# Relationship between bacterial diversity, specific urethral bacteria and incident NGU in men who have sex with women

Caitlin M Drover

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Lisa Manhart

Christine Khosropour

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## Abstract

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Caitlin M Drover

Chair of the Supervisory Committee:

Lisa Manhart

Department of Epidemiology

Background: Nongonococcal urethritis (NGU) is a common syndrome in men that is not well understood. Prior studies have investigated factors associated with prevalent NGU, but factors associated with incident NGU have rarely been examined. We evaluated potential precursors of incident NGU in a group of men who have sex with women (MSW) attending a sexual health clinic in Seattle from August 2014 to July 2018.

Methods: We conducted a nested case-control study, evaluating a subset of MSW enrolled in a cohort study designed to investigate the relationship between the male urethral microbiota and NGU. At enrollment and monthly follow-up visits, participants had a clinical examination performed at which they provided a urethral swab specimen for Gram staining and a first-void

urine sample. Participants also completed a computer-assisted self-interview (CASI) to assess history of sexually transmitted infections (STIs), sexual behaviors, and other sociobehavioral data at each visit. Case visits were defined as the visit at which an individual had an incident case of NGU. NGU was defined as elevated levels of polymorphonuclear leukocytes (PMNs) when urethral discharge was examined on a Gram-stained slide in the absence of *Neisseria gonorrhoeae* (GC), with or without symptoms such as painful urination, itching, or urethral discharge, and was considered incident if the participant did not have NGU at the prior monthly visit. We selected the visit when the case was diagnosed with incident NGU, and the preceding visit when they were NGU-negative. Controls were matched to cases on follow-up time for both time points using a 1:1 matching ratio, matching by visit number and interval between visits. Controls were NGU-negative at both matched visits. Nucleic acid amplification testing (NAAT) for GC, *Chlamydia trachomatis* (CT) and *Mycoplasma genitalium* (MG) was performed on the urine samples using Aptima assays (Hologic, Inc., San Diego, CA). To characterize the urethral microbiota, we applied broad-range 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) and sequencing to urine samples. We used Fisher's exact test for comparisons of categorical characteristics and a paired t-test for continuous characteristics. Conditional logistic regression models were used to estimate odds ratios (ORs) for the association of bacterial diversity, as measured by the Shannon Diversity Index (SDI) and species richness, as well as detection of each of four bacterial species (*Haemophilus influenzae*, *Fannyhessea vaginae, Lactobacillus iners, Streptococcus mitis*) at the visit prior to diagnosis with incident NGU.

Results: The 262 enrolled persons with follow-up data contributed 63 incident NGU events. Although cases and controls did not differ with respect to age, race/ethnicity, or NGU at

enrollment, cases had a significantly lower level of education, used condoms less frequently with their most recent sexual partner, and were more likely to have a prior history of NGU and/or CT than controls ($p \leq 0.03$ for all). After adjusting for condom use, participants with one-unit higher SDI at the visit prior to NGU diagnosis had 10.29 times the odds of incident NGU (aOR=10.29, 95% CI: 1.49-73.16, p=0.02) compared to participants with a one-unit lower SDI. Similarly, species richness at the visit prior to NGU diagnosis was associated with increased odds of incident NGU (aOR per species = 1.08, 95% CI: 1.001-1.169, p=0.047). In a multivariate model that included all four bacterial species detected at the visit prior to NGU diagnosis, *F. vaginae* was associated with 29.7 times the odds of incident NGU (aOR=29.68, 95% CI: 1.55-568.26, p=0.02) compared to those without *F. vaginae*. Neither *H. influenzae*, *L. iners,* nor *S. mitis* were significantly associated with incident NGU, although MSW with *S. mitis* were somewhat less likely to have incident NGU.

Conclusions: Higher SDI, higher species richness, and presence of *F. vaginae* were significantly associated with higher odds of incident NGU. Future work should involve larger longitudinal studies re-examining these factors' associations with incident NGU to better understand these relationships and other potential risk factors for the syndrome.

Introduction

Nongonococcal urethritis (NGU) is a commonly occurring syndrome that is not well understood, with no known cause in up to half of cases in studies testing for established causes.[1–7] NGU is characterized by symptoms such as painful urination (dysuria), itching (pruritus), or urethral discharge, along with elevated levels of polymorphonuclear leukocytes (PMNs) when urethral discharge is examined on a Gram-stained slide in the absence of *Neisseria gonorrhoeae* (GC).[8] The clinical syndrome is one of the most commonly occurring genital tract syndromes in people assigned male sex at birth, and is often caused by sexually transmitted pathogens.[3,4] There are several established causes of NGU, including *Chlamydia trachomatis* (CT), which accounts for 20% to 40% of cases, followed by *Mycoplasma genitalium* (MG), which accounts for 10 to 30% of cases.[1,3,8] Other less common causes of NGU include *Trichomonas vaginalis* (TV), herpes simplex virus (HSV), and adenovirus.[1,3,6] NGU is considered idiopathic when none of these established causes are present. Since as many as 50% of all NGU cases are idiopathic, more investigation is needed to identify other potential causes of the syndrome.

The composition of the urethral microbiota may play a role in NGU. Prior research has demonstrated that alpha diversity of the urethral microbiota trended lower in men who have sex with women (MSW) with prevalent NGU compared to those without NGU, however this finding was not statistically significant.[1] However, another study found no significant difference in diversity between men with prevalent NGU and men without NGU among both cisgender men who have sex with men (MSM) and cisgender MSW (hereafter referred to as MSM and MSW).[3] Among cisgender women, those with bacterial vaginosis were more likely to have an incident STI such as MG[9,10], CT or GC[10], suggesting that presence of certain bacterial vaginosis-

associated bacteria (BVAB) may increase susceptibility for STIs at later time points. Similarly, the composition of the urethral microbiota or presence of certain bacteria at an earlier time point may increase the likelihood that a man will develop urethritis.

