Universiteit Antwerpen

Faculteit Geneeskunde en
Gezondheidswetenschappen

INSTITUTE OF TROPICAL MEDICINE ANTWERP

Departement Klinische
Wetenschappen

Antimicrobiële resistentie in *Neisseria gonorrhoeae*

Antimicrobial resistance in *Neisseria gonorrhoeae*

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Medische Wetenschappen aan de Universiteit Antwerpen te verdedigen door

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Antwerpen, 2022

Disclaimer

Dankwoord

In de eerste plaats wens ik mijn promotor Chris Kenyon te bedanken. Chris, zonder jou was er van dit werk geen sprake geweest. Het zijn jouw enthousiasme, betrokkenheid, exemplarische wetenschappelijke skills en bovenal realiteitszin die me over de streep trokken om dit doctoraat aan te vatten. Er zijn weinig doctorandi die zo'n vliegende start hebben mogen meemaken en daarenboven in de loop van hun doctoraat nog zulke leerrijke zijsprongen hebben mogen/kunnen maken als ik tijdens zowel de COVID-19 als de apenpokken pandemie. Bedankt voor alle vrijheid en tegelijk voor je beschikbaarheid. Ik hoefde me maar om te draaien in ons bureau of op de knop te drukken in Teams …

I also thank my UAntwerpen supervisor, Surbhi Malhotra, for opening the doors of her lab to me and for sharing her critical insights from time to time. Also Britto Basil Xavier, thank you so much for helping where you could despite your tight time schedule.

Ik dank ook oprecht alle juryleden, prof. dr. Annelies Van Rie, prof. dr. Jean-Pierre Van Geertruyden, prof. dr. Steven Callens en prof. dr. Alje van Dam voor hun interesse in mijn werk en voor hun kritische lezing van deze thesis.

Jolein, jij was vanaf het begin mijn PhD-maatje. We zijn ongeveer gelijktijdig gestart en het zal nu ook snel jouw beurt zijn om je PhD te verdedigen. In de eerste maanden van ons traject liet je me vol geduld en toewijding kennismaken met de ingenieuze maar vermaledijde Morbidostat in ons labo. Het heeft me geleerd dat labowerk hard labeur is. We hebben successen en frustraties gedeeld terwijl we samen groeiden tot wetenschappers. Je bent een schat en ik zal je altijd een warm hart toedragen.

Irith, enorm bedankt om als eerste mijn thesis na te lezen en te becommentariëren. Evenzeer bedankt om mij te betrekken bij je apenpokken-

projecten. Wat een team, wat een race en wat een succes! Dit was voor mij de start van de spin-off van mijn PhD die het werk over de apenpokken geworden is.

I would also like to thank the rest of our dynamic gono-team, which has grown considerably over the past few years, for their support, critical input, helpfulness and, above all, flexibility, which has allowed all projects to run smoothly.

Verder gaat ook veel dank uit naar de collega's SOA-artsen en internisten van het ITG, alsook de klinisch biologen, wiens deuren altijd opengestaan hebben voor overleg en een brainstorm van tijd tot tijd. Ook de ITG verpleegkundigen, research nurses, medewerkers van de Clinical Trial Unit en personeel van het labo en medsec hebben een essentiële rol gespeeld in het verwezenlijken van mijn onderzoek. Zonder jullie hadden we nooit zulke kwaliteitsvolle klinische studies kunnen uitvoeren in het ITG. Ik zal ook niet vergeten hoe we ons samen tot het uiterste hebben ingezet voor het in supertempo opzetten van die andere klinische trial aan het begin van de COVID-19 pandemie.

Natuurlijk wil ik ook de vele mensen bedanken die hebben deelgenomen aan ons onderzoek, met name de mannen die voor PrEP-opvolging naar het ITG komen. Hun bereidheid om deel te nemen en inspanningen te leveren voor het algemeen welzijn is absoluut lovenswaardig. Het was en is altijd een plezier om inzichten uit te wisselen tijdens de raadpleging.

Verder wil ik Cansu bedanken voor haar steun op momenten dat het moeilijker ging, om mij bevestiging te geven wanneer ik daar nood aan had en om mijn hangriness na een lange werkdag te doorstaan.

Lieve mama en papa, jullie hebben me geleerd wat het is om hard te werken en doelen te bereiken, wat teamwork is en onvoorwaardelijke steun. Een warme thuis is de basis van alles. En tot slot Matthias, Marlies, Anke, jullie zijn drie rotsen met kleine zij-rotsjes in de branding van mijn leven.

Christophe, Oldenburg, november 2022.

Samenvatting

Seksueel overdraagbare infecties (SOI) zijn veelvoorkomend, in het bijzonder in risicogroepen zoals mannen die seks hebben met mannen (MSM) die HIV pre-exposure profylaxe (PrEP) gebruiken. Wereldwijd ziet men steeds vaker bacteriële SOI met antibioticaresistentie, zoals gonorroe. *Neisseria gonorrhoeae*, de bacterie die gonorroe veroorzaakt, is in staat antibioticaresistentie te ontwikkelen door genetisch materiaal van andere bacteriën op te nemen. Dit proces vindt vermoedelijk plaats tijdens een infectie in de keel. Deel 1 van deze thesis toont aan dat MSM die PrEP gebruiken meer resistente keelbacteriën hebben dan de algemene bevolking, zelfs als ze niet recent antibiotica gebruikten. Dit is mogelijk een gevolg van frequent antibioticagebruik in de MSM-populatie in het algemeen. Mogelijks speelt ook overdracht van (resistente) keelbacteriën tussen individuen een rol – intieme partners blijken immers een gelijkaardig keelmicrobioom te hebben. Deel 2 van deze thesis ging na of een mondspoeling als niet-antibiotisch alternatief gebruikt kan worden ter preventie van SOI of ter behandeling van gonorroe in de keel. Dit bleek in beide gevallen niet effectief. We hebben momenteel dus geen andere optie dan gonorroe met antibiotica te behandelen. Onze bevindingen bij PrEP gebruikers tonen aan dat we antibiotica spaarzaam en gericht dienen in te zetten, in het bijzonder bij MSM die PrEP gebruiken.

Summary

Sexually transmitted infections (STIs) are common, particularly in high-risk groups such as men who have sex with men (MSM) who are taking HIV pre-exposure prophylaxis (PrEP). Globally, bacterial STIs with antibiotic resistance, such as gonorrhoea, are increasingly common. *Neisseria gonorrhoeae*, the bacterium that causes gonorrhoea, is able to develop antibiotic resistance by taking up genetic material from other bacteria. This process presumably takes place during an infection in the throat. Part 1 of this thesis shows that MSM who use PrEP have more resistant throat bacteria than the general population, even if they have not used antibiotics recently. This is possibly a consequence of frequent antibiotic use in the MSM population as a whole. It is also possible that transmission of (resistant) throat bacteria between individuals plays a role – intimate partners appear to have a similar throat microbiome. Part 2 of this thesis examined whether a mouthwash can be used as a non-antibiotic alternative for prevention of STIs or for treatment of gonorrhoea in the throat. This proved to be ineffective in both cases. Therefore, we currently have no other option than to treat gonorrhoea with antibiotics. Our findings in PrEP users show that antibiotics should be used sparingly and in a targeted fashion, especially in MSM who use PrEP.

Chapter 1   **Introduction**

1.1 Sexually transmitted infections

A range of microorganisms can be transmitted by sexual activity. This includes viruses, bacteria, protozoa, and parasites.[1] The World Health Organization (WHO) estimates that 1 million sexually transmitted infections (STIs) occur worldwide, each day.[1] Four viruses, a protozoan and three bacteria cause the highest number of new infections every year.[1] These viruses are hepatitis B, herpes simplex virus, HIV and human papillomavirus (HPV).[1] The protozoan STI is trichomoniasis, and the three most common bacterial STIs are chlamydia, gonorrhoea, and syphilis.[1] While no effective therapy exists against the previously mentioned viral STIs, bacterial/protozoan STIs can be cured by antimicrobials. Nevertheless, antimicrobial resistance (AMR) has severely reduced the available treatment options, mainly in gonorrhoea.[1]

1.1.1 Bacterial sexually transmitted infections

The WHO estimated that about 374 million new infections occurred with the above-mentioned bacterial/protozoan STIs, in 2020: trichomoniasis (156 million), chlamydia (129 million), gonorrhoea (82 million), and syphilis (7.1 million).[1] The majority of these infections are asymptomatic.[1] However, besides causing local symptoms at the site of infection, they can have more severe consequences due to ascending infection (e.g. pelvic inflammatory disease, infertility, sepsis), increased risk of other STIs (HIV), or transmission from mother to child with deleterious effects on the foetus or newborn.[1]

1.1.2 Reducing the burden of bacterial sexually transmitted infections

New initiatives are needed to prevent new cases of bacterial STIs.[1] In 2016, the WHO set the goal to end STI epidemics as major public health concerns.[2] The WHO decided to primarily focus on the infections that require most urgent action, and

for which cost-effective interventions exist, namely syphilis and gonorrhoea, beside the viral infection HPV.[2] One of the aims is to reduce the global incidence of syphilis and gonorrhoea with 90% by 2030, compared to 2018 (Figure 1).[2] The WHO recognised that, apart from innovations in STI-testing and scale-up of services, new approaches would be required to prevent and treat STIs, with high efficacy and low risk of AMR.[2] Key populations constitute an important target for the control of the STI epidemic (see next section, 1.2.).

Figure 1: Incidence targets for syphilis and gonorrhoea, as defined in the WHO global health sector strategy on sexually transmitted infections 2016–2021 (from [2])
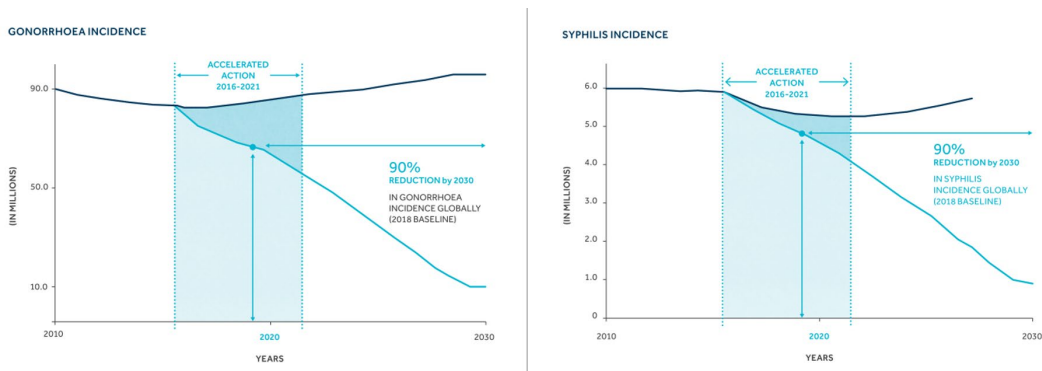
Despite the WHO's goals, current surveillance data from the USA[3] and Europe[4] show a steady increase in incidence of syphilis and gonorrhoea in the last decade (Figure 2). The SARS-CoV-2 pandemic had only limited and transient influence on the total number of cases.[5]

Figure 2: Reported cases of gonorrhoea and syphilis in Europe and the USA in the last decade. (A, B) Absolute number of cases reported to ECDC, from https://atlas.ecdc.europa.eu/public/index.asp, (C, D) Reported cases per 100 000 population reported to CDC, from https://www.cdc.gov/std/statistics/2019/overview.htm. HSM = heterosexual men; MSM = men who have sex with men
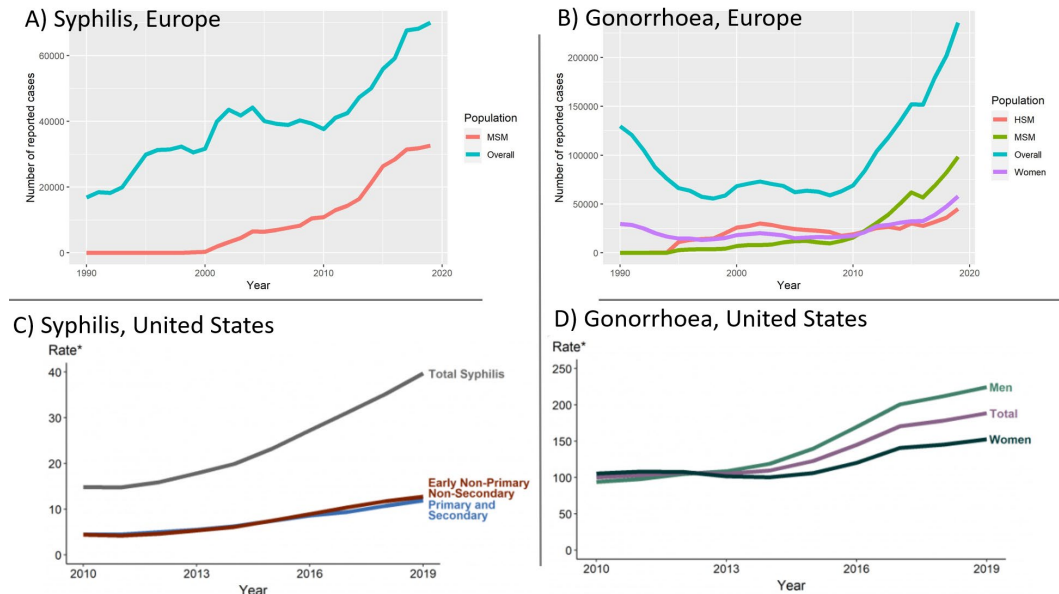
1.2 Key populations

Key populations are groups of people with a disproportionately high STI incidence which can be attributable to sexual risk behaviour, limited access to health care facilities, and stigma.[6,7] Examples of such key populations are gay, bisexual and other men who have sex with men (MSM), people who inject drugs, and sex workers.[3,6] Key populations are linked to the general population by a bridging population consisting of clients of sex workers, MSM who also have sex with women, and others who may transmit STIs between both populations (Figure 3).[7]

Figure 3: Transmission dynamics of sexually transmitted infections in a population: interactions between the core group and the general population, through a so-called bridging population (from [7])
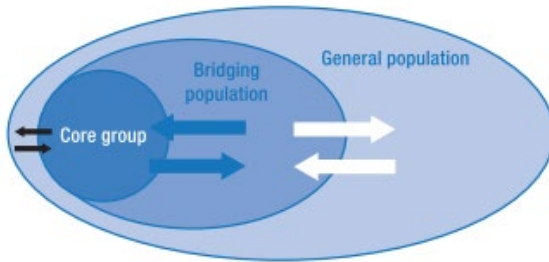
1.2.1 MSM using HIV pre-exposure prophylaxis

The main study population in this thesis are MSM using HIV pre-exposure prophylaxis (PrEP). In 2015, the WHO recommended oral PrEP as a highly effective method to prevent HIV in people at substantial risk of HIV.[8] Since then, the number of people using PrEP has been increasing, both in high- and low- income countries.[9] Whereas PrEP mitigates their risk of HIV, MSM using PrEP remain at high risk of other STIs due to their sexual risk behaviour, including high numbers of sex partners, low adherence to condom use, sexualized drug use (chemsex), or anonymous or paid sex. For example, participants of a PrEP demonstration study in Amsterdam (AMPrEP, 2015-2016) reported a median of 12 (interquartile range 6 – 25) partners per 3 months.[10] Such high rates of partner turnover and partner concurrency, combined with relatively low rates of condom use, result in very dense sexual networks with a high equilibrium prevalence of STIs.[11] Consequently, the incidence of bacterial STIs among PrEP users is high and keeps rising.[11] A meta-analysis of longitudinal data from PrEP cohorts between 2008 and 2018 found incidences, per 100 person-years, of about 21.5 (95% CI 17.9 – 25.8) for chlamydia, 37.1 (95% CI 18.3 – 25.5) for gonorrhoea, and 11.6 (95% CI 9.2 – 14.6) for syphilis.[12] Nearly half of all reported cases of primary/secondary syphilis in the USA in 2019 occurred in MSM, and gonorrhoea rates in MSM were 42 times that of heterosexual men in some areas.[3] Surveillance data from the USA and Europe show a progressive increase in the incidence of bacterial STIs among MSM USA and Europe (Figure 2).[3,13]

1.3 *Neisseria gonorrhoeae*

This work focuses primarily on *Neisseria gonorrhoeae*, or gonococcus, the bacterium causing gonorrhoea. The reason to focus on this bacterium are its high incidence in MSM along with its propensity to develop AMR, as will become clear in the next paragraphs.

1.3.1 The bacterium

Neisseria gonorrhoeae was discovered in 1879 by Albert Neisser.[14] It is a Gram negative diplococcus which belongs to the genus *Neisseria*.[14] Besides *N. gonorrhoeae*, this genus comprises a range of non-pathogenic *Neisseria species* and one other pathobiont, *Neisseria meningitidis*.[14] *N. gonorrhoeae* and *N. meningitidis* have in common that they exclusively colonise the mucosa of humans, with or without causing symptoms. Still, they evidently differ in spectrum of disease, and in contrast to *N. meningitidis*, *N. gonorrhoeae* requires close mucosal contact for transmission, and thus cannot be transmitted via respiratory droplets.[15]

Neisseria species, and *N. gonorrhoeae* in particular, have a high level of genomic plasticity: *N. gonorrhoeae* undergoes different kinds of mutations, including spontaneous mutations and the uptake of DNA fragments from plasmids, and transformation.[14] There is extensive evidence for horizontal exchange of genes between *N. gonorrhoeae* and other *Neisseria species* [14,16,17] Rapid changes in its DNA enables *N. gonorrhoeae* to survive under changing environmental conditions, such as antimicrobial exposure.[14]

1.3.2 The disease

N. gonorrhoeae colonises the human oropharynx, genitals, and anorectum, or infects the eye. Most pharyngeal and anorectal infections are asymptomatic, as are genital infections in women.[18,19] Men with a urogenital infection usually develop symptoms of urethritis within 1 to 6 days after exposure.[19] Anorectal infections may present with proctitis. In both sexes, the gonococcus evades the innate immune system and promotes inflammation.[19] It is thought that this

inflammation causes an enhanced susceptibility to HIV infection, and complications due to scarring of the higher urogenital tract (infertility, ectopic pregnancy, urethral structure).[19] In rare cases (0.06% of all reported cases in one USA surveillance report[20]), *N. gonorrhoeae* invades the bloodstream and causes disseminated infection with manifestations of skin, joint, endocarditis, or meningitis.[19]

Gonorrhoea is generally diagnosed by nucleic acid amplification testing on urine or a swab specimen of the suspected site of infection. In case of acute urethritis, intracellular diplococci can be observed in polymorphonuclear cells in urethral swab smears under direct microscopy after methylene blue or Gram staining.[21]

Treatment with effective antimicrobials usually results in resolution of symptoms within days. Patients are usually asked to notify their sexual partners and to abstain from sex in the first seven to fourteen days after treatment.[22]

1.3.3 Antimicrobial resistance

Antimicrobial resistance (AMR) is a threat to the management of all bacterial infections and was estimated to cause more than a million deaths worldwide, in 2019.[23] AMR also affects STIs, and it is of particular concern in the case of gonorrhoea. *Neisseria gonorrhoeae* is notorious for its ability to evolve AMR to all classes of antimicrobials that have been used to treat it.[24] In 2012, WHO announced a global action plan to control the spread and impact of AMR in *N. gonorrhoeae*.[6] In 2017, WHO listed *N. gonorrhoeae* as one of twelve priority pathogens that pose the greatest threat to human health because of AMR.[25]

1.3.3.1 *Timeline of antibiotics and AMR in* N. gonorrhoeae

Upon the first discovery of antimicrobials in the early- to mid-twentieth century, gonorrhoea could easily be treated with a single dose of sulphonamides or penicillin.[26] However, resistance soon evolved against these antimicrobials (Figure 4).[26] Increasing doses of penicillin were used, but by the 1980s, penicillin resistance was so widespread that treatment guidelines had to change.[26] Tetracyclines, a treatment option for penicillin-allergic patients, had been lost in

the mid-1980s due to the spread of a conjugative plasmid carrying a high-level tetracycline resistance gene.[26] Spectinomycin was also abandoned around the same time as high rates of resistance were observed.[26] Fluoroquinolones became the treatment of choice throughout the 1990s, but resistance became widespread in the 2000s.[26] By that time, macrolides had also been used in several countries, and increasing numbers of high-level macrolide resistant cases precluded the use of macrolides in monotherapy.[26] Third generation cephalosporins ("extended-spectrum cephalosporins", or ESC, referring to injectable ceftriaxone, and oral cefixime) remained the only highly effective single dose treatment option against gonorrhoea.[26] The use of low-dose oral cefixime lead to the emergence of cefixime-resistant gonococcal strains in Japan, which have spread around the world.[26] Cefixime treatment failures have been reported in Japan, and several other countries.[26] In the last few decades, gonococcal lineages with reduced susceptibility to ESC have spread across the globe.[27] Worryingly, one of these lineages, called multilocus sequence type 1901, is just one mutation away from full ceftriaxone resistance.[27] Multiple cases of ceftriaxone treatment failures have been reported in Japan, Australia, and some European countries.[26] Also extensively drug-resistant (XDR) gonococcal strains have been reported, with combined resistance against cephalosporins and several other antimicrobials.[26]

Around 2010, international guidelines recommended dual use of ceftriaxone with azithromycin (or doxycycline), hypothesising that dual therapy would delay the development and spread of gonococcal AMR.[28] Yet, in light of the increasing prevalence of gonococcal azithromycin resistance,[29] and reports of XDR gonorrhoea resistant against both antimicrobials,[30,31] this practice has become the topic of debate.[29,32] While European guidelines still recommend azithromycin + ceftriaxone dual therapy as the first choice,[22] some countries, including the Netherlands (2018 guideline)[33], the UK (2018 guideline)[34] and the USA (2020 guideline)[35] have recently changed their treatment recommendation to ceftriaxone monotherapy.

Figure 4: The emergence of antimicrobial resistance in Neisseria gonorrhoeae followed the introduction of every new antimicrobial to treat gonorrhoea, in the last century (adapted from [24]). Each bar below the timeline represents the introduction of a therapy for gonorrhoea. The end of each bar represents the moment in time when clinical and/or in vitro resistance emerged so that the effectiveness of the antimicrobial was threatened. (ESC = extended spectrum cephalosporin; PBP = penicillin binding protein; SNP = single nucleotide polymorphism; MIC = minimum inhibitory concentration)
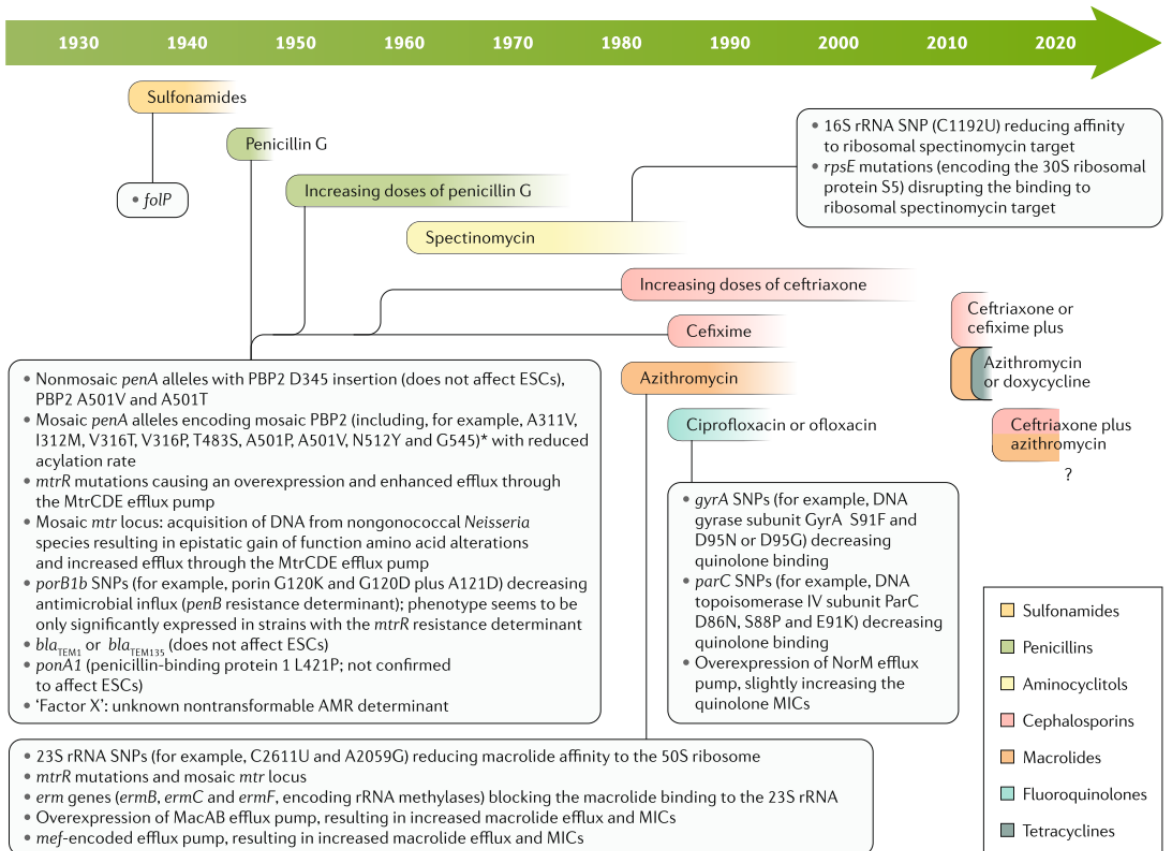
Novel antimicrobials are under evaluation for use against gonorrhoea. Ertapenem was effective against ceftriaxone-susceptible anogenital gonorrhoea in one clinical trial,[36] Zoliflodacin against anogenital,[37] and gepotidacin against urogenital gonorrhoea.[38] Still, ceftriaxone remains the only treatment option for oropharyngeal gonorrhoea, as data for the oropharynx were limited or lacking (for gepotidacin[38] and zoliflodacin[37]) or showed insufficient treatment efficacy (for ertapenem[36]). Phase III trials for zoliflodacin (ClinicalTrials.gov NCT03959527) and gepotidacin (NCT04010539) are ongoing.[39]

1.3.3.2 *Causes of AMR and relevance of men who have sex with men*

Resistance to antimicrobials probably exists for as long as bacteria exist.[40] Increasing antimicrobial exposure among humans, animals, and in the environment has however provided a selective survival benefit to bacteria that harbour resistance genes.[41] Indeed, the introduction of every new antimicrobial to the market has been followed by AMR.[41] In the same vein, phylogenomic studies have found that historical gonococcal treatment recommendations have driven the emergence and spread of resistant gonococcal lineages across the world.[27,42] Gradients in antimicrobial exposure may even explain differences in gonococcal lineages according to sexual behaviour.[43,44] Gonococcal lineage A, which is most associated with (multiple) AMR, is associated with infection in MSM.[44] The development and spread of AMR in this lineage is enhanced by the perfect storm of (a) its high level of genomic plasticity, (b) high antimicrobial exposure among MSM, and (c) opportunity to spread rapidly along a dense sexual network.[44]

(a) Genomic plasticity enabled the gonococcus to acquire an armamentarium against antimicrobial selection pressure that ranges across the entire spectrum of known resistance mechanisms: (i) decreased influx of antimicrobials (betalactams); (ii) increased efflux of antimicrobials (betalactams, fluoroquinolones, spectinomycin, macrolides); (iii) enzymatic adaptation or destruction of antimicrobials (beta lactams); (iv) antimicrobial target modification (betalactams, tetracyclines, spectinomycin, macrolides, fluoroquinolones, Figure 5).[26]

(b) Antimicrobial consumption in PrEP cohorts is high. Quarterly screening and treatment for gonorrhoea and chlamydia in PrEP cohorts results in consumptions as high as 12.05 WHO defined daily doses per 1000 individuals per day (DID) for macrolides, 2.29 DID of tetracyclines, and 0.76 DID of third generation cephalosporins.[45] This level of consumption exceeds by far that of the general population in the majority of European countries.[45]

(c) As mentioned, MSM using PrEP take part in a dense sexual network in which many participants have concurrent sexual partners. Previous research has shown that partner concurrency is key for the rapid spread of STIs within a population (Figure 6).[46]

Figure 5: Mechanisms of antimicrobial action (top) and mechanisms of antimicrobial resistance (bottom, from[47])
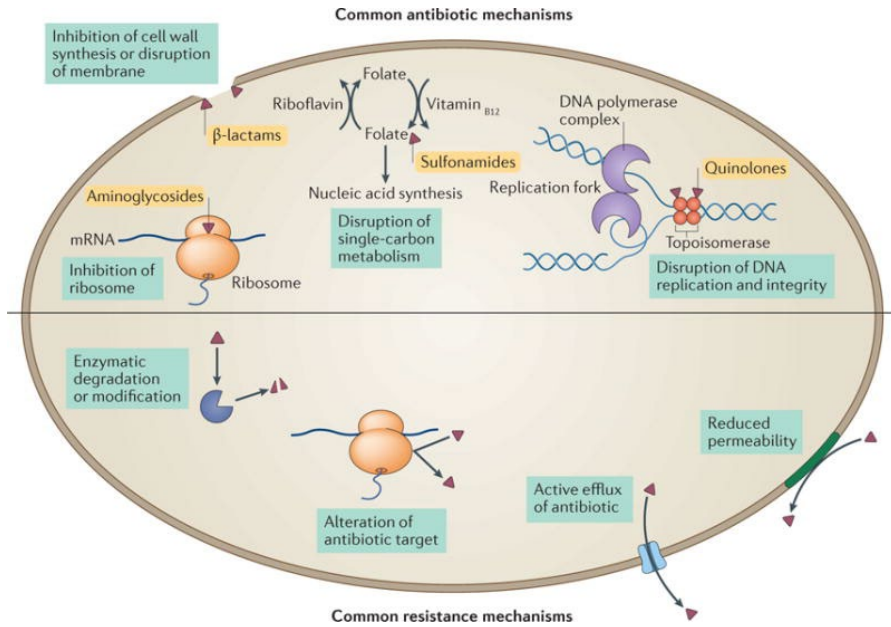

Figure 6: Small increases in partner concurrency (top row, from left to right) result in extensive increases in connectivity of individuals in a sexual network (bottom row, from[46])
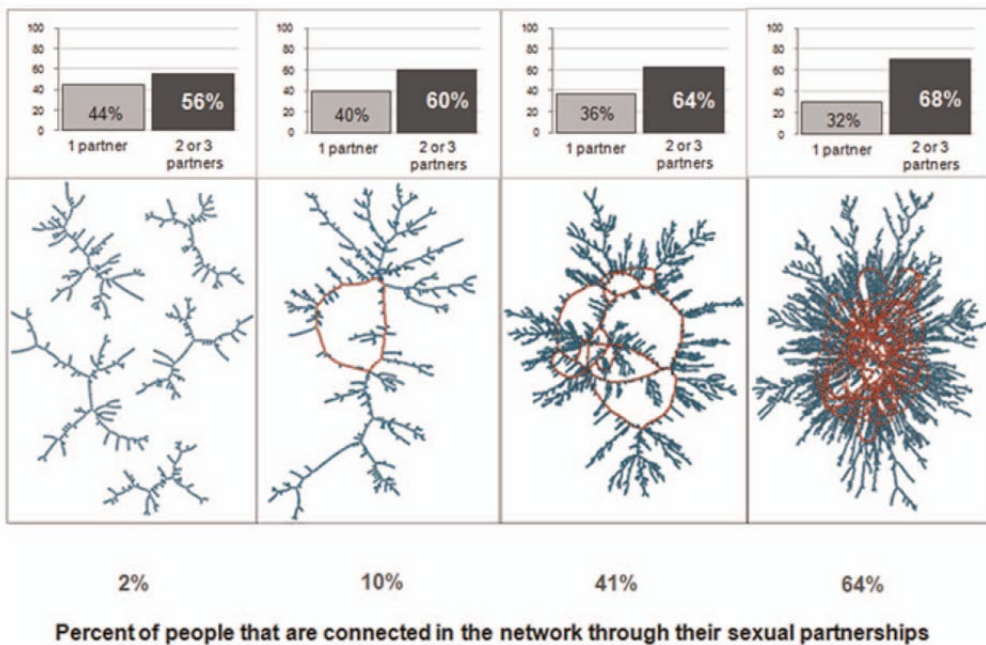
1.4 The importance of the oropharynx for gonococcal transmission and resistance

The oropharynx is one of the body sites most frequently colonized by *N. gonorrhoeae*, in MSM: prevalences vary between 0.5 and 16.5%.[12,48] Pharyngeal colonization with *N. gonorrhoeae* has several particularities, which have led to the hypothesis that the oropharynx occupies a central position in the development and spread of AMR in *N. gonorrhoeae*:[49,50]

a) *N. gonorrhoeae* can be transmitted between the oropharynx, the genitals and the anorectum by all variations of sexual activities (Figure 7).[49] In addition, kissing, fellatio (oro-penile sex) and rimming (oro-anal sex) are frequently involved in sexual activity among MSM. This gives the oropharynx a central position in the transmission dynamics of *N. gonorrhoeae*, as also supported by findings in mathematical modelling studies.[51,52] To what extent kissing contributes to the spread of gonococcal infection, remains controversial.[53,54]

b) More than 90% of oropharyngeal infections are asymptomatic.[56] Many infections thus go undetected for long periods of time, until antimicrobials are administered for another reason (e.g. after screening, partner notification), or until spontaneous clearance, which is estimated to last around 3 months[18,57]. This means that the oropharynx may act as a reservoir of infection.[58]

c) The human oropharynx is colonised by commensal bacteria, of which non-pathogenic *Neisseria species* are among the most abundant species.[59] Genetic elements can be transferred from these bacteria into an incoming *N. gonorrhoeae*, by means of transformation. The list of AMR genes for which there is evidence of transformation from commensal *Neisseria species* to *Neisseria gonorrhoeae* is growing.[17,60–64]

d) The cure rate of pharyngeal gonorrhoea is lower than at other anatomical sites.[65–67] Certain gonococcal infections may thus survive periods of antimicrobial exposure, during which selection of antimicrobial-resistant clones may occur.[50]

In conclusion, pharyngeal gonorrhoea in MSM requires special attention because it is highly prevalent, difficult to eradicate, it probably contributes to gonococcal AMR, and is supposed to play an important role in transmission.[67]

Figure 7: Main transmission dynamics of Neisseria gonorrhoea between men who have sex with men: N. gonorrhoea transmission by insertive/receptive (green) fellatio, (blue) rimming, (orange) anal sex (adapted from[55])
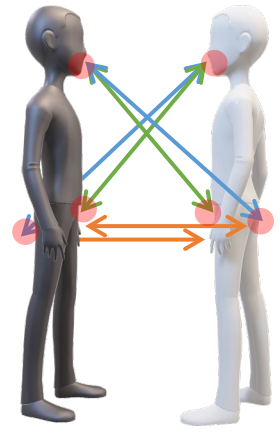
1.5 Antibiotic-sparing options to prevent or treat bacterial STIs

As mentioned, the use of antibiotics provides a selective pressure that promotes the proliferation of antibiotic resistant microorganisms. Hence, effective non-antibiotic alternatives to prevent or treat infections such as bacterial STIs are highly needed. Vaccines would be the ultimate preventive tool, but so far no effective vaccines are available against chlamydia, gonorrhoea, or syphilis.[68,69] Bacteriophages could provide a highly targeted treatment option with minimal selective pressure on bystander microorganisms. Yet, for the time being the search for bacteriophages against bacterial STIs has not been fruitful.[70,71] Topical application of antiseptics is another method to prevent/treat STIs that could have less detrimental ecological effects compared to antibiotics. The idea stems from

the pre-antibiotic era,[72] and for *N. gonorrhoeae*, preliminary studies indicate that antiseptic mouthwashes might be effective to prevent STIs or treat oropharyngeal gonorrhoea.[73] This option will be further explored in this thesis.

