The cervicovaginal neutrophil protein CRISP3 is a correlate of HIV protection

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INTRODUCTION

•Identifying immune correlates of HIV protection in the female genital tract is important for HIV prevention efforts.

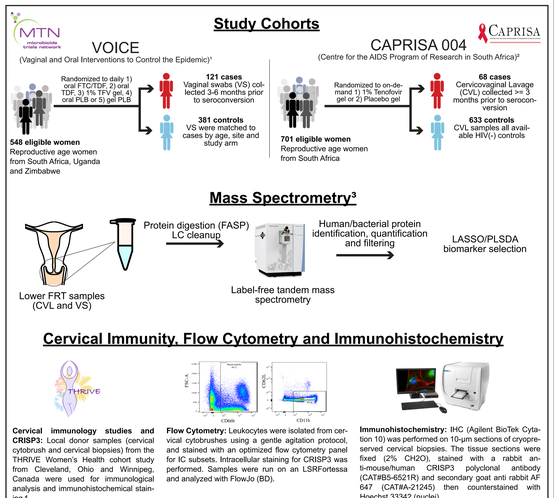
•The mucosal surface of the female genital tract is a first site of contact for HIV during sexual transmission in women. This mucosal microenvironment contains molecular and cellular components of the immune system to defend against viruses.

•Alterations to the mucosal barrier, including increased cytokines, immune cell activation, epithelial barrier disruption, and vaginal microbial dysbiosis have been linked with increased susceptibility to HIV.

•Despite this there is limited knowledge of what are the correlates of HIV protection in the female genital tract

•Herein, we used a discovery proteomics approach with machine learning to identify correlates of HIV protection from 1,249 African women enrolled in two HIV prevention trials.

METHODS



Cervical immunology studies and CRISP3: Local donor samples (cervical cytobrush and cervical biopsies) from the THRIVE Women's Health cohort study from Cleveland, Ohio and Winnipeg, Canada were used for immunological analysis and immunohistochemical staining [4].

Flow Cytometry: Leukocytes were isolated from cervical cytobrushes using a gentle agitation protocol, and stained with an optimized flow cytometry panel for IC subsets. Intracellular staining for CRISP3 was performed. Samples were run on an LSRFortessa and analyzed with FlowJo (BD).

Immunohistochemistry: IHC (Agilent BioTek Cytation 10) was performed on 10-µm sections of cryopreserved cervical biopsies. The tissue sections were fixed (2% CH2O), stained with a rabbit anti-mouse/human CRISP3 polyclonal antibody (CAT#85-65218) and secondary goat anti rabbit AF 647 (CAT#A-21245) then counterstained with Hoechst 33342 (nuclei).

RESULTS

Table 1. Clinical, behavioural and demographic characteristics of VOICE (left) and CAPRISA 004 (right) trial participants.

Variable	Total	HIV cases	HIV seronegative controls	*P* value
No. of samples*	n=548	n=136	n=412	
Treatment arm (n,%)				0.99
FTC/TDF	133 (24.3)	35 (25.7)	98 (23.8)	
TDF	119 (21.7)	30 (22.1)	89 (21.6)	
Oral placebo	129 (23.5)	31 (22.8)	98 (23.8)	
1%Tenofovir gel	22 (4.0)	5 (3.7)	17 (4.1)	
Gel placebo	145 (26.4)	35 (25.7)	110 (26.7)	
Visit Code (n,%)				0.25
3month	305 (55.7)	82 (60.3)	223 (54.1)	
6month	243 (44.3)	54 (39.7)	189 (45.9)	
Clinic Site				0.78
South Africa	514 (93.7)	128 (94.1)	386 (93.7)	
Uganda	12 (2.2)	2 (1.5)	10 (2.4)	
Zimbabwe	22 (4.0)	6 (4.4)	16 (3.9)	
Age (Mean ± SD; range)	26±4.8; 18-39	23±4.1; 18-37	25±4.9; 18-39	**6.42E-05**
FamilyPlanning Method				**2.84E-02**
Injectables	417 (76.1)	115 (84.6)	302 (73.3)	
Oral	94 (17.1)	15 (11)	79 (19.2)	
Other	37 (6.8)	6 (4.4)	31 (7.5)	
# Sex Acts (Mean ± SD; range)ª	2 ± 2; 0-20	2 ± 2; 0-11	2 ± 2.3; 0-20	0.55
# Sex Patterns (Mean ± SD; range)	1 ± 0.8; 0-11	1 ± 0.5; 0-3	1 ± 0.9; 0-11	0.75
Reported unprotected sex in last 7 days (n,%)	105 (19.2)	30 (22.1)	75 (18.2)	0.32

*38 participants removed due to low protein coverage
*2 samples had no bacterial proteins detected
•4 participants did not report sexual activity data