Specific bacteria may also play a role in NGU. *Haemophilus influenzae*, a common respiratory pathogen[11], was present and had high relative abundance in MSM and MSW with prevalent NGU, and both MSM and MSW with prevalent NGU were more likely to have *H. influenzae* detected compared to those without NGU.[1,3] In another study, *Haemophilus* was a dominant genera among men with prevalent idiopathic NGU and was present in cases four times more often than controls.[4] Conversely, *Fannyhessea vaginae* (previously *Atopobium vaginae*) and *Lactobacillus iners*, found in the human vagina[12–15], and *Streptococcus mitis* were all inversely associated with prevalent NGU in both MSM and MSW in two studies.[1,3] In contrast, there was no association of *F. vaginae* or other BVAB (including BV-associated bacterium 2 (BVAB2), *Mageeibacillus indolicus*, or *Sneathia* spp.) with prevalent NGU in two other studies, indicating the relationship between BVAB and male urethritis is not well understood.[5,16]

Notably, all of these prior studies were cross-sectional in nature and evaluated relationships with prevalent NGU. Factors associated with prevalent NGU may differ from those associated with incident NGU. To date, the relationship between bacterial diversity, species richness, as well as presence of these four specific bacteria (*H. influenzae, F. vaginae, L. iners,* and *S. mitis*) and incident NGU has not yet been evaluated. Given prior reports[1,3–5,12–15] of associations between prevalent NGU with bacterial diversity, species richness, and these four bacteria, we conducted a case-control study nested within a cohort of MSW attending a Seattle sexual health clinic. We sought to evaluate whether bacterial species diversity and richness in the male urethral environment was associated with increased risk of incident NGU, and to assess

whether *H. influenzae, F. vaginae, L. iners,* and *S. mitis* were associated with risk of incident NGU.

## Methods

### *Study Design*

We conducted a nested case-control study, analyzing previously collected data from a cohort study that was conducted in the Public Health Seattle & King County (PHSKC) Sexual Health Clinic from August 2014 to July 2018.[1,2]

### *Study Population*

MSW with and without NGU were enrolled in the parent study during clinic visits at the PHSKC Sexual Health Clinic in Seattle, Washington from August 2014 to July 2018. Eligible patients were cisgender males at least 16 years old who reported engaging in at least one sexual act with only female partner(s) in the prior 60 days. MSW were excluded if the participant was known to be HIV positive, had taken antibiotics within the 30 days prior to enrollment, had GC or recent sexual contact with someone who had GC, or could not attend follow-up visits, could not provide contact information, or could not speak English. A total of 311 eligible MSW were included in the initial cohort, with data for at least one follow-up visit available for 262 participants (84%).

### *Data Collection*

At the enrollment visit, participants had a clinical examination performed and provided a urethral swab specimen for Gram staining and a first-void urine sample. Gram staining of the

urethral discharge was performed to quantify the number of PMNs present as part of the NGU case definition. NGU was defined as either an average of at least 5 PMNs per high-power field (hpf) on a Gram-stained slide in addition to patient-reported symptoms including dysuria, pruritus, or urethral discharge, OR at least 5 PMNs/hpf in addition to clinician-observed discharge. Nucleic acid amplification testing (NAAT) for GC, CT and MG was performed on the urine samples using Aptima assays (Hologic, Inc., San Diego, CA).

Participants completed a computer-assisted self-interview (CASI) before seeing a clinician. The questionnaire asked about the participants' history of sexually transmitted infections (STIs), sexual activity history, and symptoms over the prior 2- and 12-month periods. Finally, another study-specific CASI was completed using a Research Electronic Data Capture (REDCap)[17] survey that collected sociobehavioral data about their most recent sexual encounter, including sexual behaviors and condom use.

Participants were followed for a total of 6 monthly follow-up visits, with the exception of those who had NGU at enrollment who were followed for 7 total visits to allow an extra month after receiving treatment to resolve the NGU. If participants experienced urethral symptoms between scheduled visits, they were asked to return for an additional visit to address the symptoms. The same clinical examination, tests, and CASI that were completed at the enrollment visit were completed at each follow-up visit.

Case visits were defined as the visit at which an individual had an incident case of NGU; NGU was considered incident if the participant did not have NGU at the monthly visit immediately preceding their NGU diagnosis. For these analyses, we selected the visit when the case was diagnosed with new NGU, and the preceding visit when they were NGU-negative. Controls were matched to cases on follow-up time for both time points using a 1:1 matching

ratio, matching by visit number and interval between visits. Controls were NGU-negative at both matched visits. If multiple controls could be matched to a case, we selected the control with the most complete diary data. Since there were 6 or 7 total visits for each participant, individuals could contribute multiple case visits if they had multiple NGU episodes separated by an NGU-negative visit. Individuals who were cases could also serve as controls if they were NGU-negative for the matched visits of other cases; however, cases could not serve as their own controls. The microbiota was characterized only on the subset of specimens from the matched case and control visits. We applied broad-range 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) and sequencing to the urine specimens.[1] Detailed methods have been previously described.[1] Briefly, broad-range PCR targeting the V3-V4 region of the 16S rRNA gene and sequencing on the Illumina MiSeq instrument (San Diego, CA) was conducted after DNA extraction on the case and control urine samples.

