaxone; AZM: Azithromycin



4. PARTICIPANTS, POPULATION & SELECTION

4.1 Settings, selection & recruitment

This study will be performed in MSM attending the HIV/STI Outpatient Clinic of the ITM. MSM 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, further inclusion and exclusion criteria will be evaluated.

The first act of recruitment will be considered as the moment the study doctors inform the HIV/STI physicians that potentially eligible subjects can be referred to them for screening.

4.2 Inclusion and exclusion criteria

The inclusion and exclusion criteria for this study will be as follows:

Inclusion criteria:

1. Able and willing to provide written informed consent
2. Male sex at birth
3. At least 18 years old
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)

Exclusion criteria:

1. Use of any macrolide antibiotics (azithromycin, clarithromycin, erythromycin, roxithromycin, spiramycin) in previous 6 months
2. Known contra-indications or allergy to ceftriaxone, azithromycin or lidocaine
3. Presence of any other condition, including other STIs that will (likely) require the administration of another antibiotic at the time of enrollment, as assessed by the treating physician

4.3 Sample size

A total of 42 subjects will be enrolled in the study (21 subjects in each arm). This includes a drop-out rate of 5%. Detailed calculations of the sample size can be found in the statistical section 8.4.

4.4 Randomizatio*n*

After all applicable screening assessments have been performed, subjects who have met all inclusion criteria and none of the exclusion criteria will be randomized (1:1) to one of the following groups and will receive a unique randomization number (Unblinded – Open Label study):

1) CRO/AZM (n= 21)
2) CRO (n= 21)

The randomization schedules will be prepared by an independent sponsor biostatistician. The overview of the randomization list will not be shared with the investigators until the trial database is locked. The randomization list will be prepared using SAS 9.4 (SAS Institute, Cary NC).

4.5 Withdrawal and termination of the study

Reasons for Withdrawal

In accordance with the Declaration of Helsinki, ICH Good Clinical Practice Guidelines and the Belgian Law on experiments of 2004, a participant has the right to withdraw from the study at any time for any reason without prejudice to his/her future medical care by the physician at the institution. The Investigator also has the right to withdraw patients from the study if one or more of the following events occur:

- The participant withdraws the consent
- The Investigator judges that further participation would have negative effect on the participant's health.

Handling of Withdrawals

A complete final evaluation should be made at the time of the patient's withdrawal. The Study Status Outcome form in the case report form should be completed with an explanation of why the patient is withdrawing.

Participants withdrawn from study will continue to receive standard of care for their condition. This includes, if needed, care of any adverse event or complication, whether related or not to the study procedures. The Principal Investigator (PI) will assure this standard of care is provided.

Participants will be considered lost to follow-up at study closure if they discontinued study visits without informing the study staff and could not be traced. When the investigator has no news of the participant, they must make every effort to contact him (at a minimum telephone the participant three times and send an email to them), to establish the reason for the discontinuation of treatment, and to suggest the participant comes to an end-of-study visit. If all these attempts to contact the participant fail, the investigator can then declare the participant "lost to follow-up". The investigator should document all these attempts in the corresponding medical file.

Participants informing the study staff about their withdrawal from trial will be considered as early withdrawals.

Participants have the right to withdraw their consent at any time and to ask for retrospective withdrawal of all personal data/samples related to the trial.

Termination of Study

The study may be prematurely closed or interrupted by the sponsor in case of futility or adverse health outcomes for the study participants.

If the study is prematurely terminated or suspended, the Sponsor will promptly inform the investigators/institutions, and the regulatory authority of the termination or suspension and the reason(s) for the termination or suspension. The EC's will also be informed promptly and provided the reason(s) for the termination or suspension by the Sponsor or by the investigator/institution, as specified by the applicable regulatory requirement(s).

5. STUDY PROCEDURES
5.1 Study/visit schedule

Visit 1 Day 0 = Screening and Enrollment visit:

- Check inclusion and exclusion criteria (Eligibility)
- Informed Consent Process
- Behavioral questionnaire
- Review STI history, HIV serostatus, use of PrEP (prior 12 months)
- Record previous antibiotic use (prior 12 months)
- Oral examination
- Physical examination (as indicated clinically)
- Randomization
- Sample collection for the following laboratory procedures:
    - First-void urine and anorectal swab for Ng culture
    - Ng culture will be performed from all the sites where the Ng was detected before study enrolment
    - STI screening as required
    - Oral rinse sample for microbiome/resistome analysis and for Ng culture
    - One additional anorectal swab for microbiome/resistome analysis
- Dispensing and administration of CRO and AZM (according to CRO vs CRO/AZM arms)

Visit 2 Day 14 (range 13-15 days):

- Behavioral questionnaire
- Record antibiotic use
- Record (Serious) Adverse Events ((S)AE)
- Physical examination (as indicated clinically)
- Sample collection for the following laboratory procedures:
    - First-void urine and anorectal swab for Ng culture and for Molecular Ng testing
    - Ng culture will be performed from all the sites where the Ng was detected before study enrolment
    - Oral rinse sample for microbiome/resistome analysis and for Ng culture
    - One additional anorectal swab for microbiome/resistome analysis

Unscheduled Visits:

As it is currently the case for all STI clients, participants of this study will be able to attend the ITM clinic at any point in between the scheduled visits for any health-related concerns. Participants will be encouraged to attend the clinic for any symptoms compatible with an STI.

