Rectal tenofovir gel usage is associated with changes in the mucosal proteome

Adam Burgener[1,2,3]; Laura Romas[2,1]; Kenzie Birse[2,1]; Kenneth Mayer[5]; Garrett Westmacott[6]; Irma Febo[8]; Ross Cranston[4]; Alex Carbarballo-dieguez[7]; Ian McGowan[4]

1. National HIV and Retrovirology Laboratory, JC Wilt Infectoius Disease Research Centre, Public Health Agency of Canada, CAN; 2. Medical Microbiology and Infectious Diseases, University of Manitoba, CAN; 3. Karolinska Institutet, Dept. of Medicine Solna, Center for Molecular Medicine, SWD;4. Department of Medicine, University of Pittsburgh, Pittsburgh, PA, USA 5.Harvard Medical School, Boston, MA, USA; 6. National Microbiology Lab, Public Health Agency of Canada, CAN; 7. Columbia University, New York, NY, USA. 8. University of Peurto Rico, San Juan, PR.

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Poster #269

INTRODUCTION

•Tenfovir (TFV) 1% gel was 39% effective against vaginal transmission of HIV-1[1]. Since then, rectal formulations are being explored as a prevention option.

•Vaginal formulations of TFV gel applied rectally were associated with gastrointestinal intolerance (MTN-006)[2]. This was likely due to an incompatible osmolarity and pH of the formulation with the GI mucosa.

•A reduced-glycerin vaginal formulation (RGVF) of TFV gel has been determined safe for use using epithelial sloughing, fecal calprotectin, inflammatory cytokine levels and cellular immune markers as indicators of acceptability in the rectum (MTN-007)[3].

•RGVF and other formulations of TFV 1% gel are being assessed in clinical trials for safety for rectal use in young men who have sex with men (Project Gel).

Rationale

•Traditional immune safety standards may miss important immunological or biological side effects of these gels. A systems biology analysis was recently completed to assess the effects of the RGVF gel on rectal mucosal tissues in the MTN-007 trial. A broad range RGVF-associated changes in mucosal gene/protein expression was observed that may affect immune homeostasis, mitochondrial function and epithelial cell development not seen in previous safety evaluations[3,4].

Therefore the objective of this study was to evaluate host mucosal responses through proteomics of study participants engaged in a phase 1 randomized, double-blind, multi-site, placebo-controlled trial of Tenofovir-based rectal microbicides.

Hypothesis

We have performed a comprehensive proteomic analysis to explore the impact of different formulations of TFV 1% gel within Project Gel (MTN). This will allow us to identify changes in mucosal tissues associated with gel use.

STUDY DESIGN

Table 1. Rectal microbicide formulations utilized in Project Gel.

Study participants (n=24) were divided into a control group given a HEC placebo gel, and a treatment group given the 1% TFV formulation shown below. (adapted from [5])

Product	Abbrv.	TFV Content (%w/w)	Glycerin (%w/w)	pH	Osmolarity (mmol/kg)
Hydroxyethyl cellulose placebo gel	HEC	0%	0	4.4	isotonic
1% TFV gel	TFV	1%	5	4.5	846

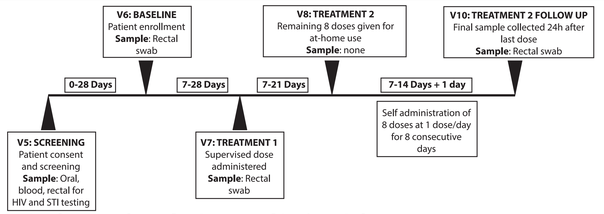


Figure 1. Study procedures for Stage 2 of Project Gel.

Participants selected for Stage 2 were screened for HIV and STIs. A baseline sample was taken 1-3 weeks prior to the first supervised dose. A sample was taken after the first dose to determine immediate effects of the gel. Eight doses of product were self-administered at home. A final sample was taken 24h after the last dose was administered.

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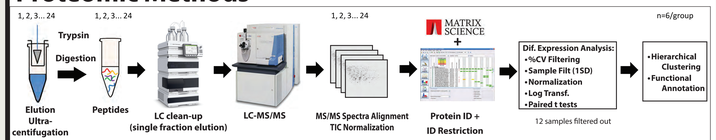


Figure 2. Proteomic analysis workflow.

Samples from 24 participants (n=12 HEC & n=12 TFV) were collected via rectal sponge. Mucosal proteins were eluted, purified via ultracentrifugation and digested into peptides. Peptides were cleaned with liquid chromatography and analyzed by label-free MS/MS. Spectra were aligned across samples. Proteins were identified with Mascot Daemon (Matrix Science) and IDs were restricted to 95% peptide and 80% protein confidence and a minimum of 2 peptides ID'd. Normalized protein abundance values were determined to be differentially expressed over time via paired t tests (Benjimani-Hochberg corr.), using changes in the HEC arm as a comparison. Hierachrical clustering (Cluster 3.0) and functional annotation (DAVID Bioinformatics) were performed.

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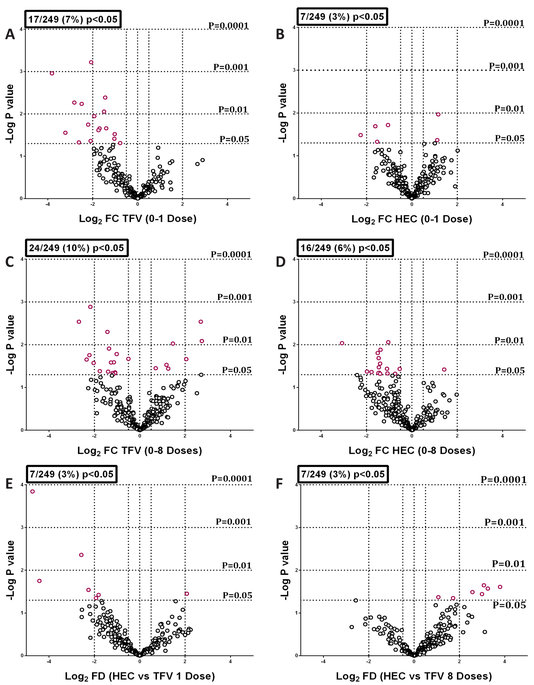


Figure 3. Effects of daily applications of either 1% Tenofovir or HEC placebo microbicide on the rectal mucosal proteome.

Volcano plots illustrating fold-change (x-axis) and statistical significance distribution (y-axis) of the proteomic datasets. Data points in maroon indicate proteins defined to be significantly differentially expressed (p<0.05; both paired and unpaired t-tests). 246 unique proteins were identified in rectal mucosal samples after variance and species filtering (human only, <25% CV in reference sample). **A and C:** Paired t tests were used to assess protein expression changes within individuals associated with short-term TFV usage, and longer-term TFV usage relative to the HEC control arm (**B and D,** respectively). **E and F:** Independent t tests compared protein expression between the TFV and HEC groups after 1 dose and 8 doses. The strongest effects associated with TFV gel use were found within individuals after 8 doses.

Figure 4. Biofunctional analysis and hierarchical clustering of proteome changes in rectal mucosal fluid after 7 day application of 1% Tenofovir gel.

A: A volcano plot illustrates fold-changes and statistical significance between proteins differentially abundant (DA) between D0-D7 (paired t-test). Those that met a p<0.05 cut-off threshold are shown in color (blue). **B:** Heatmap illustrates proteins which were differentially abundant (p<0.05, 24 proteins), between Day 1 and Day 7 after TFV-gel exposure. Clustering of proteins was generated by unsupervised average linkage hierarchical clustering using Pearson correlation coefficient as the distance metric. Both over and underexpressed proteins are observed. **C:** Pathway analysis of proteins DA was performed using GO database (DAVID). The major functions were associated with epidermis development and cytoskeletal functions. Only those with >3 factors and a p value $<10^{-4}$ (Benjimani Hochberg MCC) are shown (C) . In the HEC gel arm, although mild associations to functional categories were observed (cell activation), these did not pass multiple comparison correction threshold.

