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Asymptomatic anorectal *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections are associated with systemic CD8+ T cell activation

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ABSTRACT

Background. Oral pre-exposure prophylaxis (PrEP) has been established as a pivotal strategy in HIV prevention. However, bacterial sexually transmitted infections (STIs), such as *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG), are also highly prevalent. Although the presence of STI-related mucosal lesions is a known risk factor for HIV acquisition, the potential increase in risk associated with asymptomatic STIs is not completely understood. Recent data demonstrated higher T cell activation is a risk factor for sexually acquired HIV-1 infection. We examined the effect of asymptomatic CT and NG anorectal infection on systemic immune activation, potentially increasing the risk of HIV acquisition.

Methods. We analyzed samples from participants of *PrEP Brasil*, a demonstration study of daily oral emtricitabine/tenofovir disoproxil fumarate HIV PrEP among healthy men who have sex with men, for T cell activation by flow cytometry. We included 34 asymptomatic participants with anorectal swab for CT and/or NG infectiion while negative for other STIs, and 35 controls.

Results. We found a higher frequency of HLA-DR^{+}CD38^{+} CD8^{+} T cells (1.5 *vs.* 0.9% $p<0.005$) and with memory phenotype in the group with asymptomatic CT and/or NG infection. Exhaustion and senescence markers were also significant higher in this group. No difference was observed in the soluble CD14 levels.

Conclusion. Our findings suggest asymptomatic anorectal CT and/or NG increase systemic immune activation, potentially increasing the risk of HIV acquisition. Regular screening and treatment of asymptomatic STIs should be explored as adjuvant tools for HIV prevention.

Key words: *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, HIV, PrEP, T lymphocyte, cellular immunity, immune activation

INTRODUCTION

Oral pre-exposure prophylaxis (PrEP) is effective to prevent sexual HIV acquisition[1-4]. The implementation of PrEP in different settings raised concerns on potential increase in transmission of other sexually transmitted infections (STIs) due to an increase in condomless sexual exposure among PrEP users[5-7], even though there was no consistent evidence of such risk compensation among PrEP clinical trials participants[8, 9]]. Indeed, STIs are a public health problem with rising importance over the last decades[10]. *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) are the most prevalent STIs, with high prevalence were among men who have sex with men (MSM): 14.9% for CT and 19.2% for NG in the United States (US)[10]. In a recent study among HIV-infected and -uninfected MSM in Rio de Janeiro, the prevalence of anorectal CT was 10.0% and NG was 9.9%, with high proportion of asymptomatic infections[11]]. Asymptomatic STIs may be readily transmitted to an uninfected partner through sexual contact[12].

It is well established that an untreated STI may increase the risk of HIV acquisition[13-15]. Although in observational studies the association between bacterial STIs and HIV infection may partially result from bias due to confounders such as sexual exposure and other behavioral factors[16-18], these pathogens are known to decrease epithelial integrity and disturb mucosal immunity, directly facilitating HIV entry[19, 20]. However, the potential role of asymptomatic STIs in increasing the risk of HIV acquisition is not completely understood.

Asymptomatic STIs may impact systemic immune quiescence status by increasing T cell activation and pro-inflammatory cytokine secretion. Immune activation as a host co-factor for increased susceptibility to HIV-1 infection has long been identified[21]. Recent data suggest that reduced immune activation is associated with protection against HIV-1 acquisition in serodiscordant partners[22]. In addition, T cell activation was associated with higher HIV transmission, while higher frequency of regulatory T cells (Tregs) was a protective marker[23-27].

In this cross-sectional study, we addressed the association between asymptomatic anorectal CT and NG and immune activation in a cohort of MSM participants of a PrEP demonstration study.

