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Impact of testosterone use on the vaginal microbiota of transgender men, including susceptibility to bacterial vaginosis: study protocol for a prospective, observational study

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ABSTRACT

Introduction The effect of testosterone (T) therapy on the vaginal microbiota of transgender men (TGM) is not well characterised, although one cross-sectional study comparing the vaginal microbiota of cisgender women to TGM on T≥1 year found that, in 71% of the TGM, the vaginal microbiota was less likely to be *Lactobacillus*-dominated and more likely to be enriched with >30 other bacterial species, many associated with bacterial vaginosis (BV). This prospective study aims to investigate changes in the composition of the vaginal microbiota over time in TGM who retain their natal genitalia (ie, vagina) and initiate T. In addition, we will identify changes in the vaginal microbiota preceding incident BV (iBV) in this cohort while investigating behavioural factors, along with hormonal shifts, which may be associated with iBV.

Methods and analysis T-naïve TGM who have not undergone gender-affirming genital surgery with normal baseline vaginal microbiota (ie, no Amsel criteria, normal Nugent Score with no *Gardnerella vaginalis* morphotypes) will self-collect daily vaginal specimens for 7 days prior to initiating T and for 90 days thereafter. These specimens will be used for vaginal Gram stain, 16S rRNA gene sequencing and shotgun metagenomic sequencing to characterise shifts in the vaginal microbiota over time, including development of iBV. Participants will complete daily diaries on douching, menses and behavioural factors including sexual activity during the study.

Ethics and dissemination This protocol is approved through the single Institutional Review Board mechanism by the University of Alabama at Birmingham. External relying sites are the Louisiana State University Health Sciences Center, New Orleans Human Research Protection Program and the Indiana University Human Research Protection Program. Study findings will be presented at scientific conferences and peer-reviewed journals as well as shared with community advisory boards at participating gender health clinics and community-based organisations servicing transgender people.

Registration details Protocol # IRB-300008073.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This study will investigate the impact of testosterone (T) therapy on the vaginal microbiota of transgender men (TGM) over time with daily vaginal specimen collection for 7 days prior to T initiation and 90 days thereafter.
⇒ A combination of 16S rRNA gene sequencing and shotgun metagenomics will be used to investigate changes in the vaginal microbiota over time in this cohort, including development of incident bacterial vaginosis.
⇒ Daily diaries including data on oral, genital and anal sex, menses, antibiotic use, sex toy use, sexual partner gender(s), douching and vaginal symptoms will also be obtained for the duration of the study.
⇒ One limitation is the logistics of coordinating T therapy initiation and participant study enrolment in a region (Birmingham, Alabama, USA) with limited lesbian, gay, bisexual, transgender and queer healthcare providers.
⇒ Another limitation is the potential difficulty in recruiting and/or enrolling black TGM who may be less likely to access care or have a normal Nugent Score at screening.

INTRODUCTION

Transgender men (TGM) are assigned female sex at birth but identify as men. Some TGM undergo gender-affirming genital surgeries; however, many retain their natal genitalia (ie, vagina).[1 2] Testosterone (T) is the mainstay of gender-affirming hormone therapy for TGM.[3] Initiation of T blocks menses, thins the vaginal epithelium, and, in some cases, leads to atrophic vaginitis.[4] This phenomenon is similar to what occurs in oestrogen-deficient, postmenopausal cisgender women (CGW).[5] Additionally, the vaginal microbiota

in postmenopausal CGW shifts from *Lactobacillus* spp dominance to more diverse bacterial communities.[6 7] This acquired vaginal dysbiosis is a risk factor for acquisition of HIV and other sexually transmitted infections (STIs)[8–15] and may progress to bacterial vaginosis (BV), the most common vaginal infection.[16] Although TGM on T therapy experience oestrogen deficiency similar to postmenopausal CGW, their T levels are considerably higher. The impact of this low oestrogen/high T state on the vaginal microbiota of TGM is unknown and cannot be extrapolated from studies of postmenopausal CGW.

If and how T therapy impacts the vaginal microbiota over time in TGM remains unclear. Results of a cross-sectional study comparing the vaginal microbiota of CGW to that of TGM on T therapy ≥1 year found that, in 71% of the TGM, the vaginal microbiota was less likely to be *Lactobacillus*-dominated and more likely to be enriched with >30 other bacterial species, many associated with BV.[17] However, this study did not compare the vaginal microbiota of TGM pre-T initiation and post-T initiation to identify temporal shifts that could precede vaginal dysbiosis and/or incident BV (iBV) or identify potential causes of these shifts.

Here, we will conduct a prospective, observational study with intensive vaginal specimen collection and cutting edge metagenomic sequencing approaches to: (A) Capture the evolution of the vaginal microbiota of TGM pre-T initiation and post-T initiation, and (B) Identify changes in the vaginal microbiota that precede iBV in TGM on T. We will also investigate potential behavioural (ie, sexual activity, douching, etc) factors, along with hormonal shifts, which may be associated with iBV in this population. Our long-term goal is to optimise the health benefits of gender-affirming T therapy and mitigate its risks for TGM.