	Protein name	Gene	L2FC	p-value
	Tenofovir arm _ D0 vs D1 p<0.05 (paired *t*-test)			
CRNN_HUMAN	**Cornulin**	CRNN	-2.06	0.0006
CALM_HUMAN	**Calmodulin**	CALM	-3.82	0.0011
6PGD_HUMAN	6-phosphogluconate dehydrogenase, decarboxylating	6PGD	-1.44	0.0041
MMP9_HUMAN	Matrix metalloproteinase-9	MMP9	-2.82	0.0054
CAMP_HUMAN	Cathelicidin antimicrobial peptide	CAMP	-2.49	0.0058
CALL5_HUMAN	Calmodulin-like protein 5	CALL5	-1.49	0.0088
VIME_HUMAN	Vimentin	VIME	-1.93	0.0113
SHIL3_HUMAN	SH3 domain-binding glutamic acid-rich-like protein 3	SH3L3	-2.2	0.0179
KV305_HUMAN	Ig kappa chain V-III region WOL	KV305	-1.4	0.0219
LYPD3_HUMAN	Ly6/PLAUR domain-containing protein 3	LYPD3	-1.7	0.022
VILI_HUMAN	Villin-1	VILI	-1.74	0.0242
IL36A_HUMAN	**Interleukin 36A**	IL36A	-3.22	0.0279
CATD_HUMAN	Cathepsin D	GATD	-1.02	0.03
THIO_HUMAN	Thioredoxin	THIO	-1.04	0.0386
IF6_HUMAN	Eukaryotic translation initiation factor 6	IF6	-2.09	0.0434
RNH1_HUMAN	Ribonuclease inhibitor	RINI	-2.62	0.0471
IF2G_HUMAN	Elongation factor 1-gamma	IF1G	-0.78	0.0487
	Tenofovir arm _ D0 vs D7 p<0.05 (paired *t*-test)			
IGJ_HUMAN	Immunoglobulin J chain	IGJ	-2.18	0.0013
CALM_HUMAN	**Calmodulin**	CALM	-2.68	0.0029
FINC_HUMAN	Fibronectin	FINC	2.69	0.0029
K1C10_HUMAN	Keratin, type I cytoskeletal 10	K1C10	-1.43	0.005
WDR1_HUMAN	WD repeat-containing protein 1	WDR1	2.74	0.0082
AFAM_HUMAN	Afamin	AFAM	1.46	0.0094
FLNB_HUMAN	Filamin-B	FLNB	-1.36	0.0123
K2C1_HUMAN	Keratin, type II cytoskeletal 1	K2C1	-1.02	0.0165
CRNN_HUMAN	**Cornulin**	CRNN	-2.22	0.0175
K1C9_HUMAN	Keratin, type I cytoskeletal 9	K1C9	-0.5	0.0214
SIAS_HUMAN	Sialic acid synthase	SIAS	2.05	0.0217
CEAM5_HUMAN	Carcinoembryonic antigen-related cell adhesion molecule 5	CEAM5	-2.34	0.0222
K2C6A_HUMAN	Keratin, type II cytoskeletal 6A	K2C6A	-1.14	0.0258
CASP2_HUMAN	Caspase-14	CASPE	-1.27	0.026
K1C13_HUMAN	Keratin, type I cytoskeletal 13	K1C13	-2.03	0.0267
APOH_HUMAN	Beta-2-glycoprotein 1	APOH	1.18	0.0296
LEG4_HUMAN	Galectin-4	LEG4	0.7	0.0354
PRDX6_HUMAN	Peroxiredoxin-6	PRDX6	1.25	0.0362
IL36A_HUMAN	**Interleukin 36A**	IL36A	-1.76	0.0414
TBB4B_HUMAN	Tubulin beta-4B chain	TBB4B	-1.4	0.0426
K1C19_HUMAN	Keratin, type I cytoskeletal 19	K1C19	-1.09	0.0443
K2C5_HUMAN	Keratin, type II cytoskeletal 5	K2C5	-1.09	0.0457
CYTA_HUMAN	Cystatin-A	CYTA	-1.19	0.0457
INVO_HUMAN	Involucrin	INVO	-1.17	0.0492

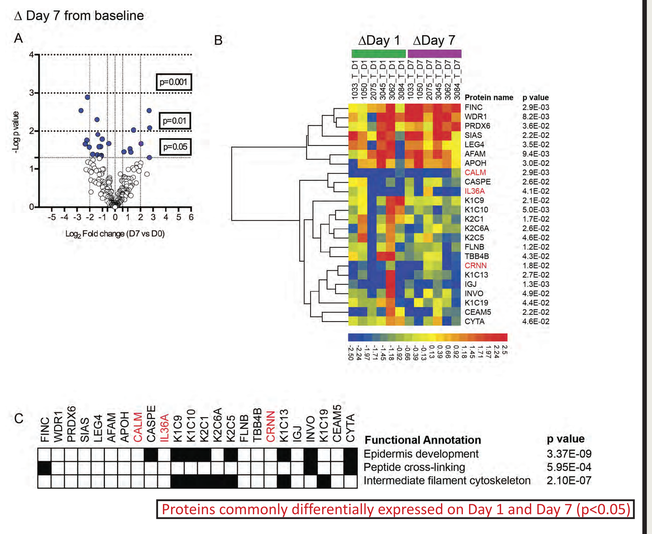
	Protein name	Gene	L2FC	p-value
	HEC arm _ D0 vs D1 p<0.05 (paired *t*- test)			
APOA1_HUMAN	Apolipoprotein A-I	APOA1	1.16	0.01
GBP2B_HUMAN	78 kDa glucose-regulated protein	GRP78	-1.05	0.02
AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	AHNK	-1.61	0.02
SPB13_HUMAN	Serpin B13	SPB13	-2.26	0.03
TBA1C_HUMAN	Tubulin alpha-1C chain	TBA1C	1.11	0.04
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	MDHM	-1.93	0.05
	HEC arm _ D0 vs D7 p<0.05 (paired *t*- test)			
ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	ZA2G	-3.04	0.01
FIBA_HUMAN	Fibrinogen alpha chain	FIBA	-3.08	0.01
1433Z_HUMAN	14-3-3 protein zeta/delta	1433Z	-1.38	0.01
KZ4_HUMAN	Keratin, type II cytoskeletal 4	K2C4	-1.50	0.02
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	MDHM	-1.47	0.02
A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	A1AG1	-1.46	0.03
FIBB_HUMAN	Fibrinogen beta chain	FIBB	-1.46	0.03
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDOA	-1.10	0.04
K1C19_HUMAN	Keratin, type I cytoskeletal 19	K1C19	-0.54	0.04
BCAS1_HUMAN	Breast carcinoma-amplified sequence 1	BCAS1	1.43	0.04
VILI_HUMAN	Villin-1	VILI	-1.98	0.04
CATD_HUMAN	Cathepsin D	CATD	-1.78	0.04
MYH14_HUMAN	Myosin-14	MYH14	-1.44	0.05
WFDC2_HUMAN	WAP four-disulfide core domain protein 2	WFDC2	-1.47	0.05
K2E_HUMAN	Keratin, type II cytoskeletal 2 epidermal	K2E	-1.08	0.05
TKT_HUMAN	Transketolase	TKT	-0.74	0.05
LG3BP_HUMAN	Galectin-3-binding protein	LG3BP	-1.40	0.05

	Protein name	Gene	L2FC	p-value
	TFV (D1-D0) vs HEC (D1-D0) p<0.05 (unpaired *t*-test)			
CALM_HUMAN	**Calmodulin**	CALM	-4.73	0.00014
MMP9_HUMAN	Matrix metalloproteinase-9	MMP9	-2.58	0.004
IL36A_HUMAN	Interleukin-36 alpha	IL36A	-4.42	0.02
TPM3L_HUMAN	Putative tropomyosin alpha-3 chain-like protein	TPM3L	-2.26	0.03
AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	AHNK	2.08	0.04
CAMP_HUMAN	Cathelicidin antimicrobial peptide	CAMP	-1.82	0.04
LYPD3_HUMAN	Ly6/PLAUR domain-containing protein 3	LYPD3	-1.93	0.045
	TFV (D7-D0) vs HEC(D7-D0) p<0.05 (unpaired *t*- test)			
FIBA_HUMAN	Fibrinogen alpha chain	FIBA	3.08	0.02
KV105_HUMAN	Ig kappa chain V-I region DEE	KV105	3.79	0.02
WDR1_HUMAN	WD repeat-containing protein 1	WDR1	3.25	0.03
AFAM_HUMAN	Afamin	AFAM	2.57	0.03
FINC_HUMAN	Fibronectin	FINC	2.99	0.04
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDO A	1.07	0.04
GFPT1_HUMAN	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1	1.72	0.04
CALM_HUMAN	**Calmodulin**	CALM	-2.57	0.0508

Biomarkers common to all comparisons (p<0.05)
Biomarkers common to both timepoints in TFV arm (p<0.05)
Biomarkers trending at significance levels (p>0.05)

Table2 a-c. Potential protein biomarkers of 1% Tenofovir or HEC gel usage in rectal mucosal fluid as identified by mass spectrometry.