MATERIALS AND METHODS

Study Participants and Procedures

A total of 546 HIV-uninfected MSM at high risk of sexually transmitted HIV from São Paulo and Rio de Janeiro were enrolled in PrEP Brasil, a 48 weeks open-label demonstration study of daily oral co-formulated emtricitabine/tenofovir disoproxil fumarate (TDF-FTC). At study entry assessments procedures included a medical assessment with physical exam, HIV rapid testing, pooled or individual HIV RNA, HBsAg, Hepatitis C antibody, syphilis serology, creatinine clearance and proteinuria (urine dipstick 1+ or more). A rectal sample was collected for CT and NG detection. Participants answered a computer assisted self-interview (CASI) including demographics,

sexual and drug use behavior questions. Eligible individuals were offered daily oral PrEP with TDF/FTC. Peripheral blood mononuclear cells (PBMC) were obtained at baseline and in case of HIV seroconversion

For this cross-sectional study, we selected all asymptomatic individuals who presented with a positive rectal swab test for CT and/or NG at study entry and had no other STIs on laboratory tests for hepatitis B and C and syphilis. Based on the sample availability, asymptomatic participants with negative rectal swab test and without any laboratory STI were randomly selected as comparison group.

Laboratory procedures

Diagnosis of CT and NG infections

CT and NG were detected with a commercially available kit (Abbott RealTime CT/NG assay, Chicago, IL), an *in vitro* polymerase chain reaction for qualitative detection of the plasmid DNA for CT and the genomic colony opacity-associated (Opa) gene for NG according to the manufacturer instructions.

Flow cytometry and Phenotype Staining Protocol

PBMC were thawed and re-suspended in supplemented RPMI1640 medium (10% fetal bovine serum). The viable cells were counted by Countess Automated Cell Counter (Invitrogen, Carlsbad, CA) and plated $1.0x10^6$ cells per well in a 96 well cell V-bottom plate. The cells were stained for live/dead kit from Invitrogen. Surface markers examined by phenotype staining and the clone used include: CD3 (UCHT1), CD4 (RPA-T4), CD8 (SK1), CD45RA (HI1000), CCR7 (3D12), HLA-DR (G46-6), CD27 (L128), CD28

(CD28.2), CD57 (NK-1), CD297 (EH12.1) and CD95 (DX2) from BD Biosciences (San Jose, CA).

All samples were stained for 30 minutes in the dark, then washed once with FACS buffer and fixed in 1% paraformaldehyde in PBS. The samples were acquired using a LSRII Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR). We defined a gate strategy (see Supplementary Figure 1, http://links.lww.com/QAD/B126) and two different scientists analyzed the data.

Soluble CD14 (sCD14) Levels

sCD14 levels were determined in stored plasma samples (−80°C) by an enzyme-linked immunosorbent assay (Human CD14 DuoSet, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The plates were read with Epoch Microplate Spectophotometer (BioTek Instruments, Winooski, VT).

Statistical Methods

For sample size estimation, a pilot study including 18 participants (9 uninfected and 9 CT and/or CG infected, based on the rectal swab results) was initially conducted to estimate mean percentage of activated CD8+ T cells in both groups; using a chi-squared test, alpha error of 0.05 and 80% power, the estimated sample size was 40 individuals per group.

Clinical and demographic characteristics of participants in each group were described using frequencies, percentages, medians and interquartile ranges. Comparisons were performed using Wilcoxon rank-sum test for continuous variables and Chi-square or Fisher's exact test for categorical variables as appropriate.

For all analyses, we assumed a two-sided alpha error of 0.05 and used the statistical software Stata version 13.1 (StataCorp. College Station, TX: StataCorp LP) and GraphPad Prism Version 6.01 (GraphPad Software, CA) and SPICE version 5.1[28].

Ethical aspects

All volunteers in the *PrEP Brasil* study have signed an informed consent form at enrollment. The study was approved by the Institutional Review Boards in each contributing site. Participant-identifiable data was maintained in locked cabined and secured electronic forms.

RESULTS

Among the 546 participants in PrEP Brasil, 31 had positive rectal swab tests for CT, 15 for NG and 6 for both CT and NG at baseline. After initial exclusion of five participants due to positive serologic test for syphilis, 34 individuals with asymptomatic positive CT and/or NG rectal swab were selected considering sample availability, along with randomly selected 35 individuals in the control group. Some samples were not available; low cell viability or positive or negative control results failing quality control standards were discarded, explaining some missing values. Demographic and clinical characteristics, were similar in both groups. Although the proportion of participants reporting receptive anal intercourse in the 3 months prior to enrollment was similar in both groups (p=0.26), CT/NG positive group had a higher median number of male sexual

partners in the same period (7, interquartile range [IQR] 2-15) compared to CT/NG negative group (2, IQR 1-7, p=0.01; Table 1).