OBJECTIVES

This study has two primary aims. Aim 1 is to investigate changes in the composition of the vaginal microbiota over time in TGM initiating T. We hypothesise that T will alter the composition of the vaginal microbiota of TGM. Based on prior data,[17] we anticipate that within 90 days of T initiation, approximately 50% of 40 TGM (n=20) with baseline normal vaginal microbiota (no Amsel criteria, normal Nugent Score 0–3 with no *Gardnerella vaginalis* morphotypes) will experience a shift towards vaginal dysbiosis (Nugent Score ≥4 on at least two consecutive days). Starting 7 days prior to T initiation and continuing for 90 days thereafter, 40 TGM will self-collect daily vaginal specimens and perform daily smears for subsequent vaginal Gram stain and Nugent Score determination.[18] Participants will also complete daily diaries to document douching, menses, sexual behaviour data, vaginal symptoms and medication use (oral and/or intravaginal) while on the study to determine their influence on the vaginal microbiota. 16S rRNA V4 (16S) sequencing[19] will be performed on all seven pre-T vaginal specimens as well as weekly specimens thereafter from each participant to determine the vaginal microbial community state type (CST) composition over time.[20] In TGM whose Nugent Scores shift to vaginal dysbiosis, additional 16S sequencing will be performed on vaginal specimens collected 7 days preshift through 3 days postshift to intensively sample this CST transition interval.[21] If multiple shifts to vaginal dysbiosis occur during the 90-day study period after T initiation, we will focus on sequencing the period around the first shift, due to budgetary constraints. Residual vaginal specimens and blood collected prior to (at enrolment) and after (days 30 and 90) T initiation will be archived to investigate additional hypotheses generated by the initial results of this study.

Aim 2 is to identify changes in the vaginal microbiota that precede iBV in TGM on T. We hypothesise that iBV could be precipitated by sexual transmission of BV-associated bacteria (BVAB) and/or other factors, including hormonal shifts, which promote the overgrowth of BVAB already present in the vaginal tract.[22 23] In a pilot study, we observed that *Lactobacillus* phages, which 16S sequencing does not detect,[24] were common in the vaginal specimens of some CGW prior to iBV.[25] In Aim 2, we will identify vaginal specimens from five TGM in Aim 1 who develop iBV (Nugent Score ≥7 for at least two consecutive days) (cases), and an equal number of controls who do not develop iBV (maintain a Nugent Score 0–6 for most of the study). We hypothesise that half (10/20) of TGM who develop vaginal dysbiosis after T initiation in Aim 1 will progress to iBV. We will use shotgun metagenomic sequencing to characterise daily vaginal specimens collected 14 days prior to the onset of iBV, the day of iBV and 3 days thereafter in cases. An equal number of vaginal specimens will be sequenced from controls, aligned with cases based on the day of T initiation. Comparison of case and control vaginal microbiota will investigate whether the introduction or bloom of bacteriophages, viral STIs and/or fungi, which are not identified by 16S sequencing approaches,[24] are associated with iBV.

METHODS AND ANALYSIS

Study recruitment

Between February 2022 and October 2023, potential participants will be recruited from clinical sites in the Birmingham, Alabama, USA metropolitan area which specialise in transgender healthcare. These include two academic medical centre clinics (ie, University of Alabama at Birmingham (UAB) Gender Health Clinic and UAB Student Health Clinic) caring for approximately 80 TGM and a community-based clinic (ie, Magic City Wellness Centre, a lesbian, gay, bisexual, transgender and queer primary care clinic) caring for approximately 180 TGM. Eligible participants will be recruited via research team members who are providers at these clinics as well as patient navigators. We will also canvas study flyers (figure 1) on the UAB campus, the Magic City Wellness Center (including their website), and throughout

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Figure 1 Study recruitment flyer. UAB, University of Alabama at Birmingham.

the Birmingham, Alabama, USA metropolitan area, post advertisements on social media channels, and encourage study participation through word of mouth. After contacting study staff and/or being approached by a provider about study participation, interested individuals will be instructed to arrange a study screening visit at the UAB Sexual Health Research Clinic (SHRC). These recruitment methods were vetted through stakeholders in the local transgender community prior to implementation and will be adapted throughout the study period to optimise community engagement.

Study design

Aim 1 is a prospective, longitudinal cohort study of TGM with normal baseline vaginal microbiota (no Amsel criteria[26] and a normal Nugent Score of 0–3 with no *G. vaginalis* morphotypes[18]) who have not undergone gender-affirming genital surgery and are T-naïve or have not been on T in the past 6 months. There are two phases: screening and enrolment.

