The Clinical Development of

Rectal Microbicides for HIV Prevention

Thesis submitted in accordance with the requirements of

the University of Liverpool for the degree of

Doctor of Medicine

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November 2013

Declaration

This thesis is the result of my own work. The material contained in it has not been presented, either wholly or in part, for any other degree or qualification.

Funding

The MTN-007 study that forms the core of this thesis was funded by the United States National Institutes of Health, National Institute of Allergy and Infectious Diseases, Division of AIDS, through the Microbicide Trials Network (Grant Number 5UM1AI068633).

Acknowledgements

The MTN-007 study was designed by Dr. Ian McGowan who was also the Principal Investigator and was responsible for the overall conduct of the study. Sample processing, flow cytometry, and gene expression studies (qRT-PCR) were developed and undertaken in Dr. McGowan's laboratory at Magee-Womens Research Institute, in the University of Pittsburgh, Pittsburgh, Pennsylvania, USA.

Other aspects of the study were conducted in collaboration with the individuals and organizations listed below.

Drs. Ross Cranston, Ken Mayer, and Craig Hoesley were the MTN-007 site investigators in Pittsburgh, Pennsylvania, USA; Boston, Massachusetts, USA; and Birmingham, Alabama, USA respectively and provided oversight for study participants enrolled at their sites. Operational support for the study was provided by Philip Andrew and his colleagues at FHI 360, Research Triangle Park, Durham, North Carolina, USA. Data management including the development and execution of the statistical analysis plan for the MTN-007 study was led by Dr. James Dai at the Statistical Center for HIV/AIDS Research and Prevention (SCHARP), University of Washington, Seattle, Washington, USA.

Microflora and Luminex cytokine/chemokine quantification was undertaken in Dr. Sharon Hillier's laboratory at Magee-Womens Research Institute, in the University of Pittsburgh, Pittsburgh, Pennsylvania, USA. Histological assessment of the rectal biopsy samples was performed by Dr. Douglas Hartman, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA. The microarray analysis, validation, and interpretation were led by Dr. Florian Hladik at the University of Washington, Seattle, Washington, USA. Statistical and bioinformatics support for interpretation of the microarray data was provided by Drs. James Dai and Raphael Gottardo, also from the University of Washington, Seattle, Washington, USA.

Sponsor oversight was provided by several colleagues at National Institutes of Health, National Institute of Allergy and Infectious Diseases, Division of AIDS including Drs. Jeanna Piper, Roberta Black, and Carl Dieffenbach. Additional support was provided by Sheryl Zwerski and Cherlynn Mathias.
Dr. Gustavo Doncel and his colleagues at CONRAD, Eastern Virginia Medical School, Norfolk, Virginia, USA are the IND holder for tenofovir gel and provided regulatory support for the MTN-007 study.

Thanks

It is said that it takes a village to raise a child and it is certainly true that it takes a metaphorical village to design, enroll, execute, and analyse a clinical trial; even a small Phase 1 trial such as MTN-007. Consequently, I would like to thank all my friends and colleagues, named and unnamed in this thesis, who helped to move the MTN-007 from a concept to a reality. I have to thank my friend Dr. Sharon Hillier, Principal Investigator of the MTN, who has enthusiastically supported the creation and expansion of an MTN research portfolio in rectal microbicide development.

My laboratory staff in Pittsburgh oversaw sample processing and conduct of the qRT-PCR and flow cytometry assays. Special thanks are due to Kathy Duffill, Laura Janocko, Shaun Burneisen, Aaron Siegel, and Alexyi Nikorov. I would also like to thank my secretary Kathy McCarthy for her help in chasing down numerous references.

I would also like to acknowledge and thank my colleagues within the NIH who provided support and encouragement, for this and other rectal microbicide trials, at a time in American history when the political desire to support this area of research was very limited.

Jim Pickett and his colleagues within the International Rectal Microbicides Advocates (IRMA) group deserve special mention and thanks. They have worked tirelessly to destigmatise anorectal sexual behavior, to lobby for

funding to support rectal microbicide research, and to disseminate research data from this new area of HIV prevention science. Special thanks are also due to Drs. Peter Anton, Craig Hendrix, Charlene Dezzutti, and Lisa Rohan who have all contributed so much to the science of rectal microbicide development.

I would also like to thank Professor David Lalloo, at the Liverpool School of Tropical Medicine, Liverpool, UK who acted as my supervisor for this MD thesis and who provided much helpful advice in navigating this project.

The burden of study investigations in rectal microbicide trials is significant and very special thanks are offered to the study participants who allow this area of research to move forward.

I would like to thank my partner, Ross Cranston, who in addition to helping me run most of my clinical trials, also provides me with love, support, encouragement, and an endless supply of astonishing meals, all of which are much appreciated. Finally, I have to acknowledge Walter the hound whose early morning walks in Schenley Park provided most of the thinking time for this thesis.

Para Ross

sin el cual no hay nada

Abstract

Introduction: Individuals practicing unprotected receptive anal intercourse are at particularly high risk of HIV infection. Men who have sex with men in the developed and developing world continue to have disproportionate and increasing levels of HIV infection. The last few years have seen important progress in demonstrating the efficacy of oral pre-exposure prophylaxis, vaginal microbicides, and treatment as prevention but there has also been significant progress in the development of rectal microbicides. The purpose of this thesis is to summarise the status of rectal microbicide research, to identify opportunities, challenges, and future directions in this important field of HIV prevention, and to describe the results of a recently completed Phase 1 rectal microbicide study (MTN-007).

Methods: MTN-007, a Phase 1, randomised, partially blinded, rectal safety study was undertaken to determine whether a reduced glycerin formulation of tenofovir 1% gel was safe and acceptable to men and women with a history of practicing receptive anal intercourse. The study was conducted at three clinical trial sites in the United States (Pittsburgh, Pennsylvania; Boston, Massachusetts; and Birmingham, Alabama). Study participants were randomized to one of three gel arms (tenofovir gel, a hydroxyethyl cellulose placebo gel, or a 2% Nonoxynol gel) or a no treatment arm and received a total of eight rectal daily doses of the study product. In addition to collecting conventional clinical safety and acceptability data, the study also included extensive mucosal safety assays to determine whether product administration was associated with changes in mucosal biology that might

predispose to increased risk of HIV acquisition associated with unprotected receptive anal intercourse.

Results: Sixty-five participants (45 men and 20 women) were recruited into the study. There were no significant differences between the numbers of ≥ Grade 2 adverse events across the arms of the study. Likelihood of future product use (acceptability) was 87% (reduced glycerin formulation of tenofovir 1% gel), 93% (hydroxyethyl cellulose placebo gel), and 63% (Nonoxynol-9 gel). Fecal calprotectin, rectal microflora, and epithelial sloughing, did not differ by treatment arms during the study. Suggestive evidence of differences was seen in histology, mucosal gene expression, protein expression, and T cell phenotype. These changes were mostly confined to comparisons between the Nonoxynol-9 gel and other study arms. Microarray analysis of the mucosal transcriptome provided preliminary evidence that topical application of tenofovir 1% gel was associated with decreased mitochondrial function within the rectal mucosa.

Conclusions: The MTN-007 study demonstrated that, using conventional criteria, tenofovir gel is safe and acceptable and should be advanced to Phase 2 development as a potential rectal microbicide. However, microarray analysis of mucosal tissue suggested that use of tenofovir gel may modulate mucosal mitochondrial function. This observation will require further evaluation in future studies.

Abbreviations and Notations

3TC	Lamivudine
ABC	Abacavir
ACASI	Audio computer-assisted self-interviews
AEs	Adverse events
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine transaminase
amfAR	The Foundation for AIDS Research
API	Active pharmaceutical ingredient
APV	Amprenavir
ARB	Anorectal biopsy
ARV	Antiretroviral
AST	Aspartate aminotransferase
AUC	Area under the curve
BUN	Blood urea nitrogen
C_{max}	Maximum plasma concentration
CAPRISA	Center for the AIDS Programme of Research in South Africa
CASI	Computer-assisted self-interview
CBC	Complete blood count
CCR5	CC chemokine receptor type 5
CDC	Centers for Disease Control and Prevention
CDS	Coital dynamic simulator
CFR	US Code of Federal Regulations
CHARM	Combination HIV Antiretroviral Rectal Microbicide
CI	Confidence interval
CJA	Conjoint analysis
CRF	Case report form
CRM	Clinical research manager
CReBBP	cAMP-response element binding protein
CRS	Clinical research site

CS	Cellulose sulphate
CT	*Chlamydia trachomatis*
CTA	Clinical trial agreement
CVL	Cervicovaginal lavage
DAIDS	Division of AIDS
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
dd-PCR	Droplet digital PCR
DLV	Delavirdine
DSMB	Data and Safety Monitoring Board
DTPA	Diethylene triamine pentaacetic acid
EC_{50}	50% effective concentration
EIA	Enzyme immunoassay
EFV	Efavirenz
ET	Eastern Time
FACTS	Follow-on African Consortium for Tenofovir Studies
FDA	US Food and Drug Administration
FDR	False discovery rates
FMO	Fluorescence minus one
FSB	Flexible sigmoidoscopic biopsy
FTC	Emtricitabine
g	Gram
GALT	Gut-associated lymphoid tissue
GC	*Neisseria gonorrhoea*
GCP	Good clinical practice
GEE	Generalized estimation equations
GLP	Good laboratory practices
GMP	Good manufacturing practices
HBB	Haemoglobin B
HBsAg	Hepatitis B surface antigen
HEC	Hydroxyethyl cellulose
HIV	human immunodeficiency virus
HIVNET	HIV Network for Prevention Trials
HPV	Human papilloma virus infection

HPTN	HIV Prevention Trials Network
hr	Hour
HRA	High resolution anoscopy
HPV	Human papilloma virus
HSV	Herpes simplex virus
IATA	International Air Transport Association
ICC	Intraclass correlation
ICH	International Conference on Harmonisation
IFA	Immunofluorescent antibody
IFN	Interferon
IL	Interleukin
IND	Investigational new drug
IoR	Investigator of record
IPCP-HTM	Integrated Preclinical/Clinical Program for HIV Topical Microbicides
IPM	International Partnership for Microbicides
IPrEx	Iativa Profilaxis PreExposicion
IRB	Institutional review board
IRMA	International Rectal Microbicide Advocates
IRR	Incidence rate ratio
IUD	Intrauterine device
IV	Intravenous
IVRS	Interactive voice response system
kg	Kilogram
LDMS	Laboratory data management system
MDP	Microbicide development program
MedDRA	Medical Dictionary for Regulatory Activities
MEMS	Micro-Electro-Mechanical-Systems
MIP	Macrophage inflammatory protein
mL	Milliliter
MMC	Mucosal mononuclear cells
MO	Medical Officer
MOP	Manual of procedures
mRNA	Messenger ribonucleic acid

MRI	Magnetic resonance imaging
MSM	Men who have sex with men
MTN	Microbicide Trials Network
MVC	Maraviroc
N9	Nonoxynol-9
NAAT	Nucleic acid amplification testing
NE	Neutrophil elastase
ng	Nanogram
NHP	Non-human primate
NIAID	National Institute of Allergy and Infectious Disease
NICHD	National Institute of Child Health and Human Development
NIH	National Institutes of Health
NIMH	National Institute of Mental Health
NL	Network Laboratory
NNRTI	Non-nucleoside reverse transcriptase inhibitor
No Rx	No treatment
NRTI	Nucleoside reverse transcriptase inhibitor
NSAID	Non-steroidal anti-inflammatory drugs
OTC	Over the counter
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PK	Pharmacokinetics
PMPA	9-[(R)-2-(phosphonomethoxy) propyl] adenine monohydrate
PMPAp	PMPA monophosphate
PMPApp	PMPA diphosphate
PrEP	Pre-exposure prophylaxis
PRS	Phone reporting system
PSP	Protocol safety physician
PSRC	Prevention Science Review Committee
PSRT	Protocol Safety Review Team

qRT-PCR	Quantitative real time reverse transcriptase polymerase chain reaction
RAI	Receptive anal intercourse
RANTES	Regulated upon activation-normal T cell expressed and secreted
RIN	RNA integrity number
RNA	Ribonucleic acid
RPR	Rapid plasma reagin
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SAE	Serious adverse event
SCHARP	Statistical Center for HIV/AIDS Research & Prevention
SDMC	Statistical and data management center
SHIV	Simian/human immunodeficiency virus
SIV	Simian immunodeficiency virus
SMC	Study monitoring committee
SMS	Short message service
SPECT/CT	Single photon emission computed tomography / computerized tomography
STI	Sexually transmitted infection
T_{max}	Time to peak concentration
Tc	Technetium
$TCID_{50}$	50% Tissue Culture Infective Dose
TDF	Tenofovir disoproxil fumarate
TDF/FTC	Tenofovir disoproxil fumarate/emtricitabine (Truvada®)
TFV	Tenofovir
UCLA	University of California at Los Angeles
ULN	Upper limit of normal
URAI	Unprotected receptive anal intercourse
US	United States
WB	Western blot
w/w	Weight for weight
µg	Microgram
µL	Microliter

µM	Micromole

Publications

Abstracts

McGowan I, Hoesley C, Cranston RD, Andrews P, Janocko LE, Dai J*, et al.* MTN-007: A Phase 1 randomized, double blind, placebo controlled rectal safety and acceptability study of tenofovir 1% gel. Abstract 34LB. *19th Conference of Retroviruses and Opportunistic Infections, Seattle, Washington* 2012.

Papers

McGowan I, Hoesley C, Cranston RD, Andrew P, Janocko L, Dai JY, Carballo-Dieguez A, Ayudhya RK, Piper J, Hladik F, Mayer K (2013) A Phase 1 randomized, double blind, placebo controlled rectal safety and acceptability study of tenofovir 1% gel (MTN-007). PLoS ONE 8: e60147.