1.6 The microbiome and resistome

1.6.1 Definitions

The microbiome denotes the microbial community in a certain habitat with specific physio-chemical properties.[74] Bacteria, archaea, fungi, algae and protists are part of the microbiota, i.e. living members of the microbiome.[74] The metagenome is the collection of genomes and genes of the microbiota.[74] The resistome represents the collection of (antibiotic and other) resistance genes present in a given environment.[47,75]

1.6.2 Methods to determine the microbiome

The microbiome can be assessed by culturing and identifying individual microorganisms present in a sample, but this approach cannot capture the full complexity and diversity of the microbiome, as it is biased towards those microorganisms that can be easily cultured.[76] Metagenomic sequencing, on the other hand, has the potential to provide a more complete overview of the microbiome, without the need for prior bacterial culturing.[76]

There are two main types of metagenomic sequencing: targeted-amplicon sequencing and shotgun metagenomic sequencing.[76] A general overview of the workflow of these techniques is given in Figure 8 and Figure 9, respectively. Targeted sequencing gives information about the presence of a pre-specified (set of) gene(s).[76] The most widely used target gene is the bacterial 16S ribosomal RNA (rRNA) subunit gene. Sequencing the hypervariable regions within this highly conserved gene allows to classify bacterial species phylogenetically.[76] 16S rRNA sequencing can thus be used to provide insight into the bacterial composition of a sample, but does not permit to make conclusions about other organisms, or the resistome.[76] In contrast, shotgun metagenomic sequencing randomly sequences fragments of DNA that can originate from any part of a microorganism's genome,

including the taxonomically informative 16S rRNA genes and coding regions for proteins that provide certain biological functions, such as resistance to antibiotics or other compounds (*i.e.* the resistome).[76,77] Shotgun metagenomics may thus provide an answer to two questions simultaneously, namely which microorganisms are present, and what are they capable of doing?[76,77] Disadvantages of shotgun metagenomic sequencing are the high computational requirements to process the sequencing data, the need to remove unwanted host DNA after sequencing, and the higher cost of sequencing compared to 16S rRNA sequencing.[76]

Figure 8: General workflow of a targeted-amplicon sequencing project, based on the example of a 16S ribosomal RNA study of the skin microbiome (from[78]).
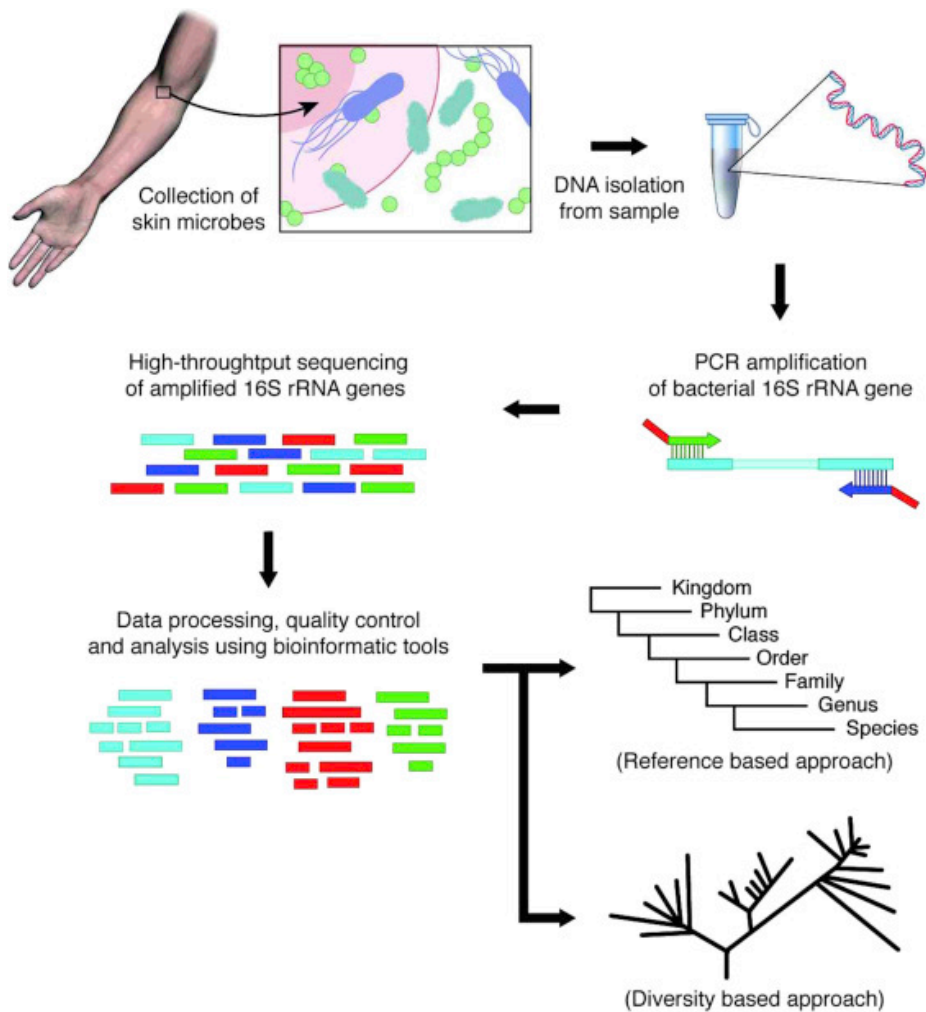
Figure 9: General workflow of a shotgun metagenomic sequencing project (from[77]).
QC = quality control
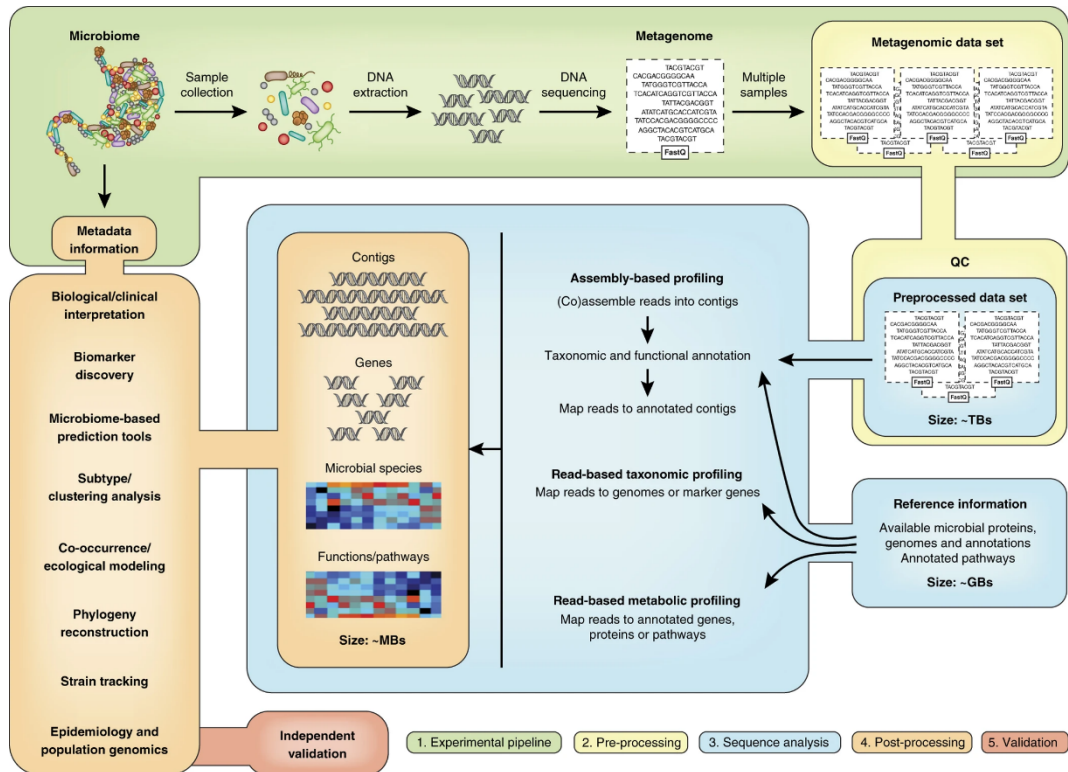
Other *-omics* approaches out of the scope of the current work but worth mentioning because of their complementary value to metagenomics are metaproteomics, metabolomics and metatranscriptomics. Metaproteomics provides insight into the protein content of a sample; metabolomics gives information about metabolites secreted or modulated by the microbiota; and metatranscriptomics gives an idea of which genes are actively being transcribed by the microbiota.[79] Together, these techniques enable a more thorough understanding of the complex dynamics of the microbiome.

1.6.3 Methods to determine the resistome

Culturing and antimicrobial susceptibility testing of specific micro-organisms is a valuable method to determine the presence of resistant organisms in a sample,

and polymerase chain reaction (PCR) or sequencing applied to the cultured isolates allow to elucidate the underlying genetic mechanisms of resistance.[80] To overcome the limitation that these methods rely heavily on the choice of the cultured organism(s), PCRs have been developed to detect resistance genes directly in a sample.[80] These PCRs are generally less costly but more biased towards known resistance genes than shotgun metagenomics.[80] As mentioned, shotgun metagenomics can provide broader information about the microbiome and resistome, at the same time.[76,80] A weakness of shotgun metagenomics in resistome research is its reliance on reference databases to identify resistance genes: resistance genes or single nucleotide variants absent in the chosen database will remain undetected.[81,82] Ultimately in this context, it is worth mentioning a newer technique which uses "targeted" probes instead of "random" metagenomic sequencing to quantify resistance and other relevant genes in a sample with higher sensitivity and specificity than shotgun metagenomics, albeit at a higher financial cost.[83,84]

1.7 Conclusion

In summary, the evolution of AMR in bacteria such as *N. gonorrhoeae* complicates the world's efforts to bring the worldwide STI epidemic under control. We need to find ways to slow down *N. gonorrhoea*'s march along the path of increasing AMR. On the one hand, we need to gain better insight into the circumstances which drive the emergence and dissemination of AMR in this bacterium. For that reason, the first part of this thesis explores the oropharyngeal microbiota in MSM as a reservoir of AMR genes. On the other hand, antibiotic-sparing alternatives to prevent or treat STIs would be highly welcome. The second part of this thesis evaluates whether antiseptic mouthwashes could be effective in that respect.

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Chapter 2   Objectives

Generally, this work addressed some of the research gaps as listed in the 2012 WHO's global action plan to control the spread and impact of AMR in *N. gonorrhoeae*.[6]

2.1 Objective 1: To assess how the oral microbiota and resistome of MSM using PrEP differs from the general population and how these differences can be explained.

Research gap[6]	Objective	Hypothesis	Type of research	Chapter
Microbiological effects (on *N. gonorrhoeae* as well as other affected bystander microorganisms) of combination therapies. Identification of factors that contribute to the emergence and spread of AMR in *N. gonorrhoeae*.	Assess how the microbiome and resistome of MSM using PrEP differ from the general population, and how antimicrobial exposure impacts *Neisseria spp.* and other bystander organisms.	MSM using PrEP are colonized with more resistant commensal *Neisseria spp*. in their oropharynx than the general population.	Prospective survey comparing the antimicrobial susceptibility profile of *Neisseria spp*. cultured from the oropharynx of MSM using PrEP with the general population.	3.1
		The oropharyngeal resistome of MSM using PrEP has a higher abundance of AMR genes, as compared to the general population.	Prospective survey comparing the resistome (the entirety of AMR genes in the microbiome, as determined by metagenomic sequencing) in the oropharynx of MSM using PrEP with the general population.	3.2
	Assess if commensal *Neisseria spp*. are shared between intimate partners	Couples share more similar commensal *Neisseria spp*. than unrelated individuals.	Analysis of publicly available data from a previously published prospective observational microbiome study to compare the similarity of oral *Neisseria spp*. within couples.	3.3

2.2 Objective 2: To evaluate if mouthwashes can be used as antibiotic-sparing options to effectively to prevent STIs or treat oropharyngeal gonorrhoea in MSM using PrEP

Research gap[6]	Objective	Hypothesis	Type of research	Chapter
The efficacy of new antibiotics or other therapeutic compounds.	Evaluate the efficacy of a mouthwash as a way to prevent bacterial STIs	A placebo mouthwash with minimal anti-gonococcal activity can be developed.	Experimental laboratory study to develop a placebo mouthwash with minimal anti-gonococcal activity for use in a clinical trial.	4.1
		A Listerine Cool Mint mouthwash cannot reduce the incidence of bacterial STIs in PrEP users.	Double blinded randomized clinical trial comparing the preventive effect of a Listerine mouthwash with the placebo mouthwash in a population of MSM using PrEP.	4.2
	Evaluate the efficacy of antiseptic mouthwashes to treat pharyngeal gonorrhoea in MSM	A chlorhexidine mouthwash cannot eradicate pharyngeal gonorrhoea.	Open label non-randomized clinical trial evaluating the therapeutic efficacy of a chlorhexidine mouthwash in MSM with pharyngeal gonorrhoea.	4.3

Chapter 3   AMR in oropharyngeal microbiota of MSM using PrEP

3.1 MSM using PrEP are colonized with more resistant commensal *Neisseria spp*. in their oropharynx than the general population.

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Antimicrobial susceptibility of commensal *Neisseria* in a general population and men who have sex with men in Belgium

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Non-pathogenic *Neisseria* are a reservoir of antimicrobial resistance genes for pathogenic *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Men who have sex with men (MSM) are at risk of co-colonization with resistant non-pathogenic and pathogenic *Neisseria*. We assessed if the antimicrobial susceptibility of non-pathogenic *Neisseria* among MSM differs from a general population and if antimicrobial exposure impacts susceptibility. We recruited 96 participants at our center in Belgium: 32 employees, 32 MSM who did not use antibiotics in the previous 6 months, and 32 MSM who did. Oropharyngeal *Neisseria* were cultured and identified with MALDI-TOF–MS. Minimum inhibitory concentrations for azithromycin, ceftriaxone and ciprofloxacin were determined using E-tests® and compared between groups with non-parametric tests. Non-pathogenic *Neisseria* from employees as well as MSM were remarkably resistant. Those from MSM were significantly less susceptible than employees to azithromycin and ciprofloxacin ($p < 0.0001$, $p < 0.001$), but not ceftriaxone ($p = 0.3$). Susceptibility did not differ significantly according to recent antimicrobial exposure in MSM. Surveilling antimicrobial susceptibility of non-pathogenic *Neisseria* may be a sensitive way to assess impact of antimicrobial exposure in a population. The high levels of antimicrobial resistance in this survey indicate that novel resistance determinants may be readily available for future transfer from non-pathogenic to pathogenic *Neisseria*.

Neisseria gonorrhoeae and *N. meningitidis* are becoming increasingly resistant to antimicrobials. For *N. gonorrhoeae* this concerns last-resort antimicrobials such as ceftriaxone and azithromycin[1,2]. Numerous studies have documented that for both species, much of this resistance has been acquired from the non-pathogenic *Neisseria* species that are a key component of a healthy oropharyngeal microbiome[3–8]. The most prominent genes involved in this transformation include *penA, mtrCDE, rplB, rplD, rplV, parC,* and *gyrA*. The acquisition of sections of these genes from non-pathogenic *Neisseria* has played an important role in the acquisition of penicillin, cephalosporin, macrolide, and/or fluoroquinolone resistance in *N. meningitidis* and *N. gonorrhoeae*[5,9,10]. Recent studies have established that uptake of DNA from non-pathogenic *Neisseria* was responsible for the majority of fluoroquinolone resistance in *N. meningitidis* and most azithromycin resistance in *N. gonorrhoeae* in Germany and the United States[4,7,11]. Non-pathogenic *Neisseria* have therefore gained interest as "canaries in the coalmine" for potential future resistance development in pathogenic *Neisseria*[9,12,13].

Despite their importance as reservoirs of antimicrobial resistance (AMR), very few studies have explored the antimicrobial susceptibilities of contemporary non-pathogenic *Neisseria*. Studies of historical isolates found that non-pathogenic *Neisseria* were generally less susceptible to antimicrobials than pathogenic *Neisseria*[9,13]. In the last decade, however, few surveys have reported data on antimicrobial susceptibility of non-pathogenic *Neisseria* isolates. Two studies reported high minimum inhibitory concentrations (MICs) for macrolides, cephalosporins

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and fluoroquinolones among *N. lactamica* isolates from children in Japan and China in 2015[14,15]. One study found 93% fluoroquinolone resistance among commensal *Neisseria* from asymptomatic *N. meningitidis* carriers in China[7]. Two other studies were surveys among men who have sex with men (MSM) visiting a sexual health clinic in Vietnam in 2016 and Belgium in 2019[8,16,17]. Both reported reduced susceptibility of non-pathogenic *Neisseria* to the antimicrobials currently used to treat gonorrhoea—azithromycin, and ceftriaxone. The high azithromycin and ceftriaxone MICs of non-pathogenic *Neisseria* among MSM is of particular concern as gonococcal AMR has frequently emerged in MSM[18–20]. MSM are also often co-colonised by *N. meningitidis* and *N. gonorrhoeae* in their pharynx[21–26].

Beyond these studies, very little is known about the epidemiology of antimicrobial susceptibilities in non-pathogenic *Neisseria*. In particular, little is known about their susceptibility in contemporary general adult populations.

It is not even known if the non-pathogenic *Neisseria* are more or less resistant in MSM than the general population and how MICs vary in relation to recent antimicrobial consumption.

Therefore, the aim of the current study was to compare the antimicrobial susceptibility of oropharyngeal *Neisseria* between MSM who recently used antimicrobials, MSM who did not, and employees of our institute as representatives of the general population in Belgium.

Methods

Survey population.
This cross-sectional survey included 64 MSM and 32 employees.

The 64 MSM participated in a single centre randomized clinical trial (PReGo) at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium in 2019–2020. PReGo was a placebo-controlled trial that assessed the efficacy of an antiseptic mouthwash (Listerine™) to prevent STIs among 343 MSM[27]. Taking HIV pre-exposure prophylaxis (PrEP) and having a history of gonorrhoea, chlamydia or syphilis in the previous two years was an inclusion criterium of that study. For the current survey, MSM were sampled at their first study visit, before administration of the PReGo study mouthwash. PReGo participants were enrolled into two groups, depending on their history of antimicrobial exposure.

Group I: MSM who recently used antimicrobials (n = 32).   The first 32 PReGo participants who used at least one antimicrobial in the previous 6 months were included in this group.

Group II: MSM who did not recently use antimicrobials (n = 32).   The first 32 PReGo participants who did not use any antimicrobial in the previous 6 months were included in this group.

Group III: Representatives of the general population: ITM employees who did not recently use antimicrobials (n = 32).   In June 2020, ITM employees were invited to participate by posters and by word of mouth. Candidates who used an antimicrobial in the previous 6 months were excluded. The first 32 eligible employees (male or female) presenting to the study team were included in this survey.

Data collection and sampling procedure.
All participants provided written informed consent prior to the collection of data and samples. Baseline characteristics were noted (including self-reported age, sex, antimicrobial use in the previous 6 months). Oropharyngeal samples were taken by a study physician who rubbed both tonsillar pillars and the posterior oropharynx with an ESwab™ (COPAN Diagnostics Inc., Italy).

Sample processing.
Culture and identification of Neisseria species.   ESwabs™ were inoculated onto Columbia Blood Agar and Modified Thayer-Martin Agar using the streak plate technique and incubated at 35–37 °C and 5% carbon dioxide. Plates were examined after 48 h and Gram negative, oxidase positive colonies were selected, enriched and stored in Skim-milk at −80 °C.

Isolates were identified to the species level using Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight mass spectrometry (MALDI-TOF MS), on a MALDI Biotyper® Sirius IVD system using the MBT Compass IVD software and library (Bruker Daltonics, Bremen, Germany). Briefly, each bacterial isolate was smeared twice on a polished steel target plate and then covered with 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution. After drying, the target plate was loaded into the instrument. The spectra were acquired in linear mode in a mass range of 2–20 kDa and subsequently compared to the library that included 9607 spectra at that time. Identification results were classified as reliable or unreliable according to recommended cut-off values of 1.7 and 2 for validated results for the genus and species levels, respectively. Only isolates belonging to the genus *Neisseria* were included in further analyses. Isolates identified as *N. macacae* were grouped into one category with *N. mucosa,* whereas isolates identified as *N. perflava* and *N. flavescens* were grouped into one category with *N. subflava*[28].

Antimicrobial susceptibility determination.   Minimum inhibitory concentrations (MICs) of *Neisseria* species to azithromycin, ceftriaxone, and ciprofloxacin were determined on GC agar plates using ETEST® (bioMérieux Marcy-l'Étoile, France) incubated for 24 h at 36.5 °C and 5–7% CO_2, and expressed in mg/L. Lack of bacterial growth during susceptibility testing resulted in missing values for that isolate.

Statistics.
Neisseria prevalence.   Prevalence was expressed as the proportion of participants from whom a certain species was isolated. Prevalence was compared between groups using Chi square tests.

	Overall (n = 96)	Employees (n = 32)	MSM who did not use antibiotics (n = 32)	MSM who used antibiotics (n = 32)	*p*-value*
Age in years, median (IQR)	35 (35–47.5)	45 (35–55)	45 (35–55)	39 (35–45)	0.21
Male sex, n (%)	74 (77.1)	10 (31.3)	32 (100.0)	32 (100.0)	< 0.001
Antibiotic exposure in the previous 6 months, n (%)	32 (33.3)	0 (0.0)	0 (0.0)	32 (100.0)	NA
β-Lactams	25 (26.0)	NA	NA	25 (78.1)	NA
Macrolides	19 (19.8)			19 (59.4)	
Fluoroquinolones	2 (2.1)			2 (6.3)	
Other	8 (8.3)			8 (25.0)	
Antibiotic exposure in the previous 1 month, n (%)	7 (7.3)	0 (0.0)	0 (0.0)	7 (21.9)	NA
β-Lactams	4 (4.2)	NA	NA	4 (12.5)	NA
Macrolides	0 (0.0)			0 (0.0)	
Fluoroquinolones	1 (1.0)			1 (14.3)	
Other	2 (2.1)			2 (6.3)	
Median number of casual sex partners in the previous 3 months	NA	NA	10.0 (4.8–15.0)	10.0 (8.0–20.0)	0.12
Used condoms with > 75% of casual anal sex partners in the previous 3 months, n (%)	NA	NA	9 (28.1)	2 (6.5)[a]	0.03
Used a mouthwash in the previous 1 month, n (%)	46 (47.9)	15 (46.9)	12 (37.5)	19 (59.4)	0.22

Table 1. Population characteristics. *NA* not applicable/not available. *Kruskal–Wallis rank sum test. [a]1 missing value.

Neisseria species richness. *Neisseria* species richness was defined as the number of different non-pathogenic *Neisseria* species per participant. Species richness was reported as median (interquartile range) and compared between groups using Kruskal–Wallis rank sum tests. If no significant differences were observed between the two groups of MSM, their data were combined.

Antimicrobial susceptibility. To enable statistical testing, MICs above the maximum or below the minimum level of the ETEST strip were simplified as follows: azithromycin MIC > 256 mg/L was recoded as 512 mg/L; ceftriaxone MIC < 0.016 mg/L as 0.008 mg/L; and ciprofloxacin MIC > 32 mg/L as 64 mg/L. If multiple colonies of the same species were isolated from the same participant, we calculated the median MIC for that species per participant. MICs were reported as median (interquartile range) and compared between groups using Kruskal–Wallis rank sum tests. If no significant differences were observed between the two groups of MSM, their data were combined. Pathogenic and non-pathogenic *Neisseria* were described and analysed separately, and subsequently stratified by species for species that were isolated at least once in each group.

In a sensitivity analysis, we used linear regression with geometric mean MIC as the outcome and two binary dependent variables: (a) being MSM/employee, and (b) antimicrobial exposure in the previous 6 months. The model was also adjusted for *Neisseria* species by the inclusion of a categorical variable.

All statistical analyses were performed with R version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria).

Ethics. Ethics approval was obtained from ITM's Institutional Review Board (1276/18 and 1351/20) and from the Ethics Committee of the University of Antwerp (19/06/058 and AB/ac/003).

The study was carried out according to the principles stated in the Declaration of Helsinki, all applicable regulations and according to the most recent GCP and GCLP guidelines. The Informed Consent Form (ICF) documents were 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).

Results

The median age of the 96 participants was 35 (IQR 35–47.5) years (Table 1). Among the employees, two thirds were female. The MSM reported a high rate of partner change and a low rate of condom use, which is compatible with the high incidence of sexually transmitted infections in the PReGo study[27]. Of the 32 MSM who used antimicrobials in the previous 6 months, 14 (43.8%) used only one class of antimicrobials, 14 (43.8%) used two different classes of antimicrobials, and four (12.5%) participants used three different classes of antimicrobials Supplementary information.

***Neisseria* prevalence.** In total 207 *Neisseria* colonies were isolated, representing seven non-pathogenic and two pathogenic species (Table 2, Fig. 1). In descending order of prevalence, we isolated the non-pathogenic species *N. subflava* (63/96, 65.6%), *N. mucosa* (14/96, 14.6%), *N. oralis* (8/96, 8.3%), *N. cinerea* (3/96, 3.1%), *N.*

	Prevalence (n/N) Participants (%)	Azithromycin (mg/L) Median (IQR)	Ciprofloxacin (mg/L) Median (IQR)	Ceftriaxone (mg/L) Median (IQR)
Pathogenic *Neisseria* spp.	27/96 (28.1)	0.5 (0.4–0.9)	0.004 (0.003–0.006)	<0.016 (<0.016–<0.016)
Neisseria meningitidis	26/96 (27.1)	0.5 (0.3–0.9)	0.004 (0.003–0.005)	<0.016 (<0.016–<0.016)
Employees	2/32 (6.3)	1.0 (0.8–1.3)	0.065 (0.034–0.095)	<0.016 (<0.016–<0.016)
MSM who used AB previous 6 months	9/32 (28.1)	0.8 (0.5–1.5)	0.004 (0.002–0.006)	<0.016 (<0.016–0.012)
MSM who used no AB previous 6 months	15/32 (46.9)	0.5 (0.4–0.5)	0.004 (0.003–0.004)	<0.016 (<0.016–<0.016)
Neisseria gonorrhoeae	1/96 (1.0)	0.125	2.0	<0.016
Employees	0/32 (0.0)	–	–	–
MSM who used AB previous 6 months	0/32 (0.0)	–	–	–
MSM who used no AB previous 6 months	1/32 (3.1)	0.125	2.0	<0.016
Non-pathogenic *Neisseria* spp.	65/96 (67.7)	3.0 (2.0–7.5)	0.032 (0.016–0.25)	0.047 (0.029–0.064)
Employees	32/32 (100.0)	3.0 (2.0–4.0)	0.023 (0.012–0.064)	0.034 (0.026–0.064)
MSM who used AB previous 6 months	19/32 (59.4)	16.0 (3.0–>256.0)	0.250 (0.141–0.500)	0.047 (0.032–0.094)
MSM who used no AB previous 6 months	14/32 (43.8)	4.0 (3.0–48.0)	0.125 (0.016–0.380)	0.047 (0.032–0.064)
Neisseria subflava	63/96 (65.6)	3.5 (2.5–16.0)	0.125 (0.016–0.380)	0.047 (0.028–0.064)
Employees	31/32 (96.9)	3.0 (2.3–4.0)	0.032 (0.016–0.197)	0.035 (0.028–0.052)
MSM who used AB previous 6 months	13/32 (40.6)	288 (3.5–>256.0)	0.380 (0.190–0.500)	0.064 (0.032–0.064)
MSM who used no AB previous 6 months	19/32 (59.4)	4.0 (3.3–72.0)	0.125 (0.022–0.380)	0.047 (0.028–0.126)
Neisseria mucosa	14/96 (14.6)	3.5 (2.3–5.5)	0.016 (0.013–0.030)	0.040 (0.032–0.064)
Employees	8/32 (25.0)	3.5 (2.8–4.5)	0.017 (0.011–0.025)	0.040 (0.032–0.072)
MSM who used AB previous 6 months	4/32 (12.5)	3.5 (2.8–6.3)	0.133 (0.015–1.688)	0.040 (0.032–0.051)
MSM who used no AB previous 6 months	2/32 (6.3)	12.6 (6.9–18.3)	0.016 (0.016–0.016)	0.063 (0.048–0.079)
Neisseria oralis	8/96 (8.3)	2.0 (1.9–3.1)	0.015 (0.012–0.018)	0.056 (0.032–0.064)
Employees	8/32 (25.0)	2.0 (1.0–3.1)	0.015 (0.012–0.018)	0.056 (0.032–0.064)
MSM who used AB previous 6 months	0/32 (0.0)	–	–	–
MSM who used no AB previous 6 months	0/32 (0.0)	–	–	–
Neisseria cinerea	3/96 (3.1)	2.0 (1.5–15.0)	0.012 (0.009–0.022)	<0.016 (<0.016–<0.016)
Employees	3/32 (9.4)	2.0 (1.5–15.0)	0.012 (0.009–0.022)	<0.016 (<0.016–<0.016)
MSM who used AB previous 6 months	0/32 (0.0)	–	–	–
MSM who used no AB previous 6 months	0/32 (0.0)	–	–	–
Neisseria elongata	3/96 (3.1)	0.5 (0.4–0.6)	0.004 (0.004–0.014)	0.047 (0.035–0.119)
Employees	3/32 (9.4)	0.5 (0.4–0.6)	0.004 (0.004–0.014)	0.047 (0.035–0.119)
MSM who used AB previous 6 months	0/32 (0.0)	–	–	–
MSM who used no AB previous 6 months	0/32 (0.0)	–	–	–
Neisseria lactamica	2/96 (2.1)	1.5 (1.3–1.8)	0.127 (0.096–0.159)	<0.016 (<0.016–<0.016)
Employees	2/32 (6.3)	1.5 (1.3–1.8)	0.127 (0.096–0.159)	<0.016 (<0.016–<0.016)
MSM who used AB previous 6 months	0/32 (0.0)	–	–	–
MSM who used no AB previous 6 months	0/32 (0.0)	–	–	–
Neisseria bacilliformis	1/96 (1.0)	2 (–)	0.125 (–)	1.5 (–)
Employees	1/32 (3.1)	2 (–)	0.125 (–)	1.5 (–)
MSM who used AB previous 6 months	0/32 (0.0)	–	–	–
MSM who used no AB previous 6 months	0/32 (0.0)	–	–	–

Table 2. Antimicrobial susceptibility of *Neisseria* isolates cultured from the oropharynx of 64 STI clinic attendees (men who have sex with men) and 32 employees of the Institute of Tropical Medicine (representing the general population) in Belgium. *AB* antibiotics, *IQR* interquartile range, *MSM* men who have sex with men, *STI* sexually transmitted infections.
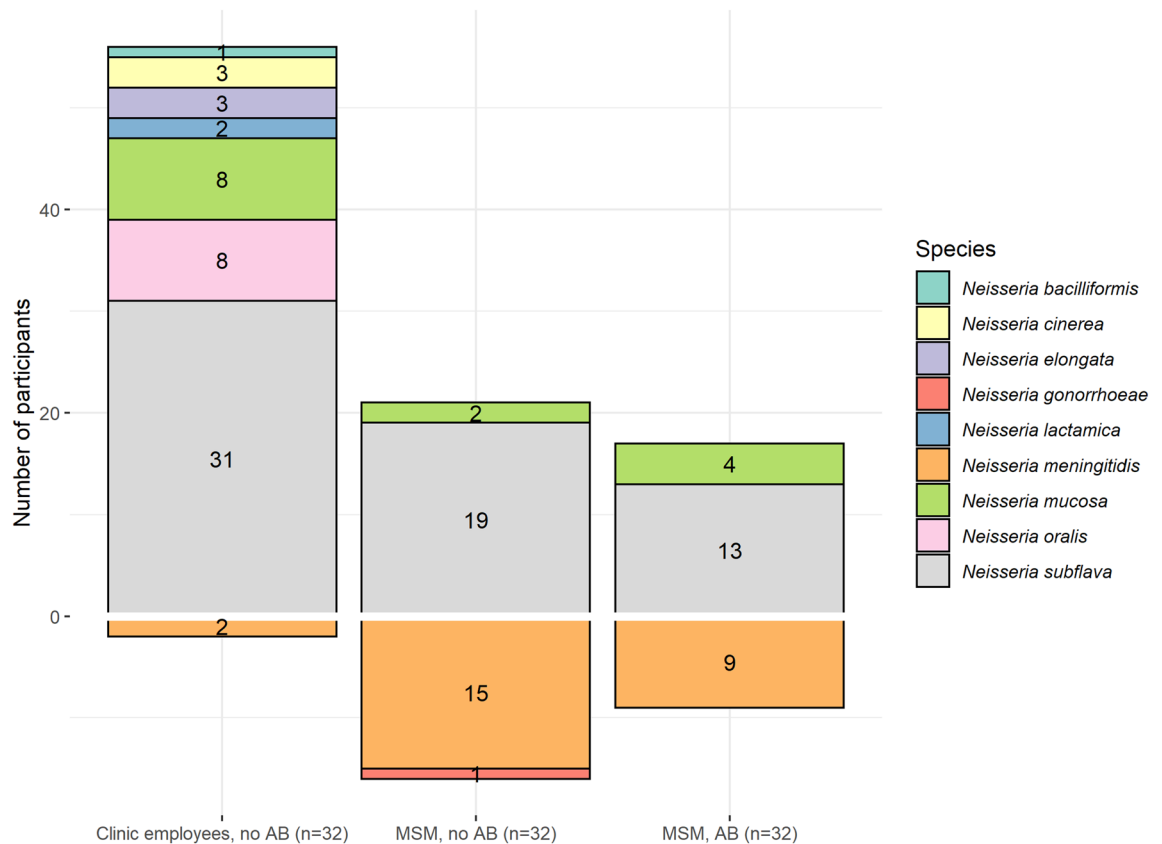

Figure 1. Prevalence and richness of *Neisseria* species, in absolute number of participants from whom the concerning species was isolated, per group. *AB* antibiotics, *MSM* men who have sex with men.

elongata (3/96, 3.1%)*, N. lactamica* (2/96, 2.1%), and *N. bacilliformis* (1/96, 1.0%). The pathogenic species were *N. meningitidis* (26/96, 27.1% prevalence), and *N. gonorrhoeae* (one isolate from a MSM, 1.0% prevalence).

The prevalence of non-pathogenic *Neisseria* was lower among MSM (51.6%) than the employees (100.0%, $p < 0.00001$, Table 2, Fig. 1), but for the pathogenic *Neisseria* this was the reverse: *N. meningitidis* was much more prevalent among MSM (37.5%) than the employees (6.3%, $p < 0.01$).

MSM who used antimicrobials in the previous 6 months were less often colonised with *N. meningitidis* (28.1%) than MSM who did not use antibiotics (46.9%), but this difference was not statistically significant ($p = 0.20$).

Richness of non-pathogenic *Neisseria* species. Co-colonisation with multiple non-pathogenic *Neisseria* species was less common among MSM (7.8% were colonised with two species) than the employees (37.5% colonised with two species and 18.8% with three species).

In addition, while all seven non-pathogenic species were isolated from the employees, only two were isolated from MSM: *N. subflava* and *N. mucosa*. The richness of non-pathogenic species was thus lower among MSM (median of 1 species, IQR 0–1) than the employees (median of 2 species, IQR 1–2, $p < 0.0001$).