For screening, TGM meeting the eligibility criteria in table 1 will provide written informed consent. They will then have a urine pregnancy test. Alternatively, participants will be able to self-report their pregnancy status if they are not comfortable having this test. A brief history will be taken to determine if they are having any current vaginal complaints (ie, discharge, odour, itching, etc). They will then self-collect one vaginal specimen for research clinician determination of the Amsel criteria (vaginal pH, whiff test and presence of ≥20% clue cells per high power field on vaginal wet mount in addition to

Table 1 Aim 1: screening inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
At least 18 years of age	Oral or intravaginal antibiotic use within the past 14 days
Assigned female sex at birth	Documented HIV infection
Presence of natal genitalia (vagina)	Pregnancy
Self-identify as transmasculine or non-binary	Use of testosterone therapy in the past 6 months
Desire to initiate IM testosterone, but willing to wait 7 days after enrolment before starting	

IM, intramuscular.

complaint of vaginal discharge)[26] and the Nugent Score.[18] A Nugent Score of 0–3 will be defined as normal vaginal microbiota, 4–6 as intermediate vaginal microbiota, and 7–10 as BV.[18] These criteria will be used to determine potential participants' eligibility for enrolment (table 2).

TGM meeting all screening eligibility criteria will provide written informed consent for the enrolment portion of the study. They will complete a study questionnaire on their sociodemographics, sexual orientation, medical and sexual history, menses, sexual behaviour data, and sexual partner history including gender(s) of sexual partners, to be entered into the Research Electronic Data Capture System (REDCap).[27 28] REDCap is a secure, web-based software platform designed to support data capture for research studies, providing an intuitive interface for validated data capture, audit trails for tracking data manipulation and export procedures, automated export procedures for seamless data downloads to common statistical packages, and procedures for data integration and interoperability with external sources. Participants will self-collect an additional vaginal specimen for chlamydia, gonorrhoea and trichomonas nucleic acid amplification testing on the Roche Cobas 6800 platform.[29 30] Standard-of-care HIV testing data will be obtained from participants' electronic medical records at their respective gender health clinic, with written permission. HIV-positive TGM will be excluded as some studies have associated those with HIV having a more diverse vaginal microbiota,[31–33] although effective antiretroviral therapy[34] and regular BV testing and treatment[35] could moderate this risk.

Table 2 Aim 1: enrolment inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
No Amsel criteria	Trichomonas on vaginal wet mount
Nugent Score 0–3 with no *Gardnerella vaginalis* morphotypes	Symptomatic vaginal yeast infection
	Positive chlamydia, gonorrhoea or trichomonas NAAT at baseline
	Positive HIV test

NAAT, nucleic acid amplification test.

Box 1 Instructions for self-collection of vaginal specimens for glass slide preparation and tube insertion

1. Remove the first 'FLOQ' swab from the packaging material by tearing off the top of the packaging. Be careful not to touch the tip of the swab.
2. Using your non-dominant hand (not your writing hand) open the labia (lips of the vaginal area) to allow entrance of the swab into the genital tract (vagina).
3. Insert the swab 2 inches into the genital tract being careful not to touch the tip of the swab anywhere else in the genital area.
4. Twist the swab several times while inside of the genital tract.
5. Remove the swab the same way that you did for insertion. Again, be careful not to touch the tip of the swab outside of the genitals.
6. Roll the swab across the length of the glass slide from left to right.
7. Place the glass slide in the slide carrier and close the top of the carrier.
8. Put the *slide* back into its bag.
9. Place this swab into the orange capped tube containing liquid.
10. Break off the handle of the swab at the score line (ie, the line pointed out to you during your visit) and discard the handle. Screw the top of the collection tube on *firmly*, but do not overtighten.
11. Put the *tube* back into its bag and keep the tube in an upright position.
12. Repeat steps 1–11 for the second and third genital swabs.
13. Store swabs in your study lunchbox in the refrigerator (at −4°C) until weekly drop-off at the University of Alabama at Birmingham Sexual Health Research Clinic.

Next, TGM will be provided with instructions for obtaining daily self-collected vaginal specimens at home. They will self-collect three vaginal specimens daily for 7 days prior to T initiation and for 90 days thereafter. One specimen will be used to smear a glass slide for subsequent Gram staining to monitor for changes in the composition of the vaginal microbiota over time. The other two specimens will be used for future 16S and shotgun metagenomic sequencing. Participants will fill out a one-page daily diary (yes/no checklist on oral, genital and anal sex, menses, oral and intravaginal antibiotic use, sex toy use, partner gender(s), douching and vaginal symptoms) for each day on the study. They will be provided with instructions on how to self-collect the specimens at home (box 1), a 1 week supply of specimen collection materials in an insulated lunch cooler, and hard copies of daily diaries. Each participant will also be asked to obtain a sampling control at the beginning of the study (ie, a blank swab opened and waved in the air for 20 s in their typical sampling environment at home). This specimen will capture any background microbial contamination from the home environment that could confound vaginal microbiota analyses.[36]

TGM will be asked to return to the UAB SHRC clinic 1 week after enrolment to turn in their daily vaginal specimens, glass slides and daily diaries, at which time they will start their T therapy (prescribed by their local gender healthcare provider per standard of care).[37] To avoid pharmacokinetic differences between T routes (transdermal or intramuscular (IM)) and doses, all participants will be prescribed weekly 50 mg IM doses of testosterone cypionate, which is a common initial regimen due to its low cost and weekly dosing. During routine care, T and oestradiol levels are not typically obtained prior to T initiation. For this study, however, serum T and oestradiol levels will be obtained from participants prior to T initiation and at 30 days and 90 days post-T initiation, and the residual serum will be stored for future research.