The overall composition of the urethral microbiota was measured by calculating the species richness and Shannon Diversity Index (SDI) for each participant at the visit during and the visit immediately preceding NGU diagnosis. We defined species richness as the total number of species present in the sample.[18] The SDI incorporates both species richness and evenness of the species present, providing a snapshot of the bacterial community.[18]

*Statistical Analysis*

Demographic and microbial characteristics of case visits and control visits were compared using Fisher's exact test for categorical characteristics and a paired t-test for continuous characteristics. When examining characteristics associated with detection of *H.*

*influenzae*, *F. vaginae, L. iners,* and *S. mitis*, Fisher's exact test was used for categorical characteristics and a t-test was used for continuous characteristics.

Prior studies have reported associations between age, race/ethnicity, education, prior history of NGU or NGU at enrollment, prior history of CT, sexual behaviors with urethral exposures, and inconsistent condom use with NGU[2,3], and these characteristics were examined as potential confounding factors. We used multivariate conditional logistic regression models to account for matched pairs and estimate odds ratios (ORs) for the association of the SDI, species richness, and detection of each of the four bacteria at the visit prior to diagnosis with incident NGU. A backwards stepwise regression was performed by identifying characteristics significantly associated with incident NGU with a p-value threshold of 0.1 for assessment in the multivariate models. Characteristics that were statistically significant at alpha≤0.05 were retained in the multivariate models. We also developed a multivariable model that included all four of the specific bacteria to adjust the estimates for the other bacteria present at that time point. Although known pathogens such as CT and MG are typically adjusted for in multivariable models of NGU, none of the controls had either bacteria detected. Therefore, participants with CT or MG at the visit concurrent with NGU diagnosis were excluded from multivariable models.

RStudio version 2022.07.1+554 was used for all statistical analyses. All participants provided written informed consent to participate in the parent study and have their data used for future analyses. The University of Washington Institutional Review Board reviewed and approved the parent study and these analyses.

## Results

The 262 enrolled persons with follow-up data contributed 63 incident NGU events. The 63 matched pairs in this analysis included 102 unique participants. Of these, microbiota data was available for 83 participants at the visit prior to NGU diagnosis and 88 participants at the visit concurrent with NGU diagnosis. Microbiota data was missing for 19 participants at the visit prior to NGU diagnosis and 14 participants at the visit concurrent with NGU diagnosis because there was insufficient bacterial DNA to perform the testing.

*Sociodemographic and Clinical Characteristics Associated with NGU*

The mean age of cases and controls did not differ (34.3 years and 33.1 years, respectively, p=0.49) (Table 1). Cases identified somewhat more frequently as non-Hispanic (NH) Black (39.7% of cases) while controls identified somewhat more frequently as NH White (47.6% of controls) (p=0.08). Cases had a significantly lower level of education compared to controls; 52.4% of cases had only achieved high school or less education, while 51.6% of controls had graduated from college (p=0.03). Controls used condoms more frequently with their most recent sexual partner compared to cases, with 36.1% of controls reporting always using condoms compared to 11.1% of cases (p=0.005). Although there were some differences in sexual behaviors between cases and controls, these were not statistically significant (p=0.33). Cases and controls were similarly likely to have NGU at enrollment (47.6% of cases vs. 49.2% of controls, p=1.0), however, cases were significantly more likely than controls to have a prior history of NGU (25.4% of cases vs. 4.8% of controls, p<0.001) and prior history of CT (47.6% of cases vs 19.0% of controls, p<0.001). Although none of the controls had CT or MG detected at the visit concurrent with NGU diagnosis, one case had CT (1.6%) and nine cases had MG (14.3%).

*Urethral Microbiota Characteristics Associated with Incident NGU*

In univariable analyses, at the visit prior to NGU diagnosis, the SDI was not significantly different between cases and controls (mean SDI of 1.50 [±0.69] among cases vs. 1.38 [±0.73] among controls, p=0.22) (Table 1). Results were similar when we assessed this at the visit concurrent with NGU diagnosis (mean SDI of 1.54 [±0.69] among cases vs. 1.38 [±0.67] among controls, p=0.19). At the visit prior to NGU diagnosis, bacterial species richness was not significantly different among cases and controls (mean species richness of 19.50 [±11.50] vs. 18.00 [±11.30], respectively, p=0.22). Results were again similar among cases and controls when we assessed species richness at the visit concurrent with NGU diagnosis, though the gap between the two groups widened slightly (mean species richness of 20.80 [±14.20] among cases vs. 17.90 [±9.53] among controls, p=0.23). Detection of *H. influenzae, L. iners,* and *S. mitis* were similarly distributed between cases and controls at both time points. However, *F. vaginae* was more common in cases at both visits, with 34% of cases vs. 11.5% of controls having *F. vaginae* detected at the visit prior to NGU diagnosis (p=0.009), and 36.5% of cases vs. 16.4% of controls having it detected at the visit concurrent with NGU diagnosis (p=0.03).

*Diversity of the Urethral Microbiota and Incident NGU - Multivariable Analyses*

After adjusting for condom use, participants with one-unit higher SDI at the visit prior to NGU diagnosis had 10.29 times the odds of incident NGU (aOR=10.29, 95% CI: 1.49-73.16, p=0.02) compared to participants with a one-unit lower SDI (Table 2). Adjusting for condom use, a one-unit higher species richness at the visit prior to NGU diagnosis had 1.08 times the odds of incident NGU (aOR = 1.082, 95% CI: 1.001-1.169, p=0.047) compared to participants

with a one-unit lower species richness. Neither race/ethnicity, age, education level, prior NGU, or prior CT were significantly associated with incident NGU in either model.