CT/NG asymptomatic anorectal infection is associated with lower CD4+ and higher CD8+ T cell percentages

Individuals with a CT and/or NG infection had lower percentage of CD4+ T cell and higher percentage of CD8+ T cell, changing the CD4+ and CD8+ T cell ratio compared to controls, as shown in Figure 1A. Differentiation pattern of CD8+ T cell revealed lower percentage of naïve (CD45RA+CD27+CCR7+) and higher percentage of effector memory (CD45RA-CD27-CCR7-) in the CT/NG infection group but no differences were found in the central memory (CD45RA-CD27+CCR7+), transitional memory (CD45RA-CD27+CCR7-) and terminal effector memory (CD45RA+CD27-CCR7-) CD8+ T cell subpopulations (Figure 1B). These results suggest a shift in CD8+ T cell differentiation to more mature forms in the group with CT/NG infection. In the CD4+ T cell compartment, we did not find any significant difference in T cell subsets comparing CT/NG infection and negative groups (Figure 1C).

CT/NG asymptomatic anorectal infection is associated with systemic CD8+ T cell activation

Individuals with CT/NG asymptomatic anorectal infection had a 1.67-fold greater percentage of HLA-DR+CD38+CD8+ T cell than the control group (1.5 *vs.* 0.9%, respectively, p=0.003; Figure 2B). We found no statistically significant difference between the two groups in the frequency of activated CD4+ T cells (0.7 *vs. 0.6%*, p=0.2;

Figure 2D). There was also no statistically significant difference in CD4+ T cell expressing a single activation marker (Supplementary Table 1, http://links.lww.com/QAD/B126).

The assessment of CD8+ T cells with a memory phenotype showed a significant difference in percentage of transitional memory subset (2.4 in CT/NG positive *vs.* 1.2 in CT/NG negative group, p=0.018) and a substantial difference in naïve (0.2 *vs.* 0.1%, p=0.09), central memory (2.9 *vs.* 1.9%, p=0.07) and effector memory (2.0 *vs.* 1.4%, p=0.06) subsets, although not reaching statistical significance (Figure 2A). We found a similar trend for some CD4+ T cell subsets (Figure 2C), but none were statistically different when comparing both groups (Supplementary Table 1, http://links.lww.com/QAD/B126).

The overall signature of activation in CD8+ and CD4+ T-cell, as depicted by differentiation stages, was not significantly different between the two groups (Figure 2B and 2D).

CT/NG positive participants present higher expression of exhaustion and senescence markers on CD8+ T cells

Percentage of CD8+ T cells with exhaustion and senescence markers were higher in individuals with CT/NG anorectal infection than in the negative group (Figure 3A). The percentage of activated CD8+ T cells expressing PD-1 (0.9 *vs.* 0.5%, p=0.009), CD95 (1.5 *vs.* 0.8%, p=0.002) and co-expressing both markers (0.9 *vs.* 0.5%, p=0.009) were significantly higher in the CT/NG positive group compared to controls. These findings were evident in the subsets of central, transitional and effector memory CD8+ T cells. As expected, we did not find the same difference in naïve and terminal effector subsets.

There were no statistically significant differences in the CD4+ T cell exhaustion and senescence markers (data not shown).

Levels of soluble CD14 (sCD14) were not significantly different between the CT/NG positive and negative groups

We did not find any significant difference in the sCD14 levels comparing CT/NG positive and negative groups (2208 ± 308 *vs*. 2286 ± 307 pg/mL, p=0.96) (Figure 3B). Mean sCD14 values were within normal range.

DISCUSSION

In this study, we examined the association between asymptomatic CT and NG anorectal infection and systemic T cell immune activation among HIV-uninfected MSM participants of PrEP Brasil. We found a baseline prevalence of CT and/or NG anorectal infection of 9.5%, similar to the prevalence described in a large national study in the US[29]. Therefore, this was an ideal environment to conduct our study. Asymptomatic CT/NG anorectal infection were associated with lower CD4+ to CD8+ T cell ratio, higher frequency of CD8+ T cell co-expressing the activation markers HLA-DR and CD38 and higher expression of exhaustion and senescence markers on CD8+ T cells.