Susceptibility of non-pathogenic *Neisseria*. The non-pathogenic *Neisseria* were significantly less susceptible (higher MICs) to all three antimicrobials than the pathogenic *Neisseria* ($p < 0.0001$ for every antimicrobial, Table 2, Fig. 2). The non-pathogenic *Neisseria* isolated from MSM had significantly higher MICs for azithromycin (7.0 mg/L, IQR 3.0–280.2) and ciprofloxacin (0.250 mg/L, IQR 0.020–0.380) compared to those from the employees (3.0 mg/L, IQR 2.0–4.0, $p < 0.0001$; and 0.023 mg/L, IQR 0.012–0.064, $p < 0.001$, respectively; Table 2, Fig. 3). The MICs for ceftriaxone were similar in both groups (0.047 mg/L, IQR 0.032–0.084 in MSM versus 0.034, IQR 0.026–0.064 in the employees, $p = 0.3$). There were no significant differences in MICs accord-
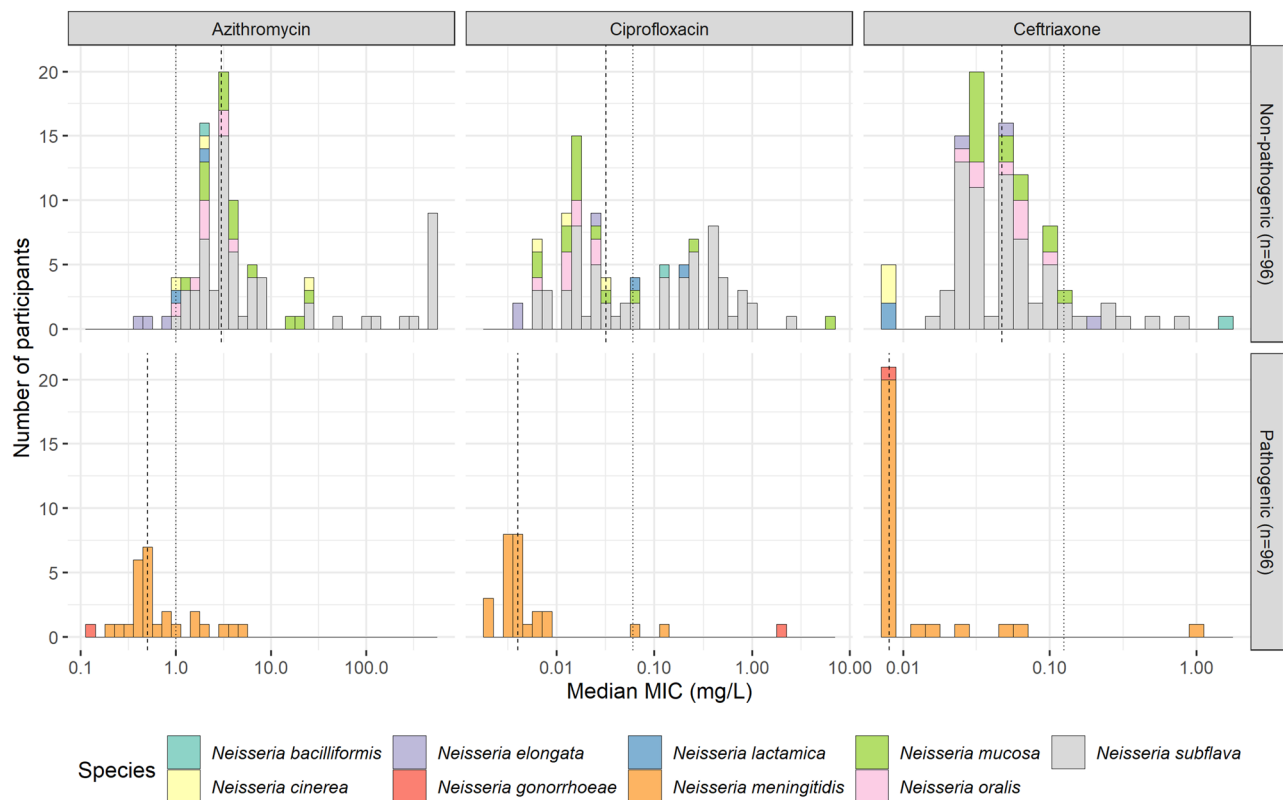
Figure 2. Minimum inhibitory concentration (MIC, mg/L) of pathogenic versus non-pathogenic *Neisseria* species isolated from all 96 participants. Numbers represent the number of participants with that specific median MIC per species. Vertical lines indicate the median of median MICs (dashed line) and the EUCAST v.11.0 cutoff for *N. gonorrhoeae* (dotted line) for each antibiotic.

ing to recent antimicrobial exposure in MSM. The stratified analysis for *N. subflava* showed similar findings. The stratified analysis for *N. mucosa* showed no significant differences in MICs between groups.

The sensitivity analysis based on a linear regression model confirmed the association between MSM and higher MICs for azithromycin (aOR 3.31, 95% CI 1.42–7.72), but estimated an additional increase with recent antimicrobial use (aOR 2.99, 95% CI 1.07–8.31).

For ciprofloxacin, the model suggested that the difference in MIC is only driven by higher MICs in those who were recently exposed to antimicrobials (aOR 3.79, 95% CI 1.49–9.59, Table 3). In addition, the model estimated an association between MSM and higher MICs for ceftriaxone (aOR 1.58, 95% CI 1.06–2.35).

Susceptibility of pathogenic *Neisseria*. For *N. meningitidis*, most isolates were highly susceptible to all three antimicrobials. According to current EUCAST breakpoints (v. 11.0), one isolate was resistant to ceftriaxone (MIC 1 mg/L) and two participants had isolates with ciprofloxacin resistance (MIC 0.125 and 0.064 mg/L).

The single *N. gonorrhoeae* isolate in this survey was susceptible to azithromycin (MIC 0.125 mg/L) and ceftriaxone (MIC < 0.016 mg/L) but resistant to ciprofloxacin (MIC 2 mg/L).

Discussion

We found that contemporary oropharyngeal non-pathogenic *Neisseria* in MSM were less susceptible to antimicrobials than those from employees representing the general population. Recent antimicrobial exposure did not entirely explain the observed differences in susceptibility. This suggests that long-term participant- or population-level antimicrobial exposure plays an important role[29]. Indeed, MSM in PrEP programs consume a large amount of antimicrobials. One of the main drivers of excessive macrolide and cephalosporin consumption among PrEP users is the practice of screening asymptomatic MSM for gonorrhoea and chlamydia[30]. In some cohorts, macrolide consumption exceeds 12 defined daily doses per 1000 individuals per day (DID)[30]. This is multiple times what is consumed by typical general populations and is above the thresholds for inducing macrolide resistance in a range of bacterial species[30,31]. Reducing the intensity of screening for gonorrhoea and chlamydia among MSM may result in a four-fold decrease in macrolide consumption[32].

Although lower than in MSM, the MICs of non-pathogenic *Neisseria* in the employees were considerably higher than in previous surveys. This is illustrated by *N. subflava*, the most prevalent species in our survey. A previous analysis of *N. subflava* isolates from the early 1980s found a considerably lower azithromycin MIC distribution (median 1.0 mg/L, IQR 0.5–2.5 mg/L) than that found in the current employees (median 3.0 mg/L, IQR 2.3–4.0 mg/L)[16].
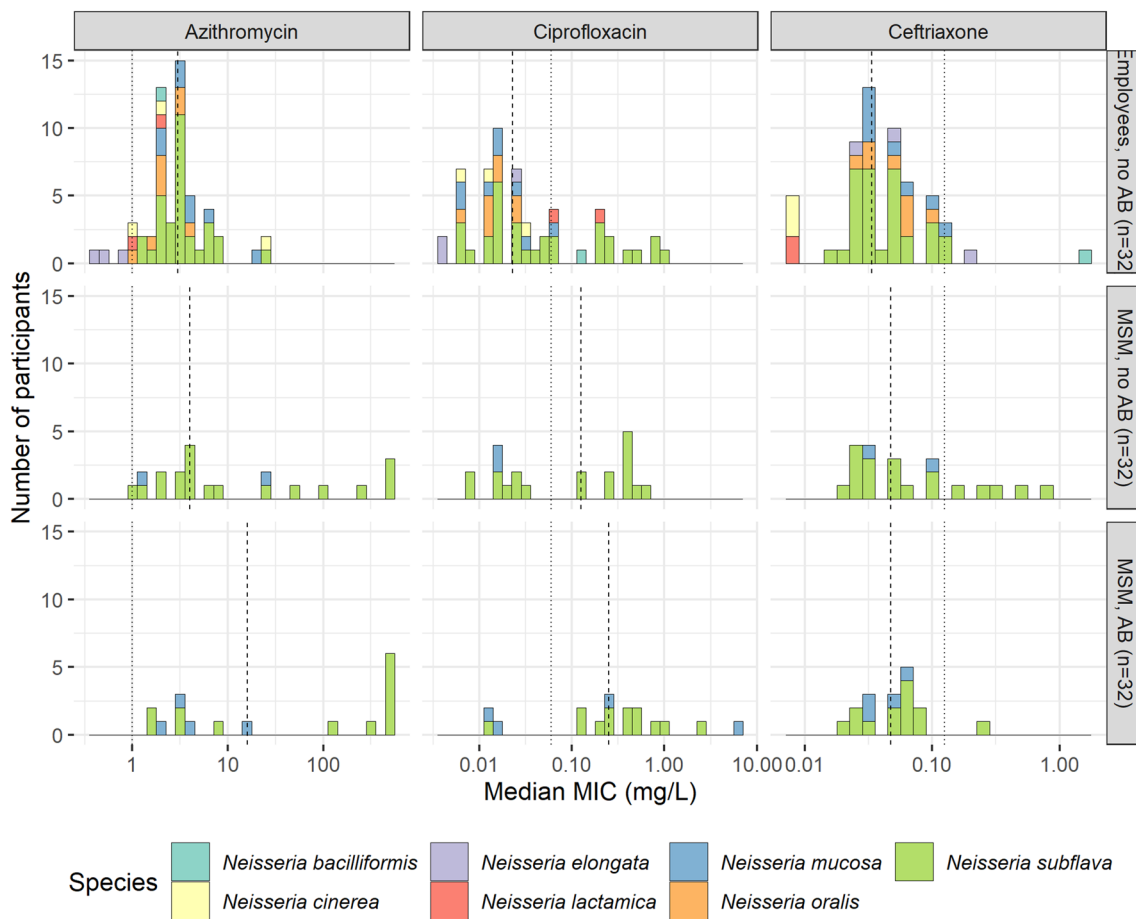
Figure 3. Minimum inhibitory concentration (MIC, mg/L) of non-pathogenic *Neisseria* species, per group. Numbers represent the number of participants with that specific median MIC per species. Vertical lines indicate the median of median MICs (dashed line) and the EUCAST v.11.0 cutoff for *N. gonorrhoeae* (dotted line) for each antibiotic.

All non-pathogenic *Neisseria*	Number of participants (%)	Ciprofloxacin		Azithromycin		Ceftriaxone	
		Unadjusted OR (95% CI)	Adjusted[A] OR (95% CI)	Unadjusted OR (95% CI)	Adjusted[A] OR (95% CI)	Unadjusted OR (95% CI)	Adjusted[A] OR (95% CI)
Population							
Employees	32 (33.3)	1 (Ref)	1 (Ref)	1 (Ref)	1 (Ref)	1 (Ref)	1 (Ref)
MSM	64 (66.7)	2.45 (1.14–5.27)*	1.69 (0.78–3.66)	4.38 (1.97–9.77)*	3.31 (1.42–7.72)*	1.66 (1.05–2.61)*	1.58 (1.06–2.35)*
Used antibiotic in the previous 6 months							
No	64 (66.7)	1 (Ref)	1 (Ref)	1 (Ref)	1 (Ref)	1 (Ref)	1 (Ref)
Yes	32 (33.3)	3.23 (1.21–8.59)*	3.79 (1.49–9.59)*	2.69 (0.97–7.47)	2.99 (1.07–8.31)*	0.75 (0.42–1.34)	0.75 (0.47–1.21)
Neisseria subflava	Number of participants (%)	Unadjusted	Adjusted[A]	Unadjusted	Adjusted[A]	Unadjusted	Adjusted[A]
Population							
Employees	31 (49.2)	1 (Ref)	NA	1 (Ref)	NA	1 (Ref)	NA
MSM	32 (50.8)	1.80 (0.75–4.33)	NA	4.07 (1.51–10.95)*	NA	1.68 (1.06–2.67)*	NA
Used antibiotic in the previous 6 months							
No	50 (79.4)	1 (Ref)	NA	1 (Ref)	NA	1 (Ref)	NA
Yes	13 (20.6)	3.34 (1.13–9.86)*	NA	4.58 (1.35–15.57)*	NA	0.78 (0.44–1.38)	NA

Table 3. Linear regression coefficients for change in geometric mean minimum inhibitory concentrations of non-pathogenic *Neisseria* for ciprofloxacin, azithromycin and ceftriaxone. *CI* Confidence Interval, *MIC* minimum inhibitory concentration, *NA* not applicable, *OR* odds ratio. *Estimate is statistically significant as the CI does not include 1. [A]Adjusted for *Neisseria* species.

In fact, the antimicrobial susceptibilities of the non-pathogenic *Neisseria* from the employees in our study were all higher than those from published reports from equivalent studies in the 1960s to the 1990s[33–37]. Of note, the earliest survey of antimicrobial susceptibility in commensal *Neisseria* that we could locate, found that 28 clinical isolates of *N. cinerea* from Germany pre-1961 were highly susceptible to penicillin (MIC range 0.00015–0.0006 mg/L)[33]. A likely explanation for this decrease in antimicrobial susceptibility over time is the level of antimicrobial consumption by the general Belgian population[38]. Macrolide consumption, for example, exceeded 3.0 DID in 2018 and 2019, which is well above a threshold of 1.3 DID which may select for resistance in pathogens like *S. pneumoniae, M. genitalium,* and *T. pallidum*[31,39]. Certain features of commensal bacteria suggest that such resistance threshold may even be lower for commensals than for pathogens. Thus, population-level antimicrobial consumption may have selected for circulating commensal *Neisseria* with elevated MICs ("Supplementary information").

The prevalence and richness of non-pathogenic *Neisseria* among MSM in our survey was lower than the employees and much lower than reported among MSM in Vietnam and the USA[8,40]. These low numbers among Belgian MSM taking PrEP could be explained by the high antimicrobial exposure of this population[30]. Similar to *N. meningitidis*, certain species of non-pathogenic *Neisseria* may be slower to acquire resistance to specific antimicrobials than other species[9,13]. For example, no isolates of *N. elongata, N. lactamica* or *N. bacilliformis* in our study had an azithromycin MIC greater than 2 mg/L, whereas the median azithromycin MIC for *N. subflava* was 3 mg/L in the employees, 8 mg/L in MSM overall and 288 mg/L in the MSM group that had used antibiotics. This high-level resistance to azithromycin in *N. subflava* has been linked to the uptake of an *msrD* gene likely from oral streptococci[41]. Other *Neisseria* species have thus far not been found to be able to take up this gene or acquire such high-level resistance to azithromycin[41]. The higher consumption of antimicrobials in this MSM PrEP cohort could thus have eliminated the most susceptible non-pathogenic *Neisseria* species and thereby have reduced *Neisseria* species richness.

Conversely, the prevalence of *N. meningitidis* in our study was higher among MSM than the employees, which corroborates other reports of *N. meningitidis* prevalences up to 42.5% among MSM[21–24]. This exceeds by some margin the prevalence in young adults across the globe[42]. *N. meningitidis* is one of the most antimicrobial susceptible *Neisseria* species, as also observed in our current study[43]. A number of genetic differences between *N. meningitidis* and other *Neisseria* have been shown to underpin the reduced capacity of *N. meningitidis* to acquire resistance to various antimicrobials[44,45].

Indeed, in our study, the prevalence of *N. meningitidis* in MSM exposed to antimicrobials was almost half that in unexposed MSM. The prevalence of *N. meningitidis* may thus temporarily decline due to the consumption of antimicrobials (as also shown in other studies[21]), but soon return to its equilibrium prevalence.

Several processes could explain the higher prevalence of *N. meningitidis* among MSM compared with members of the general population. One reason may be the high frequency of interpersonal contacts among MSM taking PrEP—like kissing and attending crowded night-clubs—during which transmission may occur[21,46]. Hypothetically, *N. meningitidis* may be more transmissible than non-pathogenic *Neisseria* and may thus outcompete the latter in recolonizing the pharynx after antimicrobial exposure. Lack of competition with other *Neisseria* species may be another explanation. A number of epidemiological, interventional and in-vitro studies have found evidence of such competition[47]. As an example, the presence of *N. lactamica* has been shown to be associated with a lower prevalence of *N. meningitidis*[48–50].

If antibiotics reduced the prevalence of species such as *N. lactamica* in MSM, this may have left this population more susceptible to colonisation by *N. meningitidis*.

This study has a number of limitations, including the small sample sizes, single centre design and the fact that the samples were not representative of all MSM or the general Belgian population. Furthermore, two experimental factors of this survey may have caused underestimation of the richness of *Neisseria* species and the spectrum of their antibiotic susceptibilities. Firstly, the study depended on culturing *Neisseria* from the posterior oropharynx and tonsils. This design would likely have missed certain non-pathogenic *Neisseria* that preferentially inhabit other parts of the pharynx[51]. Future studies could obtain samples by gargling with physiological saline to overcome this problem[51]. Secondly, only a minority of colonies grown on the agar plates were selected for species identification and MIC determination. We tried to pick at least one of each macroscopically distinct gram negative and oxidase positive colony per plate, but we may have missed particular *Neisseria* species with phenotypes similar to the sampled colonies. Metagenomic studies may also be a more sensitive way to profile the *Neisseria* microbiota and resistome than culture-based techniques. Finally, it would be instructive to repeat this study in settings with low population level antibiotic consumption.

In conclusion, we found high levels of resistance to azithromycin, ceftriaxone, and ciprofloxacin in oropharyngeal *Neisseria* among MSM and employees in Belgium. This finding is worrisome as non-pathogenic *Neisseria* provide a reservoir of resistance genes that can be readily transferred to pathogenic bacteria.

This AMR is most parsimoniously explained by excessive antibiotic exposure in the general Belgian population, but particularly in the MSM PrEP cohorts. Reduced screening for asymptomatic gonorrhoea and chlamydia may substantially reduce antimicrobial consumption by MSM.

The effect of such a policy change on the prevalence of AMR may be most easily demonstrated in the non-pathogenic *Neisseria*. Future studies may thus consider conducting regular surveys of antimicrobial susceptibility of non-pathogenic *Neisseria* in the general population and key populations such as MSM on PrEP as an early warning system of excessive antimicrobial consumption.

Data availability

All deidentified data are available as a Supplement to this manuscript. Additional related documents such as the study protocol, laboratory analysis plan, informed consent form can be obtained from the corresponding author upon reasonable request.

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Acknowledgements

Author contributions

C.K., S.A., E.B., I.D.B., J.L., C.V.D. and S.S.M.B. conceptualized the study. C.K. and C.V.D. collected the samples. S.A., J.L., I.D.B., D.M. and G.S. generated the laboratory results. J.L., C.V.D. and C.K. verified and analysed the data. C.V.D. and J.L. wrote the first draft of the manuscript. All authors reviewed and approved the final manuscript.

Funding

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-03995-1.

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3.2 The oropharyngeal resistome of MSM using PrEP has a higher abundance of AMR genes compared to the general population.

Van Dijck C, Laumen J, de Block T, Abdellati S, De Baetselier I, Tsoumanis A, et al. The oropharynx of men using HIV pre-exposure prophylaxis is enriched with antibiotic resistance genes: a cross-sectional observational metagenomic study.

Manuscript under review in "Journal of Infection".

The oropharynx of men using HIV pre-exposure prophylaxis is enriched with antibiotic resistance genes: a cross-sectional observational metagenomic study.

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Abstract

Background: Phenotypic studies have found high levels of antimicrobial resistance (AMR) among commensal *Neisseria* species in the oropharynx of men who have sex with men using HIV pre-exposure prophylaxis (MSM). This may represent a risk to pathogens like *Neisseria gonorrhoeae* which tend to develop AMR by taking up antibiotic resistance genes (ARGs) from other bacteria. We aimed to explore to what extent the oropharyngeal resistome of MSM differred from the general population.

Methods: We collected oropharyngeal swabs from 32 individuals of the general population and from 64 MSM. Thirty-two MSM had used antibiotics in the previous six months, whereas none of the other participants. Samples underwent shotgun metagenomic sequencing. Sequencing reads were mapped against MEGARes 2.0 to estimate ARG abundance. ARG abundance was compared between groups by zero-inflated negative binomial regression.

Findings: ARG abundance was significantly lower in the general population than in MSM (ratio 0·41, 95% CI 0·26 − 0·65). More specifically, this was the case for fluoroquinolones (0·33, 95% CI 0·15 − 0·69), macrolides (0·37, 95% CI 0·25 − 0·56), tetracyclines (0·41, 95% CI 0·25 − 0·69), and multi-drug efflux pumps (0·11, 95% CI 0·03 − 0·33), but not for beta-lactams (1·38, 95% CI 0·73 − 2·61). There were no significant differences in ARG abundance between MSM who had used antibiotics or not.

Interpretation: The resistome of MSM is enriched with ARGs, even without recent antibiotic use. Stewardship campaigns should aim to further reduce antibiotic consumption in MSM populations.

Introduction

Antimicrobial resistance (AMR) is a problem of increasing concern in a range of sexually transmitted bacteria, such as *Neisseria gonorrhoeae*, *Treponema pallidum*, and *Mycoplasma genitalium*. In the case of gonorrhoea, much of this resistance has resulted from the horizontal transfer of antibiotic resistance genes (ARGs) through transformation from commensal bacteria to *N. gonorrhoeae*.[1–5] The resultant resistant lineages of *N. gonorrhoeae* have spread worldwide due to antibiotic selection pressure.[6] Even the current last-line antibiotic, ceftriaxone, is losing its effectiveness against an increasing number of gonococcal isolates worldwide.[7]

Populations at high risk of STIs, such as men who have sex with men taking HIV pre-exposure prophylaxis or PrEP (henceforth referred to as MSM), have played a key role in the emergence and spread of gonococcal AMR for two reasons. Firstly, their high prevalence of pharyngeal and rectal gonorrhoea, combined with high levels of antibiotic consumption for the treatment of STIs, are thought to promote the acquisition and spread of AMR.[8,9] Secondly, the high prevalence of AMR in their oral commensal *Neisseria* spp. may increase the risk of new resistance being transferred to *N. gonorrhoeae*.[10–13] Previous attempts to characterise the resistome (i.e. repertoire of ARGs)[14] of MSM have largely been limited to pheno- and genotypic assessments of antimicrobial susceptibilities of STIs such as *N. gonorrhoeae* or specific species of commensal bacteria.[10,11,13] This approach may miss AMR determinants not present in the targeted species. Determining a population's resistome may complement more traditional AMR surveillance techniques.[15]

In the current study, we aimed to evaluate the burden of antibiotic exposure to the oropharyngeal resistome of MSM, and how their resistome differs from that of the general population.

Methods

Study population

A cross-sectional survey was conducted in which samples were collected from three populations of interest: the general population ("Employees [no AB]", n = 32), MSM who did not use antibiotics for six months ("MSM [no AB]", n = 32), and MSM who did use antibiotics in the previous six months ("MSM [AB]", n = 32). Participants representing the general population were recruited among employees at the Institute of Tropical Medicine in Antwerp, Belgium, as part of the ComCom study in 2020.[11] They were recruited by posters and word of mouth and were eligible if they had not used any antibiotics in the previous six months. Both groups of MSM were recruited among those attending our PrEP clinic, as part of the PReGo study, in 2019-2020. PReGo (Preventing Resistance in Gonorrhoea) was a randomized clinical trial among 343 MSM comparing the preventive effect of Listerine Cool Mint to a placebo mouthwash on the incidence of STIs. The study protocol and results have been published previously.[11,16] The first 32 PReGo enrollees who had not used any antibiotics in the previous six months were assigned to the group MSM [no AB], whereas the first 32 PReGo enrollees who had used at least one antibiotic within that time frame were assigned to MSM [AB]. All PReGo participants had a history of at least one bacterial STI (and thus, antibiotic treatment) in the two years prior to enrolment.

Sample size

No sample size calculation was done, as data were lacking to accurately hypothesize effect sizes: the oropharyngeal resistome of Belgian residents is largely unexplored.

Data and samples

Samples were collected as described.[11] In brief, an oropharyngeal swab was taken from each participant at the time of enrolment by rubbing both tonsillar pillars and the posterior oropharynx with a dry regular flocked swab (COPAN, Brescia, Italy). All swabs (one per participant) were transported in a cooled transport box and stored within 4 hours at -80°C until DNA extraction.[17]

DNA extraction and shotgun metagenomic sequencing

Metagenomic DNA was extracted from all swabs (n = 96) using the FastDNA™ SPIN Kit (MP Biomedicals, Irvine, CA) and quantified by a Qubit fluorometer (Life Technologies, Carlsbad, CA). Sequencing library preparation was done using Nextera XT DNA library preparation kit (Illumina Inc., USA), and libraries were sequenced using 2x250 bp Miseq and 2x150 bp NextSeq500 (Illumina Inc., USA).

Taxonomic and resistome characterization

After the initial assessment of the quality of the raw reads using FASTQC, the quality-controlled raw reads were trimmed and filtered of low-quality reads using Trimmomatic v0.39.[18,19] Next, human reads were removed by mapping the reads against the human reference genome (GRCh38, accession GCF_000001405.26) using Burrows-Wheeler Alignment (BWA-MEM) with default parameters.[20] As a final quality cut-off before downstream analysis, samples with less than 5,500 non-human reads were discarded in order to avoid issues due to the large variation in sequencing depth (Supplementary Note 1).[21]

To estimate the taxonomical abundance, non-human reads were classified with MiniKraken2_v2_8GB (https://ccb.jhu.edu/software/kraken2), followed by abundance estimation with Bracken v2.6.1.[22,23] Abundance of ARGs was estimated after alignment of non-human reads to the MEGARes 2.0 database using BWA-MEM with default settings.[24,25] Next, ResistomeAnalyzer (https://github.com/cdeanj/resistomeanalyzer) classified ARGs with a gene fraction greater than 80% into types, classes, and gene groups for further analyses. Single nucleotide polymorphisms and genes conferring resistance exclusively to non-drug compounds were not taken into account for further analysis.[26,27] ARGs were normalized by the number of bacterial reads per sample (as estimated by Bracken) and multiplied by 10^6 in order to obtain reads per million (RPM). Likely contaminants were identified by batch and correlation analysis and filtered from the taxonomic and ARG abundance profiles (Supplementary Note 2).[28] Also, species with an abundance below 0·1% in a sample were filtered from the taxonomic abundance profile of that sample.

Statistics

Statistical analyses and data visualization were performed in R 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria), with R packages phyloseq (v1.38.0), microbiome (v1.16.0), ComplexHeatmap (v2.12.0), and ggplot2 (v3.3.6). Demographic characteristics were summarized overall and by study group.

We used a zero-inflated negative binomial regression model (pscl v1.5.5) to assess associations between ARG abundance and study group. The model consisted of the total count of ARGs per sample as the outcome, study group as a categorical explanatory variable, and the logarithm of the count of bacterial reads as an offset. MSM [no AB] was considered as the reference group.

Additionally, we repeated the regression analysis per ARG class and per ARG group. For these analyses, ARG classes/groups with a prevalence below 10% were not taken into account. As a sensitivity analysis, we repeated differential abundance testing of ARGs in two sets of samples: (a) samples exclusively from male participants, and (b) samples with at least 1 non-human read (n = 94).

Diversity indices were calculated at the level of bacterial species (taxonomical data) and ARG group (resistome data). Shannon's and Inverse Simpson's indices were used to calculate within-sample diversity (alpha diversity). Alpha diversity was compared between groups by a Kruskal-Wallis test, followed by a Dunn test, if the former was significant. Beta diversity was calculated as Euclidean distances on centred log-ratio (clr) transformed abundance data. The resulting distance matrix was used to create ordination plots based on PCA, and to test statistically for differences in community composition with permutational multivariate analysis of variance (PERMANOVA, 10,000 permutations, R package vegan v2.5.7).

To test for differential abundance of bacterial taxa, we used ANCOM-BC (R package ANCOMBC v1.4.0, default parameters) at the species level.[29] Only taxa with a prevalence above 25% were taken into account for differential abundance analysis. *P*-values were corrected for multiple testing by the Benjamini-Hochberg procedure, with significance threshold $q < 0.05$.[30]

We examined the pairwise associations among bacterial genera and among ARGs by Sparse Correlations for Compositional Data (SparCC, R package SpiecEasi v1.1.2).[31] Correlations between bacterial genera and ARGs were explored using Spearman correlation analysis.

Reporting

For the reporting of this study, we followed STROBE guidelines and its 2020 extension for metagenomic studies.[32,33]

Ethics

This study was approved by ITM's Institutional Review Board (1276/18 and 1351/20) and the Ethics Committee of the University of Antwerp (19/06/058 and AB/ac/003). No study procedures were performed before obtaining written informed consent.

Role of Funders

Results

<u>Demographics</u>

The majority of participants in this study were between 30 and 59 years old (Table 1). Among the employees, 22 (68·8%) were female.

Table 1: Characteristics of study participants, demographics, antibiotic and mouthwash use

	Employees [no AB] (N=32)	MSM [no AB] (N=32)	MSM [AB] (N=32)	Total (N=96)
Age category				
20-29	5 (15·6%)	4 (12·5%)	5 (15·6%)	14 (14·6%)
30-39	9 (28·1%)	10 (31·3%)	16 (50·0%)	35 (36·5%)
40-49	9 (28·1%)	8 (25·0%)	6 (18·8%)	23 (24·0%)
50-59	8 (25·0%)	7 (21·9%)	4 (12·5%)	19 (19·8%)
60-69	1 (3·1%)	2 (6·3%)	1 (3·1%)	4 (4·2%)
70-79	0 (0%)	1 (3·1%)	0 (0%)	1 (1·0%)
Sex *				
Male	10 (31·3%)	32 (100%)	32 (100%)	74 (77·1%)
Female	22 (68·8%)	0 (0%)	0 (0%)	22 (22·9%)
Used macrolide (previous 6 months) *				
No	32 (100%)	32 (100%)	13 (40·6%)	77 (80·2%)
Yes	0 (0%)	0 (0%)	19 (59·4%)	19 (19·8%)
Used beta-lactam (previous 6 months) *				
No	32 (100%)	32 (100%)	7 (21·9%)	71 (74·0%)
Yes	0 (0%)	0 (0%)	25 (78·1%)	25 (26·0%)
Used tetracycline (previous 6 months) *				
No	32 (100%)	32 (100%)	24 (75·0%)	88 (91·7%)
Yes	0 (0%)	0 (0%)	8 (25·0%)	8 (8·3%)
Used fluoroquinolone (previous 6 months)				
No	32 (100%)	32 (100%)	30 (93·8%)	94 (97·9%)
Yes	0 (0%)	0 (0%)	2 (6·3%)	2 (2·1%)
Used other antibiotic (previous 6 months)				
No	32 (100%)	32 (100%)	32 (100%)	96 (100%)
Yes	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Used a mouthwash (previous 1 month)				
No	17 (53·1%)	20 (62·5%)	13 (40·6%)	50 (52·1%)
Yes	15 (46·9%)	12 (37·5%)	19 (59·4%)	46 (47·9%)
Samples with ≥ 5,500 non-human reads				
No	3 (9·4%)	7 (21·9%)	8 (25·0%)	15 (18·8%)
Yes	29 (90·6%)	25 (78·1%)	24 (75·0%)	78 (81·3%)

<u>Sequencing depth</u>

The number of reads per sample ranged between 26 and 18,228,775 (median 2,134,272). After trimming and removing human reads, a median of 88,120 (range 0 to 6,733,088) reads remained. Seventy-eight out of ninety-six (81·3%) samples contained a minimum of 5,500 non-human reads and were included in the analysis

(Table 1). Those 78 samples had a median of 112,964 (range 5,893 to 67,33,088) non-human reads.

Prevalence, abundance, diversity and co-occurrence of oropharyngeal ARGs

The abundance of ARGs was significantly lower among employees compared to MSM who did not use antibiotics (ratio 0·41, 95% confidence interval (CI) 0·26 – 0·65). ARG abundance did not differ significantly between the two groups of MSM (ratio 0·97, 95% CI 0·61 – 1·55, Figure 1A, Table 2).

Table 2: ARG abundance, per group, and estimated ratio of abundance by zero-inflated negative binomial regression, with MSM who received no antibiotics as the reference group.

	Estimate (95% confidence interval)		
	Employees [no AB]	**MSM [no AB]**	**MSM [AB]**
Overall	**0·41 (0·26 – 0·65)**	REF	0·97 (0·61 – 1·55)
Beta-lactams	1·38 (0·73 – 2·61)	REF	0·78 (0·41 – 1·50)
Fluoroquinolones	**0·33 (0·15 – 0·69)**	REF	0·51 (0·23 – 1·15)
Macrolides	**0·37 (0·25 – 0·56)**	REF	1·24 (0·82 – 1·86)
Multi-drug efflux pumps	**0·11 (0·03 – 0·33)**	REF	0·25 (0·06 – 1·11)
Tetracyclines	**0·41 (0·25 – 0·69)**	REF	1·35 (0·81 – 2·27)

Bold = significant (*p*-value <0·05); [AB] = used at least 1 antibiotic in the previous 6 months; [no AB] = did not use any antibiotic in the previous 6 months; MSM = men who have sex with men

Figure 1: Abundance of antibiotic resistance genes (ARG) in employees, and men who have sex with men (MSM) who did/did not use antibiotics in the previous six months, (a) overall, (b) by ARG class, and (c) by ARG group.