Gel usage was associated with changes in protein expression in both arms of this trial at early (Day 1, 1 dose) and later (Day 7, 8 doses) time points (p<0.05). **Table 2A:** The strongest effects were in the TFV arm. Cornulin, calmodulin and interleukin 36-alpha were significantly under-expressed (p<0.05) across timepoints. **Table 2B:** HEC gel usage was associated with milder proteome changes at all time points relative to TFV use. **Table 2C:** When between-arm effects were analysed (independent t tests), calmodulin was significantly under-expressed on Day 1 (p=0.00014), and was trending at Day 7 (p=0.0508). Such proteins that are consistently differentially expressed with gel use may serve ase biomarkers for future studies.



Proteins commonly differentially expressed on Day 1 and Day 7 (p<0.05)

SUMMARY

•Incorporating systems biology as a routine component of clinical trials of HIV-prevention therapies will broaden our understanding of the impact of these gels on mucosal immunity.

•The risk of HIV acquisition through the rectum is significantly higher than other sites of mucosal exposure in the absence of ART (1/1,250 vaginal, 1/2,500 penile, only 1/70 for receptive anal), which contributes to disproportionate rates of infection in at-risk MSM. Condom use is variable, and new prevention strategies are needed to block HIV spread through unprotected receptive anal intercourse (URAI).

•Several clinical studies of Tenofovir 1% topical microbicides have shown limited protection against HIV acquisition, making it a promising candidate. However, impact of this gel on the rectal mucosa and its safety is under debate.

Major Findings

•Proteomic analysis of rectal mucosal fluid was able to confidently identify 246 unique human proteins across rectal mucosal samples in Project Gel.

•The most dramatic changes to the proteome were seen in the TFV arm after 1, and to a greater extent, 8 doses of the product were administered. Down-regulated factors were associated with cell and tissue development (epidermis development, peptide cross linking and intermediate filament cytoskeleton).

•Three factors were downregulated in rectal mucosal fluid after 1 and 8 doses of TFV:

Cornulin is a regulator of cell proliferation and cell cycle arrest, it has been shown to be an important tumor suppressant in epithelial cells, and loss of this expression can lead to the development of squamous cell carcinomas[6].

Calmodulin mediates a number of enzyme and ion channel function through Ca^{2+} ion modifications. It can work with other proteins to regulate cell cycle progression through cytokenesis[7].

Interleukin-36A is a proinflammatory signalling molecule that acts on epithelial cells to promote T cell and recruitment, maturation and proliferation[8,9].

•All three factors are important mediators of cell cycle and cell proliferation, with down-regulation of one factor (cornulin) having been directly linked to tumorigenesis in epithelial tissue. This may support an earlier systems analysis by Hladik et al. (2015) linking TFV use to disregulation of epithelial cell development.

Limitations: Though proteomic methods were able to provide a comprehensive characterization the rectal mucosal fluid environment during TFV 1% gel usage, and identify several biomarkers of TFV exposure, this study was underpowered. Larger studies are needed to validate these findings.

Conclusions: These preliminary studies suggest that rectal TFV-gel usage may be associated with mucosal proteome changes related to epidermal development. Further long-term studies are warranted for validation of these biomarkers including the impact of TFV-based rectal microbicide gels on the overall mucosal proteome.

AKNOWLEDGEMENTS

We would like to acknowledge the technical support of Max Abou from the National Laboratory for HIV immunology Proteomics Unit at the JC Wilt Infectious Disease Research Centre, and Stuart McCorrster from the Mass Spectrometry Core Facility at the NML, Public Health Agency of Canada (Winnipeg, MB Canada) as well as all study participants.

National Institutes of Health

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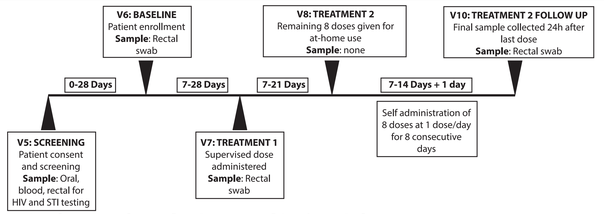


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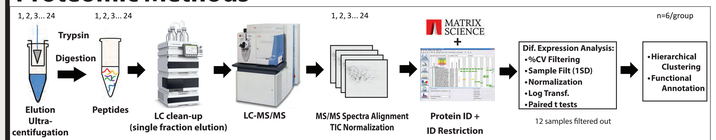


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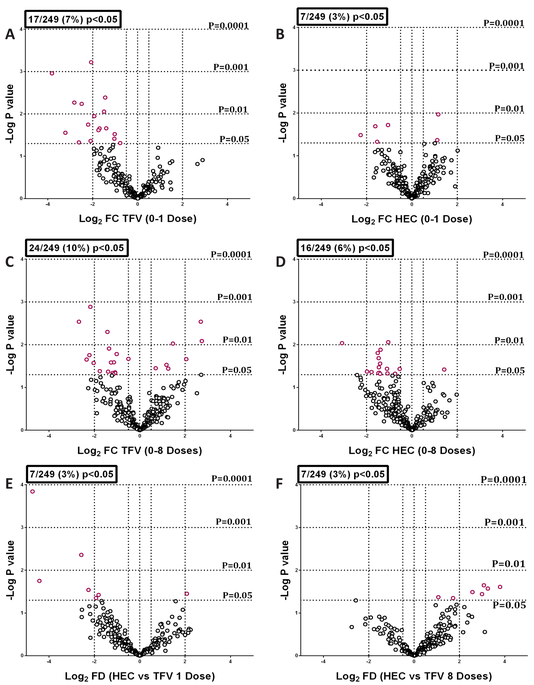


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CALM_HUMAN	**Calmodulin**	CALM	-3.82	0.0011
6PGD_HUMAN	6-phosphogluconate dehydrogenase, decarboxylating	6PGD	-1.44	0.0041
MMP9_HUMAN	Matrix metalloproteinase-9	MMP9	-2.82	0.0054
CAMP_HUMAN	Cathelicidin antimicrobial peptide	CAMP	-2.49	0.0058
CALL5_HUMAN	Calmodulin-like protein 5	CALL5	-1.49	0.0088
VIME_HUMAN	Vimentin	VIME	-1.93	0.0113
SHIL3_HUMAN	SH3 domain-binding glutamic acid-rich-like protein 3	SH3L3	-2.2	0.0179
KV305_HUMAN	Ig kappa chain V-III region WOL	KV305	-1.4	0.0219
LYPD3_HUMAN	Ly6/PLAUR domain-containing protein 3	LYPD3	-1.7	0.022
VILI_HUMAN	Villin-1	VILI	-1.74	0.0242
IL36A_HUMAN	**Interleukin 36A**	IL36A	-3.22	0.0279
CATD_HUMAN	Cathepsin D	GATD	-1.02	0.03
THIO_HUMAN	Thioredoxin	THIO	-1.04	0.0386
IF6_HUMAN	Eukaryotic translation initiation factor 6	IF6	-2.09	0.0434
RNH1_HUMAN	Ribonuclease inhibitor	RINI	-2.62	0.0471
IF2G_HUMAN	Elongation factor 1-gamma	IF1G	-0.78	0.0487
	Tenofovir arm _ D0 vs D7 p<0.05 (paired *t*-test)			
IGJ_HUMAN	Immunoglobulin J chain	IGJ	-2.18	0.0013
CALM_HUMAN	**Calmodulin**	CALM	-2.68	0.0029
FINC_HUMAN	Fibronectin	FINC	2.69	0.0029
K1C10_HUMAN	Keratin, type I cytoskeletal 10	K1C10	-1.43	0.005
WDR1_HUMAN	WD repeat-containing protein 1	WDR1	2.74	0.0082
AFAM_HUMAN	Afamin	AFAM	1.46	0.0094
FLNB_HUMAN	Filamin-B	FLNB	-1.36	0.0123
K2C1_HUMAN	Keratin, type II cytoskeletal 1	K2C1	-1.02	0.0165
CRNN_HUMAN	**Cornulin**	CRNN	-2.22	0.0175
K1C9_HUMAN	Keratin, type I cytoskeletal 9	K1C9	-0.5	0.0214
SIAS_HUMAN	Sialic acid synthase	SIAS	2.05	0.0217
CEAM5_HUMAN	Carcinoembryonic antigen-related cell adhesion molecule 5	CEAM5	-2.34	0.0222
K2C6A_HUMAN	Keratin, type II cytoskeletal 6A	K2C6A	-1.14	0.0258
CASP2_HUMAN	Caspase-14	CASPE	-1.27	0.026
K1C13_HUMAN	Keratin, type I cytoskeletal 13	K1C13	-2.03	0.0267
APOH_HUMAN	Beta-2-glycoprotein 1	APOH	1.18	0.0296
LEG4_HUMAN	Galectin-4	LEG4	0.7	0.0354
PRDX6_HUMAN	Peroxiredoxin-6	PRDX6	1.25	0.0362
IL36A_HUMAN	**Interleukin 36A**	IL36A	-1.76	0.0414
TBB4B_HUMAN	Tubulin beta-4B chain	TBB4B	-1.4	0.0426
K1C19_HUMAN	Keratin, type I cytoskeletal 19	K1C19	-1.09	0.0443
K2C5_HUMAN	Keratin, type II cytoskeletal 5	K2C5	-1.09	0.0457
CYTA_HUMAN	Cystatin-A	CYTA	-1.19	0.0457
INVO_HUMAN	Involucrin	INVO	-1.17	0.0492