Our results showed a substantial difference in the expression of activation markers for almost all CD8+ T cell subsets. Even though the differences among the memory populations were not statistically significant, the patterns were consistent with disturbance of the quiescence immune status. When naïve T cells are activated during an

infection, they proliferate and become effector T cells to fulfill their functions[30, 31]. As expected and reinforcing our hypothesis, we found a shift towards central memory and effector memory activation and a quiescence status for naïve cells.

Chronic immune activation and inflammation are well-known hallmarks of HIV infection despite effective antiretroviral therapy, and have been related to increased mortality and non-AIDS morbidity[32-34]. The level of T cell activation in blood has been also implicated in HIV acquisition risk[25, 27, 35]. It has been associated with range of possible causes from HIV-1 exposure to STIs[36-39]. In prior studies different STIs, including syphilis[16, 40, 41] and anorectal CT and NG infection have been shown to facilitate HIV acquisition[16, 17], likely through a physical disrupt in the skin or mucosal integrity along with local inflammatory conditions leading to a higher exposure of HIV-susceptible cells[19, 20]. Although STIs are an important cause of inflammatory cytokine upregulation in the genital mucosa, potentially resulting in immune activation[20, 38, 39], our findings first report the evidence of asymptomatic STIs leading to systemic immune activation, which suggests STIs could be associated with higher risk of HIV acquisition despite epithelial integrity and absent or low mucosal inflammation in asymptomatic infections. Indeed, systemic CD8+ T cell activation has been prior implicated as an independent risk factor of HIV acquisition[25].

Although CD4+ T cell activation would be an important marker of HIV susceptibility, we did not find a statistically significant difference in CD4+ T cell activation between the two groups. This might in part be justified by biological characteristics of immune responses to both pathogens. CT is an obligate intracellular agent, and the adaptive immune response is predominantly directed by CD8+ T cells. The role of CD4+ T cells

in response to intracellular pathogens is controversial. On the other hand, CD8+ T cells control intracellular infections through cytotoxicity, anti-microbial peptides, and release of cytokines[42]. NG in turn has been shown to suppress CD4+ T cells through the Opa (colony opacity-associated proteins) binding to CEACAM (carcinoembryonic Ag-related cellular adhesion molecule) family of receptors and inhibiting expression of activation markers (CD69) and proliferation[43].We could hypothesize that after initial infection with CT/NG, a low or absent mucosal inflammation in asymptomatic infections might be associated with an ineffective immune response, unable to clear off the pathogen but leading to a permanent, subtle stimulus resulting in immune activation. Although these pathogens usually cause acute infections, a potential subacute and/or chronic asymptomatic stimulus can lead in the development of CD8+ T cells that are capable of cytokine secretion yet incapable of cell division[44]. This hypothesis is in line with our findings of higher expression of exhaustion and senescence markers of CD8+ T cell in the CT/NG infection group, especially in the effector memory cells. The absence of increased level of plasma sCD14 indirectly reinforce our hypothesis that the infection of these pathogens can disturb the quiescence immune status, without evidence of surrogate markers of bacterial translocation from the gut.

Despite these relevant results, our study presents some limitations such as the cross sectional design and the inability to demonstrate the resolution of immune activation after treatment of the asymptomatic STI. The sample size might be a limitation but we do believe the effect obtained is suitable to accurately demonstrate the impact of these asymptomatic rectal infections in the activation of the CD8+ T cell compartment. We cannot rule out that residual confounders such as behavioral factors might be present and

disturb our results, although they are unlikely considering the similarity of the two groups concerning demographic characteristics. We were also unable to simultaneously address the presence of asymptomatic epithelial damage and the intensity of local immune response in the anorectal mucosa, where the infection occurs and the HIV-1 acquired infection may start. Finally, the impact of other highly prevalent and often asymptomatic infections in the immune system, like latent syphilis and HSV-2, were not addressed. HSV-2 has been associated with systemic activation and high cytokine genital levels[20, 45].