*Characteristics Associated with Presence of Specific Bacterial Species*

Of the 102 visits with microbiota data available, *H. influenzae* was detected in 11 participants, *F. vaginae* was detected in 23 participants, *L. iners* was detected in 54 participants, and *S. mitis* was detected in 76 participants (Table 3). *H. influenzae* was more commonly detected in participants who identified as NH White than in those who identified as NH Black, Hispanic (any race), or other/multiple/unknown race. Higher SDI and higher species richness were associated with detection of *F. vaginae*. *L. iners* was more commonly detected among those having greater than a high school education. Higher species richness was associated with detection of *S. mitis*.

*Bacterial Species Associated with Incident NGU*

In a univariable analysis, participants with *F. vaginae* had 11 times higher odds of incident NGU (OR: 11.00, 95% CI: 1.42-85.20, p=0.02) compared to participants who did not have *F. vaginae* (Table 4). Participants with *S. mitis* had 64% lower odds of incident NGU (OR: 0.36, 95% CI: 0.12-1.14, p=0.08) compared to participants who did not have *S. mitis*, though this association did not reach statistical significance. Neither *H. influenzae* nor *L. iners* were significantly associated with incident NGU.

In a multivariate model that included all four bacterial species detected at the visit prior to NGU diagnosis, only *F. vaginae* was significantly associated with incident NGU (Table 4). Adjusting for the three other bacteria, *F. vaginae* was associated with 29.7 times the odds

(aOR=29.68, 95% CI: 1.55-568.26, p=0.02) of incident NGU compared to those without *F. vaginae*. Adjusting for the three other bacteria, *S. mitis* was associated with lower odds of incident NGU (aOR=0.14, 95% CI: 0.02-1.19, p=0.07), but this association was not statistically significant.

## Discussion

In this case-control study of MSW attending a Seattle sexual health clinic, we found that higher SDI and higher species richness at the visit prior to diagnosis were associated with significantly increased odds of incident NGU after adjusting for condom use. Detection of *F. vaginae* at the visit prior to diagnosis was also significantly associated with increased odds of incident NGU, but there was no association with either *H. influenzae* or *L. iners*. MSW with *S. mitis* were less likely to subsequently have incident NGU, but this was not statistically significant.

In univariate analyses, a lower level of education, history of CT, and current MG were associated with incident NGU, consistent with analyses of the full cohort.[2] In addition, we also identified associations of inconsistent condom use and prior NGU with incident NGU in this matched case-control population. Despite the lack of statistical significance in this analysis, incident NGU was more common among participants who identified as NH Black. The lack of a significant association with race/ethnicity in this study was likely due to the smaller sample size compared to the full cohort analyses.

In the backwards stepwise regression models examining the associations between SDI and richness with incident NGU, only condom use (along with SDI and richness) was significantly associated with incident NGU. Using condoms correctly and consistently is widely

understood to reduce the risk of acquiring STIs[19], including those that may lead to NGU. We found that adjusting for condom use, both a higher SDI and higher species richness were associated with increased odds of incident NGU. This differs from previous findings in that SDI was somewhat lower among MSW with than without prevalent NGU[1] or was not associated with NGU among MSW.[3] However, these studies did not adjust for condom use as done here, which may explain these differences.

We found that unique characteristics were associated with the presence of each of the four bacterial species. NH White race/ethnicity was only associated with *H. influenzae*, whereas a higher level of education was associated with *L. iners*, but not the other bacteria. Higher SDI was associated with *F. vaginae*, and higher species richness was associated with both *F. vaginae* and *S. mitis*. Finally, presence of *F. vaginae* was associated with presence of *L. iners*. Both *F. vaginae* and *L. iners* are found in the vagina[5,14–16], thus vaginal sex may lead to colonization of the urethra with these bacteria. Although engaging in vaginal sex since the visit prior to NGU diagnosis was not associated with any of the four bacteria we evaluated, condomless vaginal sex has been associated with higher mean abundance of *Fannyhessia* and *L. iners* in Australian men.[3] As higher diversity of the male urethral microbiota was associated with presence of *F. vaginae*, and *F. vaginae* is associated with BV in cisgender women,[12,13,15] detection of *F. vaginae* in MSW may therefore be acquired through sexual activity with female partners who have BV. This merits further study. *H. influenzae* and *S. mitis* are respiratory/oral pathogens[11,20], and insertive oral sex has often been associated with NGU.[6,21] However, oral sex has not been directly associated with these bacteria in US[1] or Australian men[3] in cross-sectional analyses, again suggesting the need for further investigations into the role sexual behaviors play in urethral exposures and NGU acquisition.

The association of these specific bacteria with incident NGU differed from what had previously been observed with prevalent NGU. Prior studies of prevalent NGU have been unmatched case-control studies conducted among men attending sexual health clinics in Seattle, WA, USA[1], Melbourne, Australia[3], and Stockholm, Sweden[4]. Two of these studies[1,3] used methods similar to those described here to detect the presence of specific bacteria and examine associations with NGU, while the other[4] used the more sensitive species-specific qPCR. Additionally, each study used a slightly different NGU case definition. However, each of these studies included populations of both MSM and MSW, conducted stratified analyses for these associations, and included a similar age range of participants.

In this study, in both univariate analyses and multivariate analyses that adjusted for the presence of the three other bacteria, only *F. vaginae* was significantly associated with increased odds of incident NGU. This differs from prior findings that *F. vaginae* had no association[5] or was inversely associated with prevalent NGU.[1,3] Despite the statistical significance of the association between *F. vaginae* and incident NGU, the confidence intervals for *F. vaginae* were wide, likely due to small sample sizes in this study and this result should be interpreted cautiously.

*H. influenzae* was not significantly associated with incident NGU in this study, while it has previously been significantly more likely to be detected among both MSW and MSM with prevalent NGU.[1,3,4] Future work should re-examine this association in a larger sample size using the more sensitive species-specific qPCR detection method.