A schematic overview of all study assessments can be found below in Table 1.

Costs and reimbursements

Patients will be provided the antimicrobials free of charge. The patients will not be charged for any tests beyond those that are routinely conducted for patients.

Procedures and assessments

A summary of the assessments in the trial are shown in Table 1 below.

Table 1. *Schedule of Assessments*

	Visit 1 Day 0	***Visit 2 Day 14***
Eligibility	x	
Details of Ng infection - site of diagnosis, symptomatic, methods of confirmation, date	x	
Informed Consent	x	
STI history, HIV serostatus, use of PrEP	x	
Antibiotic use	x	x
Behavioral questionnaire	x	x
Oral examination	x	
Physical examination - if clinically indicated	x	x
(Serious) Adverse Event collection		x
Randomization	x	
IMP dispensing & administration	x	
Urine (Molecular Ng testing and/or Ng culture)	x	x
Anorectal swab 1 (Molecular Ng testing and/or Ng culture)	x	x
Anorectal swab 2 (Microbiome/resistome analysis)	x	x
Oral rinse (Microbiome/resistome analysis) (Molecular Ng testing and/or Ng culture)	x	x
Ng culture of the site of infection - includes assessment of antimicrobial susceptibility	x	x

Legend: Ng testing: Neisseria gonorrhoeae testing ; STI: Sexually Transmitted Infection

5.2 Obtaining informed consent

The Informed Consent Form (ICF) documents will be designed in accordance with the requirements of the Helsinki Declaration (2013), the E6 ICH GCP Guidelines (2016) and the Belgian Law on Experiment on the Human Person (2004). The ICFs will be developed in Dutch, French and English. The translation(s) will be reviewed and translation validation forms will be completed and signed by both the translator(s) and reviewer(s).

The ITM SOP for 'Obtaining the Informed Consent of clinical trials' subjects will be followed. The IC procedure will describe the purpose of the study, the procedures to be followed, the risks and benefits of participation, confidentiality issues, etc. All informed consent procedures will be conducted during the Screening and Enrolment Visit by qualified staff members identified by the PI and done in the language chosen by the participant. Participants will be informed that participation in the study is completely voluntary and that they may withdraw from the study at any time without any negative consequences. Participant Information Sheets and consent forms will be provided to the study participants for review. The participants will be given enough time to consider whether or not to participate in the study. Upon agreement on participation, the consent form will be signed in two copies, namely by the participant and by the investigator administering the consent. The participant

will receive one copy of the ICF, while the other copy will remain in the Investigator file. If a participant is unable to read or write, a signature from a witness to the informed consent discussion will be obtained.
No participant may be enrolled in the study and no study sample collection will take place until the study investigator or designee has obtained his informed consent.
Participants will be asked orally and in writing on the ICF to consent for long term storage of samples pertaining to the study (20 years after final study report) for future testing, with the exception of human genetic testing. As noted in the ICF, any genetic testing will only be directed to the microbiome and not the human genome. Future testing may include repeat and confirmatory testing to arrive at a proper diagnosis, testing that is already specified in this protocol but will be done on batched samples after all samples have been collected, or additional research. Concerning future additional researches, there is a very small likelihood of incidental findings. Before samples will be used, approval from the sponsor and the ethics committee will need to be obtained. If a participant refused long-term storage, his samples will be destroyed after the submission of the final study report. Samples will be registered in the ITM biobank.

5.3 Specific procedures and activities

The following study specific activities will be conducted at each study visit:

Physical examination:

- Oral health assessment by study physician
- Physical examinations as indicated clinically

Collection of swab:
The study physician or participant will take the anorectal swab according to clinical SOPs

Collection of rinse:
The physician will provide detailed instructions to the participant as to how to take the oral rinse sample and will supervise this specimen being taken

Collection of first-void urine sample (collected by the participant)

Behavioral Questionnaire: Visit 1 Day 0 includes antibiotic use in last year and Visit 2 Day 14 includes AZM/CRO side effects

5.4 Laboratory procedures

All laboratory procedures except for microbiome and resistome testing will be performed at ITM.

Please refer to section 5.1 Study/Visit schedule when samples are taken and below-mentioned assays are performed.