TGM will continue daily vaginal specimen collection, glass slide preparation and completion of daily diaries for 90 days after T initiation. They will deliver their specimens, glass slides and daily diaries to the UAB SHRC and obtain new supplies weekly. TGM testing positive for an STI at enrolment will be treated per standard of care[38] and dropped as STIs and their treatment may alter the vaginal microbiota.[39–42] If TGM have vaginal symptoms (eg, discharge, itching, odour, etc) while on the study, they will be encouraged to call the study site to schedule an interval, standard-of-care visit. If symptomatic BV is diagnosed during this visit (by Amsel criteria), they will be treated and dropped from the study, as the primary outcome of Aim 2 (iBV) will have been reached and they will require antibiotic treatment. If an incident STI is diagnosed during the study, participants will also be treated[38] and dropped.

All participants will be paid $20 at screening, $25 at enrolment and $20 per weekly drop-off visit (weeks 0–13). All study-related data will be entered into the REDCap database.[27]

Study setting

Participants will be enrolled at the UAB SHRC. T will be prescribed by their local gender-affirming provider as part of routine care at either of the three participating clinics. Vaginal specimens and Gram stain slides will be stored in UAB research laboratories once returned to the study site. Select vaginal specimens will be shipped to Indiana University (IU) for 16S and shotgun metagenomic sequencing by author ET. The resulting sequence data will be sent to authors JHE and CMT at the Louisiana State University Health Sciences Center (LSUHSC) in New Orleans, Louisiana, USA for bioinformatics analysis as well as to UAB (authors CAM, OTVG, AT and DL) for statistical analyses.

Data sources and variables

Online supplemental document 1 includes the REDCap case report forms (CRFs) for the screening visit, enrolment visit and daily diaries. Additional CRFs include T/oestradiol levels, an interval study visit sheet for vaginal complaints and an end-of-study document. The REDCap database for this study will be hosted by the UAB Department of Medicine Information Technology REDCap Team and managed by the UAB sexually transmitted disease (STD) Research Program Data Manager, author KJA.

Bias

While participants may experience social desirability bias, anonymity on the CRFs is expected to mitigate this bias.

DNA extraction, 16S and shotgun metagenomic sequencing methods

For Aim 1, DNA will be extracted from select vaginal specimens using the Qiagen DNeasy Blood and Tissue Kit. Controls will be included at all steps to monitor for reagent contamination. DNA quality will be monitored by gel electrophoresis and fluorescent dsDNA assays. Genomic DNA (gDNA) will be stored at −80°C until use in construction of sequencing libraries. 16S alleles spanning the V4 region will be PCR-amplified from vaginal specimen gDNA using the NEXTFLEX 16S V4 Amplicon-Seq kit 2.0 for Illumina platforms (PerkinElmer), as previously described,[43] and sequenced in pools on an Illumina MiSeq using v3 chemistry. We will examine at least 10 000 sequences/specimen since rarefaction analyses indicate that 1000–2000 sequences are insufficient to achieve saturation at a 97% operational taxonomic unit threshold.

For Aim 2, DNA extracted from select vaginal specimens will be analysed by paired end (2×150 bp) shotgun sequencing to an average depth of 330 M reads/specimen on a NovaSeq 6000 sequencer using one S4 flowcell (v1.5 chemistry). To estimate the absolute abundance of microbial reads, we will add a spike-in DNA standard, such as ZymoBIOMICS spike-in control (Zymo Research), comprising *Imtechella halotolerans* (a Gram-negative organism) and *Allobacillus halotorerans* (a Gram-positive organism), to each specimen. When spiked in at known quantities, these environmental microbes not only serve as an internal control, but their sequences can be removed bioinformatically to obtain the sample's original microbial profile. The shotgun libraries will be constructed using the NexteraXT library prep kit (Illumina).[25]

Bioinformatics analysis of 16S sequence data

Fastq files from the 16S Illumina MiSeq run will be processed through the DADA2 pipeline at LSUHSC[44] to determine amplicon sequence variants. Parameters based on quality profiles of the sequencing run will be used to retain high-quality data and trim off amplicon primers. The DADA2 algorithm will be used to build an error profile for the vaginal specimens, isolate amplicon sequence variants, and detect and remove chimaeras. The Silva database V.138[45] will be used for taxonomic classification. Sequence variants will be placed into a sequence table indicating frequency for each specimen and decontam[46] will be used with sampling controls for each participant to remove sequence variants identified

as contaminants. Phyloseq[47] will be used to calculate and visualise the alpha and beta diversity and provide a taxonomic summary of the vaginal specimens as an overview of the data. CSTs will be constructed using the Bray-Curtis distance to calculate pairwise sample distances followed by denoising of this distance matrix selecting the most significant Principal Coordinates Analysis eigenvectors.[48] Custom scripts will be written to investigate hypotheses and produce custom analyses and visualisations.

