What's Sex Got to Do With It? Understanding Potential Confounding and Exposure Misclassification in Mechanistic Sexually Transmitted Infection Research

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We conducted a prospective study of 13 heterosexual couples to understand the impact of recent condomless vaginal sex on vaginal immune marker measurement and potential exposure misclassification due to the presence of semen. All immune markers were detectable in semen and concentrations of vaginal immune markers varied by sex recency.

Keywords. cytokine; HIV; prostate-specific antigen; sexually transmitted infection.

Innate and adaptive immune factors influence human immunodeficiency virus (HIV) acquisition. Higher beta-defensin (HB)-2, interleukin (IL)-8, interferon γ -induced protein 10 (IP-10), macrophage-inflammatory protein (MIP)-1 α , MIP-1 β , RANTES, and IL-1 α and lower secretory leukocyte protease inhibitor (SLPI) concentrations have been associated with increased risk of HIV acquisition in women [1–5]. However, design challenges limit interpretation in studies that attempt to elucidate causal pathways of sexually transmitted infections (STIs), including HIV. Such studies are often nested within, or are secondary analyses of, larger cohorts and rely on existing clinical and behavioral data, which may have insufficient information for confounding control. Potential sources of confounding include but are not limited to the following: (1) undiagnosed STI; (2) vaginal hygiene practices; (3) medication use; and (4) menstrual cycle phase—all of which are known to

alter the vaginal immune and microbial environment, but for which there are often limited data for statistical control.

A less well recognized but potential source of exposure misclassification and confounding is recent vaginal sex and/or semen exposure, which may affect immune marker measurement in 2 distinct ways: attribution of detectable immune factors of male origin to the female (exposure measurement error) and/or transient modification of vaginal immune markers induced by male seminal fluid and/or the physical action of vaginal sex not related to STI/HIV acquisition—both of which could result in spurious associations between markers and STI/HIV acquisition. Seminal fluid is known to significantly affect cervical immune function in ways that are permissive for embryo implantation and development [6–9]. Immediately after exposure to seminal fluid, vaginal, cervical, and endocervical tissues significantly increase production of a host of cytokines and chemokines. Semen contains high SLPI concentrations, and ex vivo data demonstrate induction of colony-stimulating factor 2, IL-6, IL-8, IL-10, and IL-1 α after cervical explant exposure to human seminal plasma components [10]. We also showed that the presence of prostate-specific antigen (PSA) in endocervical specimens among South African women significantly modifies the effect of several cytokines, including IL-1 β , RANTES, MIP-1 β , and IL-8, on HIV acquisition [5]. Moreover, numerous studies report PSA detection frequencies of ~15%–25% across various female populations, suggesting that if vaginal sex and/or semen exposure results in such transient modification, the impact on the results of biological mechanisms studies could be significant [11, 12]. Understanding how vaginal immune environment changes that occur after vaginal sex affect marker measurement is important for interpreting mechanistic studies of STI/HIV acquisition.

METHODS

Study Design, Participants, and Ethics

We conducted a prospective cohort study of 13 sexually active, heterosexual couples in Chapel Hill, North Carolina from 2018 to 2019 to evaluate the impact of recent condomless vaginal sex on vaginal immune markers previously associated with HIV acquisition (RANTES, IP-10, IL-8, SLPI, and HB-2) to understand the potential for exposure misclassification in biological mechanism studies of STI/HIV acquisition in women.

Female and male participants completed screening visits for eligibility assessment. Participants were eligible if they (1) were between the ages of 18 and 40 (females) or ≥ 18 years of age (males), (2) were negative for HIV, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Trichomonas vaginalis*, (3) were not pregnant (females), (4) had Nugent score 1–6 (females),

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(5) were circumcised (males), (6) had only 1 sex partner that they anticipated maintaining for the duration of study participation, and (7) self-reported engagement in condomless vaginal sex. Female partners of eligible couples completed a subsequent enrollment visit to receive verbal and written instructions and supplies for home vaginal fluid collection and questionnaires to accompany each collection. Participants who had positive STI and/or HIV tests were provided results privately, offered a letter for provision to their primary care provider or a referral physician, and were counseled to disclose test results to their sexual partners.

Among enrolled couples, men provided a single semen specimen. Women collected vaginal fluid using a vaginal aspirator and completed questionnaires at home between 1 and 4 hours after last condomless vaginal sex act (recent) and ≥ 72 hours after last vaginal sex act (nonrecent). Specimens were collected twice for each time period for a total of 4 specimens per female. Women who were unable to collect vaginal fluid using the aspirator were provided swabs for collection. Specimens were not collected during menses. Specimens were stored in participants' freezers and transported to the study laboratory using study-provided coolers and ice packs. Specimens were stored at the laboratory at -80°C for 12–18 months until analysis.