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Chapter 1

Rectal Microbicide Development

1 Rectal microbicide development

1.1 Introduction

The first cases of human immunodeficiency virus (HIV) infection / acquired immunodeficiency syndrome (AIDS) were recognised in 1981 among men who have sex with men (MSM) living in North American urban centres including New York, Los Angeles, and San Francisco (1981). Subsequently, cases of AIDS were recognized in Sub-Saharan Africa (Serwadda et al., 1985) and the global HIV pandemic began to gather momentum. Over the last three decades the global epidemiology of HIV/AIDS has evolved. Incidence rates in certain populations have stabilized or even fallen. However, as can be seen in Figure 1-1, the number of new HIV diagnoses in men who have sex with men (MSM) in the United Kingdom (UK) appears to be stable or possibly increasing. Similar trends in HIV infection have been reported among MSM in most countries where data are available (Beyrer et al., 2010; Beyrer et al., 2012). A recent development is the recognition that populations of MSM with high incidence and prevalence of HIV infection can be found in most Sub-Saharan countries (Baral et al., 2009). The failure to decrease contemporary rates of HIV infection in MSM has many possible explanations. Unprotected receptive anal intercourse (URAI) between serodiscordant partners is the most efficient way to transmit HIV infection. The per contact probability of acquiring HIV infection from URAI has been estimated to be 1.4% (Jin et al., 2010; Baggaley, White, & Boily, 2010) which is approximately 18-fold higher than the risk associated with unprotected penile-vaginal intercourse (Boily et al., 2009). Unfortunately, a significant proportion of MSM are unwilling or unable to use condoms consistently

(D'Anna et al., 2012; Hensel, Rosenberger, Novak, & Reece, 2012) and not surprisingly, this practice has been associated with an increased risk of HIV acquisition (Zablotska, Prestage, Middleton, Wilson, & Grulich, 2010).

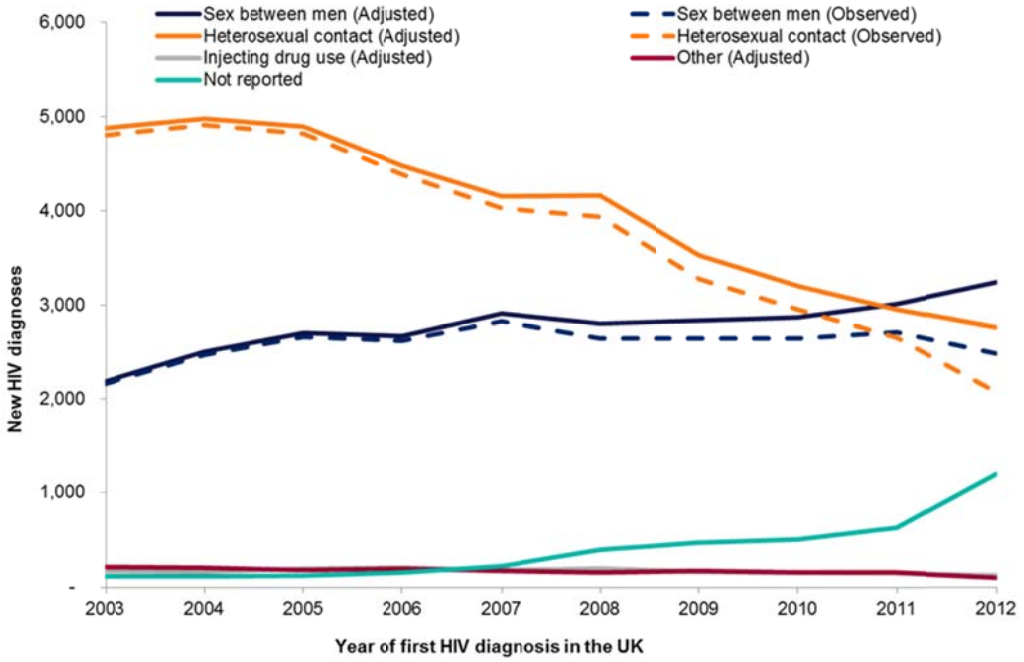


Figure 1-1 HIV diagnoses in the United Kingdom by exposure category
Data provided by Public Health England:
http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HIV/

1.2 The biology of rectal HIV-1 transmission

The specific processes underlying HIV-1 transmission are still not fully understood, but are dependent on several factors that include the stage of infection (Pilcher, Eron, Jr., Galvin, Gay, & Cohen, 2004), the presence of other sexually transmitted diseases (Cohen et al., 1997; Vernazza et al., 1997), and successful treatment of the HIV-1 infection (Baeten et al., 2012; Cohen et al., 2011; Donnell et al., 2010). The risks for HIV-1 acquisition for the receptive partner are about 10-fold higher than for the insertive partner

(Boily et al., 2009; Varghese, Maher, Peterman, Branson, & Steketee, 2002). However, these estimates are quite variable due to the factors discussed above (Baggaley et al., 2010).

The gastrointestinal tract is a rich source of HIV-1 target cells. Isolated lymphoid follicles, which serve as inductive sites for immune responses, are located throughout the colon (Koboziev, Karlsson, & Grisham, 2010). The number of follicles generally increases toward the anus with the greatest numbers found in the rectum (Langman & Rowland, 1986; Langman & Rowland, 1992). Antigen presenting cells (macrophage and dendritic cells) along with effector and regulatory T cells are found within the follicles. These cells are generally activated and express HIV-1 co-receptors, CCR5 and CXCR4, as well as soluble immune mediators (Anton et al., 2000; Poles, Elliott, Taing, Anton, & Chen, 2001; Zhang et al., 1998; McGowan et al., 2004) thus creating the perfect environment primed for HIV-1 infection.

HIV-1 can reach these activated immune cells in several ways. While micro-tears in the epithelium can occur during coitus, the envelope of HIV-1, gp120, has been shown to increase the permeability of the epithelium allowing HIV-1 enhanced access to the lamina propria (Nazli et al., 2010). Even with an intact mucosa, epithelial cells can bind and transfer HIV-1 either through active transport, transcytosis (Bomsel, 1997), or non-specifically (Dezzutti et al., 2001; Meng et al., 2002; Wu, Chen, & Phillips, 2003). Finally, dendritic cells extend dendrites through the tight-junctions of the epithelium to sample the luminal environment (Rescigno et al., 2001).

HIV-1 can take advantage of this by binding to Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and subsequently infect activated lymphocytes (Gurney et al., 2005). Once past the epithelium, HIV-1 preferentially infects local lymphocytes expressing CCR5 (Anton et al., 2000; Meng et al., 2000). Cell-free and cell-associated virus are both present in the ejaculate and so it is still not clear which form of the virus is responsible for initiating mucosal infection (Anderson et al., 2010). However, recent sequence analysis of viral RNA and integrated proviral DNA suggests that cell-free virus contributes the most to mucosal transmission (Butler et al., 2010). These early infection events are known to initiate from a single founder virus in heterosexual transmission (Keele et al., 2008; Sagar et al., 2009), but RAI is associated with a more diverse founder virus population (Li et al., 2010) likely due to direct access to underlying immune cells. This information suggests that an ideal rectal microbicide should protect the epithelium and be active against a swarm of viruses.

Ribeiro dos Santos et al. have reported the results of a non-human primate (NHP) rectal challenge study in which they used a combination of immunohistochemistry, laser capture microdissection, and a suite of molecular techniques to characterize the virological events that occurred after rectal challenge with SIVmac231 in rhesus macaques (Ribeiro Dos et al., 2011). The macaques were sacrificed at four, sixteen, and twenty four hours and two, three, and four days following rectal challenge with 7.31 log copies / mL of viral RNA. A key finding from the study was the demonstration that by four hours SIV could be identified in the rectal mucosal, and perhaps

more importantly, could also be found in the para-colic lymph nodes. The authors commented that this process of viral dissemination is faster than that seen with oral and vaginal NHP challenge studies. These findings clearly have implications for microbicide and vaccine development. A successful rectal microbicide will have to prevent this early viral dissemination and a vaccine would have to induce protective immunity both within the rectal mucosa and the draining lymph nodes.

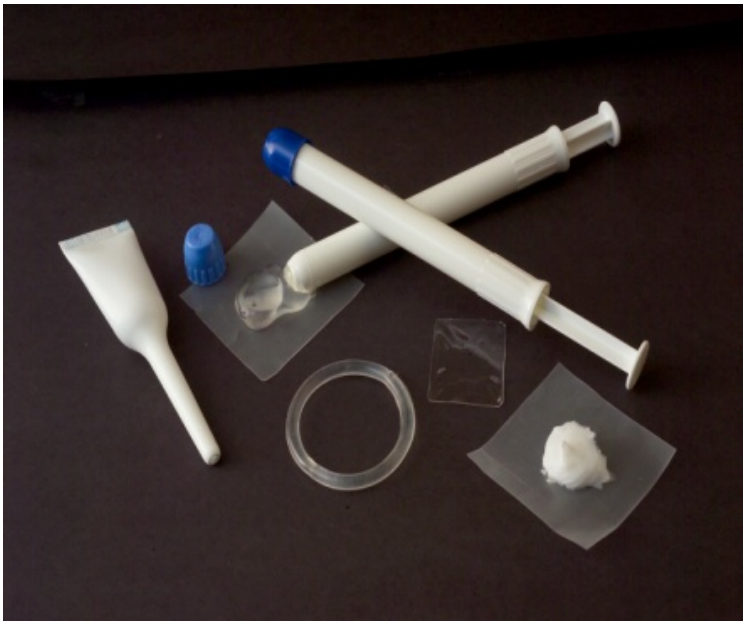
1.3 Pre-exposure prophylaxis of HIV infection

In 2010 Grant *et al*. reported the results of the iPrEx trial that demonstrated that oral pre-exposure prophylaxis (PrEP) with tenofovir disoproxil fumarate (TDF)/emtricitabine (FTC) (Truvada®, Gilead Sciences Inc., Foster City, California, USA) by MSM and transgender women was associated with a 44% reduction (95% confidence interval (CI), 15 to 63; P = 0.005) in the incidence of HIV infection (Grant et al., 2010). This important study clearly identified the potential promise of oral PrEP for HIV prevention but also identified a key challenge; among the participants randomized to receive Truvada, drug was detected in 3 of 34 participants with HIV infection (9%) and 22 of 43 seronegative controls (51%). Ideally, drug would have been detected in all the participants randomized to receive Truvada and these data suggest that in this study adherence to oral Truvada was suboptimal. The iPrEx open label extension or iPrEx OLE study will provide important data as to whether adherence patterns improve when study participants know that they are receiving active drug. Meanwhile other modalities of HIV prevention including rectal microbicides are being explored for individuals at risk of HIV infection associated with URAI.

1.4 Microbicides

Microbicides are topical products that are designed to be applied to the vaginal or rectal mucosa with the intent of preventing, or at least significantly reducing, the acquisition of sexually transmitted infections (STIs) including HIV (McGowan, 2010). The original impetus for vaginal microbicide development was to provide women with options for HIV prevention in settings where their partners were unwilling or unable to use condoms. In 1990, Zena Stein proposed the development of a “topical virucide” whose desirable properties would include the product being non-irritant, non-toxic, low-priced, and efficacious (Stein, 1990). Microbicides have been formulated as gels, creams, intravaginal rings, films, and fast dissolving tablets (Rohan & Sassi, 2009; Garg et al., 2010) (Figure 1-2).

Figure 1-2 Microbicide formulations

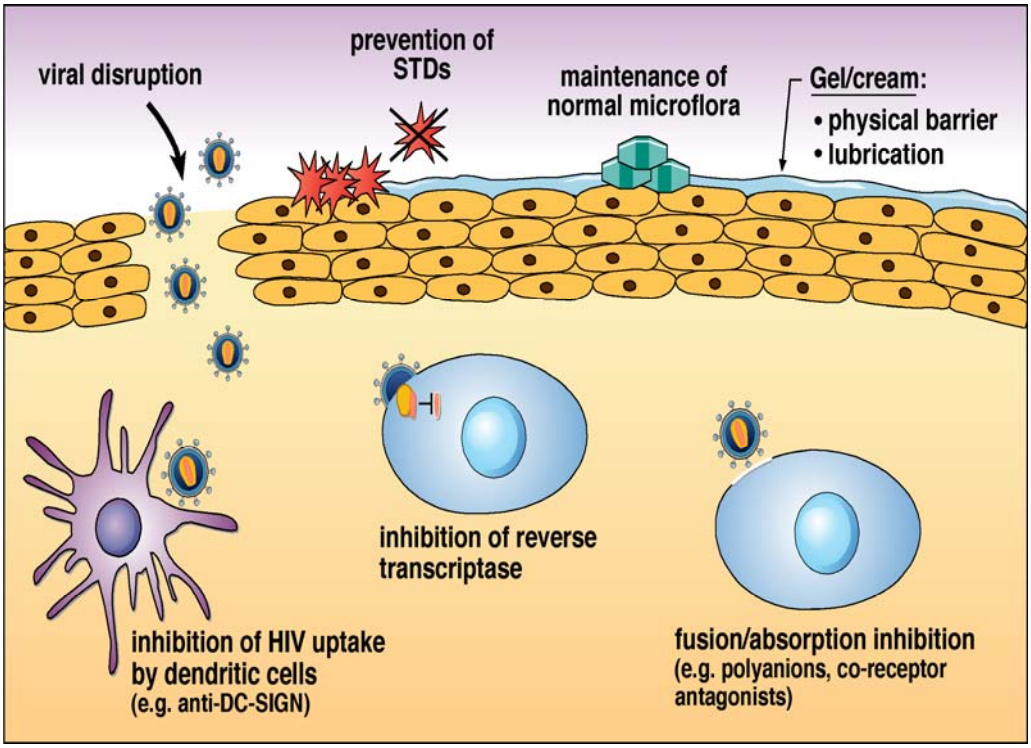


Vaginal microbicides that have been evaluated in clinical trials can be categorized by their primary mechanism of action (Figure 1-3) and are discussed below.