5.4.1. Laboratory testing performed at ITM

Laboratory testing at ITM is always performed according to their validated SOPs. How samples will be collected, processed, stored, analyzed and destroyed will be documented in the laboratory analytical plan.

Testing at the Clinical Reference Laboratory (CRL)

- Ng molecular testing will be performed at the STI Reference Laboratory on urine, oropharyngeal and anorectal samples.
- Culture of commensal oropharyngeal Neisseria and Streptococcal species
- Culture of *Neisseria gonorrhoeae* in case Ng was detected using standard techniques

The oral rinse, urine, anorectal, and swabs will be stored at -20°C until assayed. Microbial DNA will be extracted as per the current laboratory protocol from the oral rinse and anorectal swab separately according to standard laboratory procedures [61].

Morbidostat: assessing if CRO/AZM could be more resistogenic than CRO
The morbidostat will be used to test if dual therapy is more likely to transfer macrolide resistance to Ng than monotherapy [50, 51]. In this experiment we will compare the time to the emergence of low- and high-level macrolide resistance (MIC defined as >1mg/L and >256mg/L, respectively) between the two treatment arms in Ng cultured in the morbidostat following regular infusions of DNA extracts of commensal Neisseria obtained from the day 14 swabs of participants. The hypothesis we will be testing is that the commensal organisms obtained from the dual therapy arm are more likely to contain DNA that confers resistance to macrolides and that this DNA can be taken up by Ng and accelerate the acquisition of AZM resistance. The Ng morbidostat has 15 vials and they will be divided into the following groups: (1) adding day 14 DNA extracts from the cultured commensal Neisseria species. from the CRO/AZM group (3 vials); (2) adding day 14 DNA extracts from the cultured commensal Neisseria spp. from the CRO group (3 vials); (3) adding day 0 DNA extracts from the cultured commensal Neisseria spp. from the CRO/AZM group (3 vials); (4) adding day 0 DNA extracts from the cultured commensal Neisseria species. from the CRO group (3 vials); (5) adding no DNA (3 vials). All experiments will be performed with the WHO-F strain of Ng. The time to the development of AMR is defined as the time between the start of the experiment and the time that this resistance threshold is first crossed. All vials will be placed under an AZM selection pressure as described elsewhere [51].

Evaluating the effect of CRO vs. CRO/AZM on colonization resistance
We will assess if CRO/AZM diminishes the prevalence/abundance of inhibitory Neisseria and Streptococcal commensals to a greater extent than CRO. To test this hypothesis, we will use agar well diffusion assays to compare the inhibitory zone produced by oropharyngeal microbiota after receipt of either CRO/AZM or CRO.

In pilot experiments using agar overlay and agar well diffusion assays, we found that the oropharyngeal microbiota from certain individuals in the PReGo study showed strong inhibition of Ng whereas that from others had little or no inhibitory effect. We have found the agar well diffusion method to be easier to perform and the inhibitory effect to be easier to interpret via automated methods.

We will compare the colonization resistance following receipt of CRO/AZM versus CRO. This will be done using agar well diffusion assays to compare the inhibitory zone produced by the oropharyngeal microbiota obtained 14 days after receipt of either CRO/AZM or CRO for the treatment of Ng.

The experiments will be performed using 2 different Ng clinical isolates that are susceptible to this inhibitory effect of oropharyngeal microbiota as assessed by the agar well diffusion assay.

An aliquot of the oral rinse sample taken from day 14 will be used to inoculate two agar plates – a blood agar plate for the growth of Neisseria and a Streptococcus selective medium (Oxoid, Basingstoke, UK) for the growth of Streptococci. Three randomly selected individual colonies from each of these plates will be individually placed in wells in new agar plates (again Neisseria or streptococcal agars). These will then have one of the Ng isolates plated over them. The inhibitory effect of the commensals on Ng growth will be measured 24 hour later.

We will use paired samples t-tests to compare the zone of inhibition (assessed by imageJ: https://imagej.nih.gov/ij/download.html) between the CRO and CRO/AZM groups.

5.4.2. Laboratory testing at external laboratories

Resistome and microbiome profiling will be performed outsourced to an academic or commercial laboratory. All specimens taken for that purpose will be stored at the CRL at -80°C.

All laboratory procedures relating to the Ng-morbidostat will be carried out at the ITM STI Laboratory.

5.4.3. Microbiome and resistome laboratory procedures

- DNA extraction: Metagenomic DNA will be extracted from anorectal swabs via phenol-chloroform bead-beating protocol as described previously [62]. Anorectal and oropharyngeal DNA will be extracted from swabs
- DNA sequencing: oropharyngeal and anorectal DNA will be sequenced by whole metagenome shotgun sequencing. This sequencing work will be outsourced.