Bioinformatics analysis of shotgun metagenomics sequence data

For Aim 2, Kraken2 V.2.0.9-beta[49] will be used to perform a broad analysis of taxonomic classification for each shotgun metagenome specimen. Each specimen will also be processed through the bioBakery V.3 workflow[50] including KneadData to perform quality control and sequence trimming, followed by MetaPhlAn3[50] and HUMAnN3[50] analyses to identify any metagenomic pathways present. Sequencing reads identified as human by MetaPhlAan3[50] and Bowtie2[51] mapping to the most recent human host genome will be removed from the raw fastq files. Custom scripts will be written to investigate hypotheses and produce custom analyses and visualisations. For vaginal specimens with both 16S and shotgun metagenomic sequence data, taxonomic classifications will be compared.

Statistical analysis

For Aim 1, 48 TGM will be enrolled. It is estimated that approximately eight TGM will drop out of the study due to loss to follow-up (n=4) or baseline STI (n=4). This will leave a total of 40 TGM for analysis. We hypothesise that approximately 50% of these 40 TGM (n=20) will experience a shift in their vaginal microbiota towards dysbiosis (Nugent Score ≥4 for at least two consecutive days) within 90 days of T initiation. Of the 20 TGM with vaginal dysbiosis, we estimate that approximately 50% (n=10) will develop iBV during the study. This estimation is reasonable given the large proportion of TGM whose vaginal microbiota was enriched with >30 bacterial species associated with BV during a prior cross-sectional study.[17] A one-sample (two-sided) Z-test with normal approximation will have a power of 88% to detect a statistically significant (alpha=0.05) difference, assuming a 27% prevalence of BV.[52] The power will range from 93% to 81%, assuming an iBV prevalence of 25%–29%. Power calculations were performed using PASS, V.14 (NCSS, Kaysville, Utah, USA).

Data analysis will begin with descriptive statistics (age, race, sociodemographics, clinical characteristics, etc) of the cohort. Continuous variables will be reported as means with SD or medians with quartiles/ranges, depending on their distribution. Categorical variables will be reported as frequency and percentage and, for the outcome of interest, the 95% CI will be calculated. The collection of daily vaginal specimens will enable investigation of shifts in the vaginal microbiota over time among participants in the study. Characteristics/predictors of vaginal microbial shift will then be examined using risk calculations and the precision of these estimates will be examined with 95% CIs. Multivariable models will be performed using log binomial or modified Poisson regression with robust standard errors to adjust for specific predictors (eg, race/ethnicity,[20] sexual activity, partner gender(s)). These models will generate adjusted risk ratios (RRs) and accommodate for the small sample size. Model fit will be examined and covariates removed to provide stable RRs, if necessary. Statistical significance will be set at a p value of 0.05 (two-tailed). In addition, frequencies and percentages of CSTs at T initiation and at the time of vaginal microbial shift will be calculated to classify them as dominated by BVAB versus non-BV-dominated.[20] McNemar's test will be used to estimate the agreement between the times. Smaller test statistics will indicate an increased disagreement (ie, change of CST) between these time periods for within-person measurements.

For Aim 2, descriptive statistics will be reported for the dichotomised outcome of iBV; yes or no, maintaining the paired structure of the data (matched 1:1 by day of T initiation). The absolute abundance of bacterial taxa will be estimated. As the total sample size will be 10, the ability to examine longitudinal patterns in the sequence data using linear mixed models, area under the curve and repeated measures analysis of variance will be limited. Expecting skewness, non-parametrical analyses will be explored using bootstrapping to account for the correlation among repeat observations. Bootstrap CIs will be estimated using a Bonferroni correction, adjusting the 0.05 type 1 error rate for the 14 days of vaginal specimens obtained from the same participant (0.05/14=0.0036).[21]

All statistical analyses will be conducted using statistical analysis system (SAS) statistical software, V.9.4 (Cary, North Carolina, USA).

Data management and confidentiality

Participating in this study involves the disclosure of gender identity and sexual behaviour to research personnel. Every effort will be made to create a secure and trustworthy environment in the privacy of an examination room at the UAB SHRC. To mitigate potential disclosure of sensitive health information (on the daily diaries) to persons close to or cohabitating with participants, all study materials will be labelled in a generic manner and not contain any indication that the study is related to sexual health subject matter. Hard copy consent and CRF forms will be stored in study binders in a locked cabinet in a locked office at the study site. All hard copy CRF data will be entered by study personnel into the REDCap database. This database will be stored on a password-protected UAB server and managed by the UAB STD Research Program Data Manager, author KJA. Participants will be assigned a unique study identification number and no identifiers will be collected on the consent forms or the CRFs. Electronic transfer of CRF data to statisticians AT and DL will occur through the UAB intranet via Box. De-identified

sequence data generated by author ET at IU will be sent via Box to authors JHE and CMT at LSUHSC, for bioinformatics analysis, and to authors AT and DL at UAB, for statistical analysis. These data will be kept on password-protected servers on UAB-encrypted and LSUHSC-encrypted computers. Since this research does not qualify as a clinical trial, a data and safety monitoring plan is not required.