Our findings have important practical implications. As the HIV-1 epidemic continues to spread globally and a prophylactic vaccine is still a challenge, the attention has been directed to other preventive methods. There has been renewed hope in HIV prevention with the implementation of PrEP after several favorable results[1-3]. Although not conclusive, several studies indicate that an increase in sexual exposure, or risk compensation, could potentially result from a reduced perceived risk under PrEP, leading to an increase in the incidence of other STIs[2-6]. But importantly, PrEP is expected to become increasingly available in the future years and may provide an excellent opportunity to early diagnosis, periodic screening and early treatment of other STIs as an additional HIV-prevention tool. One could also speculate that the deleterious impact of asymptomatic STIs on HIV infection risk merits new approaches for diagnosis, treatment and prevention of such infections. For instance, treatment on a regular basis even without confirmed diagnosis, and even pre-exposure prophylaxis for bacterial STIs could be considered for high risk sexual behavior groups, especially taking into consideration the

high global prevalence of CT and NG infections in MSM. For this approach, the local patterns of drug-resistant STIs must be carefully considered.

In conclusion, our study showed that asymptomatic CT/NG anal infection is associated with increased systemic immune activation, which in turn was previously shown to result in increased risk of HIV acquisition. Asymptomatic STIs are typically underdiagnosed and undertreated, but this omission can be attenuated among PrEP users, for whom IST diagnosis and treatment opportunities are expanded.

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Figure 1. T cell phenotyping in individuals with anorectal CT/NG infection and uninfected controls. A, percentage of CD4+ T cells, CD8+ T cells and the CD4+ to CD8+ ratio are shown for uninfected controls (closed dots) and CT/NG infected (open dots). B and C, percentage of CD8+ and CD4+ T cell memory subsets for CT/NG infected participants and for uninfected controls, respectively, as defined by expression of CD45RA, CCR7, and CD27.

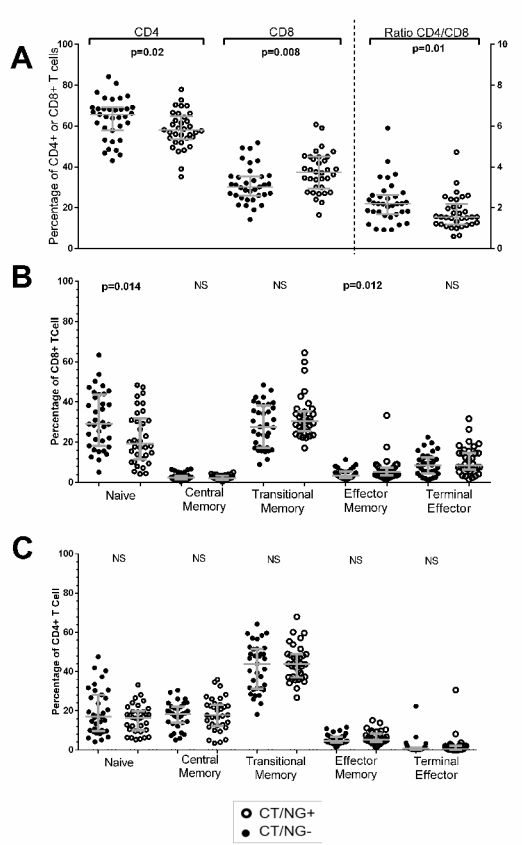

Figure 2. T cell activation. A and C, percentage of total and memory subsets of CD8+ and CD4+ T cell, respectively, co-expressing the activation markers. The activation status was phenotypically defined by the co-expression of HLA-DR and CD38 markers. B and D, pie charts illustrate the differentiation of activated CD8+ and CD4+ T cells, respectively, in the two groups (CT/NG infected and unifected controls).