While not significant, we found that *L. iners* was somewhat associated with incident NGU in both univariate and multivariate analyses, inconsistent with prior findings of an inverse association between *L. iners* and prevalent NGU among MSW.[1,3] Since these studies also used broad-range PCR and sequencing, this difference is likely related to different associations with

prevalent versus incident NGU. However, further investigations into the relationship between *L. iners* and incident NGU are warranted.

Aligning more closely with previous work[1,3], we found that *S. mitis* was inversely associated with NGU in both univariate and multivariate analyses, although neither association reached statistical significance. This was likely due to the small sample sizes in this study; future studies should re-examine this association in larger samples of MSW.

There were several limitations to these analyses. First, there may have been selection bias from the original parent study as 62% of STI clinic patients approached for study enrollment declined participation. Those who declined participation were likely different from those who agreed to participate in the study. Second, the somewhat small sample size limited statistical power to detect small effect sizes. Despite this, we were able to identify significant relationships between participant characteristics, specific bacteria, and incident NGU even in this small sample, strengthening confidence in these relationships. Third, 16S rRNA broad-range PCR was used to determine the relative abundance of each species, and this is less sensitive than species-specific quantitative PCR (qPCR). Repeating this analysis using qPCR may provide more precise results and more accurately characterize the relationships between these bacteria and NGU. Fourth, exclusion of those with CT or MG in the multivariable models due to the inability to adjust for these factors meant that these models evaluated the relationship with non-CT-non-MG NGU rather than with NGU overall. This reduced comparability to other studies of NGU that included MSW with CT and MG. Fifth, in this analysis, individual participants could contribute multiple case events, multiple control events, or be both a case and a control, but our statistical methods could not account for this correlation. Had we been able to correct for this correlation, the confidence intervals for our estimates may have been wider and

resulted in fewer significant associations. Future analyses might consider sampling without replacement to avoid multiple events from the same individuals. Finally, attendees at a single sexual health clinic may be at higher risk of NGU and other STIs compared to the general population and our results may not be generalizable to non-sexual health clinic attendees.

Despite these limitations, there are several strengths of this study. First, this study was among the first to examine incident NGU rather than prevalent NGU as previous studies have done, providing stronger evidence for causal inference. Our examination of the urethral microbiota one month prior to the diagnosis of NGU further established the temporal sequence of events leading to the development of NGU. Although a more proximal time to NGU diagnosis may be more etiologically relevant, the similar associations of the microbiota at both the visit prior to and the visit concurrent with NGU diagnosis suggest that this time frame captured the relevant changes. Second, the regular follow-up and additional symptom visits helped to ensure careful ascertainment of case status. Similarly, the detailed sociobehavioral and clinical data collected at each visit allowed us to evaluate a variety of risk factors and potential confounders.

In conclusion, we found that higher SDI, higher species richness, and presence of *F. vaginae* were associated with higher odds of incident NGU, inconsistent with previous work examining these relationships with prevalent NGU. This suggests that urethral bacteria associated with incident NGU are different from those associated with prevalent NGU. Future work should involve larger longitudinal studies re-examining these factors' associations with incident NGU using species-specific qPCR, a more sensitive method of detection, to better understand these relationships and other potential risk factors for the syndrome.

Table 1. Characteristics of men who have sex with women (MSW) attending a sexual health clinic enrolled from August 2014 - July 2018 by case (incident NGU) and control (no incident NGU) status (N=102 participants).

| | **Incident NGU Status** | | |
|---|---|---|---|
| | **Case Visits** (N=63) | **Control Visits** (N=63) | **p-value*** |
| **Age** (years), n (%) | | | |
| Mean (±SD) | 34.3 (±11.90) | 33.1 (±8.72) | 0.49 |
| 16-24 | 10 (15.9%) | 5 (7.9%) | 0.42 |
| 25-34 | 32 (50.8%) | 35 (55.6%) | |
| 35+ | 21 (33.3%) | 23 (36.5%) | |
| **Race/Ethnicity**, n (%) | | | 0.08 |
| Non-Hispanic White | 21 (33.3%) | 30 (47.6%) | |
| Non-Hispanic Black | 25 (39.7%) | 12 (19.0%) | |
| Hispanic (any race) | 4 (6.3%) | 5 (7.9%) | |
| Other/Multiple/Unknown | 13 (20.6%) | 16 (25.4%) | |
| **Education**, n (%) | | | **0.03** |
| ≤ High School/GED | 33 (52.4%) | 19 (30.6%) | |
| Some College | 11 (17.5%) | 11 (17.7%) | |
| College Grad | 19 (30.2%) | 32 (51.6%) | |
| **Condom Use**, n (%) | | | **0.005** |
| Always | 6 (11.1%) | 22 (36.1%) | |
| Usually | 17 (31.5%) | 11 (18.0%) | |
| Sometimes | 3 (5.6%) | 7 (11.5%) | |
| Never | 28 (51.9%) | 21 (34.4%) | |
| **Sexual Behaviors** since visit prior to NGU diagnosis, n (%) | | | 0.33 |
| Vaginal sex only | 8 (12.9%) | 14 (22.2%) | |
| Received oral sex +/- vaginal sex | 39 (62.9%) | 30 (47.6%) | |
| Any insertive anal sex | 5 (8.1%) | 5 (7.9%) | |
| No urethral sexual exposures | 10 (16.1%) | 14 (22.2%) | |
| **STI History** | | | |
| NGU at enrollment | 30 (47.6%) | 31 (49.2%) | 1.0 |
| Prior NGU | 16 (25.4%) | 3 (4.8%) | **<0.001** |
| Prior CT† | 30 (47.6%) | 12 (19.0%) | **<0.001** |