Ethics and dissemination

This protocol is approved through the single Institutional Review Board (IRB) mechanism by the UAB IRB (Protocol # IRB-300008073). External relying sites are the LSUHSC New Orleans Human Research Protection Program and the IU Human Research Protection Program. Study findings will be presented in scientific conferences and peer-reviewed journals as well as shared with community advisory boards for participating gender health clinics and community-based organisations servicing transgender people.

DISCUSSION

This prospective cohort study will investigate the effect of T initiation on the vaginal microbiota of TGM via intensive vaginal specimen collection over a 90-day period. This study is novel and important for several reasons. In general, sexual health research engaging TGM is lacking. To our knowledge, this is the first longitudinal study investigating the impact of T on the vaginal microbiota of TGM. Second, transgender people have limited access to gender-affirming healthcare providers, including those focusing on sexual health, particularly in the Deep South, where this study will occur (Birmingham, Alabama, USA). Community engagement in research efforts including those in this study are essential both in answering the proposed research questions as well as identifying sexual health issues faced by TGM. This study will inform future efforts on how to best engage TGM in clinical care and research related to their sexual health in this area. Given that TGM are significantly impacted by BV, HIV and other STIs,[53] investigating ways to minimise the risk for these infections could have the potential to make a huge impact on the overall sexual health of this population. An additional strength is that this study will provide an extensive repository of prospective vaginal and serum specimens from a cohort of TGM for future studies.

Despite the study strengths, there are limitations. Given the limited number of gender-affirming healthcare providers prescribing masculinising hormone therapy in the Deep South and in Birmingham, Alabama, USA, in particular, the eligibility criteria and study design may impact our ability to robustly recruit and enrol TGM. It is possible that many potential participants may either already be on T or will be otherwise ineligible to enrol in this study. Additionally, logistical challenges that could impact study eligibility may occur. Potential participants responding to study recruitment flyers may not yet have an established link with one of the local gender-affirming healthcare clinics associated with this study; wait times for new patients at these clinics can be lengthy. Additionally, potential participants may not be insured or have the funds to pay for gender-affirming hormone therapy out of pocket, not only making them unable to participate in this study but making it difficult to engage in care at one of the clinics. Another possible limitation will be recruiting and enrolling black and/or Latinx TGM. One possible reason for this is that people of colour in the South, particularly those without a cisgender identity, may harbour mistrust of the medical system and avoid engaging in care with the clinics and healthcare providers associated with this study. TGM of colour may also be less likely to have a normal Nugent Score at the screening visit, based on a higher prevalence of BV in African-American CGW,[16 54] making them ineligible.

Data statement

16S and shotgun metagenomics sequence data will be uploaded to the National Institutes of Health (NIH) Sequence Read Archive immediately upon its availability and accession numbers will be provided in publications and/or upon written request to lead author CAM. After publication of the study results, the research team will prepare final research data files that may be shared with other investigators, upon request. These data files will be archived as Excel documents or SAS data sets. The final data files will not contain any personal identifying information of participants and will only contain data collected on the study CRFs. Requests for research data must be submitted to author CAM in writing, via a standard data request form, with a justification for how the data will be used. Author CAM and UAB administrators will review all requests for research data obtained from this study. The mechanism by which the data will be made available to investigators will follow all NIH guidelines for data sharing as they evolve. At a minimum this would consist of a data use agreement that provides for commitments to use the data for research purposes only, secure the data with appropriate computer technology, obtain IRB approval, and destroy (or return) the data after analyses are complete.

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Contributors CAM, OTVG, ESK-D, KS, KJA, ICE, KJG, JHE, AT, DL, NVW, ET and CMT developed the study and the study protocol. CAM, OTVG and KJA developed the study case report forms (CRFs) for the REDCap database. The manuscript was written and revised by CAM, JAS, OTVG and CMT with input and review from all authors. All authors read and approved the final manuscript.

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Competing interests CAM has received research grant support from NIH/NIAID, Lupin Pharmaceuticals, Gilead Sciences, Visby Medical, and Abbott Molecular; is a consultant for Lupin Pharmaceuticals, BioNTech, Scynexis, and Cepheid; and has received honoraria from Visby Medical, Elsevier, Abbott Molecular, Cepheid, Roche Diagnostics, and Lupin Pharmaceuticals. OTVG has received research grant support from NIH/NIAID, Lupin Pharmaceuticals, Gilead Sciences, and Abbott Molecular and has served on a scientific advisory board for Scynexis. KJG has received research grant support from NIH/NIAID and Lupin Pharmaceuticals. The other authors have no competing interests to declare.