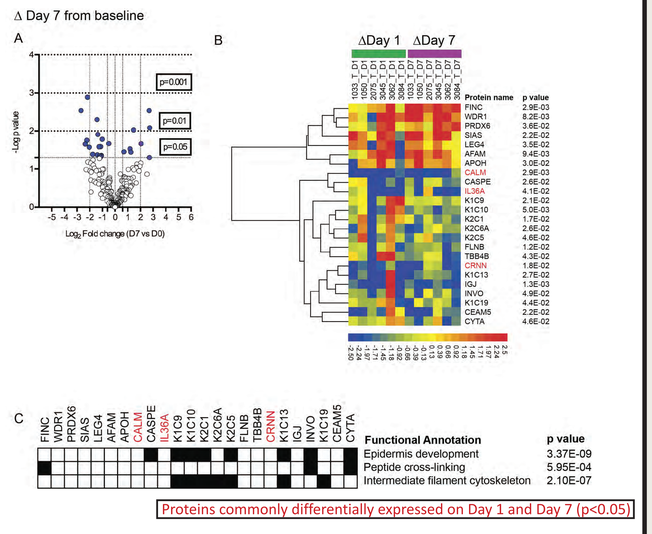
	Protein name	Gene	L2FC	p-value
	HEC arm _ D0 vs D1 p<0.05 (paired *t*- test)			
APOA1_HUMAN	Apolipoprotein A-I	APOA1	1.16	0.01
GBP2B_HUMAN	78 kDa glucose-regulated protein	GRP78	-1.05	0.02
AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	AHNK	-1.61	0.02
SPB13_HUMAN	Serpin B13	SPB13	-2.26	0.03
TBA1C_HUMAN	Tubulin alpha-1C chain	TBA1C	1.11	0.04
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	MDHM	-1.93	0.05
	HEC arm _ D0 vs D7 p<0.05 (paired *t*- test)			
ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	ZA2G	-3.04	0.01
FIBA_HUMAN	Fibrinogen alpha chain	FIBA	-3.08	0.01
1433Z_HUMAN	14-3-3 protein zeta/delta	1433Z	-1.38	0.01
KZ4_HUMAN	Keratin, type II cytoskeletal 4	K2C4	-1.50	0.02
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	MDHM	-1.47	0.02
A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	A1AG1	-1.46	0.03
FIBB_HUMAN	Fibrinogen beta chain	FIBB	-1.46	0.03
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDOA	-1.10	0.04
K1C19_HUMAN	Keratin, type I cytoskeletal 19	K1C19	-0.54	0.04
BCAS1_HUMAN	Breast carcinoma-amplified sequence 1	BCAS1	1.43	0.04
VILI_HUMAN	Villin-1	VILI	-1.98	0.04
CATD_HUMAN	Cathepsin D	CATD	-1.78	0.04
MYH14_HUMAN	Myosin-14	MYH14	-1.44	0.05
WFDC2_HUMAN	WAP four-disulfide core domain protein 2	WFDC2	-1.47	0.05
K2E_HUMAN	Keratin, type II cytoskeletal 2 epidermal	K2E	-1.08	0.05
TKT_HUMAN	Transketolase	TKT	-0.74	0.05
LG3BP_HUMAN	Galectin-3-binding protein	LG3BP	-1.40	0.05

	Protein name	Gene	L2FC	p-value
	TFV (D1-D0) vs HEC (D1-D0) p<0.05 (unpaired *t*-test)			
CALM_HUMAN	**Calmodulin**	CALM	-4.73	0.00014
MMP9_HUMAN	Matrix metalloproteinase-9	MMP9	-2.58	0.004
IL36A_HUMAN	Interleukin-36 alpha	IL36A	-4.42	0.02
TPM3L_HUMAN	Putative tropomyosin alpha-3 chain-like protein	TPM3L	-2.26	0.03
AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	AHNK	2.08	0.04
CAMP_HUMAN	Cathelicidin antimicrobial peptide	CAMP	-1.82	0.04
LYPD3_HUMAN	Ly6/PLAUR domain-containing protein 3	LYPD3	-1.93	0.045
	TFV (D7-D0) vs HEC(D7-D0) p<0.05 (unpaired *t*- test)			
FIBA_HUMAN	Fibrinogen alpha chain	FIBA	3.08	0.02
KV105_HUMAN	Ig kappa chain V-I region DEE	KV105	3.79	0.02
WDR1_HUMAN	WD repeat-containing protein 1	WDR1	3.25	0.03
AFAM_HUMAN	Afamin	AFAM	2.57	0.03
FINC_HUMAN	Fibronectin	FINC	2.99	0.04
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDO A	1.07	0.04
GFPT1_HUMAN	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1	1.72	0.04
CALM_HUMAN	**Calmodulin**	CALM	-2.57	0.0508

Biomarkers common to all comparisons (p<0.05)
Biomarkers common to both timepoints in TFV arm (p<0.05)
Biomarkers trending at significance levels (p>0.05)

Table2 a-c. Potential protein biomarkers of 1% Tenofovir or HEC gel usage in rectal mucosal fluid as identified by mass spectrometry.

Gel usage was associated with changes in protein expression in both arms of this trial at early (Day 1, 1 dose) and later (Day 7, 8 doses) time points (p<0.05). **Table 2A:** The strongest effects were in the TFV arm. Cornulin, calmodulin and interleukin 36-alpha were significantly under-expressed (p<0.05) across timepoints. **Table 2B:** HEC gel usage was associated with milder proteome changes at all time points relative to TFV use. **Table 2C:** When between-arm effects were analysed (independent t tests), calmodulin was significantly under-expressed on Day 1 (p=0.00014), and was trending at Day 7 (p=0.0508). Such proteins that are consistently differentially expressed with gel use may serve ase biomarkers for future studies.



Proteins commonly differentially expressed on Day 1 and Day 7 (p<0.05)

SUMMARY

•Incorporating systems biology as a routine component of clinical trials of HIV-prevention therapies will broaden our understanding of the impact of these gels on mucosal immunity.

•The risk of HIV acquisition through the rectum is significantly higher than other sites of mucosal exposure in the absence of ART (1/1,250 vaginal, 1/2,500 penile, only 1/70 for receptive anal), which contributes to disproportionate rates of infection in at-risk MSM. Condom use is variable, and new prevention strategies are needed to block HIV spread through unprotected receptive anal intercourse (URAI).

•Several clinical studies of Tenofovir 1% topical microbicides have shown limited protection against HIV acquisition, making it a promising candidate. However, impact of this gel on the rectal mucosa and its safety is under debate.

Major Findings

•Proteomic analysis of rectal mucosal fluid was able to confidently identify 246 unique human proteins across rectal mucosal samples in Project Gel.

•The most dramatic changes to the proteome were seen in the TFV arm after 1, and to a greater extent, 8 doses of the product were administered. Down-regulated factors were associated with cell and tissue development (epidermis development, peptide cross linking and intermediate filament cytoskeleton).