All analyses of clinical trial samples will be carried out in compliance with Good Clinical Laboratory Practice (GCLP). A Lab Analytical Plan will be available, in line with WHO-GCLP.

6. STUDY INVESTIGATIONAL PRODUCT

6.1 Purchasing, preparation and administration

The study will use two Investigational Medicinal Products (IMPs):

1) Ceftriaxone 1g IM (intramuscularly) + lidocaine 35 mg - single dose at day 0
2) Azithromycin 2g PO (per os) - single dose at day 0

These medications (CRO and AZM) will be purchased from a local pharmacy and stored at room temperature in a locked facility at ITM in accordance with good distribution and storage practice..

Instructions will be provided as to how to take the azithromycin PO and this will be given under observation [59]. The CRO will be prepared according to the product insert (SmPC) and administered IM by a qualified practitioner.

The IMPs have EC-marketing approval and are available upon medical prescription in Belgian pharmacies. SmPC documentation is available for these products.

Further details from the BCFI pertaining to the IMP is available from:

- CRO: https://www.bcfi.be/nl/chapters/12?frag=9720
- AZM: https://www.bcfi.be/nl/chapters/12?frag=9837

6.2 Participant compliance monitoring

The AZM will be given under observation. CRO will be administered by site staff.

6.3 Prior and concomitant therapy

Concomitant drugs will be noted during the visits on day 0 and day 14. Patients will be asked to contact the study team in case they require administration of an antibiotic throughout the study. The use of this antibiotic will be noted and the patient will exit the study at this point and be replaced by a new participant.

6.4 Packaging

The IMPs will retain their standard packaging. However a study label will be added to the commercial packaging with the following information:

- Study name + EudraCT number
- Contact details of the PI
- FOR CLINICAL TRIAL USE ONLY
- Sponsor name (Institute of Tropical Medicine)

This study label will be provided by the sponsor and the relabeling procedure will be performed according to the study-specific relabeling SOP. We will adhere to a stringent relabeling procedure under the supervision of a qualified pharmacist. The information on the study labels will be available in German, French and Dutch.

6.5 Reception, storage, dispensing and return

The IMPs will be stored at 15-25 °C as required for these products and as described in the SmPC. This will be done under the supervision of a qualified pharmacist.

7. SAFETY ASSESSMENT

7.1 (Serious) Adverse Events

(Serious) Adverse Events will be collected, monitored and recorded starting from the screening visit and up till the moment the patient exits the study.

Adverse Events

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)
- Nausea or vomiting (CRO, AZM)
- Abdominal pain (AZM)
- Pruritus (CRO)
- Pain at injection site (CRO)

It is the Investigator's responsibility to adequately and timely report Adverse Events to the local EC's in compliance with applicable local regulations.

Serious Adverse Events

A Serious Adverse Event (SAE) is any adverse event/experience occurring at any study drug dose that results in any of the following outcomes:

- Death;
- Life threatening (participant at immediate risk of death);
- Requires in-patient hospitalization or prolongation of existing hospitalization;
- Results in congenital anomaly/birth defect;
- Results in a persistent or significant disability or incapacity;
- An important medical event that may not result in death, be life threatening, or require hospitalization may be considered an SAE when, based upon appropriate medical judgment, the event may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

All SAEs must be:

- recorded on the appropriate SAE report form
- followed through resolution by a study clinician
- reviewed and evaluated by a study clinician.
- Follow-up until resolution (by sending updates on the SAE form)

Severity, relationship of event to study drug and outcome

All SAE's will be assessed by the clinician using a predefined grading system:

1. **Mild:** events require minimal or no treatment and do not interfere with the participant's daily activities.
2. **Moderate:** events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
3. **Severe:** events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually incapacitating.
4. **Life-threatening:** Participant at risk for death at the time of the event

Changes in the severity of an SAE will be documented to allow an assessment of the duration of the event at each level of intensity to be performed. Adverse events characterized as intermittent require documentation of onset and duration of each episode.

Assessment of causality

The investigator is obliged to assess the relationship between investigational product and the occurrence of each SAE. The investigator will use clinical judgement to determine the relationship. Alternative causes, such as natural history of the underlying diseases, concomitant therapy, other risk factors, and the temporal relationship of the event to the IMP will be considered and investigated. The investigator will also consult the drug information as needed in the determination of his/her assessment.