ARG = antibiotic resistance gene; CFX = cfx genes encoding Ambler class A beta-lactamases; ERMB/ERMF/ERMX = erm genes encoding 23S methyltransferases which transfer a methyl group to the 23S rRNA component of the bacterial ribosomes, preventing the action of macrolide, lincosamide and streptogramin (MLS) group antibiotics; LSAC = group of genes causing multi-drug resistance through an ABC-F efflux pump that confers resistance to lincomycin, clindamycin, dalfopristin and tiamulin; MDR = multi-drug efflux pumps; MEFA = mefA gene encoding an ABC efflux pump that confers resistance to MLS; streptogramins; MSRD = msrD gene encoding an ABC efflux pump conferring resistance to MLS; PATA/PATB = group of genes encoding an ABC efflux pump conferring resistance to drugs and biocides; RLMA = group of genes encoding MLS resistance through 23S rRNA methyltransferases; TET32/TETM/TETQ/TETW = genes encoding tetracycline ribosomal protection protein; TETA46/TETB46 = gene conferring tetracycline resistance through an ABC efflux pump; RPM = reads per million; [no AB] = did not use any antibiotic in the previous 6 months; [AB] = used at least one antibiotic in the previous 6 months;
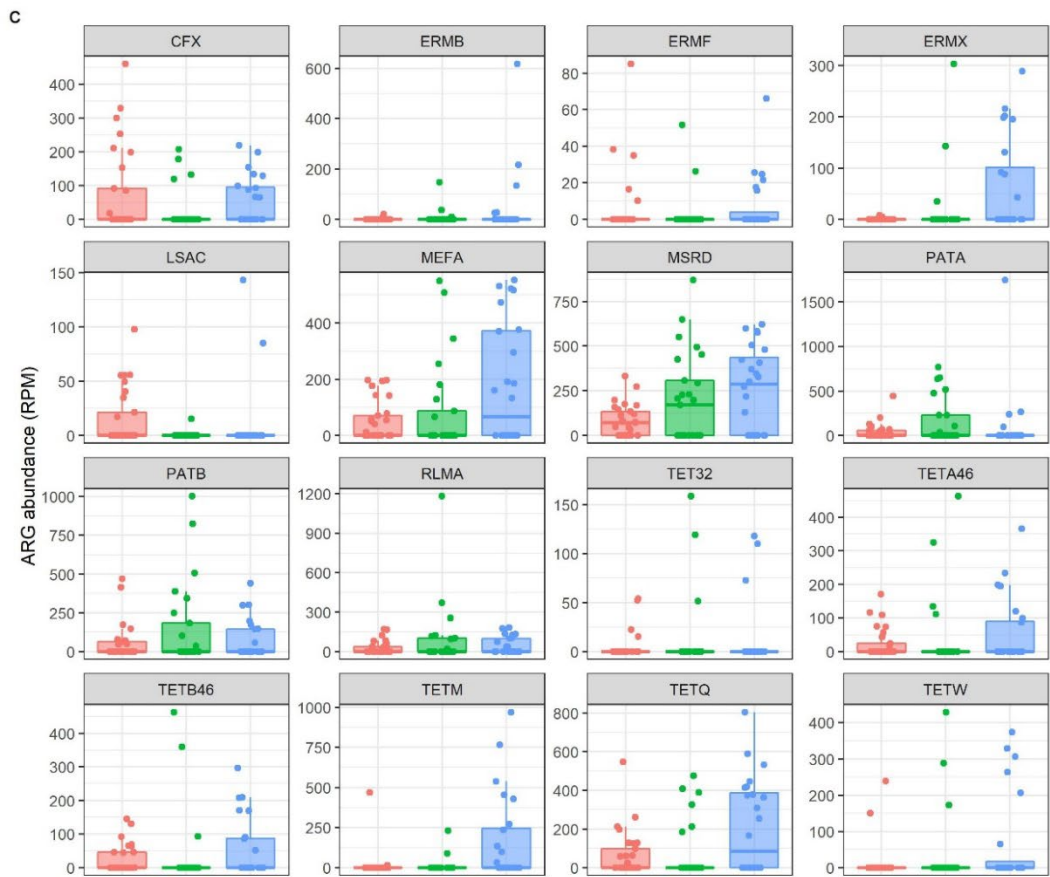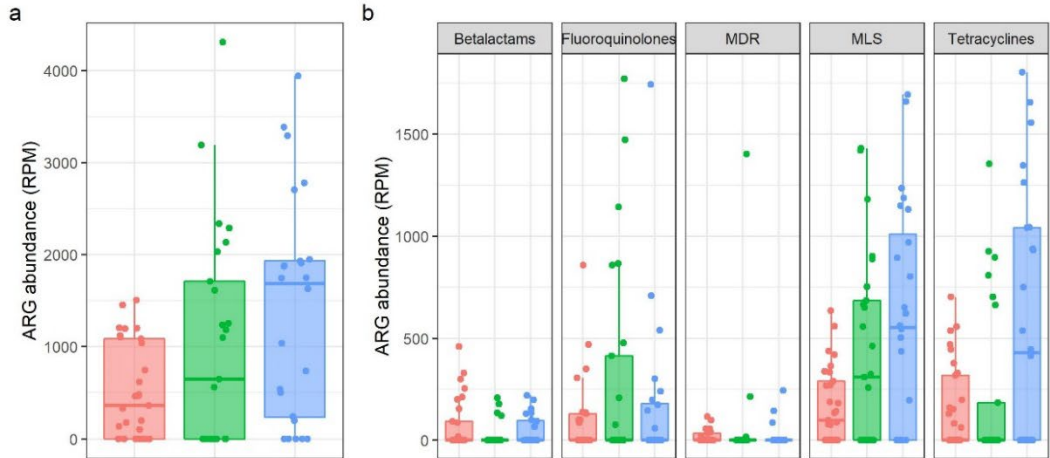
Overall, twenty-nine ARG groups were detected, and these were categorised into eight ARG classes (Figure 2). The most prevalent classes of ARGs were those conferring resistance to macrolides (MLS, including lincosamides and streptogramins: hereafter called macrolides), tetracyclines, fluoroquinolones, beta-lactams, and multi-drug efflux pumps (prevalences 61·5%, 43·6%, 37·2%, 30·8%, and 19·2%, respectively). The remaining ARG classes were those conferring resistance to phenicol, mupirocin, and aminoglycosides (prevalences 2·6%, each).

Figure 2: Relative abundance of antibiotic resistance genes, by study group.

Heatmap, clustered by Ward D, based on Euclidean distances on centred log-ratio transformed abundance data. Coloured bars below the heatmap represent antibiotic use in the previous 6 months: higher colour intensity corresponds to more recent use of the antibiotic, (range 183 to 0 days before sampling). Prevalence indicates prevalence across all samples. Ag = aminoglycosides, Bl = beta-lactams, Fq = fluoroquinolones, MDR = multi-drug efflux pumps, MLS = macrolides/ lincosamides/streptogramins, Mp = mupirocin, Ph = phenicol.

Compared to MSM, employees had a significantly lower abundance of ARGs conferring resistance to macrolides (ratio 0·37, 95% CI 0·25 – 0·56), tetracyclines (ratio 0·41, 95% CI 0·25 – 0·69), fluoroquinolones (ratio 0·33, 95% CI 0·15 – 0·69),

and multi-drug efflux pumps (ratio 0·11, 95% CI 0·03-0·33), but not beta-lactams (Table 2). There were no significant differences in abundance of ARG classes between the two groups of MSM. Findings of the two sensitivity analyses were very similar (Supplementary Table 2).

Sixteen ARG groups had a prevalence above 10%. Compared to MSM, the following ARGs were significantly less abundant among employees: macrolide resistance genes *msrD*, *mefA*, RLMA and *ermX*; tetracycline resistance genes *tetQ*, *tetA46*, *tetB46*, and *tet32*; and fluoroquinolone resistance genes *patB* and *patA* (Supplementary Table 3). Employees and MSM who used antibiotics had a significantly higher abundance of the LSAC group of multi-drug efflux pumps compared with MSM who did not use antibiotics.

Alpha diversity of ARGs did not differ significantly between groups (p = 0·9 for Shannon and inverse Simpson indices, Supplementary Figure 7A). The median Shannon index of all samples was 1·9 (IQR 0·7 to 2·6), and the median inverse Simpson index of all samples was 5·9 (IQR 2·0 to 10·1). Beta diversity of ARGs was significantly different overall (PERMANOVA p = 0.03, F = 1·84; Supplementary Figure 7B), but pairwise comparison between the study groups was not significant.

Correlation analysis with SparCC found a significantly positive correlation between several ARG groups, among which only *patA* and *patB* had a correlation coefficient above 0·5 (Supplementary Figure 8A).

Prevalence, abundance, diversity and co-occurrence of oropharyngeal microbiota. Twenty-seven bacterial genera had a prevalence above 25% in our samples (Supplementary Figure 9). *Veillonella* and *Streptococcus* were universally present in all samples. *Prevotella*, *Rothia*, *Schaalia*, and *Haemophilus* had prevalences above 90%, and *Fusobacterium* and *Gemella* had prevalences above 80%. *Neisseria* was the ninth most prevalent genus with an overall prevalence of 79·5%.

Microbial alpha diversity was highest among employees (median Shannon index 3·4, IQR 3·2 to 3·5; median inverse Simpson index 16·3, IQR 13·1 to 20·3) compared to the two groups of MSM (MSM [AB]: median Shannon index 3·3, IQR 3·1 to 3·4;

median inverse Simpson index 15·2, IQR 13·0 to 17·5; MSM [no AB]: median Shannon index 3·2, IQR 2·9 to 3·3; median inverse Simpson index 13·2, IQR 9·7 to 16·1), but these differences were not statistically significant (Shannon: p = 0·06; inverse Simpson: p = 0·07, Supplementary Figure 10A). Microbial beta diversity was significantly different overall (PERMANOVA F = 2·4, R^2 = 0·06, p <0·0001) and when comparing employees with each group of MSM (p < 0·01 for each comparison), but not between the two groups of MSM (p = 0·2; Supplementary Figure 10B). The observed differences may, however, be attributable to heterogeneity in variance (multivariate homogeneity of group dispersions p < 0·001).

Differential abundance analysis with ANCOM-BC identified three bacterial species with lower abundance in MSM compared to employees: *Fusobacterium nucleatum, Parvimonas micra,* and *Selenomonas sputigena*. (Figure 3). *Streptococcus sp. HSISS2*, *Streptococcus agalactiae* and *Actinomyces hongkongensis* were more abundant in MSM than in employees. *Selenomonas sputigena* was less abundant in MSM who used antibiotics than in MSM who did not use antibiotics. Significant differences between groups for this species were based on its structural absence in samples from MSM who did not use antibiotics.

There was a moderate level of correlation (SparCC r between 0·5 and 0·8) among the following bacterial genera: (I) *Veillonella*, *Schaalia* and *Prevotella*; (II) *Neisseria* and *Haemophilus*; and (III) *Treponema* and *Parvimonas* (Supplementary Figure 9B).

Figure 3: Differential abundance of bacterial species.

*Data are presented as effect size (log fold change compared to the reference group) and 95% confidence intervals derived from the ANCOM-BC model. Only species with adjusted p < 0·05 in at least one study group are displayed. Absence of confidence interval bars for some species is due to structural zeros, which precluded the calculation of exact p-values and confidence intervals. *significant at 5% level of significance; **significant at 1% level of significance; ***significant at 0·1% level of significance.*
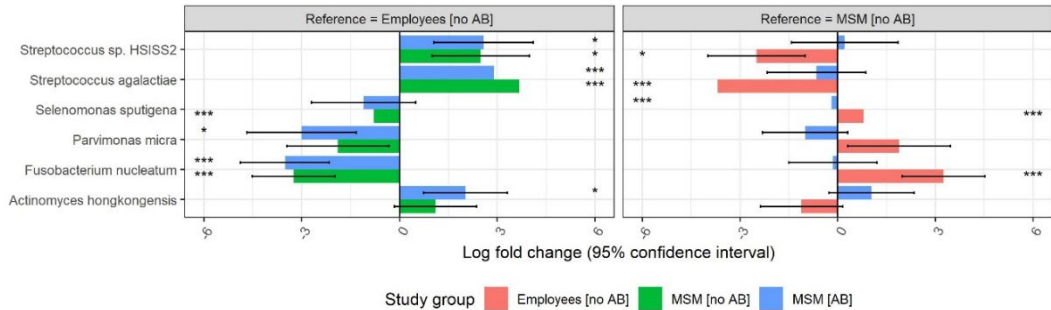
Correlation between oropharyngeal microbiota and ARGs

After adjustment for multiple comparisons, no significant correlations were identified between bacterial genera and ARG groups (Supplementary Figure 11). When considering the unadjusted *p*-values, no genus-ARG correlation had a Spearman coefficient above 0·4.

Discussion

Oropharyngeal samples from MSM contained a significantly higher abundance of resistance genes, compared to a control population. In particular, MSM carried a high abundance of genes conferring resistance to macrolides, tetracyclines, fluoroquinolones and genes encoding multi-drug efflux pumps. Remarkably, recent antibiotic use was not associated with a significant further increase in total ARG abundance among MSM, which reiterates that AMR increase post-antibiotic use is a sustained phenomenon.

These findings are in line with culture-based findings of a previously published study in the same cohort of participants at our institution.[11] In that study, we found that commensal *Neisseria spp.*, in particular, *N. subflava* from MSM were less susceptible to ciprofloxacin and azithromycin than those from employees, but that there was no significant difference in antibiotic susceptibility between isolates from MSM who had, and who had not taken antibiotics in the prior 6-months.

The similarity between the two groups of MSM in this study in terms of oropharyngeal ARG abundance and microbial beta diversity indicates that the cumulative impact of multiple antibiotic courses in the distant past has lead to a sustained increase in ARG abundance in the MSM population. Compared to this, the impact of a single antibiotic course in the last six months seems to be relatively minor. Indeed, all MSM in our study had at least one STI in the two years prior to study enrolment, for which they most likely took one or multiple antibiotics. In individuals with an overall stable gut microbiome composition, even short antibiotic exposure may cause long-term perturbations of their gut microbiome.[34] In one randomized clinical trial, a three-day course of oral azithromycin was associated with increased proportions of macrolide-resistant oropharyngeal streptococci for up to 180 days.[35] Second, population-level antibiotic exposure could be another factor contributing to the ARG-enriched resistome of MSM. Population-level antibiotic exposure has been associated with AMR in bacterial pathobionts.[36,37] As a matter of fact, MSM in PrEP programs consume multiple times the amount of antibiotics that are consumed by the general population.[38] As an illustration, 38 (59.4%) out of the 64 MSM in the current study used at least one antibiotic in the six months after enrollment.[16] This high level of antibiotic consumption is mainly a consequence of screening and treatment of asymptomatic gonorrhoeae and chlamydia infections,[38,39] and may impact the microbiome and resistome of the population as a whole. How population-level antibiotic consumption influences AMR at the individual level is incompletely elucidated, but inter-individual transmission of resistant microbiota has been hypothesized to play a role.[40] Indeed, there is increasing evidence that an individual's microbiome and resistome is shaped by their environment.[41,42] Household contacts, even when they are genetically unrelated, tend to share similar microbiomes and resistomes.[42] Food is thought to be one environmental

factor shaping the microbiome and resistome.[43] Genetics, diet, lifestyle and clinical information, however, explain less than 20% of the total microbiome variation across individuals.[44] Even though insufficiently quantified, there is strong evidence that direct or indirect transmission of microbiota between individuals shapes their microbiomes.[44] Human-to-human transmission of microbiota in addition to individual and population-level antibiotic exposure may thus explain why microbiome- and resistome- level differences between the two groups of MSM were less pronounced than those between MSM and the general population. It is possible that not only pathogens and pathobionts, but also commensal microbiota and ARGs may be shared within a network of MSM. Last, the mere use of PrEP may explain the observed differences between MSM and the general population. It has long been known that several non-antibiotic drugs have an impact on human gut microbiota.[45] More recently two observational studies have found that consumption of tenofovir-disoproxil fumarate/emtricitabine is associated with small changes in the relative abundance of individual bacterial genera in the rectum.[46,47] These studies did not evaluate if these medications affected the oropharyngeal microbiome.

Similar to other studies, we found that the oropharyngeal resistome is dominated by ARGs to macrolides and tetracyclines.[48] One of the most common ARGs in the respiratory tract is *msrD*.[49] The *msrD* gene was enriched in both groups of MSM. It encodes a ribosomal protection protein conferring macrolide resistance in bacteria such as streptococci, and is thought to act synergistically with the *mefA* efflux pump in *S. pneumoniae*.[50] *MsrD* was recently also detected in contemporary circulating strains of *Neisseria subflava* with high-level azithromycin resistance, and was likely horizontally acquired from *S. pneumoniae* or other bacteria.[50] *TetM* is another common ARG with a high abundance in MSM who received antibiotics. It is an ARG that occurs on a conjugative plasmid and confers resistance to tetracyclines. *TetM* is present in certain strains of *N. gonorrhoeae* and could thus potentially be transferred to an incoming gonococcal infection.[51] The increased abundance of ARGs among MSM may be a warning for an increased risk of horizontal gene transfer and selection of resistant strains under antibiotic pressure in MSM.

It is important to note that the observed differences in resistome between MSM and employees were only paralleled by differential abundances of a few bacterial species between those populations, rather than by major shifts in microbiota composition in terms of alpha and beta diversity. This is in agreement with gut microbiota studies in other populations which reported restoration of the microbiota composition within 1.5 months after antibiotic intake, except for some species that remained undetectable for longer periods of time.[52] In our study, among the MSM who used antibiotics, very few did so in the 1.5 months before sampling. In our study, *Fusobacterium nucleatum* was the bacterial species that had the most prominently reduced abundance among MSM. Even though this anaerobic oral pathobiont is generally susceptible to macrolides and beta-lactams,[53,54] at least one study has found that it transiently increased in relative abundance in the gut following broad-spectrum antibiotics.[52] We have not found any literature that may explain our finding of reduced abundance among MSM.

We found no clear correlation between the abundance of specific resistance genes and bacterial genera. This may be due to a lack of accuracy of the correlation analysis used.[55] Other bioinformatic analyses including assembly-based metagenomics, long-read sequencing based metagenomics or PCR-based detection of specific resistance genes may be able to better quantify and elucidate the source of the observed differences in abundance of ARGs between MSM and the general population.

Our study is the first of its kind to use shotgun metagenomics to assess the oropharyngeal microbiome and resistome of MSM. We believe that our work represents a novel and important scientific contribution. However, we also acknowledge that there are several limitations to our study. First, we were not able to control for all potential confounders.[56] While it is reasonable to assume that diet and lifestyle factors do not substantially differ between the populations of interest in our study, the impact of PrEP on the oral microbiome is uncertain. However, our study was not designed to specifically assess long-term effects of PrEP use on the microbiome. A sensitivity analysis limited to male samples gave similar results to the overall analysis, suggesting that the inclusion of women did not affect our results. Second, the findings of our study may not be generalisable

to all MSM as this study targeted high-risk MSM in particular. Despite these limitations, we believe that our study provides new and valuable insights into the potential impact of antibiotic use on the oropharyngeal microbiome and resistome of MSM.

We conclude that the resistome of MSM is enriched with ARGs. Horizontal transfer of resistance genes to incoming pathogens may drive increasing antibiotic resistance in bacteria such as *Neisseria gonorrhoeae*. Our findings, therefore, stress the importance of stewardship campaigns that aim to reduce antibiotic consumption in the MSM population to an adequate minimum. The use of antibiotics in the context of prevention, diagnosis or screening of STIs should be limited to indications for which the evidence base includes the unintended consequences of antibiotic use on the individual and population level.

Contributors

CK and SMK conceptualised the study; CK and CVD collected the samples; JL, SA, IDB, and BBX processed the samples; BBX, TdB, SSMB, JL and CVD performed bioinformatic and/or statistical analyses; AT provided statistical support, CVD drafted the manuscript; all authors revised the manuscript and approved the final version.

Declaration of Interests

There are no conflicts of interest to declare.

Acknowledgements

We thank all involved clinical and laboratory staff of the Institute of Tropical Medicine and the Laboratory of Medical Microbiology at the University of Antwerp for the collection, handling and processing of the samples.

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SUPPLEMENT

SUPPLEMENTARY NOTE 1: Exclusion of samples with low sequencing depth.

For this study, 96 samples were collected. The number of non-human reads in the 96 samples ranged between 0 and 6,733,088 (median 88,120), and the number of reads aligning to antimicrobial resistance genes per million bacterial reads (ARG-RPM) ranged between 0 and 9,534·9 (median 604·7). There was a strong positive correlation between the number of non-human reads and ARG-RPM (Spearman's rho 0·90, $p < 0·0001$), suggesting that samples with a low number of reads had insufficient sequencing depth to detect any ARGs. Indeed, ARGs were not detected in samples with less than 5,500 non-human reads (Supplementary Figure 1). After discarding those samples (n =18), the positive correlation between number of non-human reads and ARG-RPM was somewhat less pronounced (Spearman's rho 0·83, $p < 0·0001$), but this cut-off was able to separate samples with a different composition with reasonable accuracy (Supplementary Figure 2), while balancing the risk of insufficient sequencing depth versus a loss of data due to discarding too many samples.

Supplementary Figure 1: Normalized read counts of antibiotic resistance genes (ARGs, in reads per million) versus the number of non-human reads.

Every dot represents one sample. Dot size corresponds to the number of non-human reads in the sample.
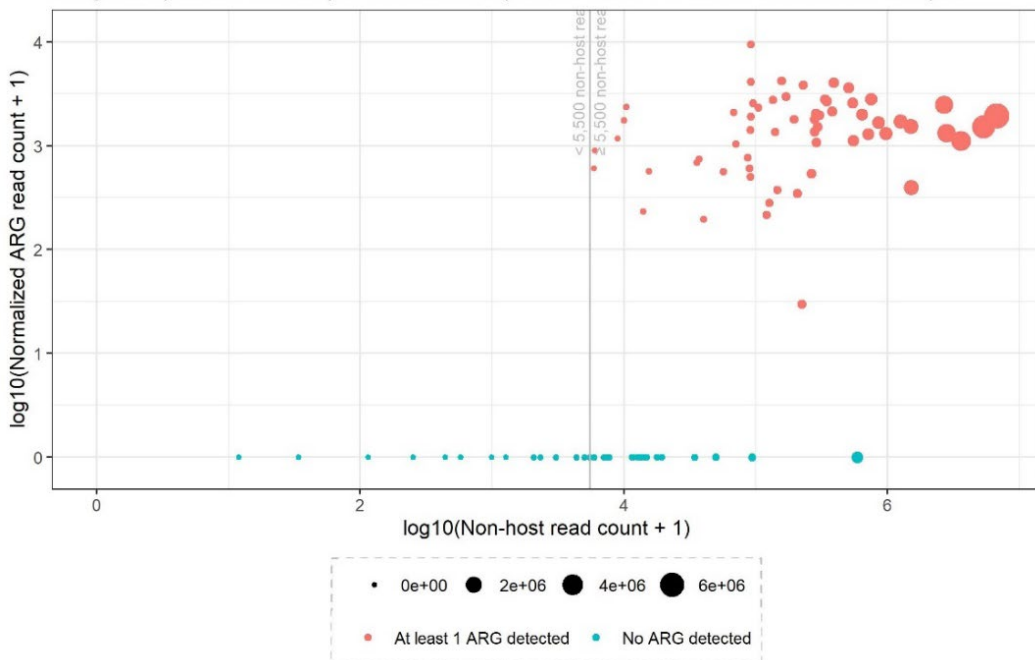
Supplementary Figure 2: Principal component analysis (PCA) of all samples, coloured according to a cut-off of 5,500 non-human reads.

PCA was based on genus-level Euclidean distances of centred log-ratio transformed taxonomic data. Every dot represents one sample. Dot size corresponds to the number of non-human reads in the sample.
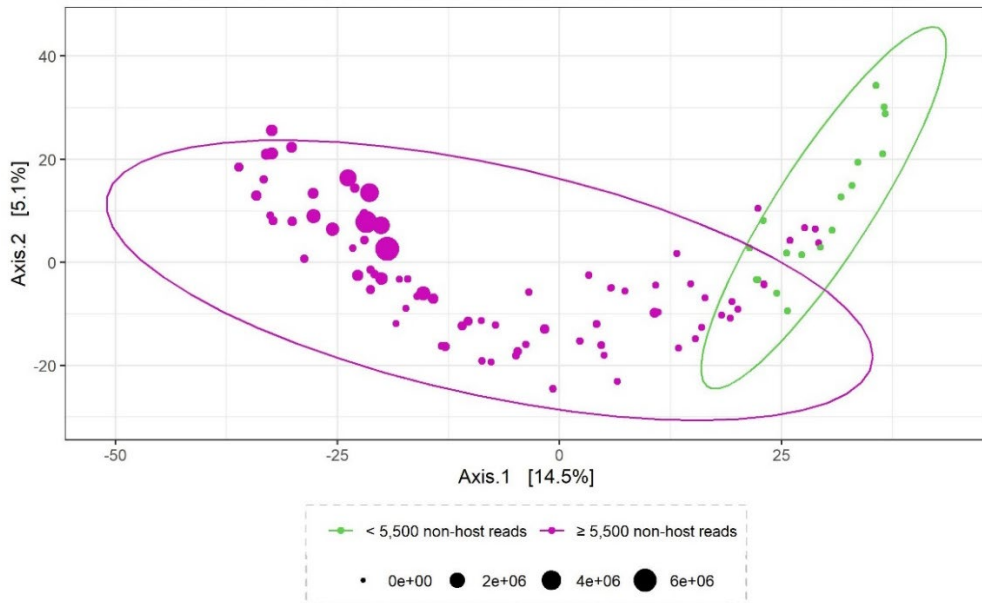
SUPPLEMENTARY NOTE 2: Removal of likely contaminant taxa/ARG

Samples were analysed in eight batches (Supplementary Table 1 and 2). Bacterial species or ARGs were considered contaminants if they complied with the following criteria: [1,2]

(1) Discordant prevalence across analysis batches: Bacterial species with a sample-wise abundance > 0·1% and prevalence of > 25% in one batch and a much lower prevalence in the remaining batches were identified as possible contaminants (Supplementary Figure 3). For ARGs, the prevalence threshold was set to 10% because of the lower prevalence of ARGs overall (Supplementary Figure 4).

(2) Correlation with other possible contaminant features: Possible contaminants identified in the previous step that correlated with each other in a Spearman correlogram were identified as likely contaminants (Supplementary Figures 5 and 6).

Likely contaminants were filtered from the taxonomic and ARG abundance profiles of their respective batches (Supplementary Table 1).

Supplementary Figure 3: Likely contaminant bacterial species, per analysis batch.

Dots represent bacterial species with a minimum abundance of 0·1%. Species with a prevalence of ≥ 25% in one batch and < 25% in the remaining batches are indicated in black and red. Annotated species (red dots) were identified as likely contaminants based on prevalence and correlation analysis.

Supplementary Figure 4: Likely contaminant antibiotic resistance genes (ARGs), per analysis batch.
Dots represent ARGs. ARGs with a prevalence of ≥ 10% in one batch and < 10% in the remaining batches are indicated in black and red. Annotated ARGs (red dots) were identified as likely contaminants based on prevalence and correlation analysis

Supplementary Figure 5: Spearman correlation of bacterial species (all analysis batches combined)

Supplementary Figure 6: Spearman correlation of antibiotic resistance genes (all analysis batches combined)
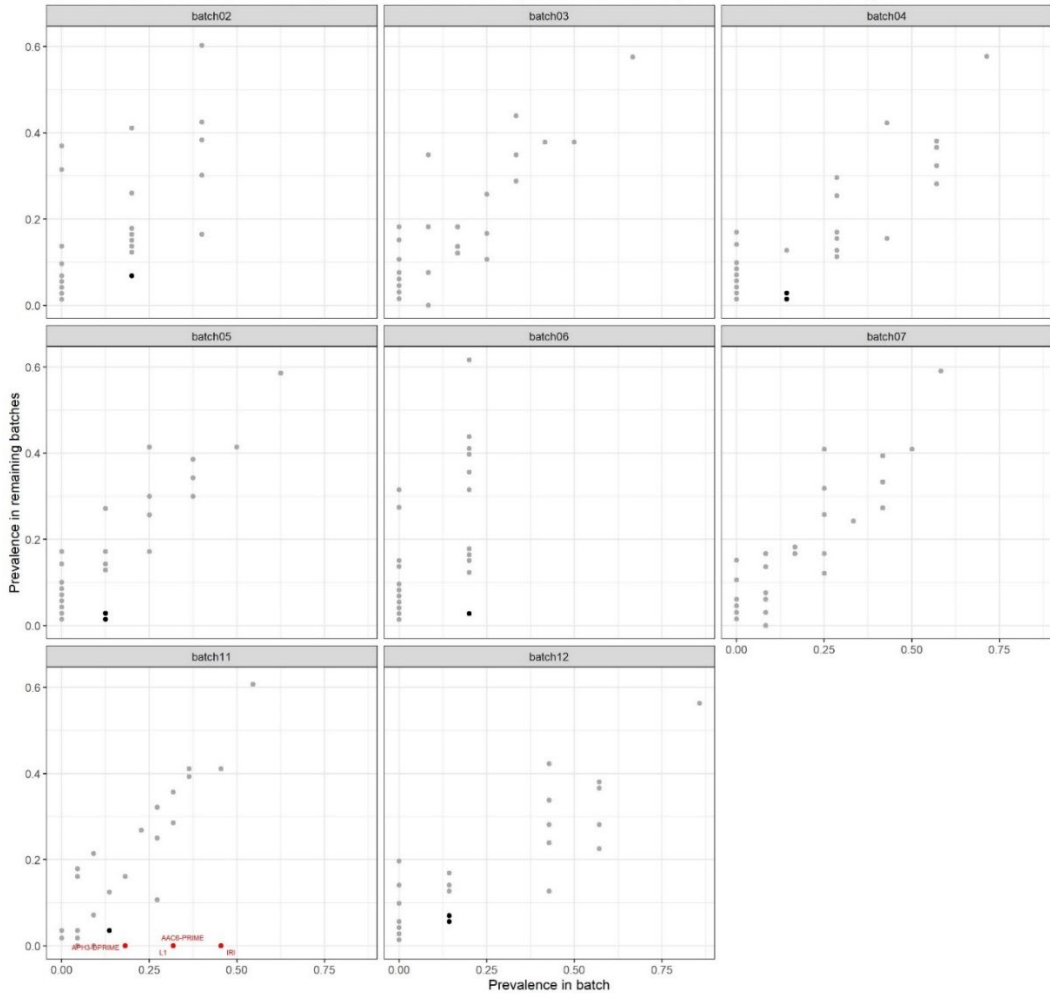
Supplementary Table 1: Bacterial species and antimicrobial resistance genes (ARGs) identified as likely contaminants, per analysis batch.

	batch02 (n = 5)	batch03 (n = 12)	batch04 (n = 7)	batch05 (n = 8)	batch06 (n = 5)	batch07 (n = 12)	batch11 (n = 22)	batch12 (n = 7)
Bacterial species								
Mesorhizobium amorphae	1	0	0	1	0	0	0	0
Mesorhizobium loti	1	0	0	1	0	0	0	0
Mesorhizobium sp. M1B.F.Ca.ET.045.04.1.1	1	0	0	1	0	0	0	0
Mesorhizobium sp. M1D.F.Ca.ET.043.01.1.1	1	0	0	1	0	0	0	0
Mesorhizobium sp. M2A.F.Ca.ET.043.02.1.1	1	0	0	1	0	0	0	0
Mesorhizobium sp. M2A.F.Ca.ET.043.05.1.1	1	0	0	1	0	0	0	0
Mesorhizobium sp. M2A.F.Ca.ET.046.03.2.1	1	0	0	1	0	0	0	0
Mesorhizobium sp. M3A.F.Ca.ET.080.04.2.1	1	0	0	1	0	0	0	0
Mesorhizobium sp. M4B.F.Ca.ET.058.02.1.1	1	0	0	1	0	0	0	0
Mesorhizobium sp. WSM1497	1	0	0	1	0	0	0	0
Mesorhizobium sp. M1E.F.Ca.ET.045.02.1.1	0	0	0	1	0	0	0	0
Ralstonia insidiosa	1	0	0	0	0	0	0	0
Streptococcus pyogenes	1	0	0	0	0	0	0	0
Mesorhizobium australicum	0	0	0	1	0	0	0	0
Mesorhizobium ciceri	0	0	0	1	0	0	0	0
Mesorhizobium huakuii	0	0	0	1	0	0	0	0
Mesorhizobium japonicum	0	0	0	1	0	0	0	0
Mesorhizobium opportunistum	0	0	0	1	0	0	0	0
Mesorhizobium soli	0	0	0	1	0	0	0	0
Mesorhizobium sp. M6A.T.Cr.TU.016.01.1.1	0	0	0	1	0	0	0	0
Mesorhizobium sp. M7A.F.Ce.TU.012.03.2.1	0	0	0	1	0	0	0	0
Mesorhizobium sp. M7D.F.Ca.US.005.01.1.1	0	0	0	1	0	0	0	0
Mesorhizobium sp. M9A.F.Ca.ET.002.03.1.2	0	0	0	1	0	0	0	0
Mesorhizobium sp. Pch-S	0	0	0	1	0	0	0	0
Ralstonia mannitolilytica	0	0	0	1	0	0	0	0
Paraburkholderia fungorum	0	0	0	0	0	0	1	0

Rhodococcus erythropolis	0	0	0	0	0	0	1	0
Rhodococcus qingshengii	0	0	0	0	0	0	1	0
Rhodococcus sp. 008	0	0	0	0	0	0	1	0
Rhodococcus sp. AQ5-07	0	0	0	0	0	0	1	0
Rhodococcus sp. BH4	0	0	0	0	0	0	1	0
Rhodococcus sp. H-CA8f	0	0	0	0	0	0	1	0
Rhodococcus sp. NJ-530	0	0	0	0	0	0	1	0
Rhodococcus sp. YL-1	0	0	0	0	0	0	1	0
Stenotrophomonas maltophilia	0	0	0	0	0	0	1	0
Stenotrophomonas sp. ASS1	0	0	0	0	0	0	1	0
Stenotrophomonas sp. PAMC25021	0	0	0	0	0	0	1	0
ARG								
APH3-DPRIME	0	0	0	0	0	0	1	0
AAC6-PRIME	0	0	0	0	0	0	1	0
L1	0	0	0	0	0	0	1	0
IRI	0	0	0	0	0	0	1	0

Supplementary Table 2: Sensitivity analyses: ARG abundance, per group, and estimated ratio of abundance by zero-inflated negative binomial regression.