1.4.1 Vaginal defense enhancers

The vaginal flora has evolved to provide a hostile environment for potential pathogens such as STIs, including HIV. A key feature of this environment is the capacity of resident lactobacilli to maintain an acidic pH. Unfortunately, in conditions such as bacterial vaginosis, lactobacilli diminish, vaginal pH rises, and these changes appear to result in increased susceptibility to acquisition of HIV infection (Myer et al., 2005). A more transient vulnerability occurs when seminal fluid, which has an alkaline pH, is deposited in the vagina. To circumvent these problems, microbicides were developed that could maintain an acidic pH (BufferGel™ and Acidform™) or that delivered lactobacilli genetically modified to release antiviral peptides such as cyanovirin (Mayer et al., 2001; van de Wijgert et al., 2001; Amaral et al., 2006; Liu et al., 2006).

Figure 1-3 Mechanism of action for microbicide candidates



McGowan, I Biologicals 2006 (McGowan, 2006)

1.4.2 Surfactants

Surfactants such as nonoxynol-9 (N9) were some of the first microbicides to be evaluated in human studies (Stafford et al., 1998). They were cheap, readily available, and in the case of N9 had a long history of use as spermicidal agents. Other surfactant microbicides evaluated in clinical trials included C31G (Savvy™) (Corner, Dolan, Yankell, & Malamud, 1988; Ballagh, Baker, Henry, & Archer, 2002), and sodium lauryl sulphate (Trottier et al., 2007; Omar et al., 2008) However, surfactant microbicides are detergents and their mechanism of action is through disruption of cell membranes (Hillier et al., 2005). Unfortunately, they do not discriminate between mammalian and viral membranes, which led to significant toxicity

issues and effectively stopped the clinical development of N9 as a vaginal microbicide.

1.4.3 Entry/fusion inhibitors

At a cellular level, HIV transmission follows an orderly process. The three steps required for cell infection are (i) attachment of virus to host cells, (ii) interaction of virus with receptors and co-receptors, and (iii) fusion of virus and host cell membranes (Shattock & Moore, 2003).

First generation anionic polymer fusion inhibitors worked by interfering with the binding of the HIV-1 V3 loop of gp120 to the CD4 receptor. Examples that have been evaluated in clinical trials include PRO-2000 (Mayer et al., 2003), dextrin sulphate (Low-Beer et al., 2002), cellulose sulfate (Malonza et al., 2005), Carraguard® (van de Wijgert et al., 2007), cellulose acetate phthalate (Lacey et al., 2010), and SPL7013 (VivaGel®) (O'Loughlin et al., 2010; McGowan et al., 2011). Small molecule agents that target the CC chemokine receptor type 5 (CCR5) HIV co-receptor have been developed and licensed for the treatment of chronic HIV infection (Maraviroc) and are being evaluated as candidate microbicides including an intravaginal ring (MTN-013; http://clinicaltrials.gov/: NCT01363037). PSC-RANTES is a synthetic CCR5 antagonist that has shown efficacy in an non-human primate vaginal challenge model (Lederman et al., 2004) and additional analogues have also shown efficacy in the NHP model (Veazey et al., 2009).

1.4.4 Replication inhibitors

Reverse transcriptase (RT) inhibitors provide the cornerstone of antiretroviral (ARV) regimens for the treatment of chronic HIV infection.

ARV microbicides act by inhibiting HIV-1 viral RT, a critical enzyme needed to convert viral RNA into DNA before integration into the host genome. In the presence of RT inhibitors this process is suppressed resulting in quantitative reductions in viral replication. ARVs are also being evaluated for oral and topical HIV-1 prevention. ARV microbicides that have been evaluated in clinical trials include tenofovir (TFV), UC781, and dapivirine (TMC-120).

1.4.4.1 Tenofovir

TFV (Gilead Sciences Inc., Foster City, CA, USA) is an adenosine nucleoside monophosphate (nucleotide) analog belonging to the class of acyclic phosphonomethylether nucleosides. The prodrug of TFV (Tenofovir disoproxil fumarate; TDF) is licensed for the treatment of chronic HIV infection (Viread®). Activation of TFV is dependent upon anabolic phosphorylation by intracellular nucleoside kinases, whose activity and availability are dependent upon the activation state of the cell. Consequently, it is unknown how long it will take lymphocytes, dendritic cells, and monocyte/macrophages in mucosal surfaces to convert TFV to its active diphosphorylated antiviral metabolite. It is possible that the absolute rate of TFV activation will depend upon a wide range of factors including the presence or absence of mucosal inflammation.

1.4.4.2 UC781

UC781 is a thiocarboxanilide non-nucleoside RT inhibitor (NNRTI) that was being developed as both a vaginal and rectal microbicide (Bunge et al., 2012; Anton et al., 2011). UC781 has reduced activity against NNRTI resistant HIV-1 (Hossain & Parniak, 2006) but remains active at concentrations of 25 μM, which is above the levels delivered by current UC781 formulations. Combinations of cellulose acetate 1,2-benzenedicarboxylate (CAP) and UC781 have been shown to provide synergistic inhibition of HIV replication (Liu, Lu, Neurath, & Jiang, 2005). Preclinical evaluation of UC781 was unremarkable (Balzarini et al., 1998), and explant studies have demonstrated inhibition of HIV-1 replication and prevention of migration of infected cells from cervical explant tissue (Fletcher et al., 2005). Unfortunately, the clinical development of UC781 as a microbicide was suspended in 2010 despite supportive safety data from completed Phase 1 studies.

1.4.4.3 Dapivirine

TMC-120 is a diarylpyrimidine NNRTI with high activity against wild type and mutant HIV (Van et al., 2004). Prevention of vaginal transmission of HIV-1 has been demonstrated in a humanized severe combined immunodeficient mouse model using TMC-120 gel (Di Fabio et al., 2001; Di Fabio et al., 2003). The International Partnership for Microbicides (IPM) is currently developing TMC-120 as a microbicide. Initial Phase 1 studies used a gel formulation but IPM is now focusing on the development of a silicone elastomer slow release vaginal ring, which offers the possibility of once monthly application (Malcolm, Woolfson, Toner, Morrow, & McCullagh, 2005;

Woolfson, Malcolm, Morrow, Toner, & McCullagh, 2006). This approach would provide women with another non-coitally dependent method of HIV-1 prevention. Two Phase 3 intravaginal ring studies (MTN-020 and IPM-027) are currently enrolling in Sub-Saharan Africa.

The advantages of the RT inhibitor class of microbicides include: (i) there are several products available, (ii) the cost of production is likely to reasonable, (iii) there is extensive proof of concept animal model studies suggesting efficacy, and (iv) RT inhibitor microbicides could be used in a non-coitally dependent fashion.

The primary disadvantage for ARV microbicides or indeed any form of ARV PrEP is the concern that their use might result in the emergence of ARV resistance (Hurt, Eron, Jr., & Cohen, 2011). This could happen in two situations. Women or men who are unaware of their HIV-1 status might use product for prolonged periods of time. The combination of repeated low dose exposure to an RT inhibitor in an individual with untreated HIV-1 infection might lead to the evolution of viral resistance. A second scenario would be an individual who seroconverts whilst receiving PrEP. This would be a particular problem if the individual were using a slow release formulation device such as an ARV intravaginal ring (Malcolm et al., 2005) or parenteral long acting ARV injection (Baert et al., 2009). In both cases it has been argued that the limited systemic absorption of RT inhibitors after mucosal application minimizes the risk of viral resistance. To date, only 3 cases of ARV resistance have occurred among 80 seroconverters on PrEP trials and

these all occurred in participants with unrecognised acute HIV infection at the time of enrollment into their respective trials (Parikh & Mellors, 2012). In contrast, a recent study from KwaZulu-Natal demonstrated that 95% of treatment naïve patients with HIV infection starting first line combination ARV therapy, who do not fully suppress their viral infection, will develop ARV resistance (Singh et al., 2011). The magnitude of PrEP-associated ARV resistance will only be quantifiable when PrEP programs are rolled out at a community level. As of yet, all the data available have been generated within clinical trials where participants are closely monitored with frequent HIV testing.

Over the last two decades a broad range of products have been evaluated as vaginal microbicides. With the exception of N9 gel, the majority of microbicide candidates were found to be safe and acceptable in Phase 1/2 studies and many were also shown to be effective when evaluated in animal models of HIV infection (McGowan, 2006; Minces & McGowan, 2010). Unfortunately when these products were studied in Phase 2B/3 effectiveness trials they proved to be either safe but ineffective (Carraguard®, C31G (SAVVY), BufferGel®, and PRO-2000 (Skoler-Karpoff et al., 2008; Peterson et al., 2007; Feldblum et al., 2008; Abdool Karim et al., 2011; McCormack et al., 2010) or they may have actually increased the risk of HIV infection (N9 and cellulose sulphate) (Van Damme et al., 2002; Van et al., 2008).

In 2010, Quarraisha and Salim Abdool Karim presented the results of the Center for the AIDS Programme of Research in South Africa (CAPRISA) 004 study (http://clinicaltrials.gov/: NCT00441298) that evaluated the safety and efficacy of TFV 1% gel in preventing HIV infection in 889 women in Kwazulu-Natal, South Africa (Abdool et al., 2010). Product was administered in a pericoital fashion. Women were requested to insert one dose of gel within 12 hours **b**efore sex and a second dose with 12 hours **a**fter sex and no more than **t**wo doses of gel in a **24**-hour period. The authors coined the term "BAT24" to describe this pattern of pericoital microbicide dosing. The CAPRISA 004 study demonstrated that the incidence of HIV infection in women randomised to receive TFV gel was reduced by 39% (95% CI, 6 to 60; P=0.017) compared to women receiving a placebo gel. Product effectiveness was increased to 54% in women with high product adherence (defined as >80% use based on returned applicator counts). In addition, product effectiveness was also related to the concentration of TFV in the genital tract; women with TFV concentrations greater than 1,000 ng/mL had a significantly lower HIV incidence rate than the placebo recipients (2.4 vs. 9.1 per 100 person years; incidence rate ratio (IRR) = 0.26, 95% CI 0.05-0.80, P = 0.01) and there was no difference in incidence rates between women receiving placebo gel and women with less than 1,000 ng/mL of TFV, IRR = 0.86, 95% CI 0.54-1.35, P = 0.51) (Karim, Kashuba, Werner, & Karim, 2011).

CAPRISA 004 was clearly a landmark study within HIV prevention research and defined several unique microbicide attributes; (i) it demonstrated that

pericoital use of a topical ARV microbicide could reduce the rate of HIV acquisition in women at significant risk of infection, (ii) product effectiveness was linked to both adherence and product concentration within the genital tract, and (iii) subsequent mathematical modeling suggested that use of TFV gel by women in South Africa was a cost-effective strategy for HIV prevention (Williams, Abdool Karim, Karim, & Gouws, 2011).

In contrast, a second PrEP effectiveness study evaluating both oral and topical TFV has generated divergent results. The Microbicide Trials Network (MTN)-003 or **V**aginal and **O**ral **I**nterventions to **C**ontrol the **E**pidemic (VOICE) study (http://clinicaltrials.gov/: NCT00705679) enrolled approximately 5,000 women who were randomised to receive TFV gel, placebo gel, TDF, TDF/emtricitabine (FTC), or placebo tablet. A Data and Safety Monitoring Board (DSMB) review of the study took place in November, 2011 and included data for the period between September 2009, when the study began, and September 2011. Based on this review, the DSMB recommended that VOICE discontinue the TFV gel and placebo gel arms, because there was no difference in effect between them in preventing HIV infection (MTN, 2012). The HIV incidence rates in the two groups were nearly identical, with a 6.1 percent incidence rate in the placebo gel group and 6.0 percent in the TFV gel group. A number of hypotheses could explain the negative results in the VOICE study. These include poor product adherence, inadequate genital tract drug concentrations, and the possibility that daily, but not pericoital, use of TFV gel may induce mucosal changes that diminish product effectiveness and/or increase the risk of HIV

acquisition (Kashuba, Patterson, Dumond, & Cohen, 2011; van der Straten, Van Damme, Haberer, & Bangsberg, 2012; Hendrix, 2012). Marrazzo et al. presented unblinded data from the VOICE trial at the 2013 Conference on Retroviruses and Opportunistic Infections in Atlanta, Georgia, USA. Unfortunately, the most likely explanation for the failure to demonstrate product effectiveness in the VOICE study was the fact that the majority of women in all of the study arms did not use the study products (Marrazzo et al., 2013).

A third effectiveness study of TFV gel is currently ongoing. The Follow-on African Consortium for Tenofovir Studies (FACTS) 001 study (http://clinicaltrials.gov/: NCT00705679) is evaluating the safety and effectiveness of TFV gel administered using the BAT24 regimen that was successful in the CAPRISA 004 study. The FACTS 001 study plans to enroll approximately 2,900 women in South Africa and hopes to be completed in 2015.

The recent, and ongoing, effectiveness studies of TFV gel have been presented in some detail because they have important implications for rectal microbicide development. It is obvious that a microbicide product, whether intended for vaginal or rectal use, will not work unless adequate quantities of the microbicide are used by individuals hoping to reduce their risk of HIV infection. It is also assumed that a successful microbicide will have to deliver the right amount of drug, in the right place, at the right time, and that therapeutic drug concentrations will persist beyond the period of viral

exposure (Hendrix, 2012). Unfortunately, it is not entirely clear what quantity of microbicide product or active pharmaceutical ingredient (API) is needed to achieve this goal. The quantity may differ by compartment (vaginal vs. rectal) and/or drug class. Recent Phase 1 microbicide trials (described in Chapter 2) are beginning to generate compartmental pharmacokinetic (PK) and pharmacodynamic (PD) data that may help define target drug concentrations prior to embarking on large and expensive microbicide effectiveness trials.

1.5 Rationale for rectal microbicide development

The rationale for the development of rectal microbicides is based upon several criteria; (i) despite almost three decades of health education and widespread availability of condoms significant proportions of MSM and other populations continue to practice URAI, (ii) the majority of individuals practicing RAI use some form of sexual lubricant and so a microbicide that had lubricant like properties would not require the type of behavioral modification associated with other forms of HIV prevention, (iii) several studies have documented interest in developing, evaluating, and the potential use of rectal microbicides by MSM, (iv) preclinical evaluation of candidate microbicides have shown protection in colorectal explant challenge studies, (v) rectal microbicides have also been shown to be efficacious in non-human primate (NHP) rectal challenge studies, and (v) rectal microbicides have been shown to be efficacious in Phase 1 clinical trials that have conducted *ex vivo* / *in vitro* colorectal explant challenge experiments. These criteria are discussed in more detail below.