The relationship of an adverse event to study drug is to be assessed according to the following definitions and can only be done by the study physician:

1. **Definitely unrelated**: Reserved for those events which cannot be even remotely related to study participation (e.g. injury caused by a third party).
2. **Unlikely**: There is no reasonable temporal association between the study drug and the AE and the event could have been produced by the participant's clinical state or other modes of therapy administered to the participant.
3. **Possible**: The suspected AE may or may not follow a reasonable temporal sequence from study drug administration but seems to be the type of reaction that cannot be dismissed as unlikely. The event could have been produced or mimicked by the participant's clinical state or by other modes of therapy concomitantly administered to the participant.
4. **Likely**: The suspected adverse event follows a reasonable temporal sequence from study drug administration, abates upon discontinuation of the drug, and cannot be reasonably explained by the known characteristics of the participant's clinical state.
5. **Definitely related**: Reserved for those events which have no uncertainty in their relationship to test drug administration: this means that a re-challenge was positive.

The outcome of each SAE must be assessed at the same visit and according to the following classification:

Recovered: The participant recovered from the event with no residual problems.

Not yet recovered: This outcome can only be used for Serious Adverse Events. The event no longer meets a 'Serious' criterion, but medical event is not yet completely cured. The remaining event should be listed as a separate adverse event in the adverse event table, and with its own outcome.

Permanent damage: The event has resulted in permanent impairment.

Ongoing: the participant is continued to be followed for the event.

Death: The participant died. This term should only be used for the event which resulted in death. Any other events which were present at the time of death, but were not the cause of death, should be listed as 'Ongoing'.

Unknown: The participant cannot be traced and no final outcome for the event could be determined.

There may be situations when an SAE has occurred, and the investigator has minimal information to include in the initial report to the Sponsor. However, it is very important that the investigator always makes an assessment of causality for every event prior to transmission of the report to the Sponsor. The investigator may change his/her opinion of causality in light of follow-up information, amending the SAE-report form accordingly.

All SAE's whether or not deemed drug related or expected must be reported immediately or within 24 hours (one working day) using the SAE Notification Form by email to:

Clinical Trials Unit
Institute of Tropical Medicine
Nationalestraat 155
B-2000 Antwerp – Belgium
Email: pharmacovigilance@itg.be

Line listings of all reported SAE's will be sent to the IRB of the ITM and the independent EC, on a yearly basis. An annual safety and status report will be sent to the Belgian Competent Authorities.

Suspected and unexpected SAE's (SUSARs) must also be reported by the sponsor to the Belgian Competent Authority (for death and life-threatening, within 7 days, for all other SUSARs within 15 days). Reporting and management of SUSARs is described in the sponsor SOP on 'Safety Reporting'.

A study safety checklist SOP will be prepared describing all responsibilities and actions that are required in case of an SAE.

7.2 Data and Safety Monitoring Board

As this study is a low intervention clinical trial with investigational products that will be used according to their market registration, no data safety monitoring board will be installed and not independent safety monitor will be appointed.

8. STATISTICAL METHODS

The statistical analysis will be described in the statistical analysis plan (SAP), written by the biostatistician, which is binding and will be finalized before database lock or before any other analysis takes place.

8.1 Study hypotheses and hypothesis

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.

8.2 Variables of interest

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

1. 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
2. the ratio of mean normalized read count of resistance determinants for each macrolide and non-macrolide antibiotic class in the day 14 oropharyngeal samples between the two treatment groups
3. The difference in proportion of oropharyngeal commensal *Neisseria* that are macrolide resistant between the two treatment groups at day 14. For each individual the proportion of oropharyngeal commensal *Neisseria* that are macrolide resistant will be calculated by dividing the number of macrolide-resistant colonies of commensal Neisseria grown on modified LB agar-azithromycin-1mg/L by the total number of commensal Neisseria colonies grown on modified LB agar without azithromycin.
4. The difference in time until acquisition of phenotypic resistance to AZM by Ng in the morbidostat upon exposure to commensal Neisseria DNA extracts from each treatment group
5. The difference in diameter of the inhibition zone of Ng during agar overlay experiments using oropharyngeal commensal Neisseria and Streptococci from day 14 samples from each treatment group

Safety outcome

The number of participants with (severe) adverse events in each treatment group.

8.3 Statistical methods

8.3.1 Analysis populations

The primary analysis and secondary efficacy analyses will be performed on all randomized participants with samples at baseline and day 14. All participants will be analyzed according to their randomization group.

8.3.2 Baseline characteristics

The number of participants screened and enrolled or excluded will be summarized according to reason for exclusion. Of the enrollees, the number of patients discontinued or lost to follow-up will be recorded by reason and time of discontinuation. These figures will be summarized in a CONSORT flow diagram.

Patients in each treatment group will be described according to baseline characteristics. The description will be in terms of medians and interquartile ranges for continuous variables and using counts and percentages for categorical variables. Differences in baseline characteristics will be noted, but no formal statistical testing will be done.

8.3.3 Primary analysis

The primary analysis of assessing a difference between the mean 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 be presented with a 95% confidence interval.

8.3.4 Secondary and tertiary analysis

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.