| | | | |
|---|---|---|---|
| **Current STI**[§] at NGU diagnosis visit, n (%) | | | |
| CT | 1 (1.6%) | 0 (0%) | 1.0 |
| MG | 9 (14.3%) | 0 (0%) | **0.003** |
| ***Microbiota at the visit prior to NGU diagnosis***[¶] (N=83) | | | |
| **Shannon Diversity Index**, mean (±SD) | 1.50 (±0.69) | 1.38 (±0.73) | 0.22 |
| **Species Richness**, mean (±SD) | 19.50 (±11.50) | 18.00 (±11.30) | 0.22 |
| **Bacteria present** | | | |
| *H. influenzae* | 5 (10.0%) | 6 (11.5%) | 1.0 |
| *F. vaginae* | 17 (34.0%) | 6 (11.5%) | **0.009** |
| *L. iners* | 31 (62.0%) | 23 (44.2%) | 0.08 |
| *S. mitis* | 33 (66.0%) | 43 (82.7%) | 0.07 |
| ***Microbiota at the visit concurrent with NGU diagnosis***[△] (N=88) | | | |
| **Shannon Diversity Index**, mean (±SD) | 1.54 (±0.69) | 1.38 (±0.67) | 0.19 |
| **Species Richness**, mean (±SD) | 20.80 (±14.20) | 17.90 (±9.53) | 0.23 |
| **Bacteria present** | | | |
| *H. influenzae* | 2 (3.9%) | 4 (7.3%) | 0.68 |
| *F. vaginae* | 19 (36.5%) | 9 (16.4%) | **0.03** |
| *L. iners* | 27 (51.9%) | 26 (47.3%) | 0.70 |
| *S. mitis* | 36 (69.2%) | 42 (76.4%) | 0.51 |

***** Fisher's exact test was used for categorical variables and a paired t-test was used for continuous variables.

[†] Two participants did not have CT history data available.

[§] One control had inconclusive results for GC and CT tests.

[¶] At the visit prior to NGU diagnosis, 13 cases and 11 controls did not have microbiota data available (N=83 participants with microbiota data). Complete microbiota data were available for 41 of the 63 pairs.

[△] At the visit concurrent with NGU diagnosis, 11 cases and 8 controls did not have microbiota data available (N=88 participants with microbiota data). Complete microbiota data were available for 44 of the 63 pairs.

Table 2. Multivariable analysis of the association of Shannon Diversity Index and species richness with incident NGU among men who have sex with women (MSW) attending a sexual health clinic enrolled from August 2014 - July 2018 (N=83 participants)[¶].

| | **Incident NGU and Shannon Diversity Index** | | **Incident NGU and Species Richness** | |
|---|---|---|---|---|
| | **Multivariable* aOR (95% CI)** | **p-value** | **Multivariable* aOR (95% CI)** | **p-value** |
| **Shannon Diversity Index** (at prior visit) | 10.29 (1.49-73.16) | **0.02** | - | - |
| **Species Richness** (at prior visit) | - | - | 1.082 (1.001-1.169) | **0.047** |
| **Condom Use** | | | | |
| Inconsistent | 1 (Ref) | Ref | 1 (Ref) | Ref |
| Consistent | 0.02 (0.001-0.41) | **0.01** | 0.010 (0.01-0.83) | **0.03** |

[¶] At the visit prior to NGU diagnosis, 13 cases and 11 controls did not have microbiota data available (N=83 participants with microbiota data).

* aOR = adjusted odds ratio. The multivariate models exclude those who had CT or MG present at the visit concurrent with NGU diagnosis as only cases had these pathogens detected at the incident NGU visit.

Table 3. Characteristics of men who have sex with women (MSW) attending a sexual health clinic enrolled from August 2014 - July 2018 associated with detection of *H. influenzae, F. vaginae, L. iners, and S. mitis* detected at the visit prior to NGU diagnosis (N=83 participants)[†].