1.5.1 Introduction

As early as 1996, Carballo-Dieguéz and Dolezal had determined that Puerto Rican MSM living in New York were using condoms inconsistently for anal sex and suggested that a safe and effective microbicidal gel that could be used rectally would be an important component of a HIV prevention strategy for MSM (Carballo-Dieguez & Dolezal, 1996). The first rectal microbicide study, the HIV Network for Prevention Trials (HIVNET)-008 study, evaluated the safety of N9 gel, and started enrollment in August 1996 (Tabet et al., 1999; Gross et al., 1999). In 1998, Gross *et al*. reported the results of a large survey that interviewed 3,257 MSM from six cities in the United States (US); Boston, Chicago, Denver, New York, San Francisco, and Seattle. The majority of the participants used lubricants for anal sex and approximately two thirds stated that they were willing to participate in rectal microbicide clinical trials (Gross, Buchbinder, Celum, Heagerty, & Seage, III, 1998). About this time community advocates began to become more involved in raising awareness about microbicide research in general and rectal microbicide development in particular (Scarce, 1999; Forbes & Harrison, 1999). In 2001, The American Foundation for AIDS Research (amfAR) convened a two day meeting in Baltimore, Maryland, USA to help develop a research and development agenda for rectal microbicides (amfAR, 2001). The remit of the conference was broad and the meeting included discussions on the prevalence and risks of anal intercourse, the anatomy, physiology, and immunology of the anorectal mucosa, the HIVNET-008 study, microbicide formulation considerations, and consumer preference. In the subsequent five years several studies took place that explored rectal

microbicide formulation preferences (gels, suppositories, and douches) from a theoretical (Carballo-Dieguez, Bauermeister, Ventuneac, Dolezal, & Mayer, 2010) and practical perspective (Carballo-Dieguez et al., 2007; Carballo-Dieguez et al., 2008). An additional study, the HIV Prevention Trials Network (HPTN)-056 protocol investigated the stability of rectal mucosal safety parameters that might be measured in future Phase 1 rectal microbicide studies (McGowan et al., 2007).

1.5.2 Prevalence of anal sex in various populations

Anal sex is a relatively common practice among MSM although prevalence rates are highly variable depending on the population studied. In a recent study from Rosenberger *et al*. that characterized sexual behavior in 24,787 gay and bisexually identified US men, anal sex was reported by 37.2% of the respondents (Rosenberger et al., 2011). In contrast, in a smaller study from California 303/398 (76%) of MSM reported anal sex within the previous 12 months (Xia et al., 2006). Unfortunately, a significant proportion of anal sex occurs without the use of condoms. The prevalence of URAI varies by the population studied but rates of greater than 30% are commonly reported from US and United Kingdom (UK) MSM (Xia et al., 2006; Lattimore, Thornton, Delpech, & Elford, 2011). Similar patterns of behaviour have been reported for MSM across the world (Beyrer et al., 2012). This includes Sub-Saharan Africa where recent studies have documented the presence of MSM in most African countries; these MSM have high rates of HIV infection and little or no access to basic HIV prevention services including the provision of condoms (Baral et al., 2009; Beyrer et al., 2010; Arnold, Struthers, McIntyre, & Lane, 2012).

There is increasing recognition that anal sex is also practiced by heterosexual couples. The National Survey of Sexual Health and Behavior (NSSHB) is a study of human sexual behavior conducted in the US by the Center for Sexual Health Promotion at Indiana University in Bloomington. The study enrolled 5,865 men and women aged 14-94 in the US. More than 20% of men ages 25-49 years and women ages 20-39 years reported anal sex in the last year. More than 40% of both men and women reported a lifetime experience of insertive or receptive anal intercourse (Herbenick et al., 2010).

Within the UK, the National Surveys of Sexual Attitudes and Lifestyles (Natsal) study has been documenting UK sexual behavior since 1990. In the most recent report (Natsal-3), interviews were completed with 15, 162 participants. The Natsal-3 survey found that the heterosexual repertoire had increased since the Natsal-2 survey. Overall, 13.4% of men and 10.5% of women had experienced heterosexual anal intercourse in the previous year and 40% of participants born after 1966 had a lifetime experience of anal sex (Mercer et al., 2013).

A number of US studies have described linkages between URAI in young women and STIs as well as illicit drug use (Risser, Padgett, Wolverton, & Risser, 2009; Gorbach et al., 2009). It is also apparent that women in the developing world are at risk of HIV infection through URAI as well as vaginal sex (Kalichman, Simbayi, Cain, & Jooste, 2009).

This brief survey of the epidemiology of anal sex suggests that this practice is common among men and women in both the developed and developing world. A significant proportion of anal sex is also unprotected. A safe and effective rectal microbicide would therefore be a valuable addition to the HIV prevention options available to individuals at risk of HIV infection through URAI.

1.5.3 Sexual lubricant use

Although sexual lubricants are not intended to prevent HIV infection their use does provide a rationale for the development of similar microbicides that do have the potential to prevent HIV infection. As discussed below, lubricant use is a common practice among men and women and products have been used with vaginal and anal intercourse. However, some lubricants have the potential to cause mucosal disruption and potentially increased vulnerability to HIV infection. Lessons learned from the assessment of lubricant safety and acceptability can help focus the development of safe, acceptable, and effective rectal microbicides.

1.5.3.1 Sexual lubricant use by women

Sexual lubricant use is a relatively common practice among women in the developed world. Products are used for a variety of reasons including managing vaginal dryness or dyspareunia or for enhancement of sexual pleasure (Sutton, Boyer, Goldfinger, Ezer, & Pukall, 2012; Herbenick et al., 2011). Herbenick *et al*. conducted a survey of 2,056 US women of whom 62% had ever used a sexual lubricant and 25.3% had used a lubricant within

the last month (Herbenick et al., 2010). Data on lubricant used by women in the developing world are more complex. There are strong cultural norms about the desirability of vaginal dryness or lubrication. Braunstein and Van de Wijgert undertook a literature review and key informant interview process that suggested that women from countries such as India, Kenya, South Africa, Thailand and Zimbabwe are concerned about excess lubrication whereas women from Brazil and the US were more concerned about vaginal dryness (Braunstein & van de Wijgert, 2005). In a recent study where women were provided with a range of sexual lubricants and asked to complete an internet based survey it was apparent that the products were used for both vaginal and anal sex (Herbenick et al., 2011). In a more detailed study, Exner *et al*. conducted in-depth interviews with 28 women who had a history of RAI. Key findings included (i) condom use for anal sex was rare; 23 of the 28 women reported no condom use during the last episode of RAI and (ii) slightly less than half the women used a sexual lubricant; the reminder used saliva and/or vaginal fluid to facilitate RAI (Exner et al., 2008). These findings support the concept of developing dual compartment microbicides.

1.5.3.2 Sexual lubricant use by MSM

Carballo-Dieguéz *et al*. conducted a survey of rectal lubricant use among 307 Latino MSM in New York during 1995/1996. The majority of study participants (94%) had experience of using lubricants with RAI and 74% used a lubricant during at least 80% of all episodes of RAI. The study also demonstrated high rates of URAI (43%) with a willingness to use rectal microbicides if available (92%) (Carballo-Dieguez et al., 2000). A second

larger study conducted among 879 MSM living in San Francisco in 2002/2003 demonstrated high levels of URAI (36%), high levels of lubricant use (89%), including significant use of N9 containing products (26%) (Carballo-Dieguez et al., 2007). The study documented variability in microbicide acceptability based on the perceived level of protection afforded by microbicides. Whereas 65% of men would consider using a rectal microbicide if it was as effective as a condom, only 15% would consider using a microbicide if it was less effective than a condom. More recently, Clark *et al*. conducted a quantitative behavioral survey among 547 MSM from Lima, Peru (Clark et al., 2013). Approximately 50% of the men reported lubricant use with RAI; reasons for non-use included: no lubricant available (32%), used pre-lubricated condoms (24%), used saliva as a lubricant (13%), or could not afford to buy lubricant (8%). URAI was common among men using lubricants (63%). In a second recent study from Latin America, Kinsler *et al*. used Conjoint Analysis (CJA) to explore consumer preferences for potential rectal microbicides among 128 MSM in Peru, Ecuador, and Brazil (Kinsler et al., 2012). Parameters used in the CJA included: cost ($0.30 vs. $5.00), effectiveness (40% vs. 80%), side effects (none vs. some), dosing (before sex vs. daily), formulation (gel vs. liquid), dosage (15 vs. 35 mL), and availability (prescription vs. over the counter (OTC)). The favored product profile was a microbicide that was; $0.30, 80% effective, no side effects, use before sex, gel formulation, 15 mL dosage, and provided through a prescription. Although CJA does not involve consumers trying products it does provide an important framework for an ideal product. In this analysis, effectiveness had the greatest impact on product acceptability;

participants chose 80% rather than 40% (the level of protection seen in the CAPRISA 004 study (Abdool et al., 2010)) and price was also significant - $0.30 representing the approximate cost of a condom in Peru, Ecuador, or Lima.

1.5.3.3 Sexual lubricants and their potential for mucosal damage

In 2007, sexual lubricant sales in the US totaled more than $110 million (Andelloux, 2011). Currently within the US, lubricants are regulated as medical devices and therefore do not require the extensive preclinical and clinical testing required for drugs. Although there are a very large number of lubricants marketed in the US they can be broken down into a number of categories (Table 1.1).

Table 1-1 Categories of sexual lubricants

Category	Examples	Comments
Petroleum-based	Petroleum jelly (Vaseline®) Mineral oil Massage oil	Compatible with polyurethane but not latex condoms
Natural oil	Vegetable oil Olive oil Coconut oil Crisco®	Compatible with polyurethane but not latex condoms
Water-based	Astroglide® KY jelly® Wet® Liquid silk® Slippery stuff®	Most commonly used lubricants
Silicone-based	Eros® Wet Platinum® Gun oil®	Hypoallergenic and condom compatible. Provides lubrication for longer than water-based lubricants

Several studies have been conducted to evaluate lubricant safety. Sudol *et al*. evaluated a number of OTC lubricants including KY-Plus (containing N9) in a mouse model wherein the mice received the test formulation followed by challenge with HSV-2 (Sudol & Phillips, 2004). In addition, mice also underwent rectal lavage, following product exposure, to determine whether formulations had the potential to cause rectal sloughing of epithelial cells. Use of KY-Plus was associated with significant cellular toxicity, enhancement of rectal HSV-2 infection, and rectal sloughing. Other, non-surfactant products such as Astroglide also increased HSV-2 infection and

rectal sloughing. Astroglide is known to be a hypersomolar gel (8,064 mOsm/kg; (Begay et al., 2011)) and in a subsequent study conducted in healthy volunteers, the use of hyperosmolar gels, including Astroglide, was associated with significant mucosal injury (Fuchs et al., 2007). Begay *et al*. evaluated a total of 41 lubricants for their osmolality, potential cytotoxicity (using an XTT assay), and antiviral activity against HIV (using TZM-bl cell lines and CXCR4 and CCR5 tropic HIV) (Begay et al., 2011). Product osmolality varied from 15 (FemGlide™) to 9,177 (ForPlay® Gel Plus) mOsm/Kg. None of the products inhibited HIV infection but four products (all Astroglide derivatives with high osmolality) significantly enhanced HIV replication. Dezzutti *et al*. recently published data on OTC lubricant safety using ectocervical and colorectal explant tissues (Dezzutti et al., 2012). All of the hyperosmolar gels were associated with cellular toxicity and epithelial damage and did not appear to have any antiviral activity (Table 1.2).The two iso-osmolar products (Good Clean Love and PRÉ) as well as the two silicone-based products (Female Condom 2 lubricant and Wet Platinum) were found to be safe in the testing algorithm.

Table 1-2 Physical characteristics of OTC lubricants

Lubricant	Osmolality (mOsm/kg)	pH	CC_{50}	ED_{50}	Therapeutic Index
Aqueous-based					
• Astroglide	6,113	4.0	0.9	0.8	1
• Elbow Grease	3,865	5.7	5.7	0.4	14
• Good Clean Love	269	4.8	>1,000	0.6	>1,000
• Gynol II	1,406	4.7	0.4	0.1	4
• ID Glide Ultra	3,150	5.2	4.8	0.8	6
• KY Jelly	2,510	4.5	11.8	5	2
• PRÉ	502	7.3	308	4.4	70
• Replens	1,875	2.9	19.8	0.5	40
• Slippery stuff	26	6.8	> 1,000	26	>40
• Sliquid Organic	106	6.8	3.0	3.9	0.8
Lipid-based					
• Boy Butter H2O	1,307	7.4	ND	ND	ND
• Boy Butter original	NA	NA	ND	ND	ND
Silicone based					
• FC 2 lubricant	NA	NA	ND	ND	ND
• Wet Platinum	NA	NA	ND	ND	ND

Adapted from Dezzutti *et al*. (Dezzutti et al., 2012); ND: Not determined; NA: Not applicable

An important, but as yet unanswered, question is whether the use of currently available lubricants could be harmful and/or increase the risk of HIV infection associated with URAI. It is known that the frequent use of an N9-based vaginal microbicide was associated with an increase in HIV acquisition in the COL1492 study (Van et al., 2002) but, with the exception of some spermicidal products, N9 is no longer used in sexual lubricants.

However, Gorbach *et al*. have suggested that consistent rectal use of lubricants (defined as use of a lubricant in the prior month) was associated with an increased risk of STIs including rectal gonorrhoea (GC)/chlamydia (CT) infection. The cross-sectional study was conducted in a very heterogeneous population including HIV-positive and negative men and women. Lubricant use was based on participant recall. A definitive, if challenging, study to answer the question as to whether lubricants could increase STI acquisition including HIV would require enrollment of an at risk population into a large prospective study. In the absence of definitive data, but based on the preclinical studies from Begay and Dezzutti (Begay et al., 2011; Dezzutti et al., 2012), it seems appropriate to counsel individuals to use iso-osmolar water soluble lubricants whenever possible.