(a) Exclusively samples from male participants (n = 74)

	Estimate (95% confidence interval)		
	Employees [no AB]	**MSM [no AB]**	**MSM [AB]**
Overall	**0·58 (0·34 – 0·98)**	REF	0·98 (0·65 – 1·47)
Betalactams	1·65 (0·98 – 2·79)	REF	0·78 (0·48 – 1·27)
Fluoroquinolones	**0·34 (0·13 – 0·93)**	REF	0·52 (0·22 – 1·24)
Macrolides	**0·46 (0·29 – 0·73)**	REF	1·24 (0·87 – 1·77)
Multi-drug efflux pumps	**0·10 (0·02 – 0·42)**	REF	0·26 (0·05 – 1·39)
Tetracyclines	**0·52 (0·32 – 0·86)**	REF	1·35 (0·89 – 2·05)

Bold = significant (*p*-value <0·05); [AB] = used at least 1 antibiotic in the previous 6 months; [no AB] = did not use any antibiotic in the previous 6 months

(b) All samples with a minimum of 1 non-human read (n = 94)

	Estimate (95% confidence interval)		
	Employees [no AB]	**MSM [no AB]**	**MSM [AB]**
Overall	**0·41 (0·26 – 0·66)**	REF	0·96 (0·60 – 1·54)
Betalactams	1·38 (0·73 – 2·60)	REF	0·78 (0·41 – 1·49)
Fluoroquinolones	**0·33 (0·16 – 0·69)**	REF	0·51 (0·23 – 1·12)
Macrolides	**0·38 (0·25 – 0·56)**	REF	1·23 (0·82 – 1·84)
Multi-drug efflux pumps	**0·11 (0·03 – 0·33)**	REF	0·25 (0·06 – 1·08)
Tetracyclines	**0·41 (0·25 – 0·69)**	REF	1·34 (0·80 – 2·25)

Bold = significant (*p*-value <0·05); [AB] = used at least 1 antibiotic in the previous 6 months; [no AB] = did not use any antibiotic in the previous 6 months

Supplementary Table 3: Abundance of ARG groups with a minimum prevalence of > 10%, and estimated ratio of abundance by zero-inflated negative binomial regression.

|	Estimate (95% confidence interval)		
	Employees [no AB]	**MSM [no AB]**	**MSM [AB]**
CFX	1·38 (0·73 – 2·61)	REF	0·78 (0·41 – 1·50)
TETQ	**0·49 (0·28 – 0·87)**	REF	1·29 (0·72 – 2·28)
TETW	0·65 (0·31 – 1·35)	REF	0·85 (0·47 – 1·52)
MEFA	**0·43 (0·24 – 0·77)**	REF	1·42 (0·79 – 2·54)
MSRD	**0·35 (0·24 – 0·51)**	REF	1·10 (0·74 – 1·63)
TETM	1·69 (0·24 – 12·08)	REF	2·63 (0·61 – 11·27)
TETA 46	**0·31 (0·15 – 0·63)**	REF	0·74 (0·35 – 1·58)
ERMX	**0·04 (0·01 – 0·12)**	REF	1·03 (0·48 – 2·19)
TETB 46	**0·26 (0·14 – 0·51)**	REF	0·52 (0·27 – 1·03)
PATB	**0·41 (0·20 – 0·85)**	REF	0·56 (0·26 – 1·20)
PATA	**0·33 (0·15 – 0·71)**	REF	1·40 (0·46 – 4·25)
RLMA	**0·35 (0·18 – 0·68)**	REF	0·51 (0·25 – 1·03)
LSAC	**3·18 (1·22 – 8·30)**	REF	**7·52 (2·40 – 23·51)**
TET 32	**0·37 (0·18 – 0·73)**	REF	1·02 (0·49 – 2·16)
ERMB	0·35 (0·04 – 2·69)	REF	4·74 (0·87 – 25·91)
ERMF	1·10 (0·40 – 3·00)	REF	0·67 (0·24 – 1·88)

Bold = significant (*p*-value <0·05); [AB] = used at least 1 antibiotic in the previous 6 months; [no AB] = did not use any antibiotic in the previous 6 months

Supplementary Figure 7: Alpha and beta diversity of antibiotic resistance genes, by study group

(A) Alpha diversity
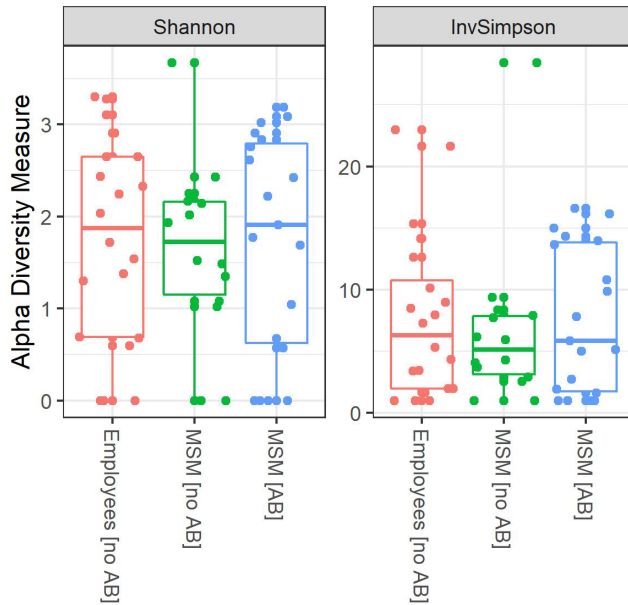
(B) Beta diversity, visualized by principle component analysis of Euclidean distances on centred log-ratio transformed abundance data.
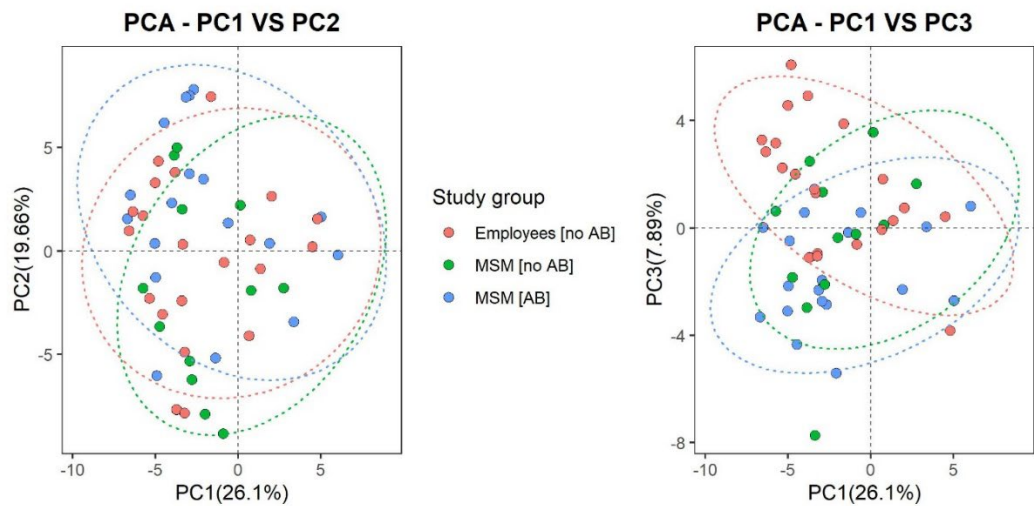
Supplementary Figure 8: Correlation analysis by Sparse Correlations for Compositional Data (SparCC).

Numbers represent correlation coefficients. Correlations with bootstrapped p-value ≥ 0·05 are left blank, and their respective correlations coefficients were set to zero.

(A) *Correlation of antimicrobial resistance genes with prevalence > 10%.*
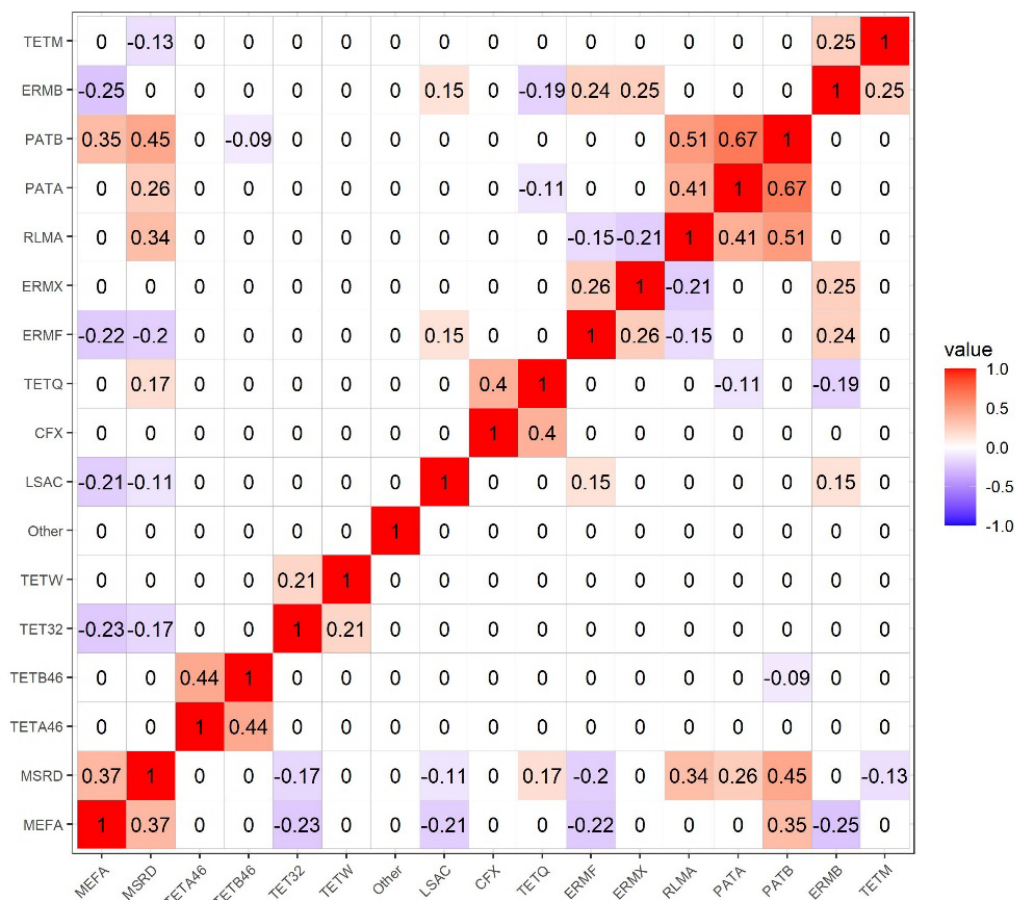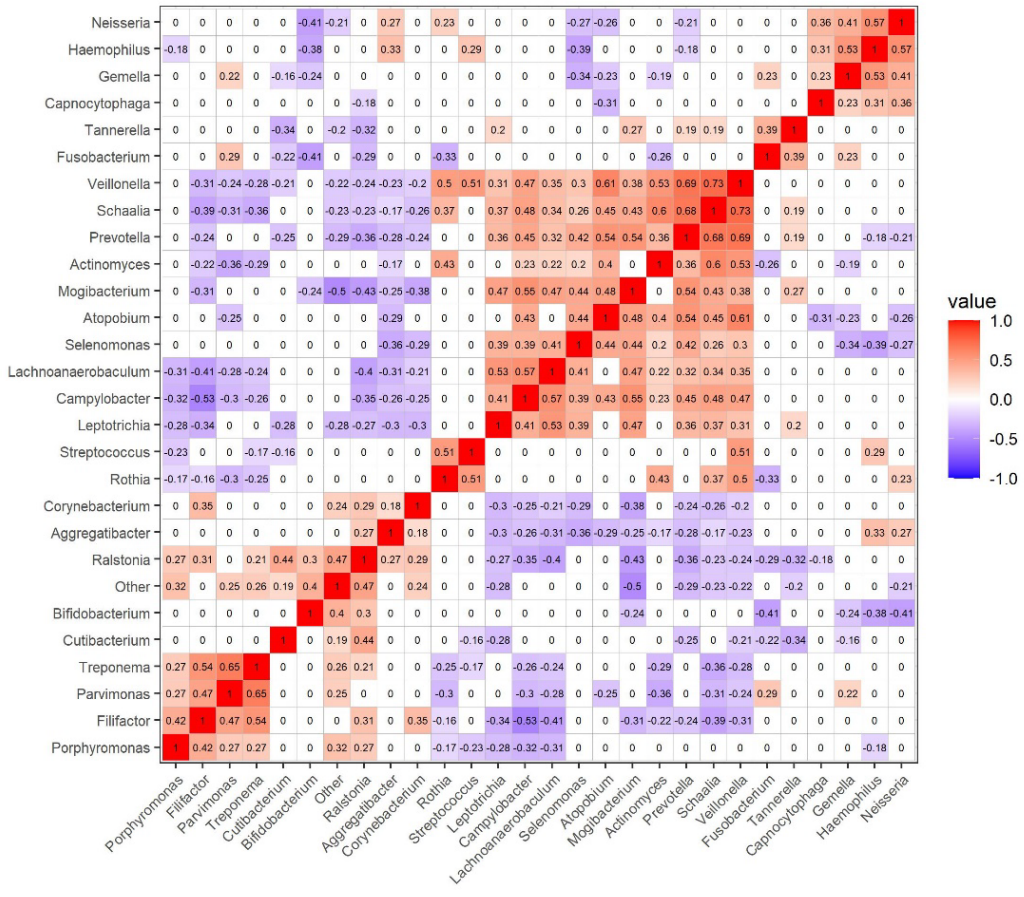
Supplementary Figure 9: Prevalence and relative abundance of oropharyngeal core bacterial genera.

Genera with a prevalence < 25% were merged into a group "Other".

(A) Prevalence plot
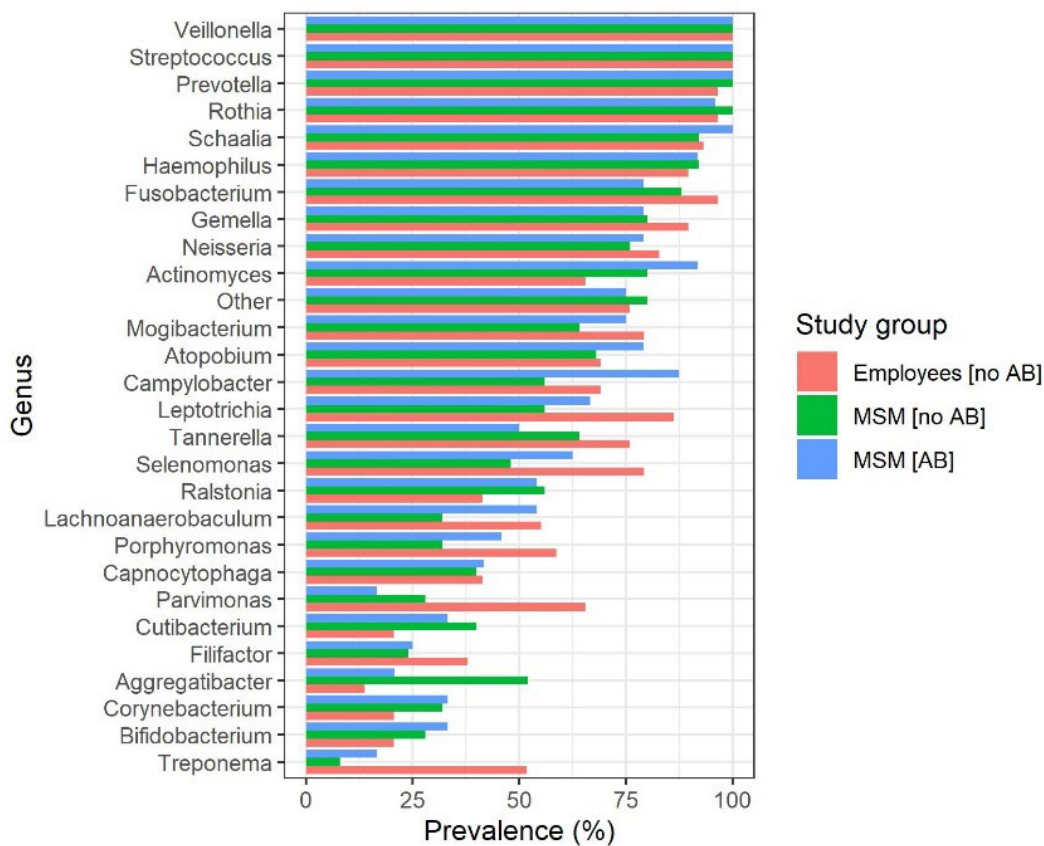
(B) Heatmap representing relative abundances, clustered by Ward D, based on Euclidean distances on centred log-ratio transformed abundance data. Coloured bars below the heatmap represent antibiotic use in the previous 6 months: the more intense the colour, the more recent the use of the antibiotic (range 183 to 0 days before sampling; grey = did not use the antibiotic)
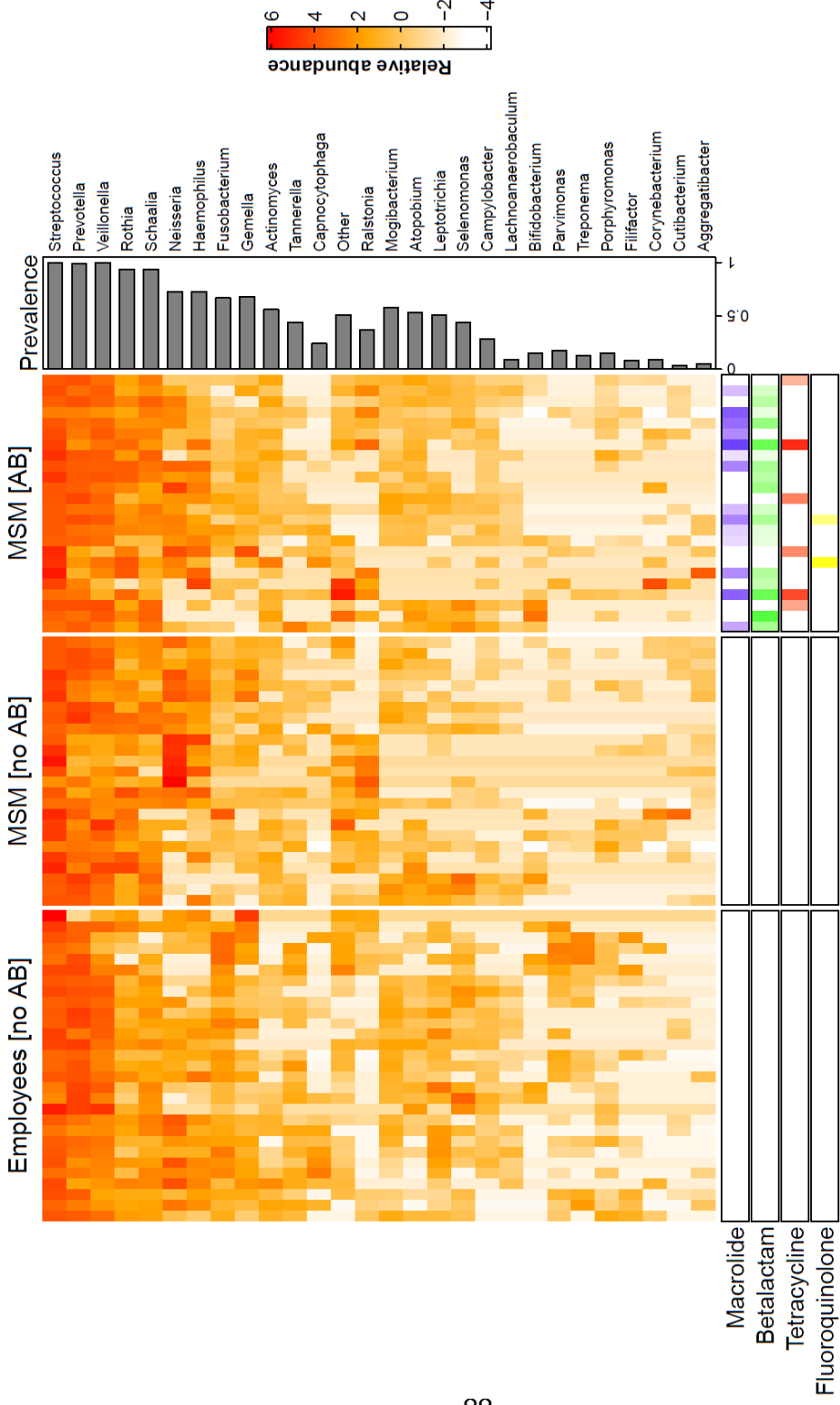
Supplementary Figure 10: Alpha and beta diversity of bacterial species, by study group

(A) Alpha diversity

(B) Beta diversity, visualized by principle component analysis of Euclidean distances on centred log-ratio transformed abundance data.
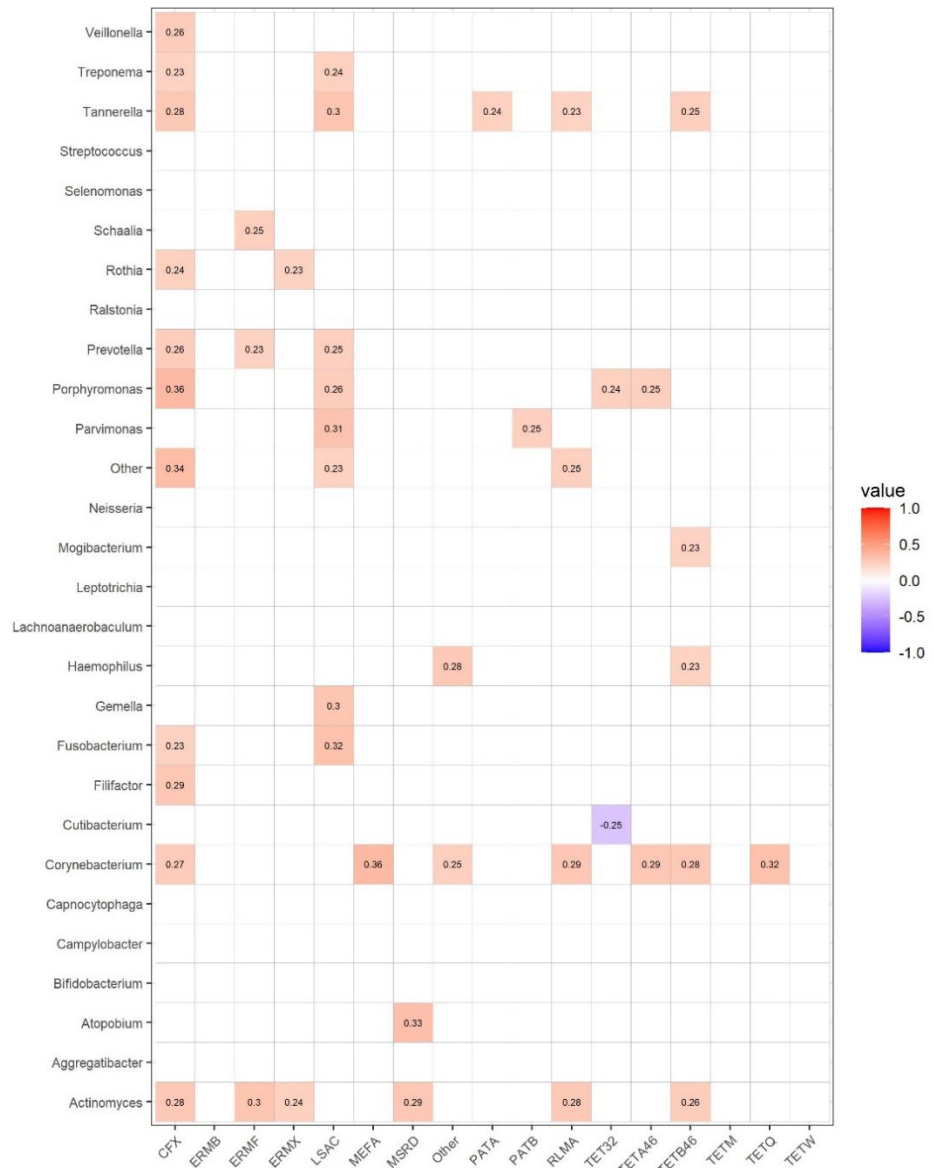
3.3 Couples share more similar commensal *Neisseria spp.* than unrelated individuals.

Van Dijck C, Laumen JGEE, Manoharan-Basil SS, Kenyon C. Commensal Neisseria are shared between sexual partners: Implications for gonococcal and meningococcal antimicrobial resistance. Pathogens. 2020;9(3):228.

Commensal *Neisseria* Are Shared between Sexual Partners: Implications for Gonococcal and Meningococcal Antimicrobial Resistance

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Abstract: Antimicrobial resistance in pathogenic *Neisseria* parallels reduced antimicrobial susceptibility in commensal *Neisseria* in certain populations, like men who have sex with men (MSM). Although this reduced susceptibility can be a consequence of frequent antimicrobial exposure at the individual level, we hypothesized that commensal *Neisseria* are transmitted between sexual partners. We used data from a 2014 microbiome study in which saliva and tongue swabs were taken from 21 couples (42 individuals). Samples were analyzed using 16S rRNA gene sequencing. We compared intimate partners with unrelated individuals and found that the oral *Neisseria* communities of intimate partners were more similar than those of unrelated individuals (average Morisita–Horn dissimilarity index for saliva samples: 0.54 versus 0.71, respectively ($p = 0.005$); and for tongue swabs: 0.42 versus 0.63, respectively ($p = 0.006$)). This similarity presumably results from transmission of oral *Neisseria* through intimate kissing. This finding suggests that intensive gonorrhea screening in MSM may, via increased antimicrobial exposure, promote, rather than prevent, the emergence and spread of antimicrobial resistance in *Neisseria*. Non-antibiotic strategies such as vaccines and oral antiseptics could prove more sustainable options to reduce gonococcal prevalence.

Keywords: commensal; *Neisseria*; gonorrhea; *meningitidis*; kissing; sharing; microbiome; transmission; antimicrobial resistance

1. Introduction

Neisseria gonorrhoeae has rapidly acquired resistance to all antimicrobials used to treat it, and there is a real risk that it may be untreatable in the near future [1]. It is increasingly appreciated that a key way it acquires this antimicrobial resistance (AMR) is via taking up resistance genes from oropharyngeal commensal *Neisseria*. The genus *Neisseria* is one of the three most abundant phyla in the human oral microbiome [2], with almost all individuals being colonized with at least one *Neisseria* species [3]. This high prevalence, in combination with extensive antimicrobial exposure, is thought to explain the extensive AMR in commensal *Neisseria* that has been found in certain populations, like cohorts of men who have sex with men (MSM) [4] and that has played an important role in the genesis of AMR in *N. gonorrhoeae* [5].

Epidemiological and modeling studies evaluating the emergence of AMR in *N. gonorrhoeae* have typically included the sexual transmission of resistant gonococci but not commensal *Neisseria* [6,7]. If resistant commensal *Neisseria* were also sexually transmitted, this would be important to take into consideration. This would be particularly important if these commensals could be transferred via highly prevalent activities such as tongue kissing. Transfer via kissing would diminish the likelihood

that traditional gonorrhea control measures would work to control the genesis and spread of gonococcal AMR. In certain instances, they may even be counterproductive. Several authors have, for example, suggested that because pharyngeal gonorrhea plays such an important role in the emergence of AMR (via horizontal gene transfer from commensals), intensive screening and treatment of pharyngeal gonorrhea in MSM should be advocated [1]. This strategy has been shown to result in extremely high antimicrobial exposure with a resultant high probability of inducing AMR in commensal *Neisseria* [8]. If these resistant *Neisseria* were then transferred via kissing and these resulted in AMR in *N. gonorrhoeae*, then intensive screening may indirectly increase rather than decrease the probability of gonococcal AMR emergence.

Concerns around the transmission of commensal *Neisseria* via kissing have emerged following increasing evidence of this mode of transmission for related bacteria. Several studies have found that kissing is a risk factor for meningococcal disease [9–11] or carriage [12–15] among students. Likewise, *N. gonorrhoeae* can be readily cultured from saliva [16–18], saliva use as a lubricant is a risk factor for rectal gonorrhea [19], kissing [20–22] as well as having a main partner with pharyngeal gonorrhea [23] may be risk factors for pharyngeal gonorrhea and a mathematical transmission model showed that oro–oral transmission is essential to generate the actual prevalence of gonorrhea among MSM [6].

Furthermore, a number of studies have found that the oral microbiome is shared between household members [24,25]. An important study by Kort et al. in 2014 demonstrated that intimate partners share a similar oral microbiome and that the degree of similarity of the salivary microbiota correlates with the kissing-frequency in the past weeks and with the time since the last kiss [26]. They calculated that an intimate kiss of 10 seconds leads to an average transfer of 10^8 bacteria from one partner to another [26].

These considerations led us to hypothesize that commensal *Neisseria* are transmitted between sexual partners. To test this hypothesis, we performed a secondary analysis of the study by Kort et al. We found that kissing partners shared more similar *Neisseria* communities than unrelated individuals.

2. Results

The dataset provided by Kort et al. [26] consisted of tongue and salivary microbiota samples taken from 21 couples visiting a Zoo in 2012. We compared the results from the entire range of 3000 operational taxonomic units (OTUs) with those from the 66 OTUs which represent members of the genus *Neisseria*. We found that pairwise comparison of samples using the Morisita–Horn dissimilarity index (MHi) did not differ significantly for analyses based on the entire versus the restricted dataset. Based on *Neisseria*-related OTUs we found the following:

1. A high pairwise similarity (an MHi value close to zero) between duplicate samples of an individual's tongue surface (MHi 0.17) and saliva (MHi 0.28) indicated that sampling was reproducible at the level of the genus *Neisseria* (Figure 1).
2. Partners' oral *Neisseria* communities sampled after a 10-second kiss were not more similar than before the kiss (saliva: average MHi 0.55 before versus 0.53 after, $p = 0.704$; surface of the tongue: average MHi 0.39 before versus 0.45 after, $p = 0.597$; Figure 1). Therefore, samples before and after kissing were combined in the subsequent analyses.
3. Partners' oral *Neisseria* communities were more similar compared to unrelated individuals. This was found for saliva (average MHi 0.54 versus 0.71, respectively, $p = 0.005$) and for samples of the tongue surface (average MHi 0.42 versus 0.63, respectively, $p = 0.006$; Figure 1).
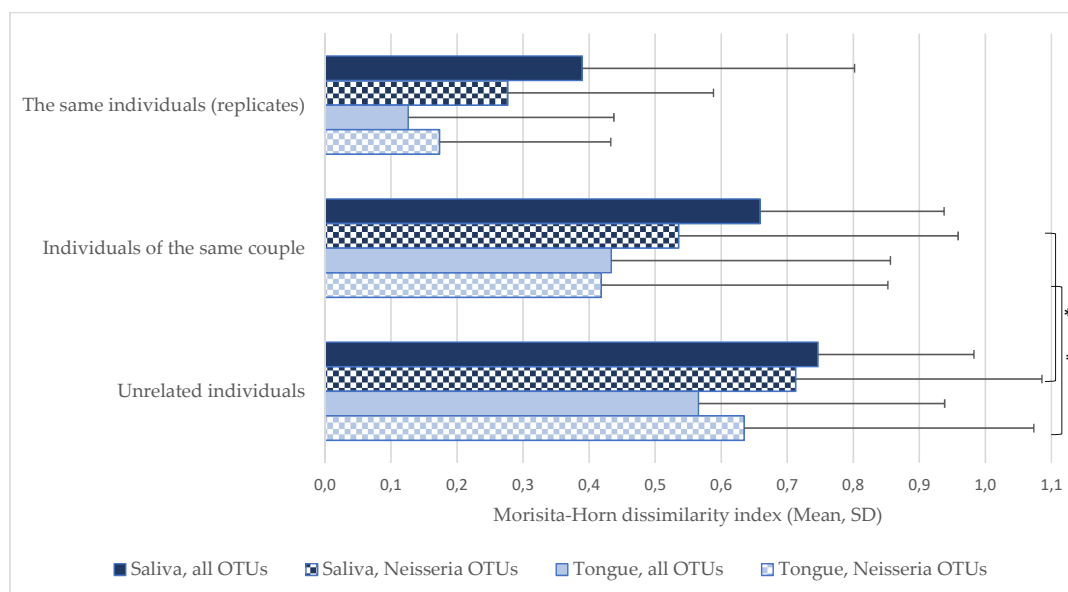
Figure 1. Morisita–Horn dissimilarity indices of samples from the same individuals, intimate partners and unrelated individuals. An index of 0 represents complete similarity whereas an index of 1 means complete dissimilarity. Each bar shows the average Morisita–Horn index, whiskers indicate standard deviations, * $p < 0.01$.

3. Discussion

Although it was already known that household members and intimate partners share oral commensal microbiota [24–26], the current analysis demonstrates that intimate partners also share similar commensal *Neisseria*. This is a logical, yet important finding, as commensal *Neisseria* are known to harbor several AMR determinants [27] that are a frequent source of AMR for pathogenic *Neisseria* [4,28,29].

Sharing of commensal *Neisseria* via this and other modalities may, therefore, explain the high prevalence of antimicrobial resistant commensal *Neisseria* in certain groups of patients. A study from Japan in 2005–2006, reported the antimicrobial susceptibility of 45 oropharyngeal *Neisseria subflava* isolates from men with urethritis and female commercial sex workers. The majority of isolates had reduced susceptibility to penicillin, tetracycline and ciprofloxacin [30]. Another study in Vietnam in 2016–2017 investigated 265 *Neisseria* isolates from 207 MSM, including 9 gonococci and 13 meningococci. Ten different *Neisseria* species were identified. Twenty-eight percent of samples had reduced susceptibility to ceftriaxone (minimum inhibitory concentration $\geq$0.125 mg/L) [4]. The reason for the high prevalence of commensal *Neisseria* with reduced antimicrobial susceptibility in these groups of patients presumably parallels the one proposed for gonorrhea: repeated cycles of reinfection/recolonization and antimicrobial exposure in individuals within a highly connected transmission-network [31].

In addition, since the pharynx is the predominant reservoir of nonpathogenic *Neisseria* in humans, it is probable that *Neisseria* are transmitted between partners by transfer of saliva, either directly (by intimate kissing or through aerosolized droplets), or indirectly (e.g., through shared fomites). The scarcity of nonpathogenic *Neisseria* within other bodily niches makes it unlikely that the skin, genital or anorectal site act as an intermediate in this transfer process. As already noted, different types of evidence suggest that pathogenic *Neisseria* species can be transmitted by kissing [6,9–23]. Our findings support to the idea that the genus *Neisseria* can be transmitted by kissing.

The limitations of this study include the following. First, the fact that partners share certain microbiota does not provide direct evidence of transmission between them. Intimate kissing may be one explanation, but we have not explored alternative means of transmission. Potential mediators of

transmission could be via fomites or animals (such as pets), or influences on the oral microbiota by environmental factors, common diet or simultaneous exposure to pathogens, toxins, mouthwashes or antimicrobials [32]. Second, identification of the oral microbiota in this study was based on the amplification of hypervariable regions V5–V7 of the 16S rRNA gene. This does not allow for the accurate identification of microbiota at the species level, nor does it provide information concerning antimicrobial susceptibility of the microbiota involved. Still, it seems reasonable to infer that sharing of specific OTUs represents sharing of a specific subset of bacterial genomes and, thus, AMR determinants within these bacteria.

The significance of this study lies in its relevance for preventing the further emergence of AMR in *N. gonorrhoeae* and *N. meningitidis*. If commensal *Neisseria* can be spread by common-place activities such as kissing, then this increases the probability that intensive gonorrhea screening in high prevalence populations such as MSM will, via increased antimicrobial exposure, promote, rather than retard, the emergence of AMR in *Neisseria*. Certain groups of at-risk populations are frequently exposed to antibiotics to treat symptomatic sexually transmitted infections. Treatment of asymptomatic cases increases this exposure even more. As most cases of anorectal and pharyngeal gonorrhea are asymptomatic, regular screening of asymptomatic patients results in a much higher number of diagnosed infections and, thus, a substantial increase in antibiotic exposure [33]. Currently, several guidelines recommend regular gonorrhea screening among MSM at high risk of infection [34,35]. The idea behind this is that treatment of all cases of gonorrhea in a population would eventually lead to a reduction (or eradication) of the pathogen from that population. There is, however, very little empirical evidence that supports this hypothesis [36]. On the other hand, increased antimicrobial exposure has been linked to AMR in gonorrhea [37,38]. This, together with the finding from the current study that *Neisseria* (including AMR determinants) may be transmitted to other individuals within a network via kissing, provides another pathway for the dissemination of AMR. Intensive screening and treatment of all positives may have a profound impact on the prevalence of AMR in commensal *Neisseria*, which could then be rapidly spread between individuals by kissing. A more prudent approach to preventing the emergence of AMR would be to reduce antimicrobial exposure as far as possible. This could include reduced screening and using non-antibiotic strategies such as vaccines and oral antiseptics to reduce gonococcal prevalence [39,40].

4. Materials and Methods

4.1. Sample Collection and Processing

In the study by Kort et al., samples were collected from 42 individuals (21 couples) visiting a Zoo in the Netherlands in 2014. A swab was taken from the anterior dorsal surface of the tongue and saliva was collected in a sterile 15 mL tube. Each participant was sampled before and after an intimate kiss of 10 s. Three couples were sampled in duplicate in order to assess reproducibility. Samples were stored at $-80\,^{\circ}$C until further processing. After DNA extraction, quantitative 16S rRNA PCR was used to generate an amplicon library based on the 16S variable regions V5-V7. Aligned 16S rRNA sequences were clustered into OTUs, defined by 97% sequence similarity. The RDP Naive Bayesian Classifier and the SILVA reference database (release 119) were used for taxonomic classification. The full study protocol is described in the original paper [26].

4.2. Availability of Data and Materials

The dataset supporting the conclusions of this article is available as a supplementary file to the paper by Kort et al. [26] For the *Neisseria*-specific analysis, the dataset was restricted to only those 66 OTUs representing members of the genus *Neisseria*.

4.3. Assessment of Community Similarity

Similarity of tongue and salivary microbiota (*β*-diversity) was determined by calculating pairwise distances with the Morisita–Horn dissimilarity index [41] using R version 3.6.1. A value of zero on this index represents complete similarity, whereas a value of one means complete dissimilarity.

4.4. Statistical Analysis

The non-parametric Wilcoxon rank-sum test in R was used to calculate the *p*-values for selected paired differences of data. Data were visualized using Microsoft Excel.

4.5. Ethics Approval and Consent to Participate

Not applicable.

Abbreviations

The following abbreviations are used in this manuscript:
AMR   antimicrobial resistance
MSM   men who have sex with men
OTU   operational taxonomic unit
MHi   Morisita–Horn index

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Mouthwashes to prevent/treat bacterial STIs

4.1 A placebo mouthwash with minimal anti-gonococcal activity can be developed.

Van Dijck C, Cuylaerts V, Sollie P, Spychala A, De Baetselier I, Laumen J, et al. The development of mouthwashes without anti-gonococcal activity for controlled clinical trials: an in vitro study. F1000Research. 2019 Sep 11;8:1620.