•Three factors were downregulated in rectal mucosal fluid after 1 and 8 doses of TFV:

Cornulin is a regulator of cell proliferation and cell cycle arrest, it has been shown to be an important tumor suppressant in epithelial cells, and loss of this expression can lead to the development of squamous cell carcinomas[6].

Calmodulin mediates a number of enzyme and ion channel function through Ca^{2+} ion modifications. It can work with other proteins to regulate cell cycle progression through cytokenesis[7].

Interleukin-36A is a proinflammatory signalling molecule that acts on epithelial cells to promote T cell and recruitment, maturation and proliferation[8,9].

•All three factors are important mediators of cell cycle and cell proliferation, with down-regulation of one factor (cornulin) having been directly linked to tumorigenesis in epithelial tissue. This may support an earlier systems analysis by Hladik et al. (2015) linking TFV use to disregulation of epithelial cell development.

Limitations: Though proteomic methods were able to provide a comprehensive characterization the rectal mucosal fluid environment during TFV 1% gel usage, and identify several biomarkers of TFV exposure, this study was underpowered. Larger studies are needed to validate these findings.

Conclusions: These preliminary studies suggest that rectal TFV-gel usage may be associated with mucosal proteome changes related to epidermal development. Further long-term studies are warranted for validation of these biomarkers including the impact of TFV-based rectal microbicide gels on the overall mucosal proteome.

AKNOWLEDGEMENTS

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Rectal tenofovir gel usage is associated with changes in the mucosal proteome

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1. National HIV and Retrovirology Laboratory, JC Wilt Infectoius Disease Research Centre, Public Health Agency of Canada, CAN; 2. Medical Microbiology and Infectious Diseases, University of Manitoba, CAN; 3. Karolinska Institutet, Dept. of Medicine Solna, Center for Molecular Medicine, SWD;4. Department of Medicine, University of Pittsburgh, Pittsburgh, PA, USA 5.Harvard Medical School, Boston, MA, USA; 6. National Microbiology Lab, Public Health Agency of Canada, CAN; 7. Columbia University, New York, NY, USA. 8. University of Peurto Rico, San Juan, PR.

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University of Manitoba

Poster #269

INTRODUCTION

•Tenfovir (TFV) 1% gel was 39% effective against vaginal transmission of HIV-1[1]. Since then, rectal formulations are being explored as a prevention option.

•Vaginal formulations of TFV gel applied rectally were associated with gastrointestinal intolerance (MTN-006)[2]. This was likely due to an incompatible osmolarity and pH of the formulation with the GI mucosa.

•A reduced-glycerin vaginal formulation (RGVF) of TFV gel has been determined safe for use using epithelial sloughing, fecal calprotectin, inflammatory cytokine levels and cellular immune markers as indicators of acceptability in the rectum (MTN-007)[3].

•RGVF and other formulations of TFV 1% gel are being assessed in clinical trials for safety for rectal use in young men who have sex with men (Project Gel).

Rationale

•Traditional immune safety standards may miss important immunological or biological side effects of these gels. A systems biology analysis was recently completed to assess the effects of the RGVF gel on rectal mucosal tissues in the MTN-007 trial. A broad range RGVF-associated changes in mucosal gene/protein expression was observed that may affect immune homeostasis, mitochondrial function and epithelial cell development not seen in previous safety evaluations[3,4].

Therefore the objective of this study was to evaluate host mucosal responses through proteomics of study participants engaged in a phase 1 randomized, double-blind, multi-site, placebo-controlled trial of Tenofovir-based rectal microbicides.

Hypothesis

We have performed a comprehensive proteomic analysis to explore the impact of different formulations of TFV 1% gel within Project Gel (MTN). This will allow us to identify changes in mucosal tissues associated with gel use.

STUDY DESIGN

Table 1. Rectal microbicide formulations utilized in Project Gel.

Study participants (n=24) were divided into a control group given a HEC placebo gel, and a treatment group given the 1% TFV formulation shown below. (adapted from [5])

Product	Abbrv.	TFV Content (%w/w)	Glycerin (%w/w)	pH	Osmolarity (mmol/kg)
Hydroxyethyl cellulose placebo gel	HEC	0%	0	4.4	isotonic
1% TFV gel	TFV	1%	5	4.5	846

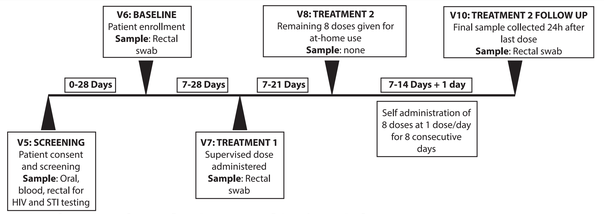


Figure 1. Study procedures for Stage 2 of Project Gel.

Participants selected for Stage 2 were screened for HIV and STIs. A baseline sample was taken 1-3 weeks prior to the first supervised dose. A sample was taken after the first dose to determine immediate effects of the gel. Eight doses of product were self-administered at home. A final sample was taken 24h after the last dose was administered.

Proteomic Methods

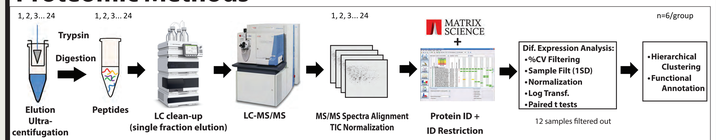


Figure 2. Proteomic analysis workflow.

Samples from 24 participants (n=12 HEC & n=12 TFV) were collected via rectal sponge. Mucosal proteins were eluted, purified via ultracentrifugation and digested into peptides. Peptides were cleaned with liquid chromatography and analyzed by label-free MS/MS. Spectra were aligned across samples. Proteins were identified with Mascot Daemon (Matrix Science) and IDs were restricted to 95% peptide and 80% protein confidence and a minimum of 2 peptides ID'd. Normalized protein abundance values were determined to be differentially expressed over time via paired t tests (Benjimani-Hochberg corr.), using changes in the HEC arm as a comparison. Hierachrical clustering (Cluster 3.0) and functional annotation (DAVID Bioinformatics) were performed.

RESULTS

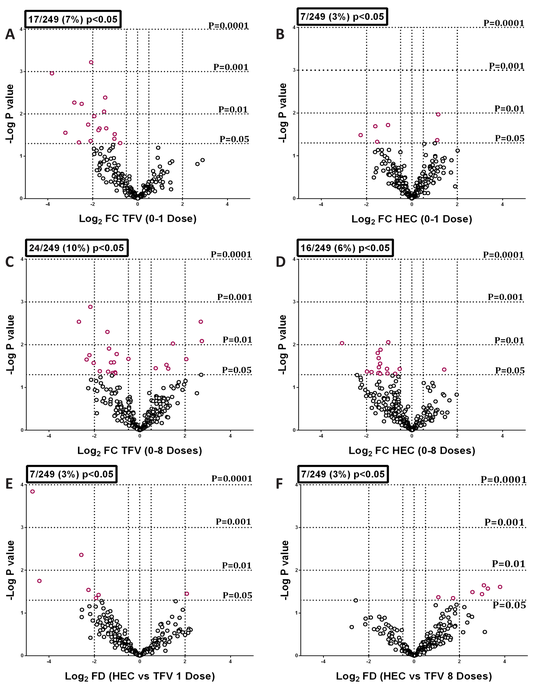


Figure 3. Effects of daily applications of either 1% Tenofovir or HEC placebo microbicide on the rectal mucosal proteome.

Volcano plots illustrating fold-change (x-axis) and statistical significance distribution (y-axis) of the proteomic datasets. Data points in maroon indicate proteins defined to be significantly differentially expressed (p<0.05; both paired and unpaired t-tests). 246 unique proteins were identified in rectal mucosal samples after variance and species filtering (human only, <25% CV in reference sample). **A and C:** Paired t tests were used to assess protein expression changes within individuals associated with short-term TFV usage, and longer-term TFV usage relative to the HEC control arm (**B and D,** respectively). **E and F:** Independent t tests compared protein expression between the TFV and HEC groups after 1 dose and 8 doses. The strongest effects associated with TFV gel use were found within individuals after 8 doses.

Figure 4. Biofunctional analysis and hierarchical clustering of proteome changes in rectal mucosal fluid after 7 day application of 1% Tenofovir gel.