The difference of proportions of resistance between the two arms will be calculated with the 95% confidence interval of the proportion difference at each time point separately. The differences in acquisition of AZM resistance and diameter of inhibition zone will be done using permutation testing and the Wilcoxon rand sum test.

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

8.3.5 Subgroup analyses

No a priori subgroup analyses are defined.

8.3.6 Multiplicity and Missing Data

No adjustment for multiplicity are needed. No interim analyses are planned. 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.

8.4 Sample size and power

We assume that CRO/AZM will result in a 2.5 fold increase in the ratio of macrolide resistance determinants compared to the CRO group. This estimate is likely a conservative estimate based on a previous study that found a seven-fold increase in this ratio following repeated AZM mass administration in Niger – the MORDOR study [39]. We have used a much lower estimate as there are important differences between the study populations in MORDOR and this study. These include children in MORDOR versus adults in ResistAZM and a low vs. very high background macrolide consumption in MORDOR and ResistAZM, respectively [29].

The sample size was calculated by simulation using permutation testing with the assumed effect size and a 5% significance level. The minimum sample size in order to obtain at least 80% power is 20

participant per group. Correcting for a drop-out rate of 5%, we will need 21 men in each arm to detect this effect size with 80% power using a two-sided significance level of α=0.05.

9. MONITORING AND QUALITY ASSURANCE

This study will be monitored by the ITM CTU (Clinical Trial Unit) in accordance with regulations applicable to clinical trials, including ICH-GCP (Good Clinical Practice) and WHO-GCLP, and sponsor-specific SOPs. The PI and involved site research staff will allocate adequate time and resources for such monitoring activities. The investigator will also ensure that the monitor or QA reviewer is given access to all the above noted study-related documents and study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.) and has adequate space and resources to conduct monitoring and source data verification (SDV).

A monitoring plan will be written to describe monitoring responsibilities and activities in detail (including percentage of SDV, timing and frequency of site visits, follow-up of findings and protocol deviations).

The sponsor will inform the Investigators concerned immediately upon notification of a pending study centers inspection by any regulatory authority or funder. Likewise, the investigator will inform the sponsor of any pending inspection.

Laboratory quality control and quality assurance:

ITM will ensure that all laboratory activities including specimen transport, processing, testing, result reporting and storage will be conducted in accordance with the clinical trial quality requirements. The laboratory will perform testing according to the SOPs which are documented in the laboratory analytical plan and will be conducted in compliance with Good Clinical Laboratory Practice Standards (GCLP), and EN-ISO 15189. Reports of laboratory test results will be forwarded to the study physician as soon as the result is available.

Trial management Group:

The PI, STI laboratory manager, study doctors and a CTU-representative will constitute the trial management group. Additional people (statistician, data manager, data reviewer, QA officer…) might join the meeting if necessary.

10. DATA MANAGEMENT

Data Management will be done in compliance with GDPR, GCP guidelines and FDA 21 CFR part 11 regulations and performed by the data manager in collaboration with the study staff at the site.

A Data Management Plan will be prepared, with following essential data management aspects:

Types of data collected

Individual, participant-level clinical trial and behavioral data will be collected by the clinical site team and held in patient files at the site. Only data defined by the study protocol will be collected. Collected data will include variables from Baseline visit (de-identified trial participant code, eligibility screening, informed consent confirmation variables, medical history, concomitant medication, randomization, lab test results, adverse events and behavioral data), Follow-Up visit data (lab test results, concomitant medication, (S)AE data), behavioral data and outcome data (final outcome).

Data collection and handling

Data will be entered at the site via study computers equipped with electronic Case Report Forms (eCRFs) developed with a GCP- and regulatory compliant clinical trials management software. Edit

checks and branching logic, programmed onto the electronic forms will validate at the point of data entry and support quality data. The electronic forms will be tested and validated before the actual data collection. The study team will be trained on the use of the eCRFs, data collection and query handling before the study start.

Data storage & preservation
All the relevant study data will be retained for a minimum of twenty years and according to applicable regulations. The trial database will be stored on a secured server at the ITM, which is physically only accessible by badge by authorized IT collaborators. The ITM has procedures in place to ensure daily backup of the server and study computers and for long-term, secure curation and preservation of data.

Confidentiality & security
The computerized system that will be used includes a robust security architecture including encryption, firewalls, antivirus software and controlled user access (username, personal password and user roles with different privileges), tracking of edits (electronic audit trail) and the use of a trustworthy electronic signature. A list of authorized users of the eCRFs, with their user role will be kept at the CTU and updated regularly.
Information of trial participants will be handled confidentially. A trial participant code (pseudonym) will be assigned to each trial participant at the earliest opportunity. Any information that could lead to the direct identification of the participant will not be included on the eCRFs, nor on any other paper documents or electronic files used for data management. The name and contact data for each participant will be kept separately and limited to authorized staff at the site.
Only pseudonymized data will be distributed amongst the study team during the study.