We conducted the study in accordance with the Declaration of Helsinki and Good Clinical Practice, and the study was approved by the Institutional review board at the University of North Carolina at Chapel Hill. Study staff obtained written informed consent from participants before conducting any study procedures.

Exposure and Outcomes

The exposure of interest in this study was condomless vaginal sex among female participants. The PSA testing (ABAcad p30 rapid immunochromatographic test; Abacus Diagnostics, West Hills, CA) was conducted on vaginal samples to corroborate timing of specimen collection relative to sex (PSA is detectable up to 48 hours after unprotected sex) [13, 14]. Outcomes measured in vaginal fluid and semen included concentrations of the following: RANTES, IP-10, and IL-8 (Human Magnetic Luminex Assay Cytokine Discovery kit; R&D Systems, Minneapolis, MN); HB-2 (Beta-Defensin [BD]-2 Human ELISA kit; Phoenix Pharmaceuticals, Burlingame, CA) [3, 5, 15]; and SLPI (Quantikine Human SLPI Immunoassay kit; R&D Systems). Immune marker concentrations in vaginal fluid and semen were normalized to volume.

Statistical Analysis

We conducted analyses using SAS version 9.4 (SAS Institute, Cary, NC). Biomarker concentrations were transformed using Box-Cox transformation and concentrations at baseline and during follow-up were summarized using descriptive statistics.

We estimated the difference in marker concentrations between specimens collected 1–4 vs ≥ 72 hours after vaginal sex using the permutation test for paired samples.

RESULTS

Twenty couples were screened, among whom 15 were eligible and 13 were enrolled. The median ages of enrolled participants were 24 and 26 for females and males, respectively. Most participants were white (85%) and not Hispanic or Latino (92%). At baseline, female and male participants reported a median of 8 and 10 monthly vaginal sex acts, respectively. Twelve (92%) had normal Nugent scores at baseline. No female participants reported using any products for intravaginal cleansing. Most female participants (85%) reported using contraception.

During follow-up, all female and male participants reported a continued sexual relationship with their enrollment partner. All female participants reported use of the same contraceptive method throughout follow-up. Female participants collected all samples over a period of 27–154 days postscreening, with a median collection period of 80 days. Female participants collected 23 of 24 expected recent samples, among which 21 had sufficient volume for analysis, and 23 of 24 expected nonrecent samples, all of which had sufficient volume for analysis. Two female participants were unable to collect vaginal fluid using the aspirator and were provided vaginal swabs for follow-up collection instead. Four follow-up samples from 1 participant were not labeled and could not be used in analyses. All male participants provided a semen sample, although 1 was not processed due to laboratory error.

We evaluated PSA in vaginal fluid of follow-up samples to evaluate participant adherence to required sampling times. Among 23 samples reported as recent, 100% had detectable PSA. Among 23 samples reported as nonrecent, PSA was not detected in 78% ($n = 18$).

All immune markers measured were detectable in male semen; IP-10, IL-8, SLPI, and HB-2 were detected in all semen samples, and RANTES was detected in 5 of 12 semen samples. The concentrations of all markers in semen overlapped with those in female vaginal samples (Figure 1). The concentrations of markers in semen were comparable to or higher than those in female vaginal samples, except for HB-2, which was lower. Recent samples had significantly higher concentrations of IP-10 and lower concentrations of HB-2 compared with nonrecent samples (Figure 1). In a sensitivity analysis excluding nonrecent samples that had detectable PSA, recent samples also had significantly higher concentrations of SLPI.

Female participants were asked whether they experienced any difficulty using the aspirator for specimen collection. Despite standardized instructions, which encouraged participants to lie flat to allow pooling of vaginal fluid for 5 minutes before collection, most participants (62%) reported difficulties including inability to collect sufficient volume and pain.

Figure 1. Side-by-side boxplots of Box-Cox-transformed immune marker concentrations in female specimens collected within 1–4 hours after vaginal sex ($n = 21$ specimens among 12 women), female specimens collected ≥ 72 hours after sex ($n = 23$ specimens among 12 women), and male semen specimens ($n = 12$ specimens among 12 men). Each box indicates the interquartile range with center bar as median and whiskers 1.5 times the quartile. (*) indicates $P < .05$.