| | ***H. influenzae*** | | ***F. vaginae*** | | ***L. iners*** | | ***S. mitis*** | |
|---|---|---|---|---|---|---|---|---|
| | **Positive** (n=11) | **Negative** (n=91) | **Positive** (n=23) | **Negative** (n=79) | **Positive** (n=54) | **Negative** (n=48) | **Positive** (n=76) | **Negative** (n=26) |
| ***AT ENROLLMENT*** | | | | | | | | |
| **Age** (years), mean (±SD) | 32.7 (±10.80) | 33.6 (±10.20) | 37.2 (±11.20) | 32.5 (±9.80) | 33.1 (±8.88) | 34.0 (±11.70) | 33.3 (±10.90) | 34.0 (±8.33) |
| **Race/Ethnicity** | | | | | | | | |
| Non-Hispanic White | **8* (72.7%)** | **35* (38.5%)** | 10 (43.5%) | 33 (41.8%) | 27 (50.0%) | 16 (33.3%) | 34 (44.7%) | 9 (34.6%) |
| Non-Hispanic Black, Hispanic (any race), or Other/Multiple/Unknown | **3* (27.3%)** | **56* (61.5%)** | 13 (56.5%) | 46 (58.2%) | 27 (50.0%) | 32 (66.7%) | 42 (55.3%) | 17 (65.4%) |
| **Education** | | | | | | | | |
| ≤ High School | 5 (45.5%) | 36 (39.6%) | 9 (39.1%) | 32 (40.5%) | **14** (25.9%)** | **27** (56.3%)** | 31 (40.8%) | 10 (38.5%) |
| > High School | 6 (54.5%) | 55 (60.4%) | 14 (60.9%) | 47 (59.5%) | **40** (74.1%)** | **21** (43.8%)** | 45 (59.2%) | 16 (61.5%) |
| **Condom Use** | | | | | | | | |
| Inconsistent | 5 (50.0%) | 64 (78.0%) | 17 (85.0%) | 52 (72.2%) | 39 (79.6%) | 30 (69.8%) | 50 (72.5%) | 19 (82.6%) |
| Consistent | 5 (50.0%) | 18 (22.0%) | 3 (15.0%) | 20 (27.8%) | 10 (20.4%) | 13 (30.2%) | 19 (27.5%) | 4 (17.4%) |
| **STI History** | | | | | | | | |
| NGU at enrollment | 5 | 43 | 9 | 39 | 21 | 27 | 37 | 11 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | (45.5%) | (47.3%) | (39.1%) | (49.4%) | (38.9%) | (56.3%) | (48.7%) | (42.3%) |
| Prior NGU | 2 (18.2%) | 11 (12.4%) | 4 (18.2%) | 9 (11.5%) | 7 (13.5%) | 6 (12.5%) | 11 (14.5%) | 2 (8.3%) |
| Prior CT | 1 (9.1%) | 28 (31.5%) | 6 (27.3%) | 23 (29.5%) | 16 (30.8%) | 13 (27.1%) | 20 (26.3%) | 9 (37.5%) |
| ***AT THE NGU DIAGNOSIS VISIT*** | | | | | | | | |
| **Sexual Behaviors** (since last visit) | | | | | | | | |
| Any vaginal sex | 10 (90.9%) | 71 (78.0%) | 21 (91.3%) | 60 (75.9%) | 46 (85.2%) | 35 (72.9%) | 57 (75.0%) | 24 (92.3%) |
| No vaginal sex | 1 (9.1%) | 20 (22.0%) | 2 (8.7%) | 19 (24.1%) | 8 (14.8%) | 13 (27.1%) | 19 (25.0%) | 2 (7.7%) |
| **Current STI** | | | | | | | | |
| Any CT or MG | 1 (9.1%) | 5 (5.6%) | 2 (8.7%) | 4 (5.1%) | 2 (3.7%) | 4 (8.5%) | 5 (6.7%) | 1 (3.9%) |
| ***AT THE PRIOR VISIT*** | | | | | | | | |
| **Bacteria Detected** | | | | | | | | |
| *H. influenzae* | - | - | 1 (4.35%) | 10 (12.7%) | 4 (7.4%) | 7 (14.6%) | 10 (13.2%) | 1 (3.9%) |
| *F. vaginae* | 1 (9.1%) | 22 (24.2%) | - | - | **20*** (37.0%)** | **3*** (6.3%)** | 16 (21.1%) | 7 (26.9%) |
| *L. iners* | 4 (36.4%) | 50 (54.9%) | **20*** (87.0%)** | **34*** (43.0%)** | - | - | 36 (47.4%) | 18 (69.2%) |
| *S. mitis* | 10 (90.9%) | 66 (72.5%) | 16 (69.6%) | 60 (75.9%) | 36 (66.7%) | 40 (83.3%) | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Shannon Diversity Index**, mean (±SD) | 1.39 (±0.45) | 1.45 (±0.74) | **1.90*** (±0.55)** | **1.31*** (±0.70)** | 1.49 (±0.73) | 1.39 (±0.69) | 1.49 (±0.74) | 1.30 (±0.60) |
| **Species Richness**, mean (±SD) | 18.70 (±11.50) | 18.70 (±11.40) | **25.40** (±11.60)** | **16.80** (±10.60)** | 19.10 (±11.50) | 18.30 (±11.30) | **20.40** (±11.90)** | **13.90** (±8.14)** |

† Column n for each species (n=102) sums to greater than the number of participants (N=83) because individuals could contribute multiple case and/or control visits.

Fisher's exact test was used for categorical variables and a t-test was used for continuous variables. Bolded associations were significant at alpha≤0.05.

* p≤0.05, ** p<0.01, ***p<0.001

Table 4. Presence of urethral bacteria at the visit prior to NGU diagnosis and incident NGU among men who have sex with women (MSW) attending a sexual health clinic enrolled from August 2014 - July 2018 (N=83 participants).

| | **Incident NGU - Univariable** | | **Incident NGU - Multivariable[†]** | |
|---|---|---|---|---|
| | **OR (95% CI)** | **p-value** | **aOR* (95% CI)** | **p-value** |
| ***H. influenzae*** | | | | |
| Absent | 1 (Ref) | Ref | 1 (Ref) | Ref |
| Present | 0.80 (0.21-2.98) | 0.74 | 7.71 (0.54-110.36) | 0.13 |
| ***F. vaginae*** | | | | |
| Absent | 1 (Ref) | Ref | 1 (Ref) | Ref |
| Present | **11.00 (1.42-85.20)** | **0.02** | **29.68 (1.55-568.36)** | **0.02** |
| ***L. iners*** | | | | |
| Absent | 1 (Ref) | Ref | 1 (Ref) | Ref |
| Present | 2.50 (0.78-7.97) | 0.12 | 2.02 (0.44-9.13) | 0.36 |
| ***S. mitis*** | | | | |
| Absent | 1 (Ref) | Ref | 1 (Ref) | Ref |
| Present | 0.36 (0.12-1.14) | 0.08 | 0.14 (0.02-1.19) | 0.07 |

* aOR = adjusted odds ratio.

[†] The multivariate model excludes those who had CT or MG present at the visit concurrent with NGU diagnosis as only cases had these pathogens detected at the incident NGU visit.