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BRIEF REPORT

REVISED The development of mouthwashes without anti-gonococcal activity for controlled clinical trials: an in vitro study [version 2; peer review: 2 approved]

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Abstract

Background: The oropharynx plays a major role in the development and spread of antimicrobial resistant *Neisseria gonorrhoeae* among men who have sex with men. Trials are currently assessing the efficacy of bactericidal mouthwashes as possible therapeutic or preventive options against these pharyngeal gonococcal infections. Controlled clinical trials require the use of a placebo mouthwash without anti-gonococcal activity. So far, no such placebo mouthwash has been described. We describe the development of a mouthwash for this purpose.

Methods: The *in vitro* anti-gonococcal activity of Corsodyl®, Listerine Cool Mint®, Biotene®, phosphate buffered saline and six in-house placebo mouthwashes was evaluated. Three gonococcal isolates from patients with pharyngeal infection were exposed to the mouthwashes for a duration ranging from 30 seconds to 60 minutes. Isolates were then plated in duplicate onto blood agar (5% horse blood) and incubated for 24 hours (5-7% CO_2, 35 ± 2°C). Growth of *N. gonorrhoeae* was scored on a five-point scale (0 = no growth, to 4 = confluent growth of colonies).

Results: Corsodyl® and Listerine Cool Mint® were bactericidal to all isolates. For the other mouthwashes, the median growth score after 60 minutes of exposure was 4 (interquartile range 4-4) for phosphate buffered saline; 1 (interquartile range 1-3) for Biotene®; and ranged between 0 and 2 for the in-house composed mouthwashes. An in-house composed mouthwash (Placebo 6) performed best, with a growth score of 2.5 (interquartile range 1-3).

Conclusions: All the evaluated potential placebo mouthwashes were bacteriostatic after gonococcal exposure of 30 to 60 minutes. In-house composed Placebo 6 showed less inhibition on gonococcal growth than Biotene® and the other in-house placebos and demonstrates, in our opinion, a good trade-off between anti-gonococcal properties and taste.

Keywords

Neisseria gonorrhoeae, gonorrhea, pharyngitis, gargle, treatment,

Open Peer Review

Reviewer Status ✓ ✓

	Invited Reviewers	
	1	**2**
version 2 (revision) 17 Feb 2020		
version 1 11 Sep 2019	✓ report	✓ report

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Any reports and responses or comments on the article can be found at the end of the article.

eradication, sexually transmitted diseases, placebo, randomized clinical
trial, mouthwash

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> **REVISED** **Amendments from Version 1**
>
> Several minor edits including clarifications and wording changes have been made throughout the paper. These are all pointed out in the responses to the reviewers' comments.
>
> **Any further responses from the reviewers can be found at the end of the article**

Introduction

The importance of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* cannot be overstated. The bacterium is renowned for its capability to acquire AMR and has developed resistance to all classes of antimicrobials used for its treatment[1]. AMR frequently emerges in core groups, such as men who have sex with men (MSM)[2]. The pharmaco-ecological theory of AMR states that this resistance is driven by two main factors: (a) frequent transmission of gonococci between individuals within a densely interconnected sexual network, and (b) excessive antimicrobial use which acts as a selection pressure on circulating gonococci to acquire AMR[3–5]. If this theory is correct, current efforts to reduce sexually transmitted infection (STI) prevalence via expanded screening and antimicrobial therapy in MSM may paradoxically be playing an important role in the promotion of gonococcal AMR[5,6].

These considerations have led to efforts to reduce the prevalence of gonococci in MSM and other core groups with non-antimicrobial products. One option is the use of an antiseptic mouthwash to decrease the oropharyngeal prevalence of gonococci (and other STIs). A modeling study showed that regular use of a mouthwash by MSM could reduce the prevalence of gonococci at different body sites[7]. A further consideration is that the oropharynx plays a central role in the emergence and spread of gonococcal AMR among MSM because of multiple reasons, which are reviewed elsewhere[8]. If a mouthwash can reduce the prevalence of oropharyngeal gonorrhoea without selecting for AMR, this may have the added benefit of reducing the probability of AMR emerging at this site[4]. Two randomized controlled trials (RCTs) are currently underway to assess whether regular mouth washing and gargling in MSM is able to reduce the cumulative incidence of gonorrhoea and other STIs. The OMEGA (Oral Mouthwash use to Eradicate GonorrhoeA) study is an RCT that assesses whether daily use of Listerine Zero® can reduce the incidence of pharyngeal gonorrhoea in a population of Australian MSM (ACTRN12616000247471)[9]. We are conducting a second RCT to assess if the use of Listerine Cool Mint® (LCM) is able to reduce the cumulative incidence of gonorrhoea (PReGo – Preventing Resistance in Gonorrhoea Study; registered at ClinicalTrials.gov with the identifier NCT03881007).

The choice of an optimal placebo is critical to the success of these RCTs. It is particularly important that a placebo is inert and has no bactericidal or bacteriostatic effect on gonococci. If it did, it would increase the probability of a false negative study outcome.

So far, no study has assessed placebo mouthwashes for this purpose. In this paper, we describe the process of developing and testing a series of candidate placebo mouthwashes. Our aim was to find the most suitable formulation for use as a placebo in the PReGo study. The major criterion we used to assess the mouthwash was its anti-gonococcal activity.

Methods

Isolates

We used three stored isolates of *Neisseria gonorrhoeae* that had been previously isolated from the oropharynx of three treatment-naive women with pharyngeal infection at the STI clinic of the Institute of Tropical Medicine, Antwerp, as part of routine gonococcal surveillance monitoring. The isolates were preserved in skimmed milk and 20% glycerol at -80°C until the experiments were performed. Antimicrobial susceptibility was determined by the agar dilution method according to Clinical & Laboratory Standards Institute[10].

Mouthwashes

The commercially available products Listerine Cool Mint® (LCM, containing alcohol and essential oils) and Corsodyl® (containing chlorhexidine 0.2%) were used to assess the isolate's susceptibility to antibacterial mouthwashes.

Biotene®, a commercially available mouthwash that does not contain alcohol, essential oils or chlorhexidine, was expected to have no antibacterial effect and was thus the first mouthwash to be evaluated as a potential placebo substance.

Subsequently, six other potential placebo mouthwashes were manufactured by a pharmacist (Sollie Pharmacy, Antwerp) based on readily available and inexpensive ingredients that are stable at room temperature. Ingredients added to create a medicinal taste were sorbitol, sodium saccharinate, benzoic acid, ethanol, mint spiritus, raspberry extract and/or elderberry extract; ingredients added as a colorant were malachite green, raspberry extract, elderberry extract or solutio viridis. Only mouthwashes with a medicinal taste, as appreciated by one of the researchers (CK), were included in the experiment. The composition of the mouthwashes is displayed in Table 1 and Table 2. Based on the properties of these ingredients, no major side effects would be expected to occur.

Phosphate buffered saline (PBS, pH 7.3 ± 0.2) was used as a negative control (inert product maintaining gonococcal viability) during every experiment.

Assessment of antibacterial effect

Each gonococcal isolate was brought into suspension in 3mL PBS at a 0.5 to 0.8 McFarland turbidity, corresponding to a concentration of 10^8 CFU/mL. From these suspensions, 100µl was then added to 900µL of each mouthwash, resulting in a concentration of 10^7 CFU/mL. After 30 seconds, 60 seconds, five minutes, 30 minutes and 60 minutes at ambient temperature (20 ± 5°C), 10µL aliquots were plated onto blood agar (5% horse blood) and incubated for 24 hours in a 6 ± 1% CO_2 environment at 35 ± 2°C. Bacterial growth was visually scored on a semi-quantitative five-point scale, as described in Figure 1. Plating was conducted in duplicate for each isolate and all bacterial growth assessments were made by a single observer.

Table 1. Ingredients of the commercially available mouthwashes, according to their product insert.

Mouthwash	Ingredients
Biotene®	purified water, glycerin, xylitol, sorbitol, propylene glycol, poloxamer 407, sodium benzoate, hydroxyethyl cellulose, methylparaben, propylparaben, flavor, sodium phosphate and disodium phosphate
Listerine Cool Mint®	aqua, alcohol 21.6%, sorbitol, poloxamer 407, benzoic acid, sodium saccharin, eucalyptol 0.092%, aroma, methyl salicylate 0.06%, thymol 0.064%, menthol 0.042%, sodium benzoate, flavor, green 3
Corsodyl®	chlorhexidine digluconate 0.2%, ethanol, peppermint flavour, polyoxyl hydrogenated castor oil, sorbitol, cochenille red dye (E 124), purified water

Table 2. Ingredients of the in-house mouthwashes.

Mouthwash	Ingredients										Total (g)
	Sorbitol (g)	Sodium saccharinate (g)	Benzoic acid (g)	Ethanol 96% (g)	Mint spiritus (g)	Malachite green[§] (g)	Raspberry extract (g)	Elderberry extract (g)	Solutio viridis[$] (g)	Aqua conservans[*] (g)	
Placebo 1	30.00	0.10	0.20	10.00	1.10	1.75				156.85	200
Placebo 2	30.00	0.10			0.66	1.75				167.49	200
Placebo 3	30.00	0.10				1.75	1.00			167.15	200
Placebo 4	30.00	0.05					1.00			168.95	200
Placebo 5	30.00	0.05						2.00		167.95	200
Placebo 6	30.00	0.10							0.70	169.20	200

[§] 100 g Malachite green contains: 0.01 g malachite green oxalate, 99.99 g aqua conservans.

[$] 100 g Solutio viridis contains: 0.3 g patent blue (E131), 0.3 g tartrazine (E102), 0.15 g sodium benzoic acid, 0.1 g tartaric acid, 99.15 g purified water.

[*] 100 g Aqua conservans contains: 0.0724 g methylparahydroxybenzoate, 0.0310 g propylparahydroxybenzoate, 0.9959 g propylene glycol, 98.901 g purified water.
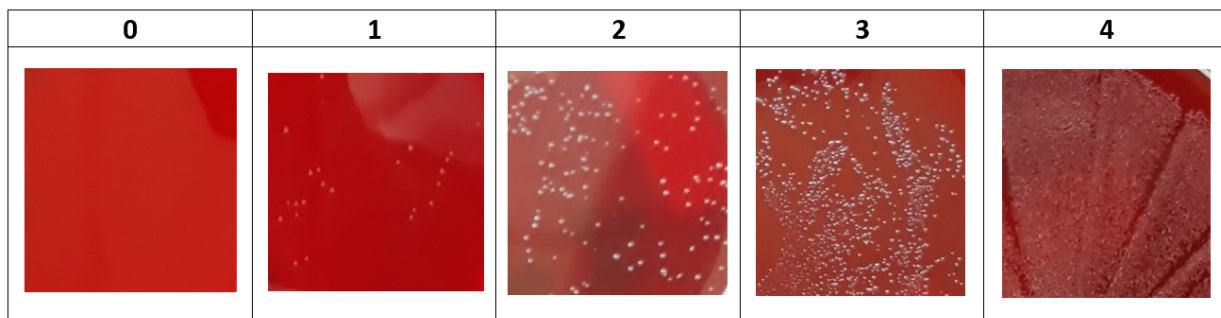
Figure 1. Five-point scale for scoring Neisseria gonorrhoeae growth on blood agar (0 = no growth; 1 = some colonies; 2 = numerous colonies; 3 = entire agar plate covered with colonies; 4 = confluent growth of colonies).

No statistical analysis was performed. This study did not involve any experiments on humans or animals and thus no ethical clearance was required.

Results

All three isolates were susceptible to ceftriaxone and spectinomycin; isolates B and C had a slightly increased minimum inhibitory concentration (MIC) for azithromycin and isolate A had a high MIC for ciprofloxacin and cefixime. None of the strains produced penicillinase (Table 3).

All isolates were fully susceptible to LCM and Corsodyl®; a full bactericidal effect was observed after an exposure of 30 seconds or longer (Table 4)[11].

Exposure to Biotene® for 30 minutes or longer was found to inhibit gonococcal growth considerably (Table 4).

Placebo 1, an ethanol-containing mouthwash was designed to have a similar color and taste as LCM® but led to almost complete inhibition of gonococcal growth even after a short duration of exposure. Placebo 2 contained no ethanol and a lower amount of mint spiritus. Yet, its bacteriostatic effect was comparable to Biotene®. In order to determine if mint spiritus or malachite green were the inhibiting factors, these ingredients were sequentially omitted in Placebo 3 and 4. Raspberry extract was added to both in order to improve the taste, but this resulted in strong inhibition of gonococcal growth in both cases. Placebo 5 contained elderberry extract instead, but substantial

Table 3. Antimicrobial susceptibility of *Neisseria gonorrhoeae* isolates used in the experiment.

Isolate	MIC values (mg/L)					
	Ciprofloxacin	Ceftriaxone	Azithromycin	Spectinomycin	Cefixime	Penicillinase
A	16.000	0.030	0.250	16.000	0.250	negative
B	0.004	0.008	0.500	16.000	0.015	negative
C	0.004	0.008	0.500	16.000	0.015	negative

MIC, Minimum Inhibitory Concentration; determined by agar dilution method according to Clinical & Laboratory Standards Institute.

Table 4. Growth of *Neisseria gonorrhoeae* after exposure to the mouthwashes.

Mouthwash	N	Median growth score (IQR) after exposure during				
		30 seconds	60 seconds	5 minutes	30 minutes	60 minutes
Listerine Cool Mint®	6	0 (0-0)	0 (0-0)	NA	NA	NA
Corsodyl®	6	0 (0-0)	0 (0-0)	NA	NA	NA
Biotene®	6	4 (4-4)	4 (4-4)	4 (2-4)	1 (1-3)	1 (1-3)
Placebo 1	6	1 (0-2)	1 (0-1)	NA	NA	NA
Placebo 2	6	4 (4-4)	4 (4-4)	3 (3-4)	3 (1-3)	1 (0-2)
Placebo 3	6	2 (2-2)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Placebo 4	6	2 (1-2)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Placebo 5	6	4 (4-4)	4 (3-4)	3 (2-3)	0 (0-0)	0 (0-0)
Placebo 6	6	4 (4-4)	4 (4-4)	4 (3-4)	3 (2-4)	2.5 (1-3)
PBS	6	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)

NA, not assessed; IQR, interquartile range; PBS, phosphate buffered saline.

gonococcal growth inhibition was seen here, too. Placebo 6 contained another type of colorant (solutio viridis) and showed the least bacteriostatic effect after 30 and 60 minutes of exposure (Table 4). During every experiment, there was full and confluent gonococcal growth after exposure to the negative control substance (PBS) (Table 4).

We noted a slight difference in susceptibility to the mouthwashes between the three tested gonococcal isolates. Isolate A was more susceptible to placebos 1–6 and Biotene® compared with isolates B and C. However, all strains showed equivalent susceptibility to LCM and Corsodyl® (Table 5 and Table 6). These differences were not assessed for statistical significance.

Discussion
The recognition of the oropharynx as a source of gonococcal transmission and the genesis of antimicrobial resistance in groups such as MSM has directed research interest towards novel non-antimicrobial methods to prevent or treat oropharyngeal gonococcal infection. Mouthwashes are one such option. In order to determine the efficacy of an intervention involving the use of a mouthwash, RCTs should be performed, and a non-bactericidal placebo is a prerequisite for these trials.

Commercially available non-alcohol containing mouthwashes (like Biotene®) are an attractive option, but our experiments suggest that exposure to Biotene® for longer than five minutes may inhibit the growth of gonococci. Mouthwashes are typically used for 60 seconds but the substantivity of its ingredients may result in the antibacterial activity of mouthwashes persisting for over six hours[12,13]. To optimize their STI preventive potential, mouthwashes could be used pre and post sex, which could lead to multiple exposures per day. These considerations triggered the search for a placebo with minimal inhibitory effect for periods of up to 60 minutes.

All three isolates in the experiment were fully susceptible to LCM and Corsodyl® and isolate A was most susceptible to all placebo mouthwashes. Although this difference in susceptibility may have been the result of random variability, we could speculate that, in the absence of overt resistance to antiseptics, there might be a mechanism that partially protected isolate B and C from the harmful effect of some of the mouthwash constituents. Isolates B and C had a reduced susceptibility to azithromycin. This might have been due to the increased expression of an efflux mechanism such as the Mtr (multiple transferable resistance) efflux pump, which is linked to resistance

Table 5. Growth of *Neisseria gonorrhoeae* after exposure to seven potential placebo mouthwashes (Biotene® and Placebo 1-6).

Isolate	N	Median growth score (IQR) after exposure during				
		30 seconds	60 seconds	5 minutes	30 minutes	60 minutes
A	14	3.5 (2-4)	3 (0-4)	1 (0-3)	0.5 (0-1)	0 (0-1)
B	14	4 (2-4)	4 (0-4)	2.5 (0-4)	0.5 (0-2.5)	0.5 (0-1.5)
C	14	4 (1-4)	4 (0-4)	3.5 (0-4)	1.5 (0-3)	0.5 (0-3)

IQR, interquartile range.

Table 6. Growth of *Neisseria gonorrhoeae* after exposure to the mouthwashes.

Isolate	Mouthwash	N	Median growth score (IQR) after exposure during				
			30 seconds	60 seconds	5 minutes	30 minutes	60 minutes
A	Listerine Cool Mint®	2	0 (0-0)	0 (0-0)	NA	NA	NA
	Corsodyl®	2	0 (0-0)	0 (0-0)	NA	NA	NA
	Biotene®	2	4 (4-4)	4 (4-4)	3 (3-3)	1 (1-1)	1 (1-1)
	Placebo 1	2	2 (2-2)	1 (1-1)	NA	NA	NA
	Placebo 2	2	4 (4-4)	4 (4-4)	3 (3-3)	1 (1-1)	0 (0-0)
	Placebo 3	2	2 (2-2)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
	Placebo 4	2	2 (2-2)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
	Placebo 5	2	4 (4-4)	3 (3-3)	2 (2-2)	0 (0-0)	0 (0-0)
	Placebo 6	2	4 (4-4)	4 (4-4)	3 (3-3)	3 (3-3)	2 (2-2)
	PBS	2	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)
B	Listerine Cool Mint®	2	0 (0-0)	0 (0-0)	NA	NA	NA
	Corsodyl®	2	0.5 (0-1)	0 (0-0)	NA	NA	NA
	Biotene®	2	4 (4-4)	4 (4-4)	2 (2-2)	1 (1-1)	1 (1-1)
	Placebo 1	2	0 (0-0)	0 (0-0)	NA	NA	NA
	Placebo 2	2	4 (4-4)	4 (4-4)	4 (4-4)	3 (3-3)	2 (2-2)
	Placebo 3	2	2 (2-2)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
	Placebo 4	2	2 (2-2)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
	Placebo 5	2	4 (4-4)	4 (4-4)	3 (3-3)	0 (0-0)	0 (0-0)
	Placebo 6	2	4 (4-4)	4 (4-4)	4 (2-4)	2 (2-2)	1 (1-1)
	PBS	2	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)
C	Listerine Cool Mint®	2	0 (0-0)	0 (0-0)	NA	NA	NA
	Corsodyl®	2	0 (0-0)	0 (0-0)	NA	NA	NA
	Biotene®	2	4 (4-4)	4 (4-4)	4 (4-4)	3 (3-3)	3 (3-3)
	Placebo 1	2	1 (1-1)	1 (1-1)	NA	NA	NA
	Placebo 2	2	4 (4-4)	4 (4-4)	4 (4-4)	3 (3-3)	1 (1-1)
	Placebo 3	2	1 (1-1)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
	Placebo 4	2	2 (2-2)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
	Placebo 5	2	4 (4-4)	4 (4-4)	3 (3-3)	0 (0-0)	0 (0-0)
	Placebo 6	2	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)	3 (3-3)
	PBS	2	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)

NA, not assessed; PBS, phosphate buffered saline.

to macrolides, as well as to many other substances like dyes and detergents[14]. We did, however, not perform genotypic assessment of the isolates used in this experiment.

After testing multiple combinations of ingredients, we found that Placebo 6 had the least bacteriostatic effect *in vitro*.

The limitations of this study include the following. First, budget and time constraints did not allow to perform any *in vivo* evaluation of the mouthwashes. It is of particular interest to know whether participants can distinguish Placebo 6 from an antibacterial mouthwash. A formal head-to-head comparison with LCM is planned as part of the PReGo study. Second, the sample size of the current experiment was too small to statistically assess differences between isolates and between mouthwashes. We may have over- or underestimated the true effect of the mouthwashes. Additionally, the experiments were performed sequentially, which may have introduced some inter-run variation. In each experiment we did however include a PBS exposed control. and plating was done in duplicate. Third, the observer who assessed bacterial growth was not blinded to the ingredients of the mouthwashes, we did not use a validated quantitative assessment method and we did no further *in vivo* or *in vitro* fitness testing of the isolates after exposure to the mouthwashes. Fourth, we used isolates from women with pharyngeal gonococcal infection, which are possibly not representative of the gonococci circulating among MSM. Their susceptibility pattern was, however, similar to that observed in most gonococcal isolates from MSM. Fifth, our *in vitro* findings are not necessarily representative of the *in vivo* setting as anatomical and biological properties may influence the effect of a mouthwash against gonococci in the throat.

Bioactive molecules in saliva may, for example, have synergistic or antagonistic effects on the mouthwash's active ingredients. Finally, we did not assess the effect of the placebo on the oropharyngeal microbiome. An increased or decreased growth of other oropharyngeal commensals might theoretically compete with gonococcal proliferation in the throat and influence gonococcal infectivity as well.

Conclusion
This experiment has shown that it is hard to develop an ideal placebo mouthwash as a range of frequently used ingredients inhibit gonococcal growth. A commercially available mouthwash like Biotene® seemed the perfect option at first but it had a bacteriostatic effect. A process of serial testing of various placebos resulted in a placebo mouthwash, which we believe demonstrates a good trade-off between anti-gonococcal properties and taste.

Data availability
Underlying data
Figshare: *In vitro* gonococcal growth after exposure to mouthwashes. https://doi.org/10.6084/m9.figshare.9757859[11].

This project contains the following underlying data:

- Data.xlsx (spreadsheet containing raw growth scores of the individual isolates after exposure to the experimental substances)

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

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4.2 A Listerine Cool Mint mouthwash cannot reduce the incidence of bacterial STIs in PrEP users.

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Antibacterial mouthwash to prevent sexually transmitted infections in men who have sex with men taking HIV pre-exposure prophylaxis (PReGo): a randomised, placebo-controlled, crossover trial

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Summary

Background Bacterial sexually transmitted infections (STIs) are highly prevalent among men who have sex with men who use HIV pre-exposure prophylaxis (PrEP), which leads to antimicrobial consumption linked to the emergence of antimicrobial resistance. We aimed to assess use of an antiseptic mouthwash as an antibiotic sparing approach to prevent STIs.

Methods We invited people using PrEP who had an STI in the past 24 months to participate in this single-centre, randomised, double-blind, placebo-controlled, AB/BA crossover superiority trial at the Institute of Tropical Medicine in Antwerp, Belgium. Using block randomisation (block size eight), participants were assigned (1:1) to first receive Listerine Cool Mint or a placebo mouthwash. They were required to use the study mouthwashes daily and before and after sex for 3 months each and to ask their sexual partners to use the mouthwash before and after sex. Participants were screened every 3 months for syphilis, chlamydia, and gonorrhoea at the oropharynx, anorectum, and urethra. The primary outcome was combined incidence of these STIs during each 3-month period, assessed in the intention-to-treat population, which included all participants who completed at least the first 3-month period. Safety was assessed as a secondary outcome. This trial is registered with Clinicaltrials.gov, NCT03881007.

Findings Between April 2, 2019, and March 13, 2020, 343 participants were enrolled: 172 in the Listerine followed by placebo (Listerine-placebo) group and 171 in the placebo followed by Listerine (placebo-Listerine) group. The trial was terminated prematurely because of the COVID-19 pandemic. 151 participants completed the entire study, and 89 completed only the first 3-month period. 31 participants withdrew consent, ten were lost to follow-up, and one acquired HIV. In the Listerine-placebo group, the STI incidence rate was 140·4 per 100 person-years during the Listerine period, and 102·6 per 100 person-years during the placebo period. In the placebo-Listerine arm, the STI incidence rate was 133·9 per 100 person-years during the placebo period, and 147·5 per 100 person-years during the Listerine period. We did not find that Listerine significantly reduced STI incidence (IRR 1·17, 95% CI 0·84–1·64). Numbers of adverse events were not significantly higher than at baseline and were similar while using Listerine and placebo. Four serious adverse events (one HIV-infection, one severe depression, one Ludwig's angina, and one testicular carcinoma) were not considered to be related to use of mouthwash.

Interpretation Our findings do not support the use of Listerine Cool Mint as a way to prevent STI acquisition among high-risk populations.

Funding Belgian Research Foundation - Flanders (FWO 121·00).

Introduction

Despite quarterly STI screening and sexual health counselling, the incidence of bacterial sexually transmitted infections (STIs) in people using HIV pre-exposure prophylaxis (PrEP) remains high.[1] This results in high levels of antimicrobial consumption, which is an important risk factor for the emergence of antimicrobial resistance (AMR) in bacteria such as *Neisseria gonorrhoeae*.[2] Specific populations including men who have sex with men (MSM) have been linked to emergence of antimicrobial

resistance.[2,3] There is an urgent need for new approaches to reduce the incidence of STIs among MSM taking PrEP that limit the emergence of AMR.

Topical antiseptics were widely used to treat and prevent STIs at the beginning of the 20th century but were largely abandoned after the discovery of antibiotics.[4] Interest in topical antiseptics has resurfaced due to increasing evidence that the oropharynx plays an important role in the transmission of STIs such as gonorrhoea and syphilis and in the genesis of gonococcal AMR.[5] So

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Research in context

Evidence before this study
We searched PubMed, up to July 14, 2020, for reports of clinical trials assessing the effect of a mouthwash on the acquisition of sexually transmitted infections. We used the search terms "mouthwash" AND "sexual" AND "trial" and found one randomised controlled trial that evaluated the therapeutic effect of Listerine Cool Mint (Johnson & Johnson, New Brunswick, NJ, USA) against culture-proven oropharyngeal gonorrhoea in men. It demonstrated that gonococcal cultures, taken 5 min after gargling, were negative in 16 (48%) of 33 men, compared with four (16%) of 25 men who gargled with normal saline (p=0·013). In addition, we identified one protocol of a randomised controlled trial that aimed to assess if once daily Listerine Zero (Johnson & Johnson) for 12 weeks could reduce the incidence of oropharyngeal gonorrhoea in men who have sex with men (OMEGA trial). The results of this study are in *The Lancet Infectious Diseases* alongside this manuscript, and showed that men who used Listerine Zero did not have a lower incidence of oropharyngeal gonorrhoea compared with those who used Biotène (GlaxoSmithKline, Brentford,

London, UK; adjusted risk difference 2·5%; 95% CI −1·8% to 6·8%).

Added value of this study
This crossover randomised controlled trial assessed if Listerine Cool Mint could reduce the incidence of syphilis, gonorrhoea, and chlamydia in men who have sex with men taking HIV pre-exposure prophylaxis. The trial was terminated before the predetermined sample size was attained, and at a point when it had a power of 60% to find the prespecified difference in primary outcome. Despite good adherence and a high incidence of bacterial STIs, we did not find that Listerine Cool Mint reduced STI incidence. We asked participants to use the mouthwash daily, before and after sex and to ask their partners to use the mouthwash before and after sex—an approach with a clear biological rationale, but one which participants noted to be particularly difficult to implement.

Implications of all the available evidence
Our study does not support the use of an antiseptic mouthwash to prevent sexually transmitted infections in high-risk groups.

far, no compelling evidence exists that topical antiseptics effectively treat or prevent STIs at any anatomical site.[6]

Studies in contemporary populations of MSM have estimated that up to half of all gonorrhoea and syphilis infections are transmitted via oral sex.[7–9] Very few MSM use condoms during oral sex.[10] Furthermore, certain attributes of the oropharynx enhance the probability of *N gonorrhoeae* acquiring AMR. Many antimicrobials have poor penetration into the oropharyngeal mucosa resulting in subtherapeutic antibiotic exposures[11] that create selection pressure for the acquisition of AMR in all *Neisseria* species, particularly in commensal *Neisseria*. Studies have demonstrated that *N gonorrhoeae* is able to take up these resistance conferring genes from commensal species via transformation.[12] Mouthwashes might reduce the risk of AMR emerging in *N gonorrhoeae* by reducing the incidence of STIs and hence antimicrobial exposure.[5]

Essential oils have a bactericidal effect by disrupting the cell wall and by inactivating essential enzymes.[13] In vitro, they are active against several bacteria including *Chlamydia trachomatis*, *Treponema* spp, and *N gonorrhoeae*.[14–17] Listerine Cool Mint (Johnson & Johnson, New Brunswick, NJ, USA; herein referred to as Listerine) is an essential-oil based mouthwash, containing menthol, thymol, methyl salicylate, and euca-lyptol in a 22% hydroalcoholic solution. A randomised controlled trial in men with culture-proven oropharyngeal gonorrhoea found that gonococcal cultures taken 5 min after gargling with 20 mL Listerine for 1 min, were negative in 16 of 33 men (48%), compared with four of 25 men (16%) who gargled with normal saline (p=0·013).[14]

Using these results, a modelling study predicted that a high efficacy mouthwash could reduce the prevalence of *N gonorrhoeae* in MSM by seven-fold.[7] Studies have also found that a mouthwash could be a popular way to reduce STI incidence in MSM.[18,19]

Two randomised controlled trials have been done to test the hypothesis that mouthwashes can reduce the incidence of STIs in MSM. The OMEGA trial[20] in Australia assessed if Listerine Zero used once daily for 12 weeks could reduce the proportion of MSM with oropharyngeal gonorrhoea. The results of this study had not been published at the time of submission of the current manuscript.

In our trial we tested a somewhat different study hypothesis: Listerine, used daily by participants, and before and after sex by participants and their partners, could reduce the incidence of gonorrhoea, chlamydia, and syphilis in MSM taking PrEP compared with a placebo mouthwash.

Methods

Study design and participants
Our study was a randomised double-blind, placebo-controlled, crossover trial designed to assess superiority of Listerine over a placebo mouthwash to prevent bacterial STIs among MSM taking PrEP. This single-centre trial took place at the outpatient STI clinic of the Institute of Tropical Medicine in Antwerp, Belgium. Ethics approval was obtained from the institute's Institutional Review Board (1276/18) and from the Ethics Committee of the University of Antwerp (19/06/058). The protocol can be viewed in the appendix (p 24).

The choice for a crossover design was justified as follows. First, a prospective observational study in our clinic's PrEP cohort in 2017–18 showed that STI incidence remained stable over time.[21] The low within-participant variation of determinants of STI incidence compared with the between-participant variation enabled us to enrol fewer participants, as strong correlation would be expected between individual STI incidence and the use of either mouthwash. Second, no substantial carry-over effect was expected, as the effect of a mouthwash on STI incidence was assumed to be reversible and short-lived (several hours only, based on a study that assessed salivary bacterial counts after use of Listerine[22]). Therefore, no washout period was required.

HIV-negative MSM aged 18 or over at follow-up for PrEP were invited to participate if they had sex with another man in the previous 12 months, if they had a documented infection with gonorrhoea, chlamydia, or syphilis in the previous 24 months, and if they were willing to comply with all study procedures. Exclusion criteria were the refusal to refrain from use of non-study mouthwashes and participation in any other clinical trial. Participants provided written informed consent at the baseline study visit.

A qualitative study (appendix p 12) was embedded within the trial to examine acceptability towards a mouthwash as an STI prevention method and to explore experienced and perceived barriers and facilitators for optimal mouthwash adherence.

Randomisation and masking

We randomly assigned participants (1:1) to start trial participation with Listerine (Listerine-placebo arm) or with placebo (placebo-Listerine arm), using block randomisation with block size eight. After 3 months, participants crossed over and received the opposite mouthwash for another 3 months. The computer-generated randomisation sequence was created by an independent statistician to ensure blinding, using SAS 9.4 (SAS Institute, Cary NC).

The placebo mouthwash was manufactured specifically for this trial after discovering that other mouthwashes such as Biotène, that have been used as placebos in similar trials,[20] have a bacteriostatic effect on *N gonorrhoeae*.[15] The placebo mouthwash we used was shown to have minimal in vitro bacteriostatic effect on *N gonorrhoeae* for up to 60 min of contact time.[15] The ingredients of this mouthwash were 30 g sorbitol, 0·10 g sodium saccharinate, 0·70 g solutio viridis (dye), and 169·20 g aqua conservans (preservative).[15] Since Listerine's colouring and flavouring agents had antiseptic effects, the same ingredients could not be used in the placebo mouthwash. As a consequence, the mouthwashes in our study differed in taste and appearance: Listerine was blue and had a mint taste, the placebo mouthwash was green and had a medicinal taste. Participants were not informed that Listerine was the active mouthwash in this study. The study mouthwashes were packaged in sequentially numbered sets of sealed, identical opaque 500 mL bottles, according to the allocation sequence. Each set of 12 bottles contained six bottles labelled A for the first 3-month period and six bottles labelled B for the second 3-month period. If the A bottles contained Listerine, then the B bottles with the same randomisation number contained the placebo mouthwash (for a participant assigned to the Listerine-placebo arm), and vice versa. Manufacturing, packaging, and labelling were done by an independent, non-blinded pharmacist.

The study physicians enrolling the participants, health-care workers providing routine STI care, data collectors, and participants were blinded to the study intervention. The allocation sequence remained concealed until final database lock at the end of the trial.

Procedures

Study visits were organised at baseline (day of randomisation), and at 3 months (follow-up visit 1) and 6 months later (follow-up visit 2), with a time window of 78 to 136 days since the previous visit. At randomisation, participants received mouthwash A. At follow-up visit 1, they were asked to return any left-over mouthwash A, and they received mouthwash B. Participants were instructed to rinse and gargle once daily for 1 min with 20 mL of the mouthwash (appendix p 2). In addition, they were asked to rinse and gargle before and after each sexual encounter, preferably while inviting their main and casual partners to use the same study mouthwash before and after sex. We asked participants to gargle as close in time as possible to the sexual act, but no time limit was defined. The rationale to request mouthwash use by partners was that participants' urogenital and anorectal STI incidence could be reduced if partners used the mouthwash before sex (figure 1).[23] This was explained to participants via a specific leaflet that could also be shared with partners (appendix p 3). Participants were required to avoid using non-study mouthwashes for the duration of the trial. Sexual behaviour and adherence to the mouthwash were assessed on a weekly basis by use of an online diary (appendix p 18).