1.5.4 The colorectal explant model

There is increasing interest in using human tissue biopsies or explants to evaluate the safety and efficacy of candidate microbicides. A wide range of tissues have been used including cervicovaginal (Greenhead et al., 2000), colorectal (Fletcher et al., 2006; Abner et al., 2005), penile (Harman, Herrera, Armanasco, Nuttall, & Shattock, 2012), and foreskin tissue (Zhou et al., 2011). Colorectal and cervicovaginal explants can be harvested from surgical resection specimens or can be collected as multiple biopsies from healthy donors. With regards to the colorectal explant system, two experimental techniques have emerged. Abner *et al*. have described the use of a polarized tissue model in which the explant is physiologically orientated in a transwell system (Abner et al., 2005). In contrast, Fletcher *et al*. have

used a non-polarized system in which the colorectal biopsies are placed on a gelfoam raft and then challenged with virus (Fletcher et al., 2006).

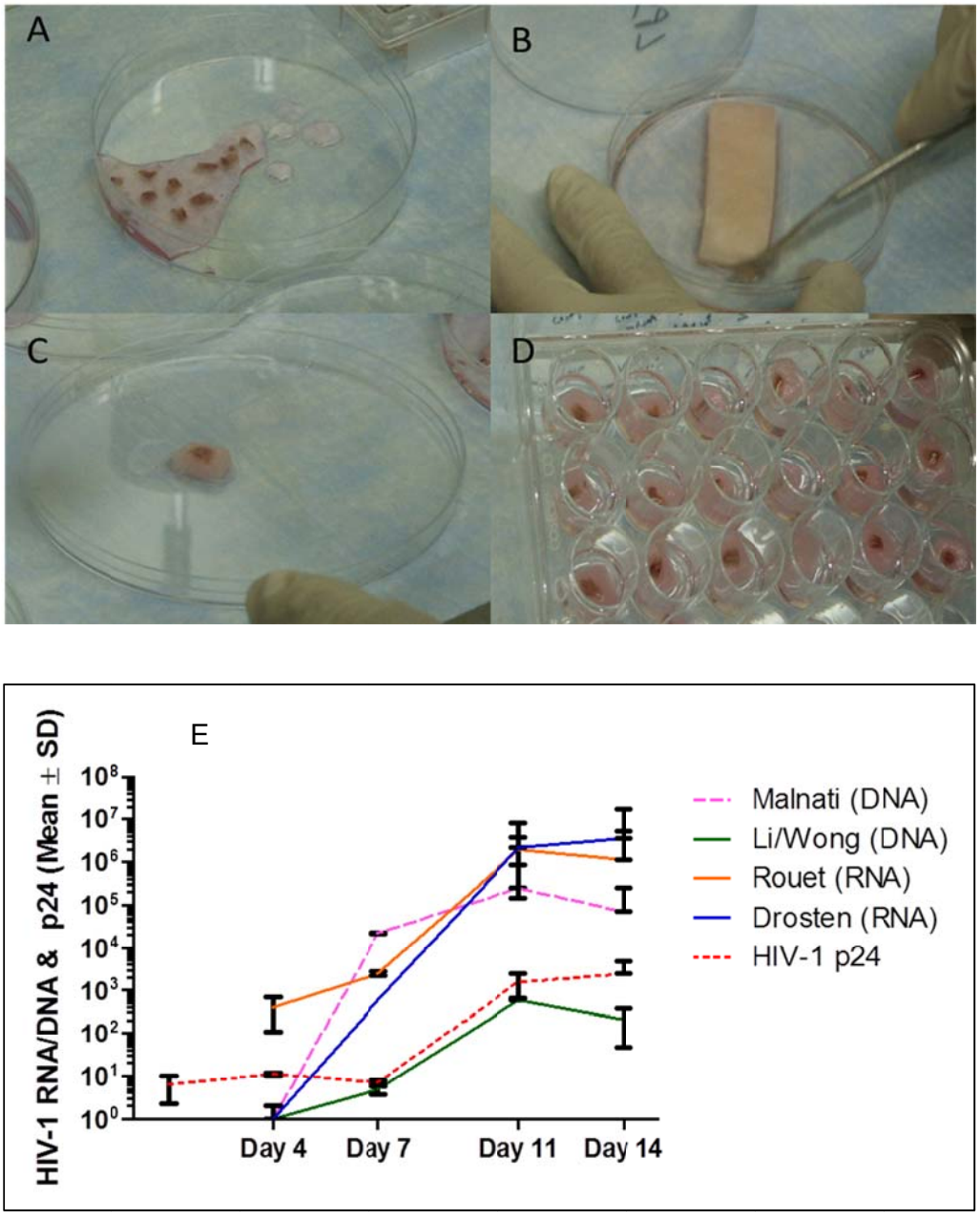


Figure 1-4 Non-polarized colorectal explant model

Non-polarized colorectal explant model. (A) Colorectal endoscopic biopsies, (B) Gelfoam raft (Pharmacia and Upjohn Company, Kalamazoo, MI, USA), (C) Explant tissue on Gelfoam rafts, (D) Explants following exposure to HIV, and (E) Kinetics of HIV replication in the explant system using quantitative DNA, RNA, and p24 antigen assays (Malnati et al., 2008; Li et al., 2010; Rouet et al., 2007; Drosten et al., 2006). (Ian McGowan, unpublished data)

Supernatant is collected over a 14 day period and evidence of infection is demonstrated by quantifying HIV-1 p24 antigen levels in the explant supernatant. It also possible to quantify accumulation of HIV-1 RNA and DNA in the explant tissue as illustrated in Figure 1-4.

Table 1-3 Microbicide candidates with colorectal explant efficacy

Product	Reference
Cellulose acetate PRO2000 SPL7013 Vena Gel UC781	(Abner et al., 2005)
PRO2000 Dextrin sulfate	(Fletcher et al., 2006)
Tenofovir	(Dezzutti et al., 2012)
C34 T20 T1249 L'644	(Harman et al., 2012)
Tenofovir Emtricitabine UC781 TMC120	(Herrera, Cranage, McGowan, Anton, & Shattock, 2011)
Saquinavir	(Stefanidou, Herrera, Armanasco, & Shattock, 2012)

These assays have characterized many potential rectal microbicide candidates including non-specific entry inhibitors (Fletcher et al., 2006), non-

nucleoside and nucleotide/nucleoside reverse transcriptase inhibitors, fusion inhibitors, and protease inhibitors (Fletcher et al., 2006; Herrera, Cranage, McGowan, Anton, & Shattock, 2009; Herrera et al., 2011; Harman et al., 2012) (Table 1-3).

Microbicide candidates have been typically evaluated as single entity agents. However, combinations of APIs are now being tested and much like systemic therapy, it appears that combinations of up to three APIs (TFV or FTC with UC781 and dapivirine) are much more potent against HIV-1 infection in colorectal tissue than single agents, even against drug resistant HIV-1 (Herrera et al., 2011). These data will help inform microbicide developers as to which candidates/combinations are optimal to pursue for further development.

1.5.5 Non-human primate rectal challenge studies

NHP infection models use two different challenge schemes that involve either a multiple low-dose or single high-dose challenge with simian immunodeficiency virus (SIV) or a chimeric SIV/HIV virus (SHIV) (Veazey, Shattock, Klasse, & Moore, 2012). Typically the virus used to evaluate microbicides is an SIV modified to express the HIV-1 envelope (SHIV) or the HIV-1 reverse transcriptase (RT-SHIV) (Pal et al., 2012).

Using the NHP model, gel formulations of an entry inhibitor, cyanovirin-N (Tsai et al., 2003), and the reverse transcriptase inhibitors, TFV and MIV-150 (Cranage et al., 2008; Singer et al., 2011) prevented rectal challenge of SHIV or RT-SHIV.

In the study by Tsai et al., macaques (*Macaca fasicularis*) were randomized to receive 1% cyanovirin (n = 5), 2% cyanovirin (n = 5), placebo (n = 5), or no gel (n = 4). Twenty minutes after a 2 mL rectal gel application the animals were challenged with 1,000 $TCID_{50}$ of SHIV89.6P (Tsai et al., 2003). All the animals receiving cyanovirin gel were protected from SHIV infection whereas the placebo gel or no gel animals all became infected with SHIV.

In the study by Cranage et al., a total of 20 Indian rhesus macaques were used to evaluate the protective efficacy of topical TFV (Cranage et al., 2008). Nine animals received rectal TFV 1% gel up to 2 hours prior to virus challenge, four macaques received placebo gel, and four macaques remained untreated. In addition, three macaques were given TFV gel 2 hours after virus challenge. Following intrarectal instillation of 20 median rectal infectious doses (MID_{50}) of a non-cloned, virulent stock of SIVmac251/32H, all animals were analysed for virus infection, by virus isolation from PBMC, quantitative proviral DNA load in PBMC, plasma viral RNA (vRNA) load by sensitive RT-PCR, and presence of SIV-specific serum antibodies by ELISA. Eight of nine macaques given TFV per rectum up to 2 hours prior to virus challenge were protected from infection (n = 6) or had modified virus outcomes (n = 2), while all untreated macaques and three of four macaques given placebo gel were infected, as were two of three animals receiving TFV gel after challenge. Moreover, analysis of lymphoid tissues post mortem failed to reveal sequestration of SIV in the protected animals. There was a strong positive association between the concentration

of TFV in the plasma 15 minutes after rectal application of gel and the degree of protection in the six animals challenged with virus at this time point. Moreover, colorectal explants from non-SIV challenged TFV-treated macaques were resistant to infection *ex vivo*, whereas no inhibition was seen in explants from the small intestine. Tissue-specific inhibition of infection was associated with the intracellular detection of TFV. Intriguingly, in the absence of seroconversion, Gag-specific gamma secreting T cells were detected in the blood of four of seven protected animals tested, with frequencies ranging from 144 spot forming cells (SFC)/10^6 PBMC to 261 SFC/10^6 PBMC (Cranage et al., 2008).

Singer et al. have evaluated the non-nucleoside reverse transcriptase inhibitor (NNRTI) MIV-150, in a carageenan formulation, in a rhesus macaque rectal challenge model (Singer et al., 2011). A total of four macaques received the MIV-150 gel either 30 minutes or four hours before rectal challenge with either 10^3 or 10^4 $TCID_{50}$ of a SHIV-RT (SIVmac239/HIV-1_{HXB2}). A control group was treated with a placebo methyl cellulose placebo gel. All the MIV-1 treated macaques exposed to 10^3 $TCID_{50}$ of the SHIV-RT were protected from infection whereas only two of the four macaques exposed to the higher dose of SHIV-RT (10^4 $TCID_{50}$) were protected from infection.

The most recent NHP rectal challenge study evaluated maraviroc (MVC) that had been formulated in a rectal specific hydrogel based formulation (Dobard et al., 2013). The MVC gel formulation was designed to have a neutral pH

and to be close to iso-osmolar. Chinese Rhesus macaques were assigned to a matching rectal placebo gel (n = 5) or MVC gel (n = 6). Twice-weekly rectal $SHIV_{SF162p3}$ challenges (500 $TCID_{50}$) were performed for 5 weeks. Gel (4 mL) was applied rectally 30 minutes before each challenge. MVC was measured in plasma 30 minutes after gel application using LC/MS. Infection was monitored by PCR of SHIV in plasma. Infected macaques continued to receive gel for an additional six weeks to monitor the potential impact of drug exposure on systemic viremia. All the placebo-treated macaques were infected after a median of four challenges. In contrast, four of six macaques receiving MVC gel remained protected after ten challenges, demonstrating high efficacy (84%, p=0.020; Fisher's exact test). Low levels of MVC (median = 4 ng/ml; range: 0-19) were detected in plasma 30 minutes after rectal dosing, suggesting MVC was rapidly released from the gel and absorbed. Plasma viremia in breakthrough infections was similar to controls.

To begin to address where rectal microbicides might distribute after dosing in a more formal way, a multi-compartment pharmacokinetic study in macaques after vaginal or rectal dosing with TFV gel was done (Nuttall et al., 2012). Macaques dosed with TFV gel vaginally showed rectal drug levels only 1 log_{10} lower than the vaginal drug levels. Both mucosal compartments were 4-5 log_{10} higher than plasma drug levels. Similar results were found when the macaques were dosed with TFV gel rectally. These data suggest that vaginal or rectal dosing of a soluble microbicide could protect against HIV-1 regardless of the route of exposure.

The NHP rectal microbicide challenge model provides compelling evidence to suggest that a rectal microbicide could provide significant protection from HIV acquisition in human efficacy studies. However, the model also illustrates that product efficacy may be impacted by the size of the NHP viral inoculum and the temporal association between when products are administered and when the animals are challenges with virus.

1.6 Rectal microbicide formulation considerations

Formulation of APIs for rectal use will likely be in a liquid or semi-solid dosage form to cover areas that are at most risk for HIV-1 exposure (Wang, Schnaare, Dezzutti, Anton, & Rohan, 2011). Preclinical testing of formulated APIs adds additional complexity because pH, osmolality, and viscosity of the product will impact the results. For instance, the polymers used in the formulation may enhance toxicity or efficacy due to smothering of individual cells or non-specifically binding HIV-1. Therefore, it is critical to include the vehicle control, the same formulation but without the API, in all assays to accommodate the impact the formulation may have on the testing results. As with unformulated APIs, testing algorithms have been developed (Rohan et al., 2010). Typically, the testing done with the formulations is to ensure the APIs activity has not been impaired and the formulation is safe. Mucosal tissues are used for testing the formulations, but are polarized, keeping the apical surface at the liquid/air interface (Rohan et al., 2010; Abner et al., 2005; Cummins, Jr. et al., 2007). The formulation with or without HIV-1 can be applied to the apical surface recapitulating the use by a person. Using this testing algorithm, it was recently reported the TFV 1% gel was hyperosmolar and consequently demonstrated epithelial fracture and

sloughing in polarized mucosal tissue (Rohan et al., 2010). The gel was reformulated to reduce the glycerin content and thus reduce the osmolality (Dezzutti et al., 2012). The reduced glycerin TFV 1% gel showed improved epithelial retention in polarized rectal and ectocervical tissue explants. These data support the clinical trial results (discussed below) that showed that gastrointestinal AEs were significantly more common when the original TFV 1% gel was used rectally compared to the reduced glycerin gel formulation (Anton et al., 2011; McGowan et al., 2012). The preclinical testing of formulations is thus important to ensure those products that move into clinical trials are safe as well as effective.