References


1. Srinivasan S, Chambers LC, Tapia KA, et al. Urethral Microbiota in Men: Association of *Haemophilus influenzae* and *Mycoplasma penetrans* With Nongonococcal Urethritis. *Clin Infect Dis*. 2020;73(7):e1684-e1693. doi:10.1093/cid/ciaa1123
2. Rowlinson E, Hughes JP, Chambers LC, et al. Incidence of Non-Gonococcal Urethritis (NGU) in Men Who Have Sex With Women (MSW) and Associated Risk Factors. *Sex Transm Dis*. 2021;48(5):341-346. doi:10.1097/OLQ.0000000000001314
3. Plummer EL, Ratten LK, Vodstrcil LA, et al. The Urethral Microbiota of Men with and without Idiopathic Urethritis. *mBio*. 13(5):e02213-22. doi:10.1128/mbio.02213-22
4. Frølund M, Wikström A, Lidbrink P, et al. The bacterial microbiota in first-void urine from men with and without idiopathic urethritis. *PLoS ONE*. 2018;13(7):e0201380. doi:10.1371/journal.pone.0201380
5. Frølund M, Falk L, Ahrens P, Jensen JS. Detection of ureaplasmas and bacterial vaginosis associated bacteria and their association with non-gonococcal urethritis in men. *PLOS ONE*. 2019;14(4):e0214425. doi:10.1371/journal.pone.0214425
6. Bradshaw CS, Tabrizi SN, Read TRH, et al. Etiologies of Nongonococcal Urethritis: Bacteria, Viruses, and the Association with Orogenital Exposure. *J Infect Dis*. 2006;193(3):336-345. doi:10.1086/499434
7. Wetmore CM, Manhart LE, Lowens MS, et al. Demographic, Behavioral, and Clinical Characteristics of Men With Nongonococcal Urethritis Differ by Etiology: A Case-Comparison Study. *Sex Transm Dis*. 2011;38(3):180. doi:10.1097/OLQ.0b013e3182040de9
8. Martin DH. Urethritis in Males. In: Holmes KK, Sparling PF, Stamm WE, eds. *Sexually Transmitted Diseases*. 4th ed. New York: McGraw Hill, 2008:987–1016.

9. Lokken EM, Balkus JE, Kiarie J, et al. Association of Recent Bacterial Vaginosis With Acquisition of *Mycoplasma genitalium*. *Am J Epidemiol*. 2017;186(2):194-201. doi:10.1093/aje/kwx043
10. Balkus JE, Manhart LE, Lee J, et al. Periodic Presumptive Treatment for Vaginal Infections May Reduce the Incidence of Sexually Transmitted Bacterial Infections. *J Infect Dis*. 2016;213(12):1932-1937. doi:10.1093/infdis/jiw043
11. Farley MM, Stephens DS, Brachman PS, Harvey RC, Smith JD, Wenger JD. Invasive *Haemophilus influenzae* disease in adults. A prospective, population-based surveillance. CDC Meningitis Surveillance Group. *Ann Intern Med*. 1992;116(10):806-812. doi:10.7326/0003-4819-116-10-806
12. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular Identification of Bacteria Associated with Bacterial Vaginosis. *N Engl J Med*. 2005;353(18):1899-1911. doi:10.1056/NEJMoa043802
13. Mendling W, Palmeira-de-Oliveira A, Biber S, Prasauskas V. An update on the role of *Atopobium vaginae* in bacterial vaginosis: what to consider when choosing a treatment? A mini review. *Arch Gynecol Obstet*. 2019;300(1):1-6. doi:10.1007/s00404-019-05142-8
14. Zheng N, Guo R, Wang J, Zhou W, Ling Z. Contribution of *Lactobacillus iners* to Vaginal Health and Diseases: A Systematic Review. *Front Cell Infect Microbiol*. 2021;11. Accessed January 24, 2023. doi:10.3389/fcimb.2021.792787
15. Srinivasan S, Hoffman NG, Morgan MT, et al. Bacterial Communities in Women with Bacterial Vaginosis: High Resolution Phylogenetic Analyses Reveal Relationships of Microbiota to Clinical Criteria. *PLOS ONE*. 2012;7(6):e37818. doi:10.1371/journal.pone.0037818

16. Zinsli KA, Srinivasan S, Balkus JE, et al. Bacterial vaginosis-associated bacteria in cisgender men who have sex with women: prevalence, association with non-gonococcal urethritis and natural history. *Sex Transm Infect*. Published online December 6, 2022. doi:10.1136/sextrans-2022-055494
17. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—A metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform*. 2009;42(2):377-381. doi:10.1016/j.jbi.2008.08.010
18. Kim BR, Shin J, Guevarra RB, et al. Deciphering Diversity Indices for a Better Understanding of Microbial Communities. 2017;27(12):2089-2093. doi:10.4014/jmb.1709.09027
19. Workowski KA, Bachmann LH, Chan PA, et al. Sexually Transmitted Infections Treatment Guidelines, 2021. *MMWR Recomm Rep*. 2021;70(4):1-187. doi:10.15585/mmwr.rr7004a1
20. Adams HM, Joyce LR, Guan Z, Akins RL, Palmer KL. *Streptococcus mitis* and *S. oralis* Lack a Requirement for CdsA, the Enzyme Required for Synthesis of Major Membrane Phospholipids in Bacteria. *Antimicrob Agents Chemother*. 2017;61(5):e02552-16. doi:10.1128/AAC.02552-16
21. Saunders JM, Mercer CH, Sutcliffe LJ, Cassell JA, Estcourt CS. Factors associated with asymptomatic non-chlamydial non-gonococcal urethritis in heterosexual men: findings from a case-control study. *Int J STD AIDS*. 2013;24(8):627-631. doi:10.1177/0956462413477554