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REFERENCES

1 Quinn VP, Nash R, Hunkeler E, *et al*. Cohort profile: study of transition, outcomes and gender (strong) to assess health status of transgender people. *BMJ Open* 2017;7:e018121.
2 James SE, Herman JL, Rankin S, *et al*. The report of the 2015 U.S transgender survey. 2016. Available: https://www.transequality.org/sites/default/files/docs/USTS-Full-Report-FINAL.PDF
3 Coleman E, Bockting W, Botzer M, *et al*. Standards of care for the health of transsexual, transgender, and gender-nonconforming people, version 7. *Int J Transgend* 2012;13:165–232.
4 Irwig MS. Testosterone therapy for transgender men. *Lancet Diabetes Endocrinol* 2017;5:301–11.
5 Muhleisen AL, Herbst-Kralovetz MM. Menopause and the vaginal microbiome. *Maturitas* 2016;91:42–50.
6 Mendling W. Vaginal microbiota. *Adv Exp Med Biol* 2016;902:83–93.
7 Cauci S, Driussi S, De Santo D, *et al*. Prevalence of bacterial vaginosis and vaginal flora changes in peri- and postmenopausal women. *J Clin Microbiol* 2002;40:2147–52.
8 Abbai NS, Nyirenda M, Naidoo S, *et al*. Prevalent herpes simplex virus-2 increases the risk of incident bacterial vaginosis in women from south africa. *AIDS Behav* 2018;22:2172–80.
9 Lokken EM, Balkus JE, Kiarie J, *et al*. Association of recent bacterial vaginosis with acquisition of mycoplasma genitalium. *Am J Epidemiol* 2017;186:194–201.
10 Brusselaers N, Shrestha S, van de Wijgert J, *et al*. Vaginal dysbiosis and the risk of human papillomavirus and cervical cancer: systematic review and meta-analysis. *Am J Obstet Gynecol* 2019;221:9–18.
11 Abbai NS, Reddy T, Ramjee G. Prevalent bacterial vaginosis infection-a risk factor for incident sexually transmitted infections in women in Durban, South Africa. *Int J STD AIDS* 2016;27:1283–8.
12 Atashili J, Poole C, Ndumbe PM, *et al*. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. *AIDS* 2008;22:1493–501.
13 Onderdonk AB, Delaney ML, Fichorova RN. The human microbiome during bacterial vaginosis. *Clin Microbiol Rev* 2016;29:223–38.
14 Gillet E, Meys JF, Verstraelen H, *et al*. Bacterial vaginosis is associated with uterine cervical human papillomavirus infection: a meta-analysis. *BMC Infect Dis* 2011;11:10.
15 Wiesenfeld HC, Hillier SL, Krohn MA, *et al*. Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection. *Clin Infect Dis* 2003;36:663–8.
16 Koumans EH, Sternberg M, Bruce C, *et al*. The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sex Transm Dis* 2007;34:864–9.
17 Winston McPherson G, Long T, Salipante SJ, *et al*. The vaginal microbiome of transgender men. *Clin Chem* 2019;65:199–207.
18 Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* 1991;29:297–301.
19 Van Der Pol WJ, Kumar R, Morrow CD, *et al*. In silico and experimental evaluation of primer sets for species-level resolution of the vaginal microbiota using 16S ribosomal RNA gene sequencing. *J Infect Dis* 2019;219:305–14.
20 Ravel J, Gajer P, Abdo Z, *et al*. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA* 2011;108:4680–7.
21 Muzny CA, Blanchard E, Taylor CM, *et al*. Identification of key bacteria involved in the induction of incident bacterial vaginosis: A prospective study. *J Infect Dis* 2018;218:966–78.
22 Schwebke JR, Muzny CA, Josey WE. Role of gardnerella vaginalis in the pathogenesis of bacterial vaginosis: a conceptual model. *J Infect Dis* 2014;210:338–43.
23 Muzny CA, Taylor CM, Swords WE, *et al*. An updated conceptual model on the pathogenesis of bacterial vaginosis. *J Infect Dis* 2019;220:1399–405.
24 Poretsky R, Rodriguez-R LM, Luo C, *et al*. Strengths and limitations of 16S rrna gene amplicon sequencing in revealing temporal microbial community dynamics. *PLoS One* 2014;9:e93827.
25 Taylor CM, Toh E, Dong Q, *et al*. *Use of shotgun metagenomics to investigate the pathogenesis of incident bacterial vaginosis. poster presentation at anaerobe 2020 (virtual); July 24, 2020*. Seattle, WA,
26 Amsel R, Totten PA, Spiegel CA, *et al*. Nonspecific vaginitis, diagnostic criteria and microbial and epidemiologic associations. *Am J Med* 1983;74:14–22.
27 Harris PA, Taylor R, Thielke R, *et al*. Research electronic data capture (redcap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009;42:377–81.
28 Harris PA, Taylor R, Minor BL, *et al*. The redcap consortium: building an international community of software platform partners. *J Biomed Inform* 2019;95:103208.
29 Marlowe EM, Gohl P, Steidle M, *et al*. *Trichomonas vaginalis* detection in female specimens with cobas® TV/MG for use on the cobas® 6800/8800 systems. *Eur J Microbiol Immunol* 2019;9:42–5.