A: A volcano plot illustrates fold-changes and statistical significance between proteins differentially abundant (DA) between D0-D7 (paired t-test). Those that met a p<0.05 cut-off threshold are shown in color (blue). **B:** Heatmap illustrates proteins which were differentially abundant (p<0.05, 24 proteins), between Day 1 and Day 7 after TFV-gel exposure. Clustering of proteins was generated by unsupervised average linkage hierarchical clustering using Pearson correlation coefficient as the distance metric. Both over and underexpressed proteins are observed. **C:** Pathway analysis of proteins DA was performed using GO database (DAVID). The major functions were associated with epidermis development and cytoskeletal functions. Only those with >3 factors and a p value $<10^{-4}$ (Benjimani Hochberg MCC) are shown (C) . In the HEC gel arm, although mild associations to functional categories were observed (cell activation), these did not pass multiple comparison correction threshold.

	Protein name	Gene	L2FC	p-value
	Tenofovir arm _ D0 vs D1 p<0.05 (paired *t*-test)			
CRNN_HUMAN	**Cornulin**	CRNN	-2.06	0.0006
CALM_HUMAN	**Calmodulin**	CALM	-3.82	0.0011
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VIME_HUMAN	Vimentin	VIME	-1.93	0.0113
SHIL3_HUMAN	SH3 domain-binding glutamic acid-rich-like protein 3	SH3L3	-2.2	0.0179
KV305_HUMAN	Ig kappa chain V-III region WOL	KV305	-1.4	0.0219
LYPD3_HUMAN	Ly6/PLAUR domain-containing protein 3	LYPD3	-1.7	0.022
VILI_HUMAN	Villin-1	VILI	-1.74	0.0242
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IF6_HUMAN	Eukaryotic translation initiation factor 6	IF6	-2.09	0.0434
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IF2G_HUMAN	Elongation factor 1-gamma	IF1G	-0.78	0.0487
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K1C9_HUMAN	Keratin, type I cytoskeletal 9	K1C9	-0.5	0.0214
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CEAM5_HUMAN	Carcinoembryonic antigen-related cell adhesion molecule 5	CEAM5	-2.34	0.0222
K2C6A_HUMAN	Keratin, type II cytoskeletal 6A	K2C6A	-1.14	0.0258
CASP2_HUMAN	Caspase-14	CASPE	-1.27	0.026
K1C13_HUMAN	Keratin, type I cytoskeletal 13	K1C13	-2.03	0.0267
APOH_HUMAN	Beta-2-glycoprotein 1	APOH	1.18	0.0296
LEG4_HUMAN	Galectin-4	LEG4	0.7	0.0354
PRDX6_HUMAN	Peroxiredoxin-6	PRDX6	1.25	0.0362
IL36A_HUMAN	**Interleukin 36A**	IL36A	-1.76	0.0414
TBB4B_HUMAN	Tubulin beta-4B chain	TBB4B	-1.4	0.0426
K1C19_HUMAN	Keratin, type I cytoskeletal 19	K1C19	-1.09	0.0443
K2C5_HUMAN	Keratin, type II cytoskeletal 5	K2C5	-1.09	0.0457
CYTA_HUMAN	Cystatin-A	CYTA	-1.19	0.0457
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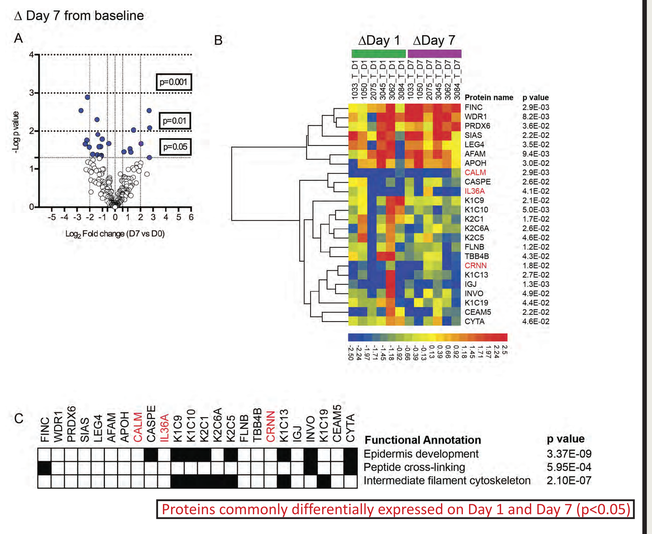
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AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	AHNK	-1.61	0.02
SPB13_HUMAN	Serpin B13	SPB13	-2.26	0.03
TBA1C_HUMAN	Tubulin alpha-1C chain	TBA1C	1.11	0.04
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	HEC arm _ D0 vs D7 p<0.05 (paired *t*- test)			
ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	ZA2G	-3.04	0.01
FIBA_HUMAN	Fibrinogen alpha chain	FIBA	-3.08	0.01
1433Z_HUMAN	14-3-3 protein zeta/delta	1433Z	-1.38	0.01
KZ4_HUMAN	Keratin, type II cytoskeletal 4	K2C4	-1.50	0.02
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	MDHM	-1.47	0.02
A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	A1AG1	-1.46	0.03
FIBB_HUMAN	Fibrinogen beta chain	FIBB	-1.46	0.03
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDOA	-1.10	0.04
K1C19_HUMAN	Keratin, type I cytoskeletal 19	K1C19	-0.54	0.04
BCAS1_HUMAN	Breast carcinoma-amplified sequence 1	BCAS1	1.43	0.04
VILI_HUMAN	Villin-1	VILI	-1.98	0.04
CATD_HUMAN	Cathepsin D	CATD	-1.78	0.04
MYH14_HUMAN	Myosin-14	MYH14	-1.44	0.05
WFDC2_HUMAN	WAP four-disulfide core domain protein 2	WFDC2	-1.47	0.05
K2E_HUMAN	Keratin, type II cytoskeletal 2 epidermal	K2E	-1.08	0.05
TKT_HUMAN	Transketolase	TKT	-0.74	0.05
LG3BP_HUMAN	Galectin-3-binding protein	LG3BP	-1.40	0.05

	Protein name	Gene	L2FC	p-value
	TFV (D1-D0) vs HEC (D1-D0) p<0.05 (unpaired *t*-test)			
CALM_HUMAN	**Calmodulin**	CALM	-4.73	0.00014
MMP9_HUMAN	Matrix metalloproteinase-9	MMP9	-2.58	0.004
IL36A_HUMAN	Interleukin-36 alpha	IL36A	-4.42	0.02
TPM3L_HUMAN	Putative tropomyosin alpha-3 chain-like protein	TPM3L	-2.26	0.03
AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	AHNK	2.08	0.04
CAMP_HUMAN	Cathelicidin antimicrobial peptide	CAMP	-1.82	0.04
LYPD3_HUMAN	Ly6/PLAUR domain-containing protein 3	LYPD3	-1.93	0.045
	TFV (D7-D0) vs HEC(D7-D0) p<0.05 (unpaired *t*- test)			
FIBA_HUMAN	Fibrinogen alpha chain	FIBA	3.08	0.02
KV105_HUMAN	Ig kappa chain V-I region DEE	KV105	3.79	0.02
WDR1_HUMAN	WD repeat-containing protein 1	WDR1	3.25	0.03
AFAM_HUMAN	Afamin	AFAM	2.57	0.03
FINC_HUMAN	Fibronectin	FINC	2.99	0.04
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDO A	1.07	0.04
GFPT1_HUMAN	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1	1.72	0.04
CALM_HUMAN	**Calmodulin**	CALM	-2.57	0.0508

Biomarkers common to all comparisons (p<0.05)
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Table2 a-c. Potential protein biomarkers of 1% Tenofovir or HEC gel usage in rectal mucosal fluid as identified by mass spectrometry.

Gel usage was associated with changes in protein expression in both arms of this trial at early (Day 1, 1 dose) and later (Day 7, 8 doses) time points (p<0.05). **Table 2A:** The strongest effects were in the TFV arm. Cornulin, calmodulin and interleukin 36-alpha were significantly under-expressed (p<0.05) across timepoints. **Table 2B:** HEC gel usage was associated with milder proteome changes at all time points relative to TFV use. **Table 2C:** When between-arm effects were analysed (independent t tests), calmodulin was significantly under-expressed on Day 1 (p=0.00014), and was trending at Day 7 (p=0.0508). Such proteins that are consistently differentially expressed with gel use may serve ase biomarkers for future studies.