1.7 Preclinical evaluation of rectal microbicides

Preclinical testing of API, has been standardized but there are subtle variations in the specific assays used based on the preferences of the laboratory doing the testing (Buckheit, Jr. & Buckheit, 2012; Lackman-Smith et al., 2008; Lard-Whiteford, 2004). Initial tests using primary immune cells, such as peripheral blood mononuclear cells, and indicator cell lines are performed to determine mechanisms of action and potency of the API against standard laboratory and primary clinical isolates of HIV-1. Some testing is now being conducted with the newly identified primary isolates known as transmitter/founder viruses from persons who acquired HIV-1 through penile-vaginal or penile-rectal coitus (Keele et al., 2008; Dezzutti et al., 2012). The incorporation of biological fluids such as semen, cervicovaginal fluid, or their simulants is also used early in the testing to ensure the API remains potent during coitus (Neurath, Strick, & Li, 2006; Patel et al., 2007).

1.8 Clinical evaluation of rectal microbicides

To date, the majority of rectal microbicide studies have evaluated either placebo formulations or been restricted to the Phase 1 rectal safety evaluation of antiretroviral candidates such as TFV or UC781 gels. A Phase 2 extended rectal safety study of TFV gel has just started screening and enrollment (MTN-017; http://clinicaltrials.gov/: NCT01687218). The general characteristics of Phase 1-3 microbicide development are outlined below.

1.8.1 Phase 1

Drug development including microbicide development involves a number of different stages. In the preclinical phase, new molecules are evaluated for safety and efficacy in cell lines and animal models. Compounds with an adequate safety profile are then advanced into Phase 1 safety studies where small groups of participants (10-30) are exposed to the product in very controlled circumstances for relatively short periods of time (1-2 weeks). The participants in Phase 1 RM studies are usually at very low risk of HIV acquisition and are asked to be sexually abstinent during the study.

1.8.2 Phase 2

On completion of Phase 1 studies, candidate microbicides are then evaluated in Phase 2 studies. Characteristically, Phase 2 microbicide studies are conducted in larger groups of sexually active participants (100-200) for three to six months and are designed to identify safety or acceptability issues associated with frequent use of the product. On completion of Phase 2 development a candidate microbicide then advances into an effectiveness (Phase 2B/3) study. This is the final phase of testing and seeks to determine

whether the product can actually reduce HIV acquisition rates in at risk populations.

1.8.3 Phase 2B/3

Phase 2B/3 evaluation of microbicides is the most arduous phase of assessment. Of necessity, the microbicide intervention has to be evaluated in populations who are already receiving a comprehensive HIV prevention package. The components of this package continue to evolve but would be expected to include diagnosis and treatment of STIs, frequent safer sex counseling, condom provision, and possibly male circumcision (UNAIDS, 2007). The net effect of these interventions is that the participants enrolled in Phase 2B/3 studies often develop a lower risk of infection than their peers not participating in the study, potentially reducing the overall HIV incidence in the study population and therefore the power to find a statistically significant result. As a consequence, Phase 2B/3 studies are usually large (2000-3000 participants), long (2-3 years), and expensive.

Chapter 2

Previous Rectal Microbicide Studies

2 Previous rectal microbicide studies

2.1 Introduction

As of October, 2013, the majority of rectal microbicide research has involved evaluation of placebo formulations or the conduct of Phase 1 rectal safety studies. Increasingly, the design of Phase 1 rectal microbicide integrates characterisation of safety, acceptability, and PK/PD endpoints.

2.2 HIVNET-008

The HIVNET-008 study was designed to assess the safety of N9 when applied one to four times daily to the rectum and penis. Twenty five HIV-negative and ten HIV-positive, seroconcordant, monogamous gay male couples were enrolled in Seattle, WA, USA. Each partner was exclusively insertive or receptive while using N9 gel and served as his own control during placebo gel use compared to during N9 gel use. The study was conducted over 7 weeks. During the first week participants used the placebo gel. Thereafter, couples used the N9 gel and the frequency of use was escalated from once daily to two applicators twice daily in the final week of the study. Despite the frequency of administration, AEs were generally mild and transient. No rectal ulcers were detected; superficial rectal erosions were noted in two HIV-negative participants. Abnormal or slightly abnormal histologic abnormalities of rectal biopsies were detected in 31 (89%) of receptive participants after N9 gel use compared to 24 (69%) of participants after 1 week of placebo gel use. Excluding participants who felt no need for an HIV prevention method, 58% said they would use N9 if approved for rectal use; 69% of receptive users reported rectal fullness and related side

effects after insertion of the gel, and 68% reported applicator-related discomfort; 59% of insertive participants found the gel too sticky (Gross et al., 1999).

2.3 HPTN-056

The purpose of this study was to evaluate the biological stability of mucosal parameters that might be used as endpoints in Phase 1 rectal safety studies (McGowan et al., 2007). Sixteen male participants were enrolled into four groups defined by HIV status, viral load, and sexual activity. Each participant underwent three flexible sigmoidoscopies at two-week intervals with collection of blood, intestinal biopsies, and rectal secretions. Intestinal histology, phenotypic characterization of mucosal mononuclear cells, cytokine mRNA profiles (RANTES, Interferon-γ (IFN-γ), and Interleukin-10 (IL-10)), and immunoglobulin secretion were assessed. Intraclass correlation (ICC) was calculated to assess endpoint stability. Qualitative histology demonstrated minimal inflammation in > 95% of biopsies and remained stable throughout the study period. ICC for the tissue cytokine mRNA measurements and several T cell phenotypic markers was > 0.7, indicating stability over time. Mucosal CD4 lymphopenia was seen in the HIV positive participants and was more pronounced in those with higher viral loads. Modest differences were observed for cytokine expression (IFN-γ) and T cell phenotype (CD3, CD4, CD8, CD19, CD4/CCR5, and CD4/CD38) between the tissue samples collected at 10 and 30 cm.

2.4 Placebo formulation studies

In 1999, Gross *et al*. published acceptability data from the HIVNET-008 study evaluating N9 as a rectal microbicide (Gross et al., 1999). Data were available for the 35 couples enrolled in the study. The majority of participants (54%) said that they would not use the product if it was approved for rectal use. Problems associated with the N9 gel included the gel being too sticky and drying out too quickly. In light of these findings, a number of microbicide placebo studies have been undertaken to assess the acceptability of potential rectal microbicide formulations including gels, enemas, and suppositories. In contrast to the majority of Phase 1 rectal microbicide studies involving formulated ARV products, where participants are usually sexually abstinent for the duration of the study, the placebo studies described below were designed to evaluate products in sexually active participants and can provide critical information about desirable characteristics for a candidate rectal microbicide.

2.4.1 Rectal microbicide volume escalation study

It is not known what volume of a microbicide gel will be needed to provide protection from HIV infection associated with URAI. The majority of vaginal microbicide studies have used approximately 4 mL of study product. However, since the vaginal compartment is a relatively enclosed anatomical space and the rectum a potentially much larger space it has been assumed that a greater volume of gel would be needed in the rectal vs. vaginal compartments. A study, undertaken by Carballo-Dieguéz et al., was conducted among HIV-uninfected MSM in Boston, Massachusetts, USA and was designed to explore the acceptability of escalating volumes of placebo

gel in MSM practicing anal sex (Carballo-Dieguez et al., 2007). The placebo gel used in the study was FemGlide® (Trumbull, Connecticut, USA), an isosmolar vaginal lubricant. The gel was administered using a syringe; each of the 18 participants received sequentially 5, 20, 35, and 50 mL of study product. The participant then used the study product at the highest volume that he had rated as acceptable during RAI. Up to 35 mL of FemGlide was found to be acceptable to study participants. Beyond this volume there were increasing reports of leaking and messiness which were associated with poor product acceptability.

2.4.2 Gel and suppository rectal microbicide preference study

In a second study from the same group, again conducted in Boston Massachusetts, USA, 77 MSM were enrolled in a cross over study where they were asked to use either a placebo gel or a suppository (Carballo-Dieguez et al., 2008). FemGlide was administered at a volume of 35 mL using an accordion type applicator and the suppository was manufactured using two polyethylene glycol bases (PEG 1450 and PEG 300) (JE Apothecary, Inc., Brookline, Massachusetts, USA). Participants were asked to insert the products up to 2 hours before anal intercourse on three separate occasions. Rectal gel was preferred over the suppository for both the recipient (75% vs. 25%, $P < 0.001$) and the partner (71% vs. 29%, $P<0.01$). Although these data suggest a clear preference for gel over suppository, the authors did comment that the suppository used in this study was quite large (8 g and 2.5 inches) and they felt that a smaller suppository might have been more acceptable.

2.4.3 Rectal microbicide formulation preference study

In a recent study, Pines *et al*. evaluated a gel, suppository, and enema in 117 HIV-uninfected males (79%) and females (21%) (Pines et al., 2012). The study was conducted in Los Angeles, California, USA and participants received an isosmolar gel (4 mL of Pre-Seed®; Spokane, Washington, USA), an isotonic enema (125 mL of Normosol-R; Hospira Inc., Lake Forest, Illinois, USA), and a suppository commonly used for the treatment of haemorrhoids (Tucks™; McNEIL-PPC Inc., Skillman, New Jersey, USA). The study products were administered in a randomized sequence and participants were asked to use each product 3-5 times over a two week period before moving on to the next product. During follow-up, 75, 76, and 66% of participants reported using the enema, gel, and suppository respectively. There was no difference in the frequency of anorectal symptoms or adverse events (AEs) by product and, based on computer-assisted self-interviews (CASI), the gel was the most acceptable product overall.

2.5 Product distribution studies

A key component of microbicide development is to determine whether the candidate microbicide is “in the right place at the right time”. Increasingly sophisticated imaging technology is being used to track semen and microbicide surrogates to understand more about their distribution and persistence following rectal administration. Craig Hendrix and his colleagues at Johns Hopkins University (JHU), Baltimore, Maryland, USA have developed this area of prevention research with a number of innovative studies (Figure 2-1).

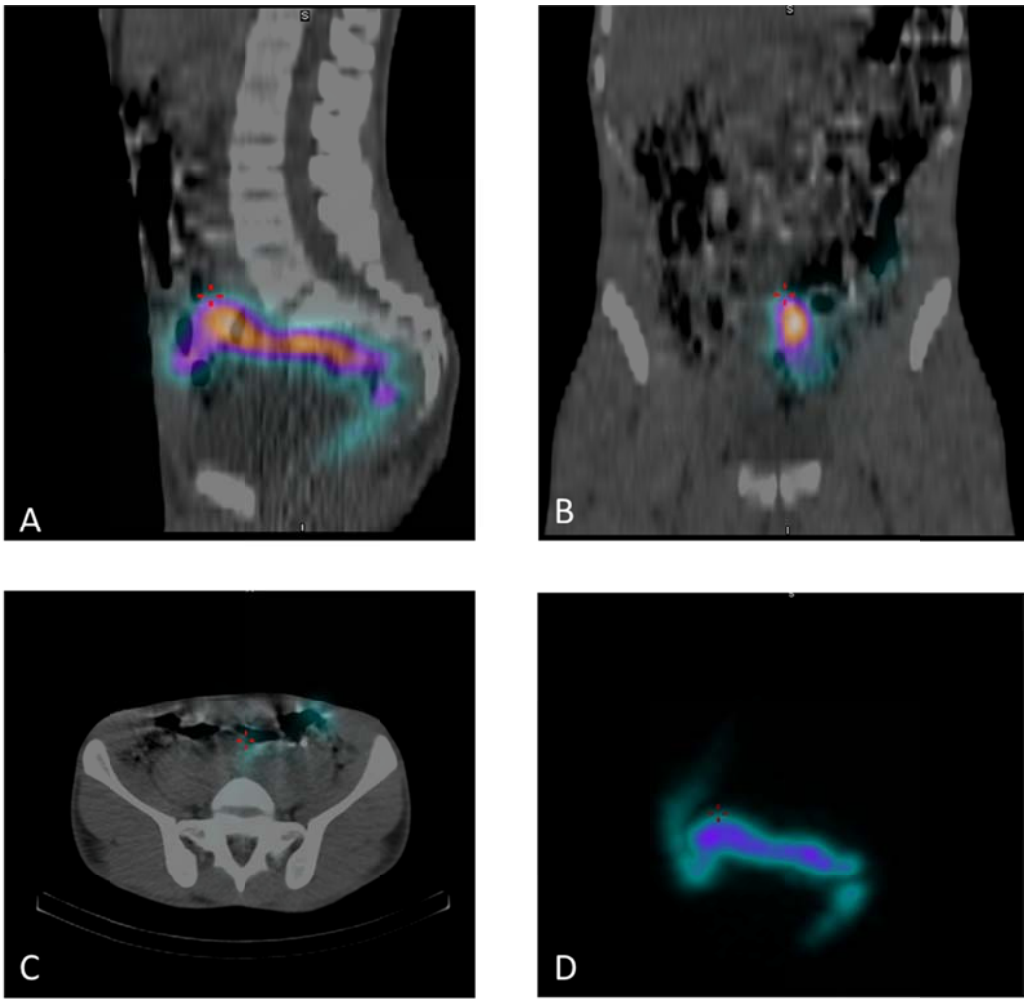


Figure 2-1 SPECT/CT images of microbicide distribution

SPECT/CT images of rectal microbicide distribution. Images were captured 2 hours after rectal administration of a placebo gel. Single-photon emission computerized tomography (SPECT) was performed using ^{111}Indium-Diethylene Triamine Pentacaetic Acid (DTPA). (A) Sagittal image, (B) Coronal image, (C) Axial image, and (D) MIP (maximal intensity projection) image displaying signal intensity from the sagittal plane with the greatest signal intensity creating a full thickness image, not a single plane. Image provided courtesy of Dr. Craig Hendrix, Professor of Medicine, Johns Hopkins University, Baltimore, Maryland, USA.