30 Van Der Pol B, Fife K, Taylor SN, *et al*. Evaluation of the performance of the cobas CT/NG test for use on the cobas 6800/8800 systems for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in male and female urogenital samples. *J Clin Microbiol* 2019;57:e01996-18.
31 Spear GT, Sikaroodi M, Zariffard MR, *et al*. Comparison of the diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women with or without bacterial vaginosis. *J Infect Dis* 2008;198:1131–40.
32 Short C-ES, Brown RG, Quinlan R, *et al*. *lactobacillus*-depleted vaginal microbiota in pregnant women living with HIV-1 infection are associated with increased local inflammation and preterm birth. *Front Cell Infect Microbiol* 2020;10:596917.
33 Borgdorff H, Tsivtsivadze E, Verhelst R, *et al*. Lactobacillus-dominated cervicovaginal microbiota associated with reduced HIV/STI prevalence and genital HIV viral load in African women. *ISME J* 2014;8:1781–93.
34 Chehoud C, Stieh DJ, Bailey AG, *et al*. Associations of the vaginal microbiota with HIV infection, bacterial vaginosis, and demographic factors. *AIDS* 2017;31:895–904.
35 Mehta SD, Donovan B, Weber KM, *et al*. The vaginal microbiota over an 8- to 10-year period in a cohort of HIV-infected and HIV-uninfected women. *PLoS One* 2015;10:e0116894.
36 Eisenhofer R, Minich JJ, Marotz C, *et al*. Contamination in low microbial biomass microbiome studies: issues and recommendations. *Trends Microbiol* 2019;27:105–17.
37 Guidelines for the Primary and Gender-Affirming Care of Transgender and Gender Nonbinary People: Department of Family & Community Medicine, University of California, San Francisco. 2016. Available: https://transcare.ucsf.edu/sites/transcare.ucsf.edu/files/Transgender-PGACG-6-17-16.pdf
38 Workowski KA, Bachmann LH, Chan PA, *et al*. Sexually transmitted infections treatment guidelines, 2021. *MMWR Recomm Rep* 2021;70:1–187.
39 van der Veer C, Bruisten SM, van der Helm JJ, *et al*. The cervicovaginal microbiota in women notified for chlamydia trachomatis infection: A case-control study at the sexually transmitted infection outpatient clinic in amsterdam, the netherlands. *Clin Infect Dis* 2017;64:24–31.
40 Martin DH, Zozaya M, Lillis RA, *et al*. Unique vaginal microbiota that includes an unknown mycoplasma-like organism is associated with trichomonas vaginalis infection. *J Infect Dis* 2013;207:1922–31.
41 Ketterer MR, Rice PA, Gulati S, *et al*. Desialylation of Neisseria gonorrhoeae lipooligosaccharide by cervicovaginal microbiome sialidases: the potential for enhancing infectivity in men. *J Infect Dis* 2016;214:1621–8.
42 Shafer WM. Does the cervicovaginal microbiome facilitate transmission of Neisseria gonorrhoeae from women to men? implications for understanding transmission of gonorrhea and advancing vaccine development. *J Infect Dis* 2016;214:1615–7.
43 NEXTFLEX® 16S V4 amplicon-seq kit 2.0 for illumina® platforms 2020. Available: https://perkinelmer-appliedgenomics.com/wp-content/uploads/2020/4203-02-NEXTflex-16S-V4-Amplicon-Seq-Kit-2.0.pdf
44 Callahan BJ, McMurdie PJ, Rosen MJ, *et al*. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;13:581–3.
45 Quast C, Pruesse E, Yilmaz P, *et al*. The silva ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;41:D590–6.
46 Davis NM, Proctor DM, Holmes SP, *et al*. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 2018;6:226.
47 McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;8:e61217.
48 DiGiulio DB, Callahan BJ, McMurdie PJ, *et al*. Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci USA* 2015;112:11060–5.
49 Wood DE, Lu J, Langmead B. Improved metagenomic analysis with kraken 2. *Genome Biol* 2019;20:257.
50 Beghini F, McIver LJ, Blanco-Míguez A, *et al*. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with biobakery 3. *Elife* 2021;10:e65088.
51 Langmead B, Salzberg SL. Fast gapped-read alignment with bowtie 2. *Nat Methods* 2012;9:357–9.
52 Peebles K, Velloza J, Balkus JE, *et al*. High global burden and costs of bacterial vaginosis: a systematic review and meta-analysis. *Sex Transm Dis* 2019;46:304–11.
53 Van Gerwen OT, Jani A, Long DM, *et al*. Prevalence of sexually transmitted infections and human immunodeficiency virus in transgender persons: a systematic review. *Transgend Health* 2020;5:90–103.
54 Muzny CA, Sunesara IR, Austin EL, *et al*. Bacterial vaginosis among African American women who have sex with women. *Sex Transm Dis* 2013;40:751–5.