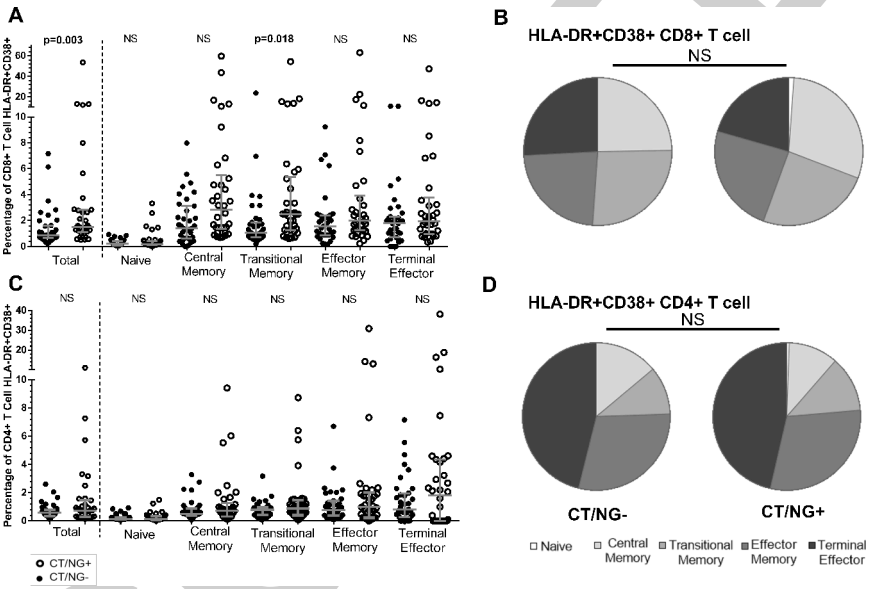

Figure 3. A, Expression and co-expression of activation, exhaustion and senescence markers in total and memory subsets of CD8+ T cells. The difference of CD8+ T cell expression of exhaustion markers (PD-1+), activation markers (CD95+, CD38+ and HLA-DR+) and senescence markers (CD57+ and CD28-) between CT/NG infected participants and uninfected controls. All statically significant differences observed are associated with a higher expression in the CT/NG infected group over the control group. **B, Comparison between the levels of sCD14 in plasma in the CT/NG infected and uninfected controls**. Bars represents the mean and standard deviation.

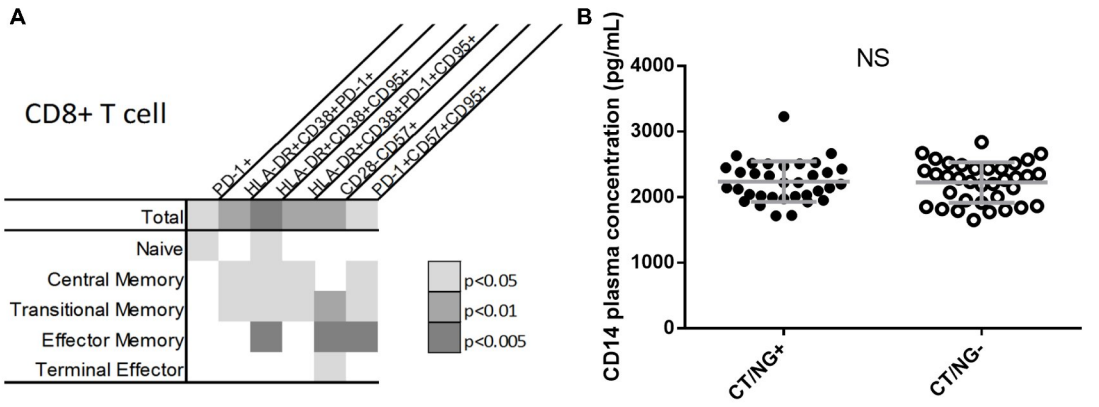

Table 1. Demographics of the study population.

	STI** positive N=34	STI** negative N=35	p-value*
Swab positive			
Chlamydia trachomatis	22 (65%)		
Neisseria gonorrhoeae	9 (26%)		
Both	3 (9%)		
Age at enrollment (SD) †****	29 (25-34)	29 (24-36)	0.93
Ethnicity			
White	21 (64%)	23 (70%)	0.60
Non-white	12 (36%)	10 (30%)	
HIV-positive male sexual partner in the last 3 months*	14 (44%)	18 (58%)	0.26
Number of male sexual partners in the last 3 months†	7 (2-15)	2 (1-7)	0.01
Had receptive anal intercourse in the last 3 months&	14 (42%)	17 (52%)	0.26
Alcohol use&	19 (58%)	23 (70%)	0.31
Smoking#	15 (50%)	15 (48%)	0.90

**STI = sexually transmitted infection; SD = standard deviation.

†Continuous variables are shown as medians and interquartile ranges

*Data available for 63 participants

& Data available for 66 participants

Data available for 61 participants