In their first study, Hendrix *et al*. administered a hydroxy ethyl cellulose (HEC)-based microbicide surrogate and a viscosity-matched semen surrogate to three participants (Hendrix et al., 2008). Each participant underwent seven phases of evaluation wherein the impact of variables such as enema administration, semen and microbicide surrogate, and coital

simulation on product distribution were evaluated. Both the microbicide and semen surrogates were labeled with gadolinium-diethylene triamine pentaacetic acid (DTPA) (as a magnetic resonance imaging (MRI) contrast agent) and ^{99m}Technetium-sulphur colloid (for single photon emission computed tomography (SPECT) imaging).

The microbicide surrogate was administered using a syringe and the semen surrogate was administered via a commercially available artificial phallus or coital dynamic simulator (CDS) that was modified to allow delivery of the semen surrogate through a triple lumen catheter. The semen surrogate was delivered after a five minute period of insertion and removal of the device to simulate RAI. MRI studies conducted at one and four hours following product administration demonstrated presence of signal throughout the rectum with the highest signal being seen dependent/posterior aspect of the rectum (participants remained supine throughout the procedure). In contrast, SPECT imaging demonstrated more proximal product migration. At four hours post product exposure 4/16 studies demonstrated signal in the descending colon, two of which reached the splenic flexure.

In a second study, the JHU team evaluated the distribution of cell-free and cell-associated HIV surrogates in the colon after simulated RAI in six men (Louissaint et al., 2012). In this study autologous leukocytes, collected from study participants were radiolabelled with ^{111}Indium-oxine as the cell associated surrogate and sulphur colloid particles were labeled with ^{99m}Technetium-sulphur as the cell-free surrogate. Both products were mixed

with 3 mL of autologous semen and delivered to the rectum via a CDS after 5 minutes of simulated RAI. SPECT/CT images were acquired at 1, 4, 8, and 24 hours post dosing. In this study cell-associated and cell-free surrogates generally co-localized, were largely confined to the rectosigmoid colon, and signal persisted for up to 24 hours in the absence of defecation. Furthermore, cell-free and cell-associated surrogates were found in tissue biopsies collected from the study participants.

In the third published study, the group further evaluated microbicide and semen surrogate colonic distribution in eight participants using the methodological approach described above (Cao et al., 2012). Participants sequenced through three phases: (i) Phase I: Microbicide / Coital Simulation / No Semen, (ii) Phase II: No Microbicide / Coital Simulation / Semen, and (iii) Phase III: Microbicide / Coital Simulation / Semen. Three novel PK parameters to describe product distribution were developed: (i) Maximum distance (D_{max}): the distance of the proximal endpoint of the radiosignal (drug or pathogen), (ii) Distance at concentration maximum (D_{Cmax}): location within the colon with the highest drug or pathogen density/signal, and (iii) Mean residence distance (D_{ave}): the mean distance within the colon throughout which the drug or pathogen is distributed at the time of the observation. In this study, gel distribution was limited to the rectosigmoid in 84% (26/31) of the studies although 16% (5/31) studies had proximal spread to descending colon. Comparison of D_{max}, D_{Cmax}, and D_{ave} across different phases of the study did not generate any significant differences suggesting that microbicide and semen surrogates were generally co-localized.

In their most recent study, Leyva et al. conducted an evaluation of enemas as potential vehicles for microbicide delivery (Leyva et al., 2013). Three formulations were assessed, a hyperosmolar enema (Fleet Phosphate Enema [2,100mOsmol/kg], Fleet Laboratories, Lynchburg, VA, USA), a hypoosmolar enema (distilled water), and an isoosmolar enema (Normosol-R enema [294mOsmol/Kg], Hospira, Inc., Lake Forest, IL, USA). The study was conducted in a crossover fashion so that the nine study participants were exposed to all three formulations in a randomised order. Enemas were radiolabeled with ^{99m}Technetium (Tc)-DTPA to assess enema distribution in the colon using SPECT/CT imaging. Plasma ^{99m}Tc-DTPA was measured to assess mucosal permeability. Sigmoidoscopic colon tissue biopsies were collected to assess mucosal injury as well as tissue penetration of the ^{99m}Tc-DTPA. Acceptability was assessed after each product use and at the end of the study. SPECT/CT imaging showed that the isoosmolar enema had greater proximal colonic distribution (up to the splenic flexure) and greater luminal and colon tissue concentrations of ^{99m}Tc-DTPA when compared to the other enemas. Only the hyperosmolar enema caused sloughing of the colonic epithelium. In permeability testing, the hypoosmolar enema had higher plasma ^{99m}Tc-DTPA compared to the hyperosmolar and isoosmolar enemas, respectively. Acceptability was generally good with no clear preferences among the three enema types. The team concluded that enemas have potential as rectal microbicide vehicles.

Collectively, these highly innovative studies suggest that the rectosigmoid is the area of highest viral exposure following RAI and that rectal microbicides

also appear to co-localize in this area. Obviously, these data need to be interpreted with some degree of caution; the participants are undergoing simulated RAI and receiving microbicide and semen surrogates. Actual semen and specific microbicide products may have different patterns of distribution. However, this methodological approach is now being used to study distribution of actual rectal microbicides in the **C**ombination **H**IV **A**ntiretroviral **R**ectal **M**icrobicide (CHARM) Program (Section 2.9).

2.6 RMP-01

In the Rectal Microbicide Program (RMP)-01 study, thirty six HIV-1 seronegative, sexually-abstinent men and women were enrolled in Los Angeles, CA, USA and randomized into a double-blind, placebo-controlled trial comparing UC781 gel at two concentrations (0.10% and 0.25%) with a placebo gel (1:1:1). Safety and acceptability were the primary study endpoints. Changes in colorectal mucosal safety biomarkers and UC781 plasma drug levels were secondary endpoints. *Ex vivo* explant infectibility with HIV-1 was an ancillary study endpoint. Samples were collected at enrollment, after a single rectal dose of study product, and after seven daily doses. The majority of AEs were mild. Product acceptability was high, including likelihood of future use. No changes in mucosal safety biomarkers were identified. Plasma levels of UC781 were not detected. *Ex vivo* infection of biopsies using two titers of HIV-1_{BaL} showed marked suppression of HIV-1 p24 in tissues exposed *in vivo* to 0.25% UC781. Ideally the product would have been advanced into Phase 2 development but the IND sponsor (CONRAD; http://www.conrad.org/) terminated the UC781 development program.

2.7 RMP-02/MTN-006

Eighteen participants were enrolled from Pittsburgh, PA, USA and Los Angeles, CA, USA. All participants received a single 300mg dose of TDF and were then randomized 2:1 to receive a single then seven daily doses of TFV 1% gel or the HEC placebo gel. Safety endpoints included clinical AEs and mucosal safety biomarkers. Participants were assessed at enrollment, after single doses of oral TFV and study gel, and after seven daily doses of study gel. Blood and colonic biopsies were collected for PK analysis and *ex vivo* challenge with HIV-1. No serious AEs were reported. However, AEs, especially gastrointestinal AEs, were significantly increased with seven-day use of the TFV 1% gel. Only 25% of participants liked the TFV gel; however, likelihood of use, if the product was somewhat protective, was high (75%). No significant mucosal injury was detected. Tissue TFV diphosphate (TFV-DP) C_{max} 30-minutes after single rectal exposure was 112-times greater than single oral-exposure with tissue Cmax in 7-day exposure 5-times greater than single rectal-exposure. Seven-day exposure to rectal TFV was associated with significant suppression of explant infection. Increased AEs suggested that the vaginal formulation of TFV 1% gel used rectally was not entirely safe nor fully acceptable, suggesting a need for improved formulations.

2.8 Project Gel

The National Institutes of Health (NIH) has funded a project entitled “Microbicide safety and acceptability in young men” that attempts to evaluate rectal microbicide safety, adherence, and acceptability in young ethnic minority MSM in Boston, MA, USA; Pittsburgh, PA, USA; and San Juan, PR,

USA. The study design has two stages: a clinical and behavioral evaluation (Stage 1A) with an acceptability and adherence trial (Stage 1B), followed by a Phase 1 randomized, double-blind, multi-site, placebo-controlled safety trial (Stage 2). The first 120 eligible participants who complete Stage 1A and report unprotected RAI in the previous 3 months will continue on to Stage 1B. During Stage 1B, participants will be given condoms and a placebo gel to use during receptive anal intercourse. Over a three month period they will report the frequency of product use and be interviewed about the acceptability of the product. The first 24 participants who complete Stage 1B will be eligible to participate in Stage 2 where they will be randomized to receive an actual microbicide (reduced glycerin (RG)-TFV 1% gel) or matched placebo. It is hoped that data from this study will provide unique insights into the acceptability, safety, and adherence of rectal microbicides in young MSM.

2.9 The CHARM Program

The CHARM Program will develop and evaluate a combination ARV rectal specific product. TFV and maraviroc (MVC) are the two lead compounds and the ultimate goal is to develop a TFV/MVC combination product. Two Phase 1 studies, CHARM-01 and CHARM-02 will start in 2013. CHARM-01 will assess the safety, acceptability, and PK/pharmacodynamic (PD) profile of three TFV gel formulations; the original TFV 1% gel used in vaginal microbicide studies, the RG-TFV 1% gel, and a rectal specific TFV gel. CHARM-02 will evaluate the safety, PK, and distribution of the same three gels. Similar techniques have been used to characterize the distribution of semen surrogates and microbicide products in the presence and absence of

simulated receptive anal intercourse (Louissaint et al., 2012; Cao et al., 2012). Collectively, these studies will provide unique data on the influence of formulation characteristics, including osmolality, and product safety, PK/PD, and distribution.

2.10 Current concepts in the design of rectal microbicide studies

From a contemporary perspective, the first rectal microbicide study conducted by Tabet et al. (Tabet et al., 1999) was unusual as it enrolled both HIV-positive and HIV-negative participants. The study involved dose escalation and participants were sexually active. As rectal microbicide development has become more focused on ARV products a number of study design changes have occurred. All participants enrolled in Phase 1 ARV rectal microbicides are screened for HIV infection and only HIV-negative participants are enrolled in the study. This approach arises from concerns that an HIV-positive participant, especially one who is not receiving combination ARV therapy, exposed to a single agent ARV microbicide such as TFV gel, might develop HIV resistance to products used for treatment (Abbas, Hood, Wetzel, & Mellors, 2011). In addition, Phase 1 rectal microbicide studies involving the first exposure of the product in man are conducted in sexually abstinent populations. Emergent AEs can then be attributed to product use rather than coital associated trauma or infection. Initial product exposure is limited to 1-2 weeks of daily dosing to limit the potential for mucosal damage. In contrast, during the HIVNET-008 study of N9, participants received up to six weeks of product exposure.

2.10.1 Safety

As with all clinical trials, emergent AEs in rectal microbicide studies are captured through participant interview/examination and events are documented and classified using the Medical Dictionary for Regulatory Activities (MedDRA) coding system. The severity of AEs is graded using the NIH/National Institute of Allergy and Infectious Diseases (NIAID)/Division of AIDS (DAIDS) "Table for Grading the Severity of Adult and Pediatric Adverse Events, Version 1.0, December 2004" (Clarification August 2009; http://rsc.tech-res.com/safetyandpharmacovigilance/gradingtables.aspx). In August 2009, NIH/DAIDS published an addendum to the 2004 DAIDS AE table: "Addendum 3: Rectal Grading Table for Use in Microbicide Studies" (available in Appendix 1, Section 9.1). This table provided a mechanism to capture the types of AEs (primarily anorectal and gastrointestinal) that might be anticipated to occur in rectal microbicide trials and was in fact developed by a number of clinical trials physicians working in this area of HIV prevention research.

Colposcopic inspection of the cervicovaginal mucosa is a standard component of Phase 1/2 vaginal microbicide trials and is used to detect mucosal inflammation and/or epithelial disruption that might occur secondary to product application. Microbicide-specific atlases have been developed to assist with training of staff in performing colposcopy within microbicide trials (Bollen, Kilmarx, & Tappero, 2004). However, a number of recent studies have questioned the utility of colposcopy compared to simple naked eye examination of the cervicovaginal mucosa (Mauck, Weiner, Lai, & Schwartz,

2012; Chirenje et al., 2012). High resolution anoscopy (HRA) is an analogous technique used to provide magnified images of the anorectal mucosa. HRA is used primarily to diagnose and treat human papilloma virus (HPV) associated anal dysplasia that is commonly found in MSM with HIV infection (Jay, 2011). HRA is also being evaluated for its utility in identifying microbicide induced mucosal damage involving the anal canal and the distal rectum (Project Gel, Section 2.8). One practical limitation of HRA is that, depending on individual anorectal anatomy, it can only image the distal 6-9 cm of the anorectum (Ross Cranston MD, personal communication). It is know from microbicide distribution studies that, following application, a candidate microbicide can spread in a retrograde fashion into the rectosigmoid colon, occasionally into the descending colon, and rarely as far as the splenic flexure (Hendrix et al., 2008; Cao et al., 2012). As a consequence, the majority of Phase 1 rectal microbicide studies have used flexible sigmoidoscopy to image the distal rectum as well as to collect mucosal samples for histology, safety biomarkers, and PK/PD samples. In theory, a flexible sigmoidoscope can reach the splenic flexure which is located approximately 45-60 cm from the anorectal margin. However, in the rectal microbicide studies conducted to date, the most proximal level for sample collection and observation has been 30 cm from the anorectal margin. Assessment of the endoscopic appearances of the colorectal mucosa is not a routine component of rectal microbicides studies. Overt lesions such as ulceration will be documented but capturing generalized changes such as “mild inflammation” are not particularly helpful. It is known from the inflammatory bowel disease (IBD) literature that there is very high

variability in the recognition of mild disease activity between observers (Travis et al., 2012; Dhanda, Creed, Greenwood, Sands, & Probert, 2012) and variable correlation between endoscopic evidence of inflammation and actual histopathological evidence of inflammation (Gomes, du Boulay, Smith, & Holdstock, 1986; Osada et al., 2008).