Both the sponsor and site will also see to it that the necessary measures are taken to ensure that all data management documents (such as the Data Management Plan, and procedures) and IT devices and equipment (computers, server) are kept secure (in closed cupboards, closed and/or badge-controlled offices or rooms).

Data integrity and quality
Ensuring data integrity and data quality is an essential task throughout the trial, carried out by competent study staff who by preference are assigned to specific roles in data management and ICT (such as the data manager, data reviewer, monitor, IT helpdesk). The study specific Data Management Plan (DMP) and ICT and Data Management Standard Operating Procedures (SOPs) are amongst the essential documentation which will guide and support the trial conduct. Good practices during the design of the eCRFs, such as use of coding, check boxes, question input masks and drop-down menus will enhance the data quality. Aside from automatic edit checks, a specific Data Validation Plan with manual checks will also be performed to identify out of range data, missing data and inconsistent data. Medical event terms will be coded and standardized using the Medical Dictionary for Regulatory Activities (MedDRA).

Data sharing
After study completion and publication of results, anonymized individual participant data may be shared by means of a managed access procedure and along a transparent decision-making process, with: (i) Completion of a data request form, (ii) Evaluation by a data access committee, (iii) Data sharing Agreement, (iv) Secure transfer of anonymized data, and (v) Additional metadata which clarify the research data. Metadata will include the study protocol, questionnaire(s), data dictionary. Specifics about data sharing will be included in the Informed Consent Form. To this end, the ITM Data Sharing Policy will be adhered to.
All research data processing will be performed in compliance with the European General Data Protection Regulation 2016/679 (GDPR). ITM is the controller of the data processing and the lawful basis for the processing of participant personal data will be the public interest.

11. ETHICAL ISSUES

The study design randomizes individuals to receive one of two treatment schemas – CRO and CRO/AZM. Treatment with CRO is the preferred therapy for Ng according to the UK, USA and Netherlands Ng guidelines whereas treatment with CRO/AZM is the preferred treatment according the Belgian and IUSTI Europe guidelines. Both treatments have been given to a large number of individuals without any large concerns raised about their safety or efficacy. As such we do not think that there is evidence to regard either or both of these treatment regimens as unethical.

Participants will be required to return for a post-treatment visit. This is standard practice and explicitly recommended in the IUSTI Europe Ng treatment guidelines.

Participants will not be renumerated, but participants will not be charged any costs additional to those that result from their routine case management. In particular, participants will not be charged any costs additional to those that result from their routine case management. In fact by virtue of not paying for the antimicrobials used, participating in the study will cost them less than routine care.

11.1 Ethical and regulatory review

This clinical trial will be submitted for formal review and approval to the Institutional Review Board of the ITM, the designated Belgian EC and Competent Authorities of Belgium (FAMPH). The trial will be submitted to the FAMPH pilot project for the EU Clinical Trial Regulation 536/2014, which includes review by an independent Ethical Committee. No study-specific interventions will take place before written approval by the Ethics Committee(s) has been obtained and the local regulatory requirements have been complied with, and the signature of the clinical study protocol of each contractual party involved have been obtained.

The study will be carried out according to the principles stated in the Declaration of Helsinki as amended in 2013 and any further updates, all applicable national and international regulations and according to the most recent ICH-GCP guidelines.

The study will also be included in the EudraCT public registry prior to the start of participant recruitment.

11.2 Protocol amendments

Once the final clinical study protocol has been issued and signed by the authorized signatories, it cannot be informally altered. Protocol amendments have the same legal status and must pass through the appropriate steps before being implemented. Any substantial change must be approved by all the bodies and EC's that have approved the initial protocol, prior to being implemented, unless it is due to participant's safety concerns (in which case the immediate implementation can be necessary for the sake of participant's protection. In case modifications to the protocol or amendment are requested by any local EC/CA during the review process, these must be discussed and agreed upon with the Sponsor prior to any resubmission incorporating those changes.

11.3 Informed consent

No participant may be enrolled into the study until the Investigator or designee has obtained the written informed consent form (see section 5.2).

11.4 Confidentiality

All the participant data will be pseudonymised in all collection tools, the CRF and database by means of a unique subject assigned participant study number. The study documents which can identify the participants, e.g. the ICF's, , study source medical record, will only be accessible to the relevant study staff, study monitors, auditors and inspectors under confidentiality agreements.