DISCUSSION

Our results demonstrate that innate immune markers previously associated with HIV acquisition in women, including RANTES, IP-10, IL-8, SLPI, and HB-2, are detectable in male semen. This finding suggests that studies which attempt to elucidate biological mechanisms of STI/HIV infection through analysis of female genital tract samples may be subject to exposure misclassification when female participants have had recent condomless vaginal sex. Concentrations of immune markers in vaginal specimens collected within 1–4 hours tended to be higher compared with those collected >72 hours after sex. However, due to the study design, we cannot determine whether the observed concentration increases after recent sex is due to a biological increase in female markers and/or the additive effects of markers in female and male partner samples. If female innate immune markers increase after recent sex, it is possible that recent sex may confound observed associations between innate immune markers and STI/HIV acquisition in mechanistic studies. It is also possible that changes in vaginal microbiota over time could have influenced our results. However, we expect any such impact to be minimal given the limited time between baseline BV measurement and final follow-up sample collection.

CONCLUSIONS

Given our small sample size, limited sampling, and small number of measured markers, additional research is needed to confirm,

and expand upon, these findings. Nonetheless, the detection of all markers in semen suggests that mechanistic studies should use methods to objectively determine vaginal sex recency (eg, PSA, y-chromosome) among participants. An improved understanding of whether, and how, the female genital tract immune environment is modified by recent sex is critical to the interpretation of studies of biological mechanisms underlying STI/HIV acquisition.

Notes

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References

1. Dezzutti CS, Richardson BA, Marrazzo JM, et al.; MTN Biomedical Sciences Working Group; HPTN 035 Protocol Team. Mucosal *Escherichia coli* bactericidal activity and immune mediators are associated with HIV-1 seroconversion in women participating in the HPTN 035 trial. *J Infect Dis* **2012**; 206:1931–5.
2. Roberts L, Passmore JA, Williamson C, et al. Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression. *AIDS* **2010**; 24:819–31.
3. Morrison C, Fichorova RN, Mauck C, et al. Cervical inflammation and immunity associated with hormonal contraception, pregnancy, and HIV-1 seroconversion. *J Acquir Immune Defic Syndr* **2014**; 66:109–17.
4. Baeten JM, Donnell D, Ndase P, et al.; Partners PrEP Study Team. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med* **2012**; 367:399–410.
5. Deese J, Masson L, Miller W, et al. Genital cytokine elevation is associated with HIV infection among African women. *HIV Research for Prevention*. Chicago, IL, **2017**.
6. Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res* **2005**; 322:43–52.
7. Schjenken JE, Robertson SA. The female response to seminal fluid. *Physiol Rev* **2020**; 100:1077–117.
8. Sharkey DJ, Glynn DJ, Schjenken JE, Tremellen KP, Robertson SA. Interferon-gamma inhibits seminal plasma induction of colony-stimulating factor 2 in mouse and human reproductive tract epithelial cells. *Biol Reprod* **2018**; 99:514–26.

9. Sharkey DJ, Macpherson AM, Tremellen KP, Mottershead DG, Gilchrist RB, Robertson SA. TGF- β mediates proinflammatory seminal fluid signaling in human cervical epithelial cells. *J Immunol* **2012**; 189:1024–35.
10. Denison FC, Grant VE, Calder AA, Kelly RW. Seminal plasma components stimulate interleukin-8 and interleukin-10 release. *Mol Hum Reprod* **1999**; 5:220–6.
11. Deese J, Masson L, Miller W, et al. Injectable progestin-only contraception is associated with increased levels of pro-inflammatory cytokines in the female genital tract. *Am J Reprod Immunol* **2015**; 74:357–67.
12. Gallo MF, Behets FM, Steiner MJ, et al. Validity of self-reported ‘safe sex’ among female sex workers in Mombasa, Kenya–PSA analysis. *Int J STD AIDS* **2007**; 18:33–8.
13. Macaluso M, Lawson L, Akers R, et al. Prostate-specific antigen in vaginal fluid as a biologic marker of condom failure. *Contraception* **1999**; 59:195–201.
14. Hobbs MM, Steiner MJ, Rich KD, et al. Good performance of rapid prostate-specific antigen test for detection of semen exposure in women: implications for qualitative research. *Sex Transm Dis* **2009**; 36:501–6.
15. Masson L, Passmore JA, Liebenberg LJ, et al. Genital inflammation and the risk of HIV acquisition in women. *Clin Infect Dis* **2015**; 61:260–9.