At every study visit, information on STI diagnoses in the preceding period was recorded. Adverse events were also recorded at every study visit, both by self-report (eg, tooth and mouth problems) and physician-assessment (eg, tooth discolouration, caries). In addition, data pertaining to mouthwash use and adherence, sexual behaviour, and attitude towards mouthwash use were collected by computer assisted self-interviews (CASIs, appendix p 19). The CASI at follow-up visit 2 additionally assessed how many of the participants' partners used the mouthwash. Samples were collected to screen for HIV, syphilis, chlamydia, and gonorrhoea. HIV testing was done with a validated HIV screening algorithm.[24] Syphilis was diagnosed using Macro-Vue RPR Card (RPR; Becton Dickinson, Sparks, MD, USA) and a TPA assay (Ortho-Clinical Diagnostics, Rochester, NY, US), interpreted
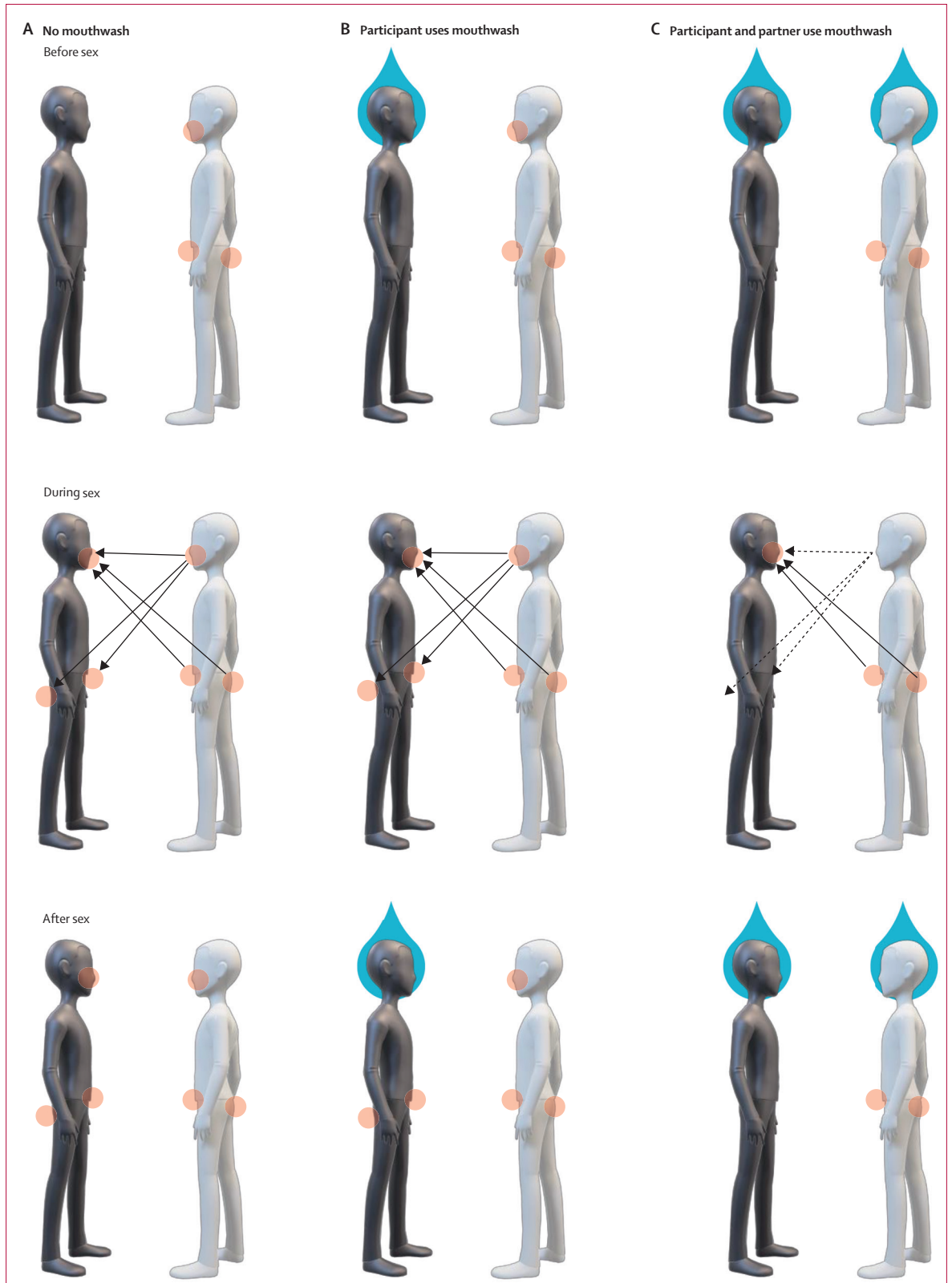
according to currently used European case definitions.[25] The Abbott RealTime *C trachomatis*/*N gonorrhoeae* assay was used for molecular testing on first-void urine, self-collected anorectal swabs and physician-collected oropharyngeal swabs according to the manufacturer's instructions. *N gonorrhoeae* positive samples were confirmed with in-house, real-time PCR.[26]

During the study, we did 15 semistructured interviews at follow-up visit 2. Interviewees were purposively selected by their self-reported acceptability and adherence as reported in the CASIs.

Outcomes

The primary outcome was the combined incidence of all diagnoses of syphilis, plus gonorrhoea, plus chlamydia detected at any anatomical site since the previous study visit. Even though there was more evidence that Listerine could be efficacious in the prevention of gonorrhoea than of syphilis or chlamydia, this composite outcome was chosen on the basis of the fact that the oropharynx plays a role in transmission of each of these STIs. Multisite infections of the same STI were counted only once at each visit. Secondary outcomes were the proportion of participants diagnosed with either oropharyngeal gonorrhoea, chlamydia at any site, or syphilis; adherence to the mouthwash (daily and before and after sex); and safety. Gonorrhoea at any site was not a predefined outcome, and it was added after an unexpectedly low total number of oropharyngeal gonorrhoea infections was observed.

Statistical analysis

On the basis of an estimated 50% reduction in the incidence of gonorrhoea and syphilis, and 30% of chlamydia, a total sample size of 288 participants (144 per allocation arm) was calculated to detect the prespecified effect size at a 0·05 significance level and a power of 90%. The sample size was adjusted for an anticipated 18% dropout rate yielding a final sample size of 352 participants.

No interim analysis was planned in the initial study protocol, but one was necessitated by the COVID-19 pandemic, which affected Belgium from March 2020 onwards. From March 18, all individuals were required to stay at home, and to avoid contact outside their family as much as possible. All non-urgent medical consultations such as PrEP consultations were postponed indefinitely as of March 18, 2020 (appendix p 23). Casual sexual contacts would not be compatible with the national requirements of physical distancing. This change in study context raised significant ethical issues with continuing a study that advocated the use of a mouthwash during casual sexual contacts.[27] For these reasons, recruitment and follow-up were halted on March 18, 2020, and an interim analysis was done by an unblinded statistician on April 23, 2020. At that moment, 343 participants were enrolled, of whom 161 were still in the study. No information on treatment allocation at individual participant level was provided to the study team. The power of the study was calculated retrospectively for the number of participants as of April 23, 2020 and revealed a power of 60% to find the prespecified difference in primary outcome. The interim analysis found that Listerine was not associated with reduced incidence of bacterial STIs, but with an increased incidence in pharyngeal gonorrhoea. A decision was therefore taken to terminate the study prematurely. Recruitment was not resumed after March 18, 2020, and final follow-up was on June 15, 2020.

Continuous characteristics were presented as medians and interquartile ranges and categorical ones as counts and percentages. The primary efficacy analysis was based on a mixed-effects Poisson regression model, with the count of bacterial STIs as the outcome variable, mouthwash (placebo versus Listerine), time on Listerine or placebo and period (period 1 versus period 2) as fixed effects, and a random intercept for each participant. In the final model, time on Listerine or placebo was dropped as it did not improve the model fit. Adjustment for mouthwash adherence was done by inclusion of a binary variable indicating the composite of either greater than 75% adherence to the use of mouthwash daily or before and after sex to the model. The secondary outcomes of the individual STIs (binary variables indicating oropharyngeal gonorrhoea, chlamydia, or syphilis) were analysed using mixed-effects logistic regression models, with mouthwash (placebo versus Listerine) and period (period 1 versus period 2) as fixed effects, and a random intercept for each participant. In an additional analysis that was not prespecified in the protocol, we adjusted all models for mouthwash adherence, the number of casual partners, and condom use. An overview of the composition of all models is provided in appendix (p 8). In all analyses, an all-available-case strategy was followed if data were missing.

Figure 1: **STI transmission routes from or to the oropharynx and potential impact of mouthwash**
Before sex a study participant (left in each panel) is assumed to be free of STIs, as a consequence of screening and treatment of STIs in the oropharynx, anorectum, and urethra at the beginning of each study period. His sexual partners (right in each panel) can be infected with chlamydia, gonorrhoea, or syphilis in the oropharynx, anorectum, or urethra (red dots). During sex (middle row), these STIs can be transmitted to the study participant through oral sex (including kissing, orogenital, and oroanal sex).[23] (A) If neither the study participant, nor his partners use mouthwash, then oral sex can result in STI transmission from his partner's oropharynx to the study participant's oropharynx, anorectum, and urethra. (B) If only the study participant uses a mouthwash before and after sex, then oral sex can result in STI transmission from his partner's oropharynx to the study participant's anorectum and urethra, but not to his oropharynx. (C) If both the study participant and his partner use a mouthwash before and after sex, then all STI transmission by oral sex to the participant's anorectum, urethra, and oropharynx could be prevented. Note that STI transmission to the participant's urethra or anorectum would still be possible by insertive or receptive anal sex (not displayed). STI=sexually transmitted infection.
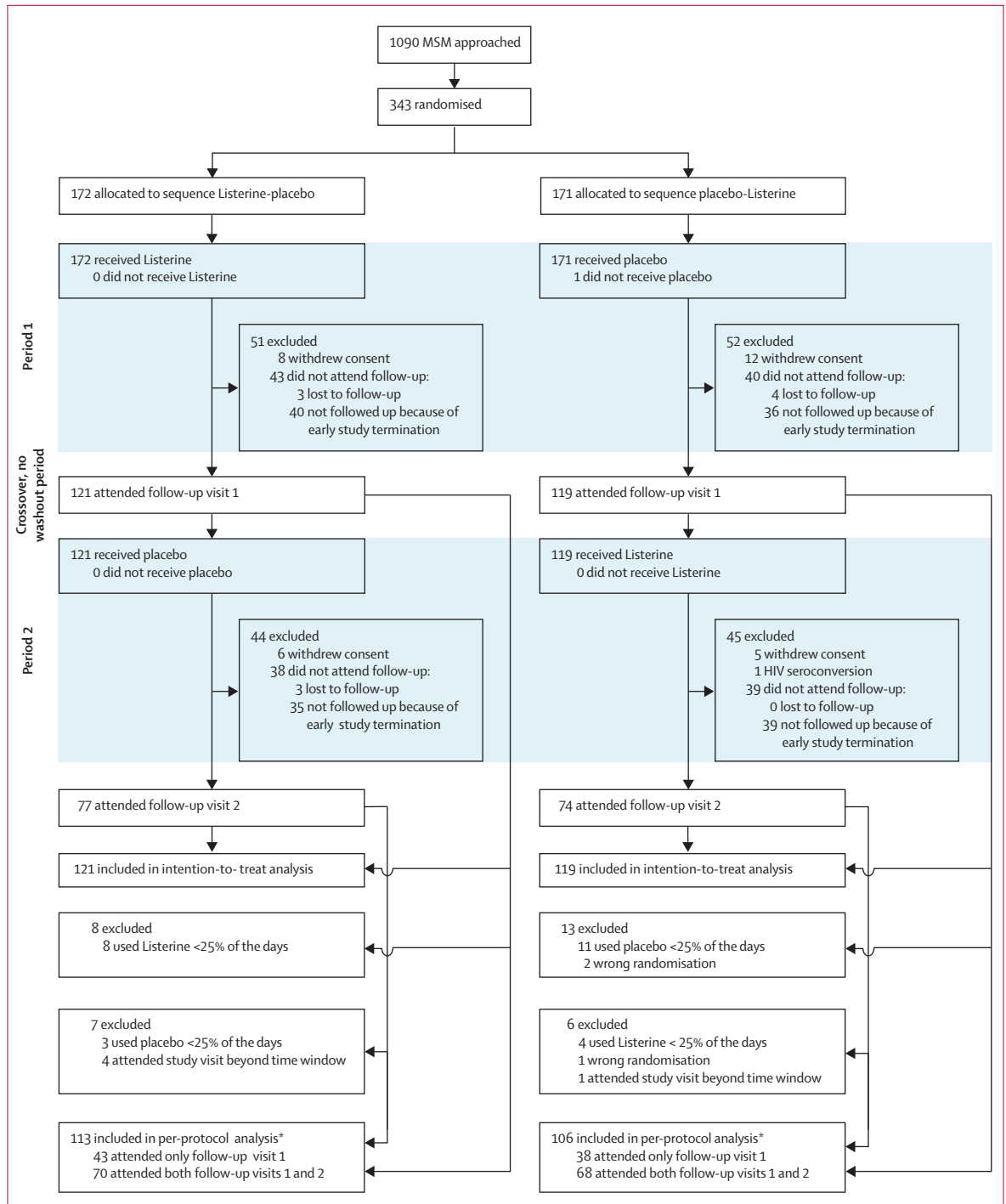
Figure 2: **Trial profile**

*Data from period 1 were included in per-protocol analysis if participants were protocol adherent during study period 1; data from period 2 were included in per-protocol analysis if participants were protocol adherent during study period 2.

The primary analysis used the intention to treat data set; all primary and secondary efficacy endpoints were also analysed by use of a per-protocol approach. Participants who did not comply with the inclusion and exclusion criteria, did not receive the assigned mouthwash, used the mouthwash less than 25% of the days, or performed follow-up visits outside the prespecified time window were excluded from the per-protocol analysis. The low number of observed adverse events did not justify statistical testing between allocation arms.

All analyses were done with R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria), using the package *lme4* for the mixed-effects models. Details of the analyses were documented in the statistical analysis plan before database lock and release of treatment allocation codes for analysis. There was no independent data monitor with access to unblinded data.

Our trial is registered at ClinicalTrials.gov, NCT03881007.

Role of the funding source

The funder had no role in trial design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Of 1090 MSM approached between April 3, 2019, and March 13, 2020, at Institute of Tropical Medicine STI clinic, 343 were enrolled: 172 were assigned to the Listerine-placebo arm and 171 to the placebo-Listerine arm. Exclusion was mainly due to non-eligibility (eg, no STI history in the previous 24 months), not being interested, or frequent travelling (for which carrying of the mouthwash was perceived to be impractical). One participant did not receive the allocated mouthwash (figure 2). 103 (30·0%) of 343 participants did not attend follow-up visit 1, and 192 (56·0%) did not attend follow-up visit 2: reasons were premature termination of the study due to COVID-19 in 150 (78·1%) of 192 participants and withdrawal of consent in 31 (16·1%). Numbers of non-attendance were fairly equally distributed over allocation arms and study periods. Primary outcome data were available for 121 (70·3%) of 172 participants in the Listerine-placebo arm and for 119 (69·6%) of 171 participants in the placebo-Listerine arm.

Baseline characteristics (table 1) were well balanced between the two allocation arms. Median age was 40 years (IQR 32-47·5). 217 (63·3%) of 343 participants had history of gonorrhoea in the 24 months before enrolment, 171 (49·9%) had history of chlamydia, and 141 (41·1%) had history of syphilis. Participants had a median of one (IQR 0–1) main partner, and ten (IQR 5–20) casual partners in the 3 months before enrolment. Participants reported a higher number of casual partners during the first study period than at baseline in both allocation arms (table 2). This number dropped back to baseline during the second study period in the Listerine-placebo arm, but not in the placebo-Listerine arm.

At baseline, 112 (33·8%) of 331 participants used condoms with less than 25% and 86 (26·0%) with more than 75% of their casual partners (table 1, appendix p 4). There was little change in condom use over the course of the study (table 2). About two-thirds of participants used mouthwash on more than 75% of the days (table 2). More than a third of participants used mouthwash

	Listerine-placebo arm (n=172)	Placebo-Listerine arm (n=171)
Age, years	39 (31–48)	40 (34–47)
STI history in past 24 months		
Chlamydia	89 (51·7%)	82 (48·0%)
Gonorrhoea	100 (58·1%)	117 (68·4%)
Syphilis	75 (43·6%)	66 (38·6%)
Behavioural characteristics		
Main partners	1 (0–2)	1 (0–1)
Casual partners in past 3 months	10 (5–19)	10 (5–20)
Used condoms with <25% of casual partners	50/167 (29·9%)	62/164 (37·8%)
Used condoms with 25–49% of casual partners	42/167 (25·1%)	34/164 (20·7%)
Used condoms with 50–74% of casual partners	31/167 (18·6%)	26/164 (15·9%)
Used condoms with ≥75% of casual partners	44/167 (26·3%)	42/164 (25·6%)
Mouthwash use before participation		
Used a mouthwash in the past month	80/170 (47·1%)	73/167 (43·7%)
Ever used a mouthwash with idea to prevent STIs	18/170 (10·6%)	17/167 (10·2%)

Data are median (IQR), n (%), n/N (%); Listerine=Listerine Cool Mint; STI=sexually transmitted infection.

Table 1: Baseline participant characteristics

before sex with more than 75% of casual partners, and almost a third used it after sex with more than 75% of casual partners. Adherence was similar in each allocation arm and in every study period (appendix p 4, 5). The self-reported behavioural data obtained from the weekly diaries were similar to those obtained from the 3-monthly CASIs (appendix p 9). More specifically, reported condom use was very similar, whereas reported adherence to the mouthwash was slightly lower in the diaries compared with the CASIs.

In the ITT population, the STI incidence rate in the Listerine-placebo arm was 140·4 per 100 person-years during the Listerine period, and 102·6 per 100 person-years during the placebo period. In the placebo-Listerine arm, the STI incidence rate was 133·9 per 100 person-years during the placebo period, and 147·5 per 100 person-years during the Listerine period. The incidence of bacterial STIs did not correlate significantly with the use of Listerine compared with placebo (IRR 1·17, 95% CI 0·84–1·64; table 3, appendix p 10). Adjustment for adherence did not change this conclusion, whether based on data from the CASIs (IRR 1·21, 95% CI 0·85–1·71) or from the weekly diaries (IRR 1·19, 95% CI 0·84–1·68). Likewise, similar effect estimates were found after additional adjustment for risk behaviour (appendix p 10).

A significantly higher proportion of participants had oropharyngeal gonorrhoea when using Listerine than when using placebo (table 3). Listerine use was not

	Listerine-placebo arm		Placebo-Listerine arm	
	First period Listerine (n= 121)	Second period placebo (n= 77)	First period placebo (n= 119)	Second period Listerine (n= 74)
Duration of the study period (days)	98 (94—106)	98 (91—112)	98 (92–106)	98 (91—107)
Behavioural characteristics				
Main partners	1 (0–1)	0 (0–1)§	1 (0–1)	1 (0–1)*
Casual partners in past 3 months	14·5 (7–25·75)	10 (4–25)	15 (6–26)	14 (5–25)
Used condoms with <25% of casual partners in past 3 months	39/90 (34·2%)	25/70 (35·7%)	53/114 (46·5%)	36/70 (51·4%)
Used condoms with 25–49% of casual partners in past 3 months	26/90 (22·8·%)	24/70 (34·3%)	22/114 (19·3%)	14/70 (20·0%)
Used condoms with 50–74% of casual partners in past 3 months	27/90 (23·7%)	11/70 (15·7%)	18/114 (15·8%)	9/70 (12·9%)
Used condoms with ≥75% of casual partners in past 3 months	22/90 (19·3%)	10/70 (14·3%)	21/114 (18·4%)	11/70 (15·7%)
Mouthwash adherence				
Participants used mouthwash daily ≥75% of the time	80/116 (69·0%)	46/71 (64·8%)	79/116 (68·1%)	47/74 (63·5%)
Participants used mouthwash before sex with ≥75% of casual partners	49/114 (43·0%)	24/70 (34·3%)	41/114 (36·0%)	25/70 (35·7%)
Participants used mouthwash after sex with ≥75% of casual partners	45/114 (39·5%)	21/70 (30·0%)	34/114 (29·8%)	21/70 (30·0%)
Partners asked to use study mouthwash throughout study	..	4 (1–8·5)	..	4 (2–7·3)
Partners used mouthwash before sex throughout the study	..	2 (1–5·5)	..	3 (0·8–7)

Data are median (IQR), n/N (%). Listerine=Listerine Cool Mint. *Mann-Whitney U test p value=0·043.

***Table 2:* Behavioural characteristics and mouthwash adherence during study**

	Listerine-placebo arm		Placebo-Listerine arm		Effect measure (95% CI)			
	First period Listerine (n=121)	Second period placebo (n=77)	First period placebo (n=119)	Second period Listerine (n=74)	Crude	p value	Adjusted for mouthwash adherence*	p value
Any† bacterial STI, n‡ (IR)	47 (140·4)	22 (102·6)	44 (133·9)	30 (147·5)	IRR 1·17 (0·84–1·64)	0·359	aIRR 1·19 (0·85–1·69)	0·317
Oropharyngeal gonorrhoea, n§ (%)	8 (6·6%)	0 (0·0%)	2 (1·7%)	3 (4·1%)	OR 5·78 (1·52–136·56)	0·024	..	..
Chlamydia, n§ (%)	19 (15·7%)	10 (13·0%)	18 (15·1%)	11 (14·9%)	OR 1·09 (0·61–1·95)	0·774	..	..
Syphilis, n§ (%)	2 (1·7%)	4 (5·2%)	6 (5·0%)	4 (5·4%)	OR 0·59 (0·20–1·63)	0·323	..	..

Data are n (%), IRR/OR (95% CI). IR=incidence rate per 100 person-years; IRR=incidence rate ratio; Listerine=Listerine Cool Mint; OR=odds ratio. *Incidence rate ratio for STI incidence while using Listerine versus placebo, adjusted for adherence, defined as the composite of either >75% adherence to daily, before sex, or after sex use of mouthwash. †Composite of gonorrhoea, chlamydia, and syphilis at any anatomical site. ‡Number of infections. §Number of participants with this diagnosis.

***Table 3:* Outcomes in the intention-to-treat population**

significantly associated with the total proportion of gonorrhoea cases at any anatomical site (OR 1·48, 95%CI 0·81–2·83; appendix p 10). There was no significant association between Listerine versus placebo use and the proportion of chlamydia and syphilis cases (table 3). All per-protocol analyses showed similar results to the ITT analyses (appendix p 11).

The participants' attitudes towards the intervention deteriorated during the study. Although 323 (95·6%) of 338 participants had positive attitudes towards use of the study mouthwash at baseline, the proportion of participants with positive attitudes decreased to 121 (85·2%) of 142 by follow-up visit 2 (appendix p 6–7). At baseline, only 44 (13·1%) of 335 participants expected that using the mouthwash would be burdensome, whereas by follow-up visit 2, 50 (45·5%) of 110 found that using the mouthwash had been burdensome. Interviewees typically expressed positive attitudes towards the mouthwash for the prevention of STIs, on the understanding that it would prove to be effective, as the following interviewee explains:

"if that [mouthwash] helps, I'd like that very much, [...] yeah, like I said before, the more ways you can avoid [STIs], the better hey, the safer that you feel."

(Interviewee 14, 43 years old)

Interviewees explained that it was much easier to use daily, than before and after sex. Proposing its use to sex partners was considered particularly difficult:

"Asking one's partner [to mouthwash] is the most difficult, definitely if it's in a group context. Uhm, before and after kind of depends, because sometimes you suddenly have this date and you have to move [to the place where you will have sex]. Huh your mouth is not rinsed, and there is no mouthwash available [at this place] and a sexual contact is quite impulsive. And then the easiest [daily use] is of course yes, it's like brushing your teeth ... if it's right in front of your nose, yes, then, it should basically succeed."

(Interviewee 15, 50 years old)

Among 139 respondents at follow-up visit 2, 120 (86·3%) asked at least one partner throughout the study to use the mouthwash. Overall, participants asked a median of four (IQR 1–7·5) partners to use the mouthwash, while a median of two (IQR 1–6) of their partners actually used the study mouthwash (table 2).

Numbers of adverse events (self-reported teeth problems, physician-assessed tooth discolouration, or caries) were similar while using Listerine and placebo and were not significantly higher than at baseline (table 4). Four serious adverse events were reported: one HIV-infection, one severe depression, one Ludwig's angina, and one testicular carcinoma. None of these were considered to be related to use of the mouthwash (table 4).

Discussion

This randomised controlled trial did not find that Listerine used daily and before and after sex reduces incidence of bacterial STIs in a cohort of MSM taking PrEP. This finding did not change after adjusting for adherence to the mouthwash or when considering the incidence of chlamydia, syphilis, or oropharyngeal gonorrhoea separately. This finding was in line with the results of the OMEGA trial[28] (published with our paper in *The Lancet Infectious Diseases*), which showed that men who used Listerine Zero did not have lower incidence of oropharyngeal gonorrhoea compared with those who used Biotène.

Although based on small numbers, our study found that a higher number of oropharyngeal gonorrhoea infections were detected in each Listerine period than in the placebo periods. This raises the possibility that Listerine could do more harm than good. Hypothetically, damage to the oropharyngeal mucosa or microbiome might have enhanced, rather than reduced, the risk of gonorrhoea transmission from or to the oropharynx in this study. In vitro and clinical studies have found

	Events reported at baseline	Events reported during the Listerine period	Events reported during the placebo period
Ludwig's angina	..	1	..
Severe depression	..	..	1
Testicular carcinoma	..	..	1
HIV infection	..	..	1
Teeth or mouth problems in past 3 months*	54/338 (16·0%)	23/189 (12·2%)	20/187 (10·7%)
Tooth discolouration†	44/343 (12·8%)	26/195 (13·3%)	18/196 (9·2%)
Caries†	28/343 (8·2%)	19/195 (9·7%)	12/196 (6·1%)

Data are counts, or n/N (%). *Self-reported on 3-monthly computer-assisted self-interview. †As assessed by study physician.

***Table 4:* Safety outcomes**

persuasive evidence that various commensal *Neisseria* species can inhibit the growth or carriage of *N gonorrhoeae* or *Neisseria meningitidis*.[29,30] If Listerine were to eradicate these protective *Neisseria* species, then it might paradoxically increase the risk for gonococcal and meningococcal infections. This finding could be interpreted as in keeping with results of studies showing that certain vaginal microbicides increased the risk for HIV and genital ulcers.[31]

The main limitation of this study was the premature termination due to the COVID-19 pandemic, which reduced study power from 90% to 60% thereby creating a risk for a false negative result. The observation of a larger incidence of bacterial STIs in the Listerine than the placebo periods (including a statistically significant increase in pharyngeal gonorrhoea), and the fact that the lower limit of the 95% CI for the primary outcome analysis was 0·84, suggest that even if Listerine were effective, its effect size would be small. We consider it unlikely that study continuation would have changed the overall conclusions of this study. A second limitation is the fact that the primary outcome of our study was not validated in previous studies. The effect size of the primary outcome might have been diluted by the inclusion of the combined incidence of syphilis, gonorrhoea, and chlamydia at all anatomical sites. Although there is some in vitro[14,15] as well as in vivo[14] evidence to expect an effect of Listerine on gonorrhoea incidence, for chlamydia and syphilis the in vitro evidence is more sparse,[16,17] and in vivo data are absent. In addition, the inclusion of STI incidence in the urogenital and anorectal site made the primary outcome heavily dependent on mouthwash use by sexual partners. A third limitation was that the placebo mouthwash we used in this study was not identical in taste and appearance to Listerine.[15] We cannot exclude that any unintended participant unblinding occurred after crossover, and that

participants who felt protected by the use of Listerine were more adherent to that mouthwash, intentionally exhibited more pronounced sexual risk behaviour, and were more frequently exposed to STIs during that period compared with the placebo period. Participant unblinding could explain the observed differences in the second versus the first study period of mainly the Listerine-placebo arm when it comes to the number of casual partners, but not condom or mouthwash use. Even though additional analyses showed no substantial change in effect size after adjustment of all statistical models for mouthwash adherence, number of sexual partners, or condom use (appendix p 9), residual confounding due to participant unblinding could have biased our study toward the null hypothesis.

This study also had several strengths. We evaluated a novel STI prevention method in a real-world setting, where the study population was representative of a typical PrEP cohort.[1] Our study design was complementary to that of the OMEGA study. The OMEGA study assessed the impact of a daily mouthwash on the incidence of gonorrhoea. Arguably the use of a mouthwash before sex by one's partner is crucial to maximise the efficacy of the intervention (argument outlined in figure 1). This provided the rationale for our study design, in which we asked all participants to invite their partners to use the mouthwash before and after sex. On the basis of our results, asking a partner to mouthwash before sex would only seem to be feasible in a small proportion of MSM. We also went to substantial effort to develop a placebo with as small a bacteriostatic effect on *N gonorrhoeae* as possible.[15]

In addition, the crossover design allowed us to include fewer participants than would have been the case in a parallel design study. As mentioned above, it is unlikely that any substantial carryover effect occurred. All regression models accounted for the paired nature of the design and for the period effect (ie, the possibility that a participant's outcome differs between two subsequent study periods, related to factors independent of the intervention). We cannot exclude the possibility that STI diagnoses led to discontinuation of study participation in between study visits. However, the low number of participants that were lost to follow-up after STI diagnoses makes it improbable that selective dropout had a substantial impact on the data.

We are planning to assess the impact of Listerine on the oropharyngeal microbiome and resistome, including populations of commensal *Neisseria* species, in a subgroup of the participants of this trial. Listerine-type mouthwashes might eradicate beneficial commensal *Neisseria* species and other bacteria that might in-turn negate any beneficial effect on the carriage of pathogenic *Neisseria* species. Other mouthwashes might not have this deleterious effect. One option would be to use the methylated-DNA extracts from commensal *Neisseria* species that can be selectively toxic to the pathogenic *Neisseria* species.[29,32]

In conclusion, the current study does not support the use of Listerine to prevent the acquisition of gonorrhoea, chlamydia, and syphilis in MSM. As such, the study provides useful guidance to those MSM who currently report using various mouthwashes for this purpose.[18] Although we cannot extrapolate to other populations such as sex workers, we consider it unlikely that Listerine would prevent STIs in other high-risk populations. Finally, our results do not mean that a mouthwash would be ineffective as treatment for isolated oropharyngeal gonorrhoea. Two clinical trials are currently ongoing to assess if a mouthwash can be used as an antibiotic-sparing way to treat oropharyngeal gonorrhoea (ACTRN12618001380280 and EudraCT 2019-003604-12).

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4.3 A chlorhexidine mouthwash cannot eradicate pharyngeal gonorrhoea.

Van Dijck C, Tsoumanis A, De Hondt A, Cuylaerts V, Laumen J, Van Herrewege Y, et al. Chlorhexidine Mouthwash Fails to Eradicate Oropharyngeal Gonorrhea in a Clinical Pilot Trial (MoNg). Sex Transm Dis. 2022;49(2):e38–41.

Included in this thesis is the final peer reviewed version of the manuscript. The article was published in Sexually Transmitted Diseases:

Van Dijck C, Tsoumanis A, De Hondt A, et al. Chlorhexidine Mouthwash Fails to Eradicate Oropharyngeal Gonorrhea in a Clinical Pilot Trial (MoNg). Sex Transm Dis 2022;49:e38–41. doi:10.1097/olq.0000000000001515

Chlorhexidine mouthwash fails to eradicate oropharyngeal gonorrhea in a clinical pilot trial (MoNg)

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Abstract

This single-arm open-label pilot trial in Antwerp, Belgium was ended early in accordance with the protocol since twice daily gargling with chlorhexidine 0.2% for six days failed to eradicate *Neisseria gonorrhoeae* from the oropharynx of asymptomatic men who have sex with men (n=3, efficacy of 0%, 95% CI $0 - 56.1\%$).

Introduction

The threat of multidrug resistant *N. gonorrhoeae* (Ng) has motivated the search for alternative, antibiotic-sparing treatment options against this pathogen. The oropharynx gained particular interest as the source of emergence and spreading of resistant Ng due to factors such as poor antimicrobial penetration into the pharyngeal mucosa[1–3]. As a consequence, several authors have proposed that antiseptic mouthwashes may be used to prevent or treat oropharyngeal gonorrhea[1,4,5].

In vitro studies have established that Ng is highly susceptible to killing by mouthwashes such as Listerine®[6,7] and chlorhexidine[8–10], and a randomized controlled trial in men with culture-positive oropharyngeal gonorrhea found that Ng could not be cultured in a significant proportion of men five minutes after a single gargling session with Listerine®[7]. Despite these initial encouraging results, three randomized clinical trials have found that Listerine® failed to prevent (PReGo[4] and OMEGA1[11]) or treat (OMEGA2[5]) oropharyngeal gonorrhea.

While experiments in mice have suggested that chlorhexidine might effectively prevent genital Ng infection[12], data for preventive or therapeutic efficacy on human oropharyngeal gonorrhea are lacking. Nevertheless, chlorhexidine's strong *in vitro* bactericidal effect suggests that it could be more effective than Listerine® in killing Ng in the oropharynx[6,8,13,14].

The aim of this single arm, open-label pilot study was therefore to assess whether a chlorhexidine mouthwash could eradicate Ng from the oropharynx of asymptomatic men.

Materials and Methods

Subjects

Participants were recruited at the STI clinic of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium. Eligible candidates were adult men who had sex with at least one male partner in the last 12 months who returned to the clinic for treatment of asymptomatic oropharyngeal gonorrhea. This diagnosis was made by nucleic acid amplification test (NAAT) on an oropharyngeal swab taken during routine STI screening, maximum 14 days prior to enrollment. Excluded were men with signs or symptoms of gonorrhea or any other STI, men who needed to take antibiotics for any indication in the previous 14 days or at the time of inclusion and men with a contra-indication for chlorhexidine/Corsodyl®. Participants had to abstain from sex, kissing and the use of non-study mouthwashes throughout the study.

Ethics approval was provided by the Belgian Federal Agency for Medicines and Health Products (CTR Pilot 132) on February 11, 2020. Participants provided written informed consent before trial enrollment. The trial protocol was registered at the European Clinical Trial Register (EudraCT 2019-003604-12). The full protocol is available as a supplement to this manuscript.

Study design & study procedures

This was an open-label single arm clinical trial. Participants had to attend six study visits: day 1 (baseline), day 2, day 3, day 5, day 7 and day 14. At baseline,

oropharyngeal swabs were taken for Ng culture and NAAT to confirm positivity at the start of the study. Anorectal and urine samples were taken to exclude concomitant rectal and/or urethral chlamydia and gonorrhea if these sites had not been screened in the previous 7 days. The chlorhexidine mouthwash-gargle protocol was started immediately after sampling without awaiting the results of these tests as they typically have a turnaround time of multiple days in our clinic. Participants received two 200 mL bottles of chlorhexidine 2 mg/mL (Corsodyl®, GlaxoSmithKline Consumer Healthcare s.a., Avenue Pascal 2/4-6, 1300 Wavre, Belgium). They were asked to rinse and gargle with 20 mL of the mouthwash for 60 seconds and to repeat this after a 15-minute interval. Fifteen minutes after the second gargle, another oropharyngeal swab was taken for Ng culture. Participants had to continue using the mouthwash at home for 60 seconds once every morning and every evening for the next five days, *i.e.* twelve gargle sessions in total. At every study visit, oropharyngeal swabs were taken for Ng culture and NAAT. All oropharyngeal samples were taken by a study physician who rubbed both tonsillar pillars and the posterior pharyngeal wall with one flocked swab. Participants reported their adherence to the mouthwash protocol, sexual behavior and antibiotic use in an online questionnaire on days 7 and 14.

Participants could exit the trial before completion in case their baseline Ng culture turned out to be negative or if they needed antibiotics for another indication (e.g. anorectal/urethral chlamydia detected in a sample taken at baseline). Participants who exited the trial before documentation of a negative oropharyngeal Ng culture and NAAT were contacted to receive antibiotics as recommended per the current treatment guidelines for gonorrhea[15].

Laboratory procedures
Oropharyngeal swabs underwent Ng NAAT by Abbott Real-Time CT/NG assay (Abbott m2000sp and m2000rt, Abbott Molecular Inc. Des Plaines, IL, USA). Ng positive samples were confirmed with an in-house PCR based on a previously described primer set[16]. The Abbott delta cycle (DC) value of confirmed Ng positive samples was used as an indicator of Ng bacterial load. The Abbott delta cycle (DC) value is the difference in cycle threshold value between the sample and a cutoff control. The cutoff control is a reference sample with Ng DNA concentration at the

lower limit of detection and thus has a high cycle threshold value. Consequently, samples with a high bacterial load will have high DC values and vice versa.