Privacy by Design principles will be adhered to with regard to personal data processing and maximizing confidentiality. Data collection will be minimized as much as possible and will be proportionate to achieve the goals of the study. Each participant will be assigned a unique personal identification number (PID), which will be used on all study forms. This identification number will be linked to the person's name and contact information in a central "participant identification list". The identification list and documents containing the names and/or signatures of participants (such as consent forms) will be kept separately from all other study documents containing participant data, that will only be identified by the participant identification numbers. All study documents will be stored in lockable rooms or cabinets with access limited to study staff.

Names of the participants will not appear on any reports or publications resulting from this study. The findings from this study may be published in a medical/scientific journal. The study participants will never be identified by name. After the study is completed, participants will be informed of the study results by the Principal Investigator or designee.

The Sponsor provides all study documents to the Investigators and his/her appointed staff in confidence. Materials may not be disclosed to any party not involved in the study, unless written permission from the Sponsor or ITMs Data Access Committee with regard to the sharing of data for secondary research.

11.5 Risks and benefits

No major risks are anticipated for the participants. There may be a small risk of breach of privacy during the study. Because we are collecting data pertaining to sexual behavior and STIs, any breach of privacy could have serious consequences for participants. We are aware of this risk and have designed the study in a way to minimize this risk. In particular we will follow strict protocols to maintain patient confidentiality (details provided in section 11.4).

The principal benefit for the participants is to contribute to optimizing the treatment of Ng in a way which minimizes the emergence and spread of AMR in Ng and other bacteria.

11.6 Compensation for participation

There will be no direct compensation for the study participation.

11.7 Insurance

As required by the Belgian law on experiments on the human person of May 7th 2004, the sponsor has obtained a no-fault liability insurance (with Amlin Insurance S.E., insurance policy number LXX099279) covering any harm, injury or (material) damage which may occur to study participants and which may be directly or indirectly caused by their participation in the trial. The insurance provisions will also be mentioned during the informed consent discussion and in the ICF.

12. DISSEMINATION OF RESULTS, INTELLECTUAL PROPERTY

All study documents are provided by the Sponsor to the Investigators and his/her appointed staff in confidence. None of this material may be disclosed to any party not directly involved with the study, without written permission from the Sponsor.

Reporting and publication of the study data will be done in accordance with the CONSORT statement (check http://www.consort-statement.org/consort-statement/) and ITM's publication policy.

13. ARCHIVING

The sponsor and Investigator must maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be verified. The relevant (essential) documents are those documents which individually and collectively permit to assess the conduct of the trial, the quality of the data produced and the compliance with GCP standards and applicable regulatory requirements. The Investigator's File should at least contain all the (essential) documents as listed in the procedure "Set up and maintenance of the Investigator Trial File". A copy of all source data and Case Report Forms must always be kept on site. The PI is responsible for ensuring a secure and appropriate location for his Investigator's File and any other trial related documentation present at site, as well as for ensuring that only site staff that is competent and delegated to work for the trial has got access to the files.

All the relevant study documentation should be retained for a minimum of 20 years after completion of the study, as set out by the current Belgian law. The Sponsor should be informed prior to destruction of the files.

After completion of the study, the IF will remain available for internal audits and/or inspections of regulatory authorities for a period of 25 years.

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15. LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
AZM	Azithromycin
CA	Competent Authorities
CASI	Computer Assisted Self-Interview
CI	Coordinating Investigator
CRL	Clinical Reference Laboratory, ITM
CRO	Ceftriaxone
Ct	Chlamydia trachomatis
DMP	Data Management Plan
DVP	Data Validation PlanD
(e-)CRF	(electronic) Case Report Form
EC	Ethical Committee
ECG	Electrocardiogram
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAMHP	Federal Agency on Medicines and Health Products
GCP	Good Clinical Practice
GCLP	Good Clinical Laboratory Practice
GDPR	General Data Protection Regulation
HGT	Horizontal Gene Transfer
IM	Intramuscularly
IMI	Intramuscular Injection
ITM	Institute of Tropical Medicine
IC(F)	Informed Consent (Form)
ICH	International Conference on Harmonization
(I)EC	(Independent) Ethics Committee
IMP	Investigational Medicinal Product
IRB	Institutional Review Board
ITM	Institute of Tropical Medicine
LCM	Listerine Cool Mint
MIC	Minimum Inhibitory Concentration
MTB	Mycobacterium tuberculosis
MSM	Men who have Sex with Men
NAAT	Nucleic Acid Amplification Test
Ng	*Neisseria gonorrhoeae*
PI	Principal Investigator
PrEP	HIV Pre-Exposure Prophylaxis

PO	Per Os
QA	Quality Assurance
QC	Quality Control
RCT	Randomized Clinical Trial
(S)AE	(Serious) Adverse Event
SAP	Statistical Analysis Plan
SDV	Source Data Verification
STI	Sexually transmitted infection
SOP	Standard Operating Procedure
TMG	Trial Management Group
WHO	World Health Organization