Variable	Total	HIV cases	HIV seronegative controls	P value
	n=701	n=68	n=633	
Median adherence during trial >50% (n, %)	275 (39.2)	23 (36.5)	252 (36.0)	0.7
Any STI during trial (n, %) ¤	138 (35.48)	21 (46.67)	117 (30.08)	0.1
Vaginal insertion ever reported during trial (n, %)	126 (17.97)	10 (15.87)	116 (16.55)	0.7
Age at enrolment (Mean ± SD; range)	23.85±5.17)	23.02±4.39)	23.94±5.23)	0.3
TFV arm (n, %)	350 (49.93)	24 (38.1)	326 (46.5)	0.0637
Rural (n, %)	513 (73.18)	42 (66.67)	471 (67.19)	0.2
Given Birth (n, %)	554 (79.03)	48 (76.19)	506 (72.18)	0.6
Condom use (n, %)				0.3
Always	200 (28.5)	20 (29.4)	180 (28.4)	
Most times	129 (18.4)	15 (22.1)	114 (18.0)	
Occasionally	245 (36.2)	22 (32.4)	232 (36.7)	
Never	118 (16.8)	6 (8.8)	112 (17.7)	
Contraception use (n, %) †				0.3
DMPA	460 (65.6)	48 (70.6)	412 (65.1)	
NET-EN	124 (17.7)	8 (11.8)	116 (18.3)	
COC	97 (13.8)	7 (10.3)	90 (14.2)	
Any cervical ectopy (n, %) ‡	49 (7.25)	3 (4.84)	46 (6.8)	0.6
Antibiotic use in past 30 days (n, %)	55 (7.85)	6 (9.52)	49 (6.99)	0.6
Sex acts in past 30 days (Mean ± SD; range)	8.28±5.56)	7.44±6.3)	8.36±5.85)	0.4
Sex acts in past 7 days (Mean ± SD; range)	1.95±(3.14)	1.68±(2.15)	1.98±(3.22)	0.4

¤ 312 women were not tested for STI's during the trial
γ 4 women have no information about specific contraception use at visit, 16 women were using other forms of birth control, including IUD, hysterectomy or condoms only
† 22 women had no information at the visit of cervical ectopy

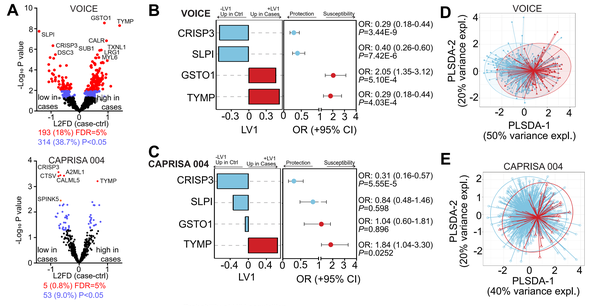
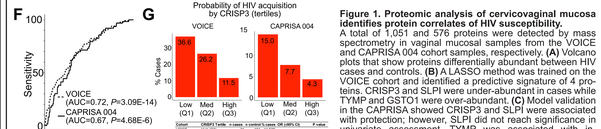


Figure 1. Proteomic analysis of cervicovaginal mucosa identifies protein correlates of HIV susceptibility.
A total of 1,051 and 576 proteins were detected by mass spectrometry in vaginal mucosal samples from the VOICE and CAPRISA 004 cohort samples, respectively. **(A)** Volcano plots that show proteins differentially abundant between HIV cases and controls. **(B)** A LASSO method was trained on the VOICE cohort and identified a predictive signature of 4 proteins. CRISP3 and SLPI were under-abundant in cases while TYMP and GSTO1 were over-abundant. **(C)** Model validation in the CAPRISA showed CRISP3 and SLPI were associated with protection; however, SLPI did not reach significance in univariate assessment. TYMP was associated with increased probability of seroconversion in each cohort. **(D&E)** PLSDA validation shows separation of cases and controls by the predictive model. **(F)** AUC (Area Under the Curve) evaluation of the model. **(G)** Probability of seroconversion associated with CRISP3 levels showed a dose-effect in CAPRISA 004 participants.



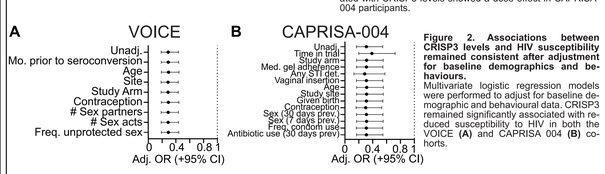


Figure 2. Associations between CRISP3 levels and HIV susceptibility remained consistent after adjustment for baseline demographics and behaviours.
Multivariate logistic regression models were performed to adjust for baseline demographic and behavioural data. CRISP3 remained significantly associated with reduced susceptibility to HIV in both the VOICE **(A)** and CAPRISA 004 **(B)** cohorts.