Oropharyngeal swabs were cultured on blood agar plates and incubated at 35°C (±2°C) and 5-7% CO_2. Suspected colonies were sub-cultured on blood agar and oxidase-positive gram-negative diplococci underwent confirmation by the above-mentioned in-house PCR[16]. Etests were used to determine minimum inhibitory concentrations (MICs) for ceftriaxone, azithromycin and ciprofloxacin on one Ng colony isolated from each participant at baseline and on day 7.

Outcomes

The primary outcome was Ng culture (positive/negative) at day 7. The primary analysis population included all participants with a positive Ng culture at baseline.

The secondary outcome was the evolution of Ng bacterial load (represented by Abbott DC value) between day 1 and day 7. The secondary analysis population included all study participants with a positive Ng NAAT at baseline.

Samples taken before study enrollment were not taken into account for the primary or secondary analysis.

Statistical analyses and interim analysis

Target sample size was 100 participants of whom at least 20 were expected to be culture positive for Ng at baseline and complete the trial. Being a pilot study, the intention was to continue enrollment only if an interim analysis showed at least 80% efficacy in the first five participants of the trial.

The primary outcome was expressed in counts and proportions with two-sided Wilson's confidence intervals. The secondary outcome was analyzed using a mixed effects linear regression model that included participant number as a random effect. Statistical analyses were done using R, version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Trial enrollment took place between October 2020 and March 2021. Ten men with asymptomatic oropharyngeal gonorrhea were referred to the study team, of whom six met the inclusion criteria, were enrolled and commenced twice daily mouthwash/gargling (Figure 1).

Figure 1: Study flowchart


```
┌──────────────────────────────────┐
│ MSM with oropharyngeal gonorrhea │
│ referred to study team and assessed │
│ for eligibility (n=10)           │
└──────────────────────────────────┘
          │
          │         ┌──────────────────────────────────┐
          ├────────▶│ Excluded (n=4)                   │
          │         │    Declined participation (n=3)  │
          │         │    Unable to attend study visits (n=1) │
          │         └──────────────────────────────────┘
          ▼
┌──────────────────────────────────┐
│ Enrolled and received            │
│ chlorhexidine mouthwash (n=6)    │
└──────────────────────────────────┘
          │
          │         ┌────────────────────────────────────────────────┐
          ├────────▶│ Exited trial before day 7 (n=3)                │
          │         │   Negative Ng NAAT and culture at baseline (n=1) │
          │         │   Positive Ng NAAT but negative culture at baseline (n=2)* │
          │         └────────────────────────────────────────────────┘
          ▼
┌──────────────────────────────────┐
│ Attended day 7 visit and included in │
│ primary analysis (n=3)*          │
└──────────────────────────────────┘
          │
          │         ┌────────────────────────────────────────────────┐
          ├────────▶│ Exited trial before day 14 (n=1)               │
          │         │   Needed antibiotic for treatment of urethral chlamydia (n=1) │
          │         └────────────────────────────────────────────────┘
          ▼
┌──────────────────────────────────┐
│ Attended day 14 visit (n=2)      │
└──────────────────────────────────┘
```

* included in secondary analysis (n=5)

Among the three participants with a positive Ng NAAT and culture at baseline, none had a negative Ng culture by day 7 (0%, 95% CI 0 – 56.1%). Even if two more participants completed the study and chlorhexidine successfully eradicated Ng from their oropharynx, the upper limit of the 95% confidence interval would fall below 80% (efficacy of 40%, 95% CI 11.8 – 76.9%). The trial was ended in

accordance with the study protocol since it would prove impossible to attain 80% efficacy based on the first five trial participants.

Median age of the participants was 35.5 (IQR 31.25 - 40.5) years. All participants indicated that they complied with all gargle sessions and that they did not tongue kiss or have sexual intercourse during trial participation. Minimum inhibitory concentrations of Ng isolates were similar at baseline and day 7 (Supplementary Table S1).

A linear mixed effects regression model estimated that there was little change in Abbott DC value over time (increase of 0.03 per day; 95% CI -0.29 – 0.35, Figure 2).

No adverse events were reported during the study.

Figure 2: Evolution of the bacterial load* of Neisseria gonorrhoeae (Ng) in the oropharynx of men who have sex with men using a chlorhexidine 0.2% mouthwash twice daily for 6 days (day 1 – 6).
* represented by the Delta Cycle (DC) value of Abbott Nucleic Acid Amplification Test (NAAT)
The vertical line indicates the end of the chlorhexidine mouthwash-gargle protocol
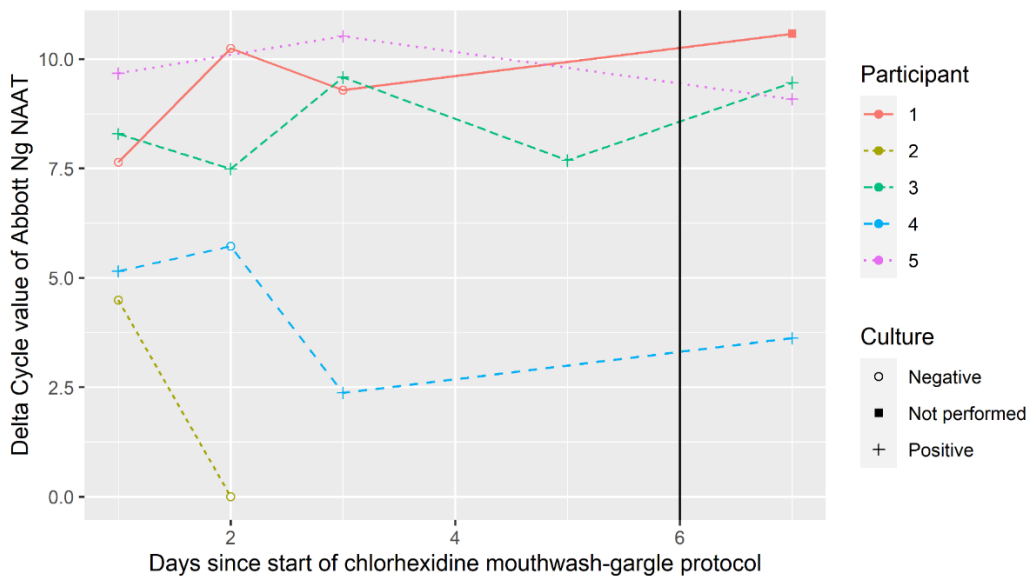
Discussion

Twice daily gargling with chlorhexidine 0.2% for six days failed to eradicate Ng from the oropharynx in three culture positive trial participants. A significant weakness of this study was the small amount of available data. The upper bound of the 95% confidence interval of the primary outcome was however 56.1%, which is far below the efficacy of contemporary antibiotic therapies. A recent systematic review, for example, reported an overall efficacy of 100% (95% CI 98.2 – 100.0%) for a range of antibiotics to achieve culture conversion in oropharyngeal gonorrhea[3]. We consider it unlikely that a larger sample size would produce a culture conversion rate close to that produced by antibiotics. Further corroborating evidence of the lack of efficacy of chlorhexidine comes from the fact that Abbott DC values did not decrease over time. Finally, as noted above, a trial of Listerine® mouthwash had similarly negative results[5].

Several factors may explain the failure of antiseptic mouthwashes to eradicate Ng from the oropharynx. First, a mouthwash/gargle (and even an oral spray[5,17]) may not be able to reach the target region in the oropharynx where Ng resides. This region is not well defined, but includes at least both tonsillar pillars and the posterior oropharyngeal wall[18]. Moreover, Ng may mainly reside intracellularly, where it could be protected from the activity of an antiseptic agent but not from an antibiotic. This concept is supported by microscopic studies which confirmed the intracellular location of Ng in tonsillectomy specimens[19] and by the high efficacy of ceftriaxone against susceptible oropharyngeal gonorrhea despite its limited bio-availability in saliva (saliva:plasma ratio below 0.004)[2]. A further possibility is that Ng may form a protective biofilm in the oropharynx as has been observed in the cervix[20]. In addition, even though chlorhexidine's substantivity is about 7 hours in saliva[21], its bactericidal effect may be attenuated by food[10,21] or may be too short-lived for effective eradication of Ng[22]. Last, we did not check chlorhexidine susceptibility of Ng isolates in this study, but considered resistance unlikely as Ng was highly susceptible to chlorhexidine in previous studies[8–10].

The use of chlorhexidine is also not without risks. Evidence is mounting that chlorhexidine can have adverse effects on blood pressure[23] and oral microbiota

including a shift towards chlorhexidine resistant bacteria[22]. Based on these findings we conclude that the available evidence argues strongly against the further consideration of mouthwashes such as chlorhexidine in the treatment of oropharyngeal gonorrhea.

Acknowledgements

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Chapter 5   **Discussion**

In view of the range of antimicrobials *N. gonorrhoeae* has evolved resistance against, there is little doubt that the bacterium will also develop AMR against future, novel antimicrobials.[1] Questions remain on how the risk of further emergence of AMR in *N. gonorrhoeae* can be mitigated. As mentioned in the introduction, MSM using PrEP and their oropharyngeal microbiota are at the centre of the discussion, because of their key role in the emergence and spread of resistant gonorrhoea.[2] In this thesis, we assessed how the oral microbiota and resistome of MSM using PrEP differs from the general population and how these differences can be explained (chapter 3). Next, we evaluated whether mouthwashes could be used as antibiotic-sparing options to prevent STIs or treat oropharyngeal gonorrhoea (chapter 4).

5.1 AMR in oropharyngeal microbiota of MSM using PrEP

In chapter 3, we examined the prevalence of AMR and composition of the resistome of the oropharyngeal microbiota of MSM using PrEP. We hypothesized that MSM using PrEP are colonized with more resistant commensal *Neisseria spp.* in their oropharynx than the general population. We confirmed this hypothesis in chapter 3.1., and we also showed that recent antimicrobial exposure among MSM could not fully explain the observed differences in susceptibility of commensal *Neisseria spp.* between MSM and the general population. Next, we evaluated if the oropharyngeal resistome of MSM using PrEP had a higher abundance of AMR genes, as compared to the general population (chapter 3.2). Again, we confirmed this hypothesis. We found that the resistome in MSM using PrEP was enriched with genes that confer resistance to several antimicrobial classes, independent of recent antimicrobial exposure. These findings suggest that there may be an increased risk of horizontal gene transfer of AMR genes towards pathogens such as *N. gonorrhoeae* in MSM using PrEP, and that antimicrobial consumption of the general MSM population is of more importance than individual antimicrobial consumption.[3]

Besides these important findings, in chapter 3.3 we also showed that couples share more similar commensal *Neisseria spp.* than unrelated individuals. These results add to the evidence that commensal *Neisseria spp.* are transmitted between individuals, which may explain some of the population-level effect of antimicrobial consumption on AMR.

Taken together, chapter 3 of this thesis conveys an important warning. Frequent and repeated intake of antimicrobials in a population like MSM using PrEP may have an important long-term impact on their oropharyngeal microbiome, which extends beyond the individual level if commensal AMR is shared between individuals. Incoming infections with *N. gonorrhoea* and other pathogens in MSM using PrEP are more likely to enter an environment that is inhabited by resistant commensal bacteria. This may increase the risk that *N. gonorrhoeae* acquires new AMR through transformation, and highlights the need to rationalise antimicrobial use in the MSM-PrEP population. The use of antimicrobials in the context of prevention, diagnosis or screening of STIs should be limited to indications for which the evidence base includes the unintended consequences of antimicrobial use on the individual and population level. In addition, there is a high need of alternative, non-AMR inducing options to prevent and treat STIs, as discussed in chapter 4.

5.2 Antibiotic-sparing options to prevent or treat bacterial STIs

In chapter 4, we set out to evaluate mouthwashes as antibiotic-sparing options to prevent or treat bacterial STIs in MSM using PrEP. We first developed a mouthwash with minimal anti-gonococcal activity, a challenging task in view of the antimicrobial properties of several food dyes.[4] We then hypothesised that an antiseptic mouthwash (Listerine Cool Mint®) can reduce the incidence of bacterial STIs in PrEP users. Unfortunately, we (and others[5]) could not confirm this hypothesis. Similarly, the hypothesis that a chlorhexidine mouthwash can eradicate pharyngeal gonorrhoea was refuted by ourselves and the research team of Chow et al.[6]

Some authors argue that topical antiseptics may still have a role to play in the prevention of gonorrhoea transmission, because of several reasons. Current studies may, for example, have been underpowered to detect a reduction in oropharyngeal gonorrhoea incidence,[7] oral sprays might more effectively reach the tonsils and posterior pharyngeal wall to kill gonococci colonizing those surfaces compared to mouthwashes,[8] or mouthwashes may still have the potential to reduce the transmission potential from an already infected partner towards others.[5,9,10] Before deploying new studies with topical antiseptics for STI prevention or treatment, we would need to expand our knowledge about their effects on the oropharyngeal mucosa, microbiome and resistome,[4] and the natural history of oropharyngeal gonorrhoea, including the intracellular life of *N. gonorrhoeae* in neutrophils.[11] There is another important limitation to the use of mouthwashes in the context of sex: proposing a mouthwash to one's partners is not sexy.[10]

5.3 Strengths and limitations

The strengths and limitations specific to each study were mentioned in their respective chapters. An important strength of this thesis is the prospective setup of most included studies. This allowed for a more unbiased and complete data collection than would have been the case in a retrospective study. The two studies of the oropharyngeal microbiota and resistome of MSM using PrEP in chapter 3 were innovative, for example because they compared findings in MSM with another population (which had not been done before), or because they applied techniques that had not been used for that purpose in this population yet (shotgun metagenomic sequencing). Similarly, the two clinical trials in chapter 4 were novel: at the time they were set up and executed, no clinical trial had ever reported on the effectiveness of a mouthwash to prevent or treat STIs. In the meanwhile, two studies in Australia, published in 2020, have confirmed our findings that mouthwashes are ineffective in preventing or treating oropharyngeal gonorrhoea among men.[5,6] This indicates that our findings were robust as they could be reproduced in a different region of the world using somewhat different methodologies. Nevertheless, it should be borne in mind that all studies except for those in chapter 3.3 and 4.1 were performed in a single STI clinic in a particular

subset of high-risk MSM. On the one hand, this single-centre setup has the advantage of homogeneity in study procedures and data collection. On the other hand, observations such as the differences between the resistome of PrEP users and the general population may be more or less pronounced in settings with lower or higher levels of antimicrobial consumption in the general population, respectively. In addition, the MSM included in our studies were selected among the PrEP-users at highest risk for STIs, and their sexual behaviour and resistome may not be representative for all MSM. While PrEP-cohorts in western Europe are a highly-counselled and rather homogeneous group in terms of sexual behaviour,[12] MSM who do not use PrEP constitute a very heterogeneous group which includes MSM with HIV, MSM without HIV who are at high risk of STIs but not engaged in care for any reason, and MSM at low risk of STIs. We do not expect any major differences in effectiveness of mouthwashes against STIs in other populations besides those studied, but there could be differences in resistome with less AMR being expected in MSM with few partners, or – potentially – in high-risk MSM not taking part in an STI screening program. Another important limitation of the surveys in chapter 3 is that they do not quantify the risk that commensal AMR confers to AMR in *N. gonorrhoeae*. Future genomic and experimental studies will need to establish to what extent AMR genes in the resistome of MSM can be transformed into the genome of *N. gonorrhoeae*.

5.4 Conclusions and future prospects

We can draw two important conclusions from this thesis. First, high levels of antimicrobial exposure in MSM using PrEP have consequences that extend beyond the short term, and probably beyond the individual that was exposed. Hence, we must be prudent when deciding on indications for antimicrobial use in PrEP cohorts. Second, antiseptic mouthwashes cannot replace antimicrobials to treat oropharyngeal gonorrhoea, nor can they be used to prevent STIs. In the absence of effective vaccines, our current anti-STI armamentarium thus remains limited to STI screening, sexual risk counselling, condoms and antimicrobials.[13]

We must take actions to preserve the effectiveness of antimicrobials in the years to come. Reducing unnecessary antimicrobial consumption is key in that respect. This encompasses antimicrobial use for STIs, as well as other community-acquired infections. In a secondary analysis of the PReGo data, we found that MSM with more than ten partners within a three-month period used almost 1.5 times the amount of antibiotics compared to those with less partners.[14] This illustrates that we need to keep endorsing sexual risk reduction in populations at high risk of STIs. Furthermore, we need to critically appraise what we consider standard clinical practice. As a first example, empirically treating asymptomatic contacts of gonorrhoea and chlamydia cases without awaiting results of STI testing was shown to be highly inefficient in several studies.[15–17] One before-after study estimated that non-empiric treatment of asymptomatic gonorrhoea/chlamydia contacts can reduce antimicrobial consumption fivefold.[15] A second example is the still unanswered question whether "seek-and-destroy" is the optimal strategy to reduce prevalence of gonorrhoea and chlamydia among MSM: several guidelines recommend three-monthly three-site gonorrhoea/chlamydia screening in MSM using PrEP, but a before-after study estimated that such a screening regimen results in a fourfold increase in macrolide consumption.[18] Currently, a randomised controlled trial is ongoing to evaluate the risks and benefits of gonorrhoea/chlamydia screening in asymptomatic MSM using PrEP in Belgium (GonoScreen Study, ClinicalTrials.gov NCT04269434). A third example to reduce macrolide consumption in PrEP populations is to treat *N. gonorrhoeae* with ceftriaxone alone, instead of combining ceftriaxone with azithromycin as is current practice in most European countries. Indeed, it is uncertain whether the addition of azithromycin prevents AMR in *N. gonorrhoeae*, and its use must be balanced against the currently increasing prevalence of gonococcal macrolide resistance.[19,20] For that reason, the CDC, as well as British and French guidelines now recommend ceftriaxone monotherapy as the first line treatment option for gonorrhoea.[21–23] As a last remark, the introduction of novel antimicrobials to the market should be done wisely, taking into account their potential to select for AMR. The MAGIcIAN project (https://www.magician-amr.eu) is notable in this context: it uses advanced data science and machine learning techniques to model the most sustainable way to use last-resort or novel antimicrobials. For pathogens like *N. gonorrhoeae*, which tend to acquire AMR through horizontal gene transfer

from commensal bacteria, it may be important to include the impact of novel antibiotics on the commensal resistome in the overall risk assessment.

I would like to conclude this thesis with the following. In a survey among MSM in the United Kingdom in 2019, the majority of respondents indicated that they did not see AMR in STIs as an immediate threat, and that it did not influence sexual decision making.[24] Indeed, the threat of AMR may be still too distant to influence everyone's sexual risk perception, but this thesis illustrates that the threat is real and that we currently have few innovative approaches to counter it. It is our duty as clinicians to convey this message towards the population it relates to. Feeding back the findings of our studies to PrEP users in our outpatient clinic has resulted in many interesting conversations and is a way of mapping out the care pathway together with rather than for the population we are working with.

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List of abbreviations

AMR   Antimicrobial resistance

ARG   Antibiotic resistance gene

ESC   Extended spectrum cephalosporins

HIV   Human immunodeficiency virus

MSM   Men who have sex with men

NGS   Next generation sequencing

PCR   Polymerase chain reaction

PrEP   (HIV) pre-exposure prophylaxis

STI   Sexually transmitted infections

XDR   Extensively drug resistant

Curriculum vitae

Personal Information

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Work Experience

04/2019 - present **PREDOCTORAL RESEARCHER**
Institute of Tropical Medicine, Antwerp (Belgium)

04/2019 - present **SPECIALIST IN INTERNAL MEDICINE (INFECTIOUS DISEASES)**
Institute of Tropical Medicine, Antwerp (Belgium)

08/2013 – 04/2019 **RESIDENT IN INTERNAL MEDICINE**

04/2018 - 02/2019	Antwerp University Hospital
10/2017 - 03/2018	Leuven University Hospital
08/2015 & 04/2016 - 09/2017	Antwerp University Hospital
08/2014 - 07/2015	Hospital GZA St. Vincentius, Antwerp
08/2013 - 07/2014	Hospital AZ Nikolaas, Sint-Niklaas

Education and Training

2013 - 2019 **MASTER IN SPECIALIST MEDICINE – INTERNAL MEDICINE**
Interuniversity course on antibiotic stewardship organized by the University of Antwerp, Ghent, and Leuven, Belgium

2009 - 2013 **MASTER IN MEDICINE (GREAT DISTINCTION)**
University of Antwerp, Belgium

2006 - 2009 **BACHELOR IN MEDICINE (GREAT DISTINCTION)**
University of Antwerp, Belgium

Additional training:

05/2021	**FUNDAMENTALS OF CLINICAL STUDIES: GCP & BEYOND**
	Institute of Tropical Medicine Antwerp, Belgium
2018 - 2021	**CERTIFICAT INTERUNIVERSITAIRE EN INFECTIOLOGIE ET MICROBIOLOGIE CLINIQUE** (GREAT DISTINCTION)
	Interuniversity course on infectious diseases and clinical microbiology organized by the Université Libre de Bruxelles (ULB), l'Université Catholique de Louvain (UCL) and Liège University (ULG), Belgium
2018 - 2019	**BIJZONDERE OPLEIDING ANTIBIOTICABELEID**
	Universiteit Leuven, Universiteit Antwerpen, Universiteit Gent
2015 - 2016	**POSTGRADUATE CERTIFICATE IN TROPICAL MEDICINE & INTERNATIONAL HEALTH** (GREATEST DISTINCTION)
	Institute of Tropical Medicine Antwerp, Belgium
2009 - 2010	**ELECTROCARDIOGRAPHY POST-ACADEMIC TRAINING** (DISTINCTION)
2000 - 2006	**GENERAL SECONDARY SCHOOL, LATIN-MATHEMATICS** (GREAT DISTINCTION)
	Sint-Michielscollege Brasschaat, Belgium

Awards & Grants

2022	**TRAVEL GRANT**
	32nd European Congress of Clinical Microbiology and Infectious Diseases, Lisbon (Portugal)
2021	**SCHOLARSHIP**
	STI & HIV 2021 World Congress: Sexual Diversity and the City (virtual)
2019	**ATTENDANCE GRANT**
	10th International Summer Course on Research Methodology and Ethics in Health Sciences, Koç University, Istanbul (Turkey)
2018	**BEST THESIS AWARD – MASTER OF SPECIALIST MEDICINE**
	"Antibiotic stewardship interventions in hospitals in low-and middle-income countries: a systematic review" Supervisors: E. Vlieghe MD PhD, J. Cox MD PhD
2013	**BEST THESIS AWARD – MASTER OF MEDICINE**
	"Study of the physiopathology of portal hypertension induced by severe steatosis" Supervisors: S. Francque MD PhD, P. Michielsen MD PhD

Scientific conferences

Oral presentations

04/2022   32nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Lisbon, Portugal, April 23-26, 2022.   "*Ceftriaxone monotherapy for the treatment of gonorrhoea in the Netherlands was not associated with any decrease in cephalosporin susceptibility*"

11/2021   Eighth International Meeting on Emerging Diseases and Surveillance (IMED), virtual, November 4-6, 2021.   "*Antimicrobial susceptibility of commensal Neisseria in the general population and men who have sex with men in Belgium: a cross sectional survey.*"

07/2021   STI & HIV 2021 World Congress: Sexual Diversity and the City, virtual, July 14-17, 2021.   "*Could number of partners be a risk for antimicrobial resistance? Higher macrolide consumption amongst a core group of PrEP users.*"

Poster presentations

04/2022   32nd ECCMID, Lisbon, Portugal, April 23-26, 2022.   "*The oropharynx of men using HIV pre-exposure prophylaxis is enriched with antimicrobial resistance genes.*"

07/2021   31st ECCMID, virtual, July 9-12, 2021.   "*Higher gonococcal bacterial load in Mycoplasma genitalium co-infection.*"

10/2017   10th European Congress on Tropical Medicine and International Health (ECTMIH), Antwerp, Belgium, October 16-20, 2017. "*Antibiotic stewardship in low- and middle-income countries: a systematic review.*"

12/2013   18th Annual Congress of Internal Medicine, Brussels, Belgium, December 13, 2013.   "*Recurrent hypoglycaemia in a non-diabetic patient.*"

Other conference and workshop attendances

06/2022   DGI-DZIF Joint Annual Meeting of the German Society of Infectious Diseases and the German Center for Infection Research (Stuttgart, Germany)

01/2022   The Neisseria gonorrhoeae Research Society 2022 Virtual Conference

04/2021   Microbiome Data Analysis Workshop, Hasselt University (virtual)

10/2020   The Neisseria gonorrhoeae Research Society 2020 Virtual Conference

10/2020   ID Week 2020 (Infectious Diseases Society of America – IDSA, virtual)

09/2020   IUSTI congress 2020: "Taming the tide of STIs & HIV"(virtual)

09/2020   Summer School "Modelling Infectious Diseases and Health Economics"(University of Antwerp, Belgium, virtual)

06/2019	10th International Summer Course on Research Methodology and Ethics in Health Sciences, Koç University, Istanbul (Turkey)
04/2019	29th ECCMID, Amsterdam (The Netherlands)
07/2018	ESCMID Summer School, Paris (France)
04/2018	28th ECCMID, Madrid (Spain)
03/2018	"Boerhaave Nascholing" infectious diseases course of the Dutch Society of Infectious Diseases, Noordwijkerhout (The Netherlands)
2011	Multidisciplinary course HIV/aids (Institute of Tropical Medicine Antwerp)
07/2011	Summer School Health and Migration, Ghent University (Belgium)
03/2011	Global Health Short Course, Ghent University (Belgium)

Societal Memberships & Activities

2021 - present **Deutsche Gesellschaft für Infektiologie (DGI)**
Regular member

2017 - present **EUROPEAN SOCIETY OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ESCMID)**
Regular member
2019-2021: Belgian national representative for the Trainee Association of ESCMID
2022-present: secretary of the ESCMID study group on Host and Microbiota Interaction (ESGHAMI)

2017 - present **BELGIAN SOCIETY OF INFECTIOUS DISEASES AND CLINICAL MICROBIOLOGY (BVIKM)**
Regular member

2020 - present **INTERNATIONAL UNION AGAINST SEXUALLY TRANSMITTED INFECTIONS (IUSTI)**
Associate member

2020 - present **NEISSERIA GONORRHOEAE RESEARCH SOCIETY (NGORS)**
Regular member

Bibliography

Publications as first author

- <u>Van Dijck C,</u> Kenyon C. No evidence of reduced cephalosporin susceptibility of circulating strains of N. gonorrhoeae in the Netherlands despite nearly a decade of recommending ceftriaxone monotherapy. Sex Transm Infect. Accepted for Publication.
- <u>Van Dijck C</u>, Hens N, Kenyon C, Tsoumanis A. The roles of unrecognized monkeypox cases, contact isolation and vaccination in determining epidemic size in Belgium. A modelling study. Clin Infect Dis Published Online First: 2022.
- <u>Van Dijck C</u>, De Baetselier I, Kenyon C, Coppens J, Michiels J, de Block T et al. Retrospective Detection of Asymptomatic Monkeypox Virus Infections among Male Sexual Health Clinic Attendees in Belgium. Nat Med 2022. (shared first authorship)
- <u>Van Dijck C,</u> Laumen JGE, Abdellati S, De Baetselier I, Serrano G, Manoharan-Basil SS, Bottieau E, Martiny D, Kenyon C. Antimicrobial susceptibility of commensal Neisseria in a general population and men who have sex with men in Belgium. Sci Rep. 2022 Jan 7;12(1):9. (shared first authorship)
- <u>Van Dijck C</u>, De Baetselier I, Cuylaerts V, Buyze J, Laumen J, Vuylsteke B, Kenyon C. Gonococcal bacterial load in PrEP users with Mycoplasma genitalium coinfection. Int J STD AIDS. 2021 Nov 2.
- <u>Van Dijck C</u>, Tsoumanis A, De Hondt A, Cuylaerts V, Laumen J, Van Herrewege Y, Florence E, De Baetselier I, Kenyon C. Chlorhexidine mouthwash fails to eradicate oropharyngeal gonorrhea in a clinical pilot trial (MoNg). Sex Transm Dis. 2021 Jul 17.
- <u>Van Dijck C</u>, Tsoumanis A, Rotsaert A, Vuylsteke B, Van den Bossche D, Paeleman E, et al. Antibacterial mouthwash to prevent sexually transmitted infections in men who have sex with men taking HIV pre-exposure prophylaxis (PReGo): a randomised, placebo-controlled, crossover trial. Lancet Infect Dis. 2021;3099(20).

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- Van Dijck C, Laumen JGE, Manoharan-Basil SS, Kenyon C. Commensal Neisseria Are Shared between Sexual Partners: Implications for Gonococcal and Meningococcal Antimicrobial Resistance. Pathogens. 2020 Mar 19;9(3):228.
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- Van Dijck C, Laumen J, Zlotorzynska M, Manoharan-Basil SS, Kenyon C. Association between STI screening intensity in men who have sex with men and gonococcal susceptibility in 21 States in the USA : an ecological study. Sex Transm Infect. 2020;1–4.
- Van Dijck C, Vlieghe E, Cox JA. Antibiotic stewardship interventions in hospitals in low-and middle-income countries: a systematic review. Bull World Health Organ. 2018;96(4):266-80.
- Van Dijck C, Van Esbroeck M, Rutsaert R. A 54-year-old Philippine sailor with fever and jaundice. Acta Clinica Belgica. 2016; 1-4.

Publications as co-author

- González N, Abdellati S, De Baetselier I, Laumen JGE, Van Dijck C, de Block T, et al. Alternative Pathways to Ciprofloxacin Resistance in Neisseria gonorrhoeae: An In Vitro Study of the WHO-P and WHO-F Reference Strains. Antibiotics. 2022;11(4).
- Vanbaelen T, Van Dijck C, Laumen J, et al. Global epidemiology of antimicrobial resistance in commensal Neisseria species: A systematic review. Int J Med Microbiol 2022; 312: 151551.
- de Block T, González N, Abdellati S, Laumen JGE, Van Dijck C, De Baetselier I, et al. Successful Intra- but Not Inter-species Recombination of msr(D) in Neisseria subflava. Front Microbiol. 2022 Mar 30;13:855482.

- Kenyon C, Vanbaelen T, <u>Van Dijck C</u>. Recent insights suggest the need for the STI field to embrace a more eco-social conceptual framework: A viewpoint. Int J STD AIDS. 2022 Jan 4.

- Laumen JGE, <u>Van Dijck C</u>, Manoharan-Basil SS, et al. Sub-Inhibitory Concentrations of Chlorhexidine Induce Resistance to Chlorhexidine and Decrease Antibiotic Susceptibility in Neisseria gonorrhoeae. Front Microbiol. 2021;12:776909. Published 2021 Nov 25.

- Manoharan-Basil SS, Laumen JGE, <u>Van Dijck C</u>, De Block T, De Baetselier I, Kenyon C. Evidence of Horizontal Gene Transfer of 50S Ribosomal Genes rplB, rplD, and rplY in Neisseria gonorrhoeae. Front Microbiol. 2021 Jun 10;12:683901.

- Rotsaert A, Reyniers T, <u>Van Dijck C</u>, Vuylsteke B, Kenyon C. Proposing an antibacterial mouthwash to prevent gonorrhoea is not sexy. Lancet Infect Dis. 2021 Jul;21(7):909.

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- Kenyon C, Manoharan-Basil SS, <u>Van Dijck C</u>. Is There a Resistance Threshold for Macrolide Consumption? Positive Evidence from an Ecological Analysis of Resistance Data from Streptococcus pneumoniae, Treponema pallidum, and Mycoplasma genitalium. Microb Drug Resist. 2021 Aug;27(8):1079-1086.

- Kenyon C, Laumen J, <u>Van Dijck C</u>, De Baetselier I, Abdellati S, Manoharan-Basil SS, et al. Gonorrhoea treatment combined with population-level general cephalosporin and quinolone consumption may select for Neisseria gonorrhoeae antimicrobial resistance at the levels of NG-MAST genogroup:

An ecological study in Europe. J Glob Antimicrob Resist. 2020;23(October):377–84.

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- Romaen K, <u>Van Dijck C</u>, Paridaens K, Detienne S. Carotido-caverneuze fistel vier weken na een hoofdtrauma. Tijdschr. voor Geneeskunde 2015, 71(21): 1415-1418.

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Posters, abstracts and oral presentations

- <u>Van Dijck C</u>, De Baetselier I, Kenyon C, Florence E, Vandenbruaene M, Apers L, et al. Monkeypox screening in a Belgian sexual health clinic identifies cases outside the case definition. Poster presentation at the joint BVIKM, NVMM and NVII symposium, Bruges, 7-8.11.2022.

- <u>Van Dijck C</u>, Kenyon C, Ceftriaxone monotherapy for the treatment of gonorrhoea in the Netherlands was not associated with any decrease in

cephalosporin susceptibility. Oral presentation at ECCMID 2022, 32nd European Congress of Clinical Microbiology and Infectious Diseases, Lisbon, 23-26.04.2022.

- <u>Van Dijck C</u>, Laumen J, de Block T, Abdellati S, Malhotra Kumar S, Kenyon C, Manoharan-Basil SS, Xavier BB, The oropharynx of men using HIV pre-exposure prophylaxis is enriched with antimicrobial resistance genes. Poster presentation at ECCMID 2022, 32nd European Congress of Clinical Microbiology and Infectious Diseases, Lisbon, 23-26.04.2022.

- <u>Van Dijck C</u>, Laumen J, Abdellati S, De Baetselier I, Martiny D, Kenyon C. Low Macrolide and Fluoroquinolone Susceptibility in Non-pathogenic Neisseriae in the General Belgian Population and STI Clinic Attendees. Poster presentation at the Eighth International Meeting on Emerging Diseases and Surveillance, IMED 2021; Int J Infect Dis 2022; 116: S2.

- <u>Van Dijck C</u>, De Baetselier I, Cuylaerts V, Buyze J, Laumen J, Vuylsteke B, Kenyon C. Higher gonococcal bacterial load in Mycoplasma genitalium co-infection, Poster presentation at ECCMID 2021, 31st European Congress of Clinical Microbiology and Infectious Diseases, online edition, 9-12.07.2021.

- <u>Van Dijck C</u>, Tsoumanis A, Vanbaelen T, Laumen J, Kenyon C. Could number of partners be a risk for antimicrobial resistance? Higher macrolide consumption amongst a core group of PrEP users. Oral presentation at STI & HIV World Congress, Amsterdam, 14-17.07.2021.

- <u>Van Dijck C</u>, Vlieghe E, Cox JA. Systematic literature review of antibiotic stewardship interventions in hospitalized patients in low-and middle-income countries. Poster presentation at ECTMIH 2017, 10th European Congress on Tropical Medicine and International Health, Antwerp, 16-20.10.2017. Tropical Medicine and International Health 2017, 22 (Supplement 1): 190.

- <u>Van Dijck C</u>, Bogaerts E, Rutsaert R, Vermeiren S. Transfusies onder de loep: correcte indicatie en tijdstip van toediening? Poster session 'Transfusiecomités in actie', BeQuint 2016, Annual symposium Belgian Quality in Transfusion, Brussels, 2.06.2016.

- <u>Van Dijck C</u>, Coremans P, De Meester J. Recurrent hypoglycaemia in a non-diabetic patient. Poster presentation at the Annual Congress of the Belgian Society for Internal Medicine 2013, Brussels, 13-14.12.2013. Acta Clin Belg 2013 Dec; 68(6):459.

- Francque S, Kwanten W, <u>Van Dijck C</u>, D'Hondt M, De Winter B, Van Marck E, Pelckmans P, Michielsen P.   Increased intrahepatic resistance in severe steatosis is partially dynamic, associated with endothelial dysfunction and mediated by increased intrahepatic endothelin and thromboxane A2 production. Poster presentation at The Liver Meeting® 2012, 63rd Annual Meeting of the American Association for the Study of Liver Diseases, Boston, 9-13.11.2012. Hepatology 2012; 56 (4, Suppl): 859A 1413.