Proteins commonly differentially expressed on Day 1 and Day 7 (p<0.05)

SUMMARY

•Incorporating systems biology as a routine component of clinical trials of HIV-prevention therapies will broaden our understanding of the impact of these gels on mucosal immunity.

•The risk of HIV acquisition through the rectum is significantly higher than other sites of mucosal exposure in the absence of ART (1/1,250 vaginal, 1/2,500 penile, only 1/70 for receptive anal), which contributes to disproportionate rates of infection in at-risk MSM. Condom use is variable, and new prevention strategies are needed to block HIV spread through unprotected receptive anal intercourse (URAI).

•Several clinical studies of Tenofovir 1% topical microbicides have shown limited protection against HIV acquisition, making it a promising candidate. However, impact of this gel on the rectal mucosa and its safety is under debate.

Major Findings

•Proteomic analysis of rectal mucosal fluid was able to confidently identify 246 unique human proteins across rectal mucosal samples in Project Gel.

•The most dramatic changes to the proteome were seen in the TFV arm after 1, and to a greater extent, 8 doses of the product were administered. Down-regulated factors were associated with cell and tissue development (epidermis development, peptide cross linking and intermediate filament cytoskeleton).

•Three factors were downregulated in rectal mucosal fluid after 1 and 8 doses of TFV:

Cornulin is a regulator of cell proliferation and cell cycle arrest, it has been shown to be an important tumor suppressant in epithelial cells, and loss of this expression can lead to the development of squamous cell carcinomas[6].

Calmodulin mediates a number of enzyme and ion channel function through Ca^{2+} ion modifications. It can work with other proteins to regulate cell cycle progression through cytokenesis[7].

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Limitations: Though proteomic methods were able to provide a comprehensive characterization the rectal mucosal fluid environment during TFV 1% gel usage, and identify several biomarkers of TFV exposure, this study was underpowered. Larger studies are needed to validate these findings.

Conclusions: These preliminary studies suggest that rectal TFV-gel usage may be associated with mucosal proteome changes related to epidermal development. Further long-term studies are warranted for validation of these biomarkers including the impact of TFV-based rectal microbicide gels on the overall mucosal proteome.

AKNOWLEDGEMENTS

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Rectal tenofovir gel usage is associated with changes in the mucosal proteome

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University of Manitoba

Poster #269

INTRODUCTION

•Tenfovir (TFV) 1% gel was 39% effective against vaginal transmission of HIV-1[1]. Since then, rectal formulations are being explored as a prevention option.

•Vaginal formulations of TFV gel applied rectally were associated with gastrointestinal intolerance (MTN-006)[2]. This was likely due to an incompatible osmolarity and pH of the formulation with the GI mucosa.

•A reduced-glycerin vaginal formulation (RGVF) of TFV gel has been determined safe for use using epithelial sloughing, fecal calprotectin, inflammatory cytokine levels and cellular immune markers as indicators of acceptability in the rectum (MTN-007)[3].

•RGVF and other formulations of TFV 1% gel are being assessed in clinical trials for safety for rectal use in young men who have sex with men (Project Gel).

Rationale

•Traditional immune safety standards may miss important immunological or biological side effects of these gels. A systems biology analysis was recently completed to assess the effects of the RGVF gel on rectal mucosal tissues in the MTN-007 trial. A broad range RGVF-associated changes in mucosal gene/protein expression was observed that may affect immune homeostasis, mitochondrial function and epithelial cell development not seen in previous safety evaluations[3,4].

Therefore the objective of this study was to evaluate host mucosal responses through proteomics of study participants engaged in a phase 1 randomized, double-blind, multi-site, placebo-controlled trial of Tenofovir-based rectal microbicides.

Hypothesis

We have performed a comprehensive proteomic analysis to explore the impact of different formulations of TFV 1% gel within Project Gel (MTN). This will allow us to identify changes in mucosal tissues associated with gel use.

STUDY DESIGN

Table 1. Rectal microbicide formulations utilized in Project Gel.

Study participants (n=24) were divided into a control group given a HEC placebo gel, and a treatment group given the 1% TFV formulation shown below. (adapted from [5])

Product	Abbrv.	TFV Content (%w/w)	Glycerin (%w/w)	pH	Osmolarity (mmol/kg)
Hydroxyethyl cellulose placebo gel	HEC	0%	0	4.4	isotonic
1% TFV gel	TFV	1%	5	4.5	846

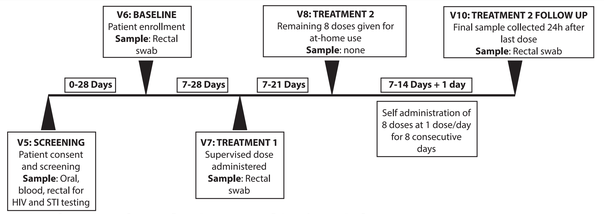


Figure 1. Study procedures for Stage 2 of Project Gel.

Participants selected for Stage 2 were screened for HIV and STIs. A baseline sample was taken 1-3 weeks prior to the first supervised dose. A sample was taken after the first dose to determine immediate effects of the gel. Eight doses of product were self-administered at home. A final sample was taken 24h after the last dose was administered.

Proteomic Methods

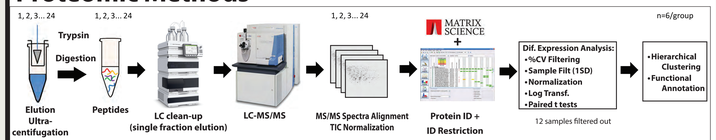


Figure 2. Proteomic analysis workflow.

Samples from 24 participants (n=12 HEC & n=12 TFV) were collected via rectal sponge. Mucosal proteins were eluted, purified via ultracentrifugation and digested into peptides. Peptides were cleaned with liquid chromatography and analyzed by label-free MS/MS. Spectra were aligned across samples. Proteins were identified with Mascot Daemon (Matrix Science) and IDs were restricted to 95% peptide and 80% protein confidence and a minimum of 2 peptides ID'd. Normalized protein abundance values were determined to be differentially expressed over time via paired t tests (Benjimani-Hochberg corr.), using changes in the HEC arm as a comparison. Hierachrical clustering (Cluster 3.0) and functional annotation (DAVID Bioinformatics) were performed.

RESULTS

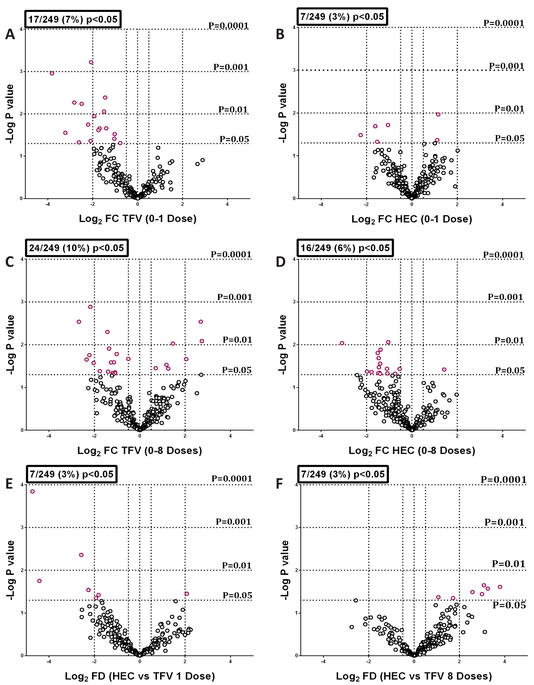


Figure 3. Effects of daily applications of either 1% Tenofovir or HEC placebo microbicide on the rectal mucosal proteome.

Volcano plots illustrating fold-change (x-axis) and statistical significance distribution (y-axis) of the proteomic datasets. Data points in maroon indicate proteins defined to be significantly differentially expressed (p<0.05; both paired and unpaired t-tests). 246 unique proteins were identified in rectal mucosal samples after variance and species filtering (human only, <25% CV in reference sample). **A and C:** Paired t tests were used to assess protein expression changes within individuals associated with short-term TFV usage, and longer-term TFV usage relative to the HEC control arm (**B and D,** respectively). **E and F:** Independent t tests compared protein expression between the TFV and HEC groups after 1 dose and 8 doses. The strongest effects associated with TFV gel use were found within individuals after 8 doses.

Figure 4. Biofunctional analysis and hierarchical clustering of proteome changes in rectal mucosal fluid after 7 day application of 1% Tenofovir gel.