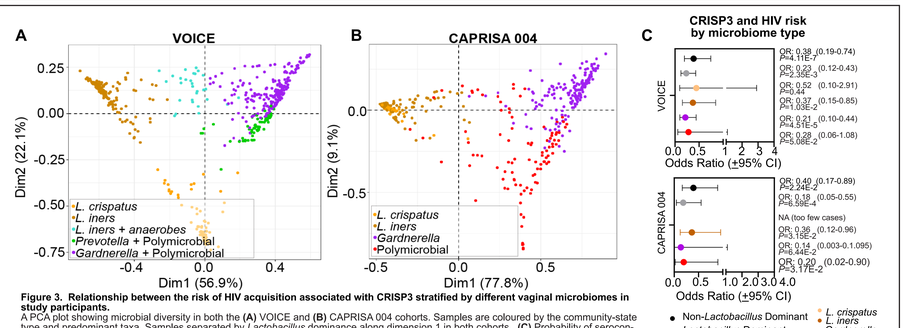


Figure 3. Relationship between the risk of HIV acquisition associated with CRISP3 stratified by different vaginal microbiomes in study participants.
A PCA plot showing microbial diversity in both the **(A)** VOICE and **(B)** CAPRISA 004 cohorts. Samples are coloured by the community-state type and predominant taxa. Samples separated by *Lactobacillus* dominance along dimension 1 in both cohorts. **(C)** Probability of seroconversion was lower with above-median levels of CRISP3 within *Lactobacillus* dominant and non-*Lactobacillus* dominant women, as well as by community types.

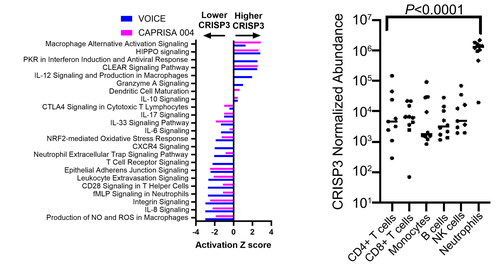


Figure 4. The relationship between molecular pathways in cervicovaginal mucosa and the level of CRISP3.
Molecular pathways in cervicovaginal mucus significantly associated with CRISP3 levels ($P<0.05$), as identified by Ingenuity pathway software (Qiagen). Higher CRISP3 associated with increased activation of innate immune pathways, and lower CRISP3 associated with activation of pro-inflammatory pathways.

Figure 5. Intracellular CRISP3 levels across immune cell lineages.
Peripheral blood immune cells were isolated from healthy donors and analyzed by mass spectrometry. Neutrophils showed the highest expression of CRISP3 in relation to other cell types.

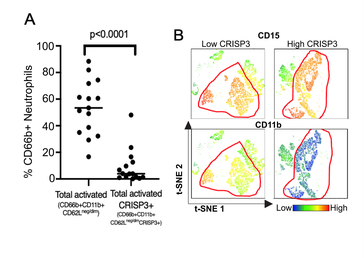


Figure 6. Detection of CRISP3 in cervical neutrophils by flow cytometry.
(A) Flow cytometry analysis shows cervical CRISP3+ neutrophils have lower activation (CD66b+CD11b+CD62Lneg/dim); **(B)** t-SNE plots showing relationship between CRISP3 levels in vaginal mucus and cervical neutrophil phenotype. Neutrophils were gated based on CD15 expression, and CD11b indicates an activation marker.

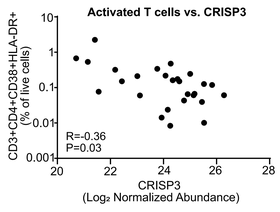


Figure 7. CRISP3 levels associate with activated CD4+ T cells.
CRISP3 levels detected in cervicovaginal fluid of healthy donors and activated cell levels (CD3+CD4+CD38+HLA-DR+) within matched cytobrushes showed a negative correlation.

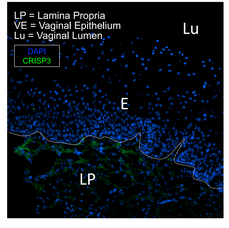


Figure 8. Fluorescence immunohistochemistry of CRISP3 in cervical tissue.
This image shows staining of CRISP3 in the cervical epithelium (Green: CRISP3, Blue: nuclei), and shows protein expression to localize to the basal layer of the epithelium.

DISCUSSION

In this study of two independent cohorts of African women, we identified CRISP3 as a novel vaginal mucosal neutrophil protein associated with reduced HIV susceptibility.

CRISP3 associated with 3.5-fold reduced risk of HIV acquisition, and upwards of 5-fold when comparing highest vs. lowest quartiles in one study cohort.

Protection associated with CRISP3 is apparent across different vaginal microbiome types.

CRISP3+ is present in cervical neutrophils, and CRISP3+ neutrophils show a reduced immune activation phenotype.

Immunohistochemical staining showed CRISP3 was expressed in the basal layer of the cervical epithelium, in proximity to initial sites of HIV infection.

HIV protection associated with CRISP3 may be mediated by increased innate immunity and reduced HIV target cell activation.

CRISP3 is a 28kDa glycoprotein that belongs to a family of cysteine-rich proteins. It has been shown to be present in peroxidase-negative secretory granules of neutrophils. Previous publications have suggested it has anti-viral activity against HCV[5], and it may affect calcium mobilization, remodeling of the ECM, and adhesion/proliferation of epithelial cells[6].

Given these data, more research on CRISP3 function in immunity and associated neutrophil phenotypes in the context of HIV prevention trials and strategies is warranted.

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