A: A volcano plot illustrates fold-changes and statistical significance between proteins differentially abundant (DA) between D0-D7 (paired t-test). Those that met a p<0.05 cut-off threshold are shown in color (blue). **B:** Heatmap illustrates proteins which were differentially abundant (p<0.05, 24 proteins), between Day 1 and Day 7 after TFV-gel exposure. Clustering of proteins was generated by unsupervised average linkage hierarchical clustering using Pearson correlation coefficient as the distance metric. Both over and underexpressed proteins are observed. **C:** Pathway analysis of proteins DA was performed using GO database (DAVID). The major functions were associated with epidermis development and cytoskeletal functions. Only those with >3 factors and a p value $<10^{-4}$ (Benjimani Hochberg MCC) are shown (C) . In the HEC gel arm, although mild associations to functional categories were observed (cell activation), these did not pass multiple comparison correction threshold.

	Protein name	Gene	L2FC	p-value
	Tenofovir arm _ D0 vs D1 p<0.05 (paired *t*-test)			
CRNN_HUMAN	**Cornulin**	CRNN	-2.06	0.0006
CALM_HUMAN	**Calmodulin**	CALM	-3.82	0.0011
6PGD_HUMAN	6-phosphogluconate dehydrogenase, decarboxylating	6PGD	-1.44	0.0041
MMP9_HUMAN	Matrix metalloproteinase-9	MMP9	-2.82	0.0054
CAMP_HUMAN	Cathelicidin antimicrobial peptide	CAMP	-2.49	0.0058
CALL5_HUMAN	Calmodulin-like protein 5	CALL5	-1.49	0.0088
VIME_HUMAN	Vimentin	VIME	-1.93	0.0113
SHIL3_HUMAN	SH3 domain-binding glutamic acid-rich-like protein 3	SH3L3	-2.2	0.0179
KV305_HUMAN	Ig kappa chain V-III region WOL	KV305	-1.4	0.0219
LYPD3_HUMAN	Ly6/PLAUR domain-containing protein 3	LYPD3	-1.7	0.022
VILI_HUMAN	Villin-1	VILI	-1.74	0.0242
IL36A_HUMAN	**Interleukin 36A**	IL36A	-3.22	0.0279
CATD_HUMAN	Cathepsin D	GATD	-1.02	0.03
THIO_HUMAN	Thioredoxin	THIO	-1.04	0.0386
IF6_HUMAN	Eukaryotic translation initiation factor 6	IF6	-2.09	0.0434
RNH1_HUMAN	Ribonuclease inhibitor	RINI	-2.62	0.0471
IF2G_HUMAN	Elongation factor 1-gamma	IF1G	-0.78	0.0487
	Tenofovir arm _ D0 vs D7 p<0.05 (paired *t*-test)			
IGJ_HUMAN	Immunoglobulin J chain	IGJ	-2.18	0.0013
CALM_HUMAN	**Calmodulin**	CALM	-2.68	0.0029
FINC_HUMAN	Fibronectin	FINC	2.69	0.0029
K1C10_HUMAN	Keratin, type I cytoskeletal 10	K1C10	-1.43	0.005
WDR1_HUMAN	WD repeat-containing protein 1	WDR1	2.74	0.0082
AFAM_HUMAN	Afamin	AFAM	1.46	0.0094
FLNB_HUMAN	Filamin-B	FLNB	-1.36	0.0123
K2C1_HUMAN	Keratin, type II cytoskeletal 1	K2C1	-1.02	0.0165
CRNN_HUMAN	**Cornulin**	CRNN	-2.22	0.0175
K1C9_HUMAN	Keratin, type I cytoskeletal 9	K1C9	-0.5	0.0214
SIAS_HUMAN	Sialic acid synthase	SIAS	2.05	0.0217
CEAM5_HUMAN	Carcinoembryonic antigen-related cell adhesion molecule 5	CEAM5	-2.34	0.0222
K2C6A_HUMAN	Keratin, type II cytoskeletal 6A	K2C6A	-1.14	0.0258
CASP2_HUMAN	Caspase-14	CASPE	-1.27	0.026
K1C13_HUMAN	Keratin, type I cytoskeletal 13	K1C13	-2.03	0.0267
APOH_HUMAN	Beta-2-glycoprotein 1	APOH	1.18	0.0296
LEG4_HUMAN	Galectin-4	LEG4	0.7	0.0354
PRDX6_HUMAN	Peroxiredoxin-6	PRDX6	1.25	0.0362
IL36A_HUMAN	**Interleukin 36A**	IL36A	-1.76	0.0414
TBB4B_HUMAN	Tubulin beta-4B chain	TBB4B	-1.4	0.0426
K1C19_HUMAN	Keratin, type I cytoskeletal 19	K1C19	-1.09	0.0443
K2C5_HUMAN	Keratin, type II cytoskeletal 5	K2C5	-1.09	0.0457
CYTA_HUMAN	Cystatin-A	CYTA	-1.19	0.0457
INVO_HUMAN	Involucrin	INVO	-1.17	0.0492

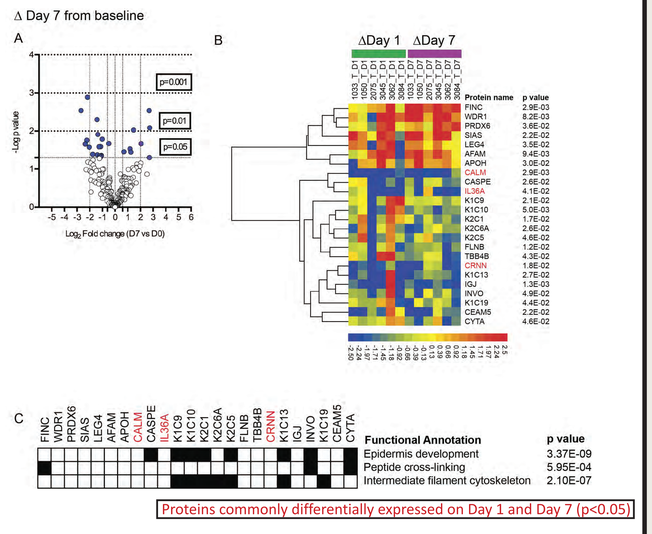
	Protein name	Gene	L2FC	p-value
	HEC arm _ D0 vs D1 p<0.05 (paired *t*- test)			
APOA1_HUMAN	Apolipoprotein A-I	APOA1	1.16	0.01
GBP2B_HUMAN	78 kDa glucose-regulated protein	GRP78	-1.05	0.02
AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	AHNK	-1.61	0.02
SPB13_HUMAN	Serpin B13	SPB13	-2.26	0.03
TBA1C_HUMAN	Tubulin alpha-1C chain	TBA1C	1.11	0.04
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	MDHM	-1.93	0.05
	HEC arm _ D0 vs D7 p<0.05 (paired *t*- test)			
ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	ZA2G	-3.04	0.01
FIBA_HUMAN	Fibrinogen alpha chain	FIBA	-3.08	0.01
1433Z_HUMAN	14-3-3 protein zeta/delta	1433Z	-1.38	0.01
KZ4_HUMAN	Keratin, type II cytoskeletal 4	K2C4	-1.50	0.02
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	MDHM	-1.47	0.02
A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	A1AG1	-1.46	0.03
FIBB_HUMAN	Fibrinogen beta chain	FIBB	-1.46	0.03
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	ALDOA	-1.10	0.04
K1C19_HUMAN	Keratin, type I cytoskeletal 19	K1C19	-0.54	0.04
BCAS1_HUMAN	Breast carcinoma-amplified sequence 1	BCAS1	1.43	0.04
VILI_HUMAN	Villin-1	VILI	-1.98	0.04
CATD_HUMAN	Cathepsin D	CATD	-1.78	0.04
MYH14_HUMAN	Myosin-14	MYH14	-1.44	0.05
WFDC2_HUMAN	WAP four-disulfide core domain protein 2	WFDC2	-1.47	0.05
K2E_HUMAN	Keratin, type II cytoskeletal 2 epidermal	K2E	-1.08	0.05
TKT_HUMAN	Transketolase	TKT	-0.74	0.05
LG3BP_HUMAN	Galectin-3-binding protein	LG3BP	-1.40	0.05

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•Proteomic analysis of rectal mucosal fluid was able to confidently identify 246 unique human proteins across rectal mucosal samples in Project Gel.

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