In the absence of validated indices of microbicide induced mucosal injury, the rectal microbicide research community has taken an expansive view on including multiple potential biomarkers of mucosal injury within the design of Phase 1/2 studies. These biomarkers are summarized in Table 2-1 and discussed in more detail in Chapter 5.

Table 2-1 Mucosal safety assays in rectal microbicide studies

	Rectal Microbicide Studies			
Mucosal Assay	**RMP-01**	**RMP-02 / MTN-006**	**MTN-007**	**MTN-017**
Histology	X	X	X	X
Epithelial sloughing	X	X	X	
Fecal calprotectin	X	X	X	
Luminex	X	X	X	
qRT-PCR	X	X	X	X
Microarray			X	X
Rectal microflora	X	X	X	

In general, the biomarkers have been chosen because they have been used in previous animal or human vaginal microbicide studies (e.g. Luminex® assessment of chemokines/cytokines (Fichorova, Tucker, & Anderson,

2001)) or because they are closely linked to the initial events in HIV infection (e.g. the proportion and activation phenotype of gut-associated lymphoid tissue (GALT) CD4+/CCR5+ T cells (Cicala, Arthos, & Fauci, 2011)).

2.10.2 Acceptability

Rectal microbicides will only play an important role in HIV prevention if the target populations find them acceptable and use them correctly and consistently (Elias & Coggins, 2001; Severy & Newcomer, 2005; Mantell et al., 2005). Although there has been some discussion concerning whether acceptability studies should be postponed until efficacy of a product is demonstrated, others (Morrow & Ruiz, 2008; Mantell et al., 2005; Tolley & Severy, 2006) have convincingly defended the wisdom of integrating acceptability research in early clinical phases of microbicide development. Morrow and Ruiz state that Phase 1 trial participants "are an invaluable source of information regarding acceptability [for] they constitute the handful of individuals with actual product use experience and, thus, are in the best position to provide feedback on actual product characteristics and how these factors may influence individuals' willingness to initiate and maintain product use over time" (Morrow & Ruiz, 2008). They suggest that these trials assess a variety of factors, including product scent, color, and texture; clarity of instructions and ease of product preparation and application; qualities of product during and after use; frequency and timing of use; and related covariates, such as history of lubricant use, frequency of anal and vaginal sex, and relationship communication. Rosen et al. (Rosen et al., 2008) and Morrow and Ruiz (Morrow & Ruiz, 2008) propose the use of mixed methods (quantitative and qualitative) to assess the different factors. This advice is

particularly sound in the case of small trials for which the utility of quantitative findings alone often has been limited (Bentley et al., 2000; Morrow et al., 2003; El-Sadr et al., 2006).

Three recent papers have made important contributions to our knowledge of the acceptability of rectal microbicides. Importantly, the observations were based on interviews with participants who had actually used experimental rectal products rather than a theoretical discussion of product acceptability. A National Institute of Child Health and Human Development (NICHD) funded trial found that a sexually active cohort of middle aged MSM rated volumes up to 35 mL of gel acceptable for use during anal intercourse (Carballo-Dieguez et al., 2007). In a second study, MSM appeared to prefer microbicide gels rather than rectal suppositories (Carballo-Dieguez et al., 2008). Acceptability data from a Phase 1 safety study of UC781 gel, an ARV microbicide gel, found the product to be highly acceptable and the majority of participants said that they would use such a product if it was commercially available (Ventuneac et al., 2009).

2.10.3 Adherence

A fundamental concept in clinical trials is that product effectiveness is determined by the combination of the product's innate efficacy and the degree to which the product is used by study participants. Non-adherence to product use can dramatically decrease the observed product effectiveness. As one example, in the iPrEx study of Truvada, the intention-to-treat (ITT) analysis of efficacy in reducing HIV acquisition was only 42% (Grant et al., 2010) whereas the efficacy in a nested case-control study of

participants with detectable drug estimated the efficacy to be as high as 92%. The iPrEx investigators have concluded that only 18% of participants were taking PrEP on a daily basis (Amico, 2012). The negative outcome of the FemPrEP study (evaluating Truvada PrEP in African women) was attributed to product non-adherence (Van et al., 2012). At the time of study discontinuation, 95% of women reported usually or always using the study drug. However, in the subset of participants randomized to receive Truvada who seroconverted, TFV was only detected in 7 of 27 (26%) women. These studies demonstrate two important features of PrEP studies; participants are not always able to give accurate estimates of product use and secondly PK levels can be an important marker of product use.

As concerns have increased over non-adherence in PrEP studies, there is increasing interest in using behavioral techniques to improve product adherence (Amico et al., 2012; Amico, 2012) as well incorporating objective measures of adherence into clinical trial design. These objective measure include electronic monitoring of product use with medical event monitoring systems (MEMS) (Moench, O'Hanlon, & Cone, 2012) or Wisebags™(van der Straten et al., 2013), techniques to determine whether microbicide applicators have been placed into the vagina (Moench et al., 2012), measurement of systemic and compartmental drug levels (Hendrix et al., 2013; Minnis et al., 2013) and demonstration of antiviral activity in body fluids (Keller et al., 2011). More recent techniques include tagging microbicide gels with secondary esters such as 2-butyl acetate that once metabolized in the body can be measured in breath (Morey et al., 2012).

To date, adherence monitoring in rectal microbicide studies has depended on self-reported behavior as well as PK monitoring. However, ongoing and planned rectal microbicide studies, in addition to conventional adherence monitoring (self-report assessed through interviews and computer assisted self-interview (CASI) and PK monitoring), are including newer techniques such as interactive voice response systems (IVRS) (Project Gel, Section 2.8), Wisebags (CHARM Program, Section 2.9), and short message service (SMS) diaries (MTN-017).

2.10.4 Pharmacokinetics

Characterisation of the PK profile of novel APIs is a routine component of drug development and is also a common feature of HIV prevention trials. Intense PK monitoring routinely occurs in Phase 1/2 studies whereas less frequent sampling schedules are employed in later stage studies. In the context of HIV prevention studies, Phase 1/2 PK studies help address the question as to whether ARV exposure in the cervicovaginal or rectal compartment is sufficient to out distance and outlast the virus at the site of infection (Hendrix, 2012). In combination with PD assessments (discussed in Section 2.10.5), PK data can be used to generate PK/PD models that have the potentials to provide insights as to whether candidate PrEP or microbicide agents are likely to demonstrate efficacy in Phase 2B/3 effectiveness trials. PK data can also be used as an important adherence biomarker in PrEP studies.

To date, the majority of PK data generated in HIV prevention trials have focused on TFV gel, oral TDF, and TDF/FTC. TFV is a monophosphorylated adenine nucleoside analogue RTI that requires two phosphorylation steps, undertaken by nucleoside kinases, to form the active moiety, TFV diphosphate (TFV-DP). TFV has a plasma half-life of 17 h compared to TFV-DP which has a half-life of 150-180 h in peripheral blood mononuclear cells (PBMCs) (Kearney, Flaherty, & Shah, 2004; Hawkins et al., 2005). TDF is an esterified prodrug with increased bioavailability compared to TFV. FTC is a cytidine nucleoside analogue RTI and requires three phosphorylation steps, undertaken by deoxycytidine kinase and cellular kinases, to form the active moiety, FTC triphosphate (FTC-TP). FTC has a half-life in plasma of 8-10 h and FTC-TP has a half-life in PBMC of 29-56 h (Blum, Chittick, Begley, & Zong, 2007; Wang et al., 2004).

Several studies have evaluated the compartmental distribution of TFV, TFV-DP, FTC, and FTC-TP. Patterson *et al*. characterized the PK profile of a single oral dose of TDF/FTC to 12 healthy men and women (Patterson et al., 2011). Blood plasma, genital secretions, and cervicovaginal and rectal tissue biopsies were collected over the subsequent 14 days. TFV and FTC were measured in blood and genital secretions. TFV-DP and FTC-TP were measured in the tissue samples. TFV and FTC were detected in plasma throughout the 14-day period. The concentration of FTC was 27-fold higher in genital secretions compared to plasma. However, TFV was only 2.5-fold higher in genital secretions compared to plasma. Rectal tissue TFV-DP was 100-fold higher than cervicovaginal tissue levels and persisted throughout

the 14 days. FTC-TP levels in the vaginal biopsies were 10-15-fold higher than rectal tissue but only persisted for 2 days after oral dosing. The RMP-02/MTN-006 study compared the safety, acceptability, and PK/PD profile of a single 300 mg oral dose of TDF to rectal TFV 1% gel (single and seven daily doses)(Anton et al., 2012). Plasma TFV levels were 23-fold greater after oral compared to rectal dosing but conversely, rectal TFV-DP levels were 10-fold higher after rectal compared to oral dosing. The study also enrolled female participants and demonstrated that rectal application of TFV 1% gel resulted in measurable levels of TFV in cervicovaginal fluid; an observation also made by Nuttall *et al.* in a recent NHP study (Nuttall et al., 2012). The MTN-001 study enrolled 144 African and US participants in a cross-design where all participants received 6 week sequences of oral TDF, vaginal TFV gel, and a combination of oral TDF and vaginal TFV gel (Hendrix et al., 2011; Minnis et al., 2012). Oral administration of TDF resulted in 60-fold higher plasma levels of TFV whereas vaginal dosing with TFV gel resulted in 130-fold higher levels of TFV-DP in vaginal tissue compared to oral dosing with TDF. Rectal fluid levels of TFV were also higher after vaginal than after oral dosing.

Collectively, these studies demonstrate that oral PrEP with TDF/FTC results in higher systemic exposure than after topical dosing with TFV gel. However, topical dosing with TFV gel results in higher tissue levels of TFV-DP than after oral dosing. Oral TDF/FTC appears to preferentially deliver TFV-DP to the rectal vs. cervicovaginal tissues whereas FTC-TP is higher in the cervicovaginal tissues compared to rectal tissues after oral dosing. Finally,

topical TFV gel application in the rectal compartment can also result in TFV exposure in the cervicovaginal compartment.

The majority of available PrEP PK data relate to TFV and TDF/FTC. However, the CCR5 antagonist, maraviroc (Selzentry®, ViiV Healthcare, North Carolina, USA (Dorr et al., 2005)), is also being considered as both an oral (HPTN-069, http://clinicaltrials.gov/: NCT01505114) and a topical agent for HIV prevention (as a vaginal ring (MTN-013) and a rectal microbicide (CHARM Program, Section 2.9). Brown et al. conducted a PK study in 12 HIV-negative men who received 300 mg of maraviroc twice daily for 8 days (Brown et al., 2011). Rectal tissue maraviroc area under the curve (AUC) was 7.5 to 26-fold higher than blood plasma. In a second study in women, maraviroc AUC in cervicovaginal fluid following oral dosing was only 1.9 to 2.5-fold higher than blood plasma (Dumond et al., 2009). Maraviroc has the potential to be an important drug for HIV prevention since greater than 95% of new HIV infections are transmitted by CCR5 (R5) tropic viruses (Meng et al., 2002; Moore, Kitchen, Pugach, & Zack, 2004). One note of caution was raised by a recent NHP study conducted by the Center for Disease Control and Prevention (CDC), Atlanta, USA in which 10 macaques received oral maraviroc (44 mg/kg) and were challenged rectally with SHIV162p3 (Massud et al., 2013). Despite similar PK profiles to human PK studies of maraviroc (including high concentrations of maraviroc in rectal secretions (median = 2,329 ng/mL vs. median = 451 ng/mL in blood), 5/6 macaques receiving maraviroc and 3/4 control macaques were infected with SHIV162p3. One possible explanation for these NHP data is that there are eight amino acid

differences between the macaque and human CCR5 receptor. Although both receptors bind maraviroc, disassociation of the maraviroc from the macaque CCR5 receptor was 10-fold faster than from the human CCR5 receptor (Napier et al., 2005). Additional studies, including explant challenge, will be required to further assess the utility of maraviroc as a PrEP agent.

These preliminary PK observations may have profound implications for the success or failure of PrEP regimens. As one example, Hendrix has argued that since the median ARV levels in the FEM-PrEP study (with a relative risk reduction in HIV infection of 0.0 (Van Damme et al., 2012) were similar to the iPrEx study (with a relative risk reduction in HIV infection of 0.42), it is possible that the divergent study outcomes resulted from higher rectal PK exposure in the iPrEx study where the route of HIV acquisition was rectal (Hendrix, 2012). A second example, again provided by Hendrix, is that the failure to demonstrate efficacy of the vaginal TFV gel arm in the VOICE study and the modest results from the CAPRISA 004 study cannot be purely explained by non-adherence. Based on PK data from the MTN-001 study, even with poor adherence, Hendrix estimates that the vaginal tissue concentrations in the VOICE and CAPRISA 004 studies would be 10-fold higher than those seen in the Partner's PrEP study that reported a relative risk reduction of 0.75. Hendrix further asserts that there may be a dose-variable effect that diminishes the effectiveness of topical microbicides i.e. more is not always better. Marrazzo et al. have recently presented the preliminary results of the VOICE study (Marrazzo et al., 2013). Neither oral nor topical TFV use was associated with protection from HIV infection and

PK data suggest that these results were a reflection of poor product adherence. In some of the VOICE clinical sites product adherence was below 20%. These results clearly emphasize the important of conducting PK assessments throughout all phases of oral PrEP and microbicide development.

2.10.5 Pharmacodynamics

In the context of HIV infection, a common PD relationship is the fall in plasma viral load that usually occurs when ARV combination therapy is started by a treatment naïve individual who is chronically infected with HIV infection. The magnitude of the PD response can be modulated by the ARV dose used to treat the individual. In the context of HIV prevention the PD concept is less obvious because the goal is to prevent an event (HIV acquisition) happening rather than treating a chronic condition. However, PK/PD relationships can be demonstrated in PrEP studies. As one example, Karim et al. showed that, within women enrolled in the CAPRISA 004 study, those participants randomised to receive TFV gel who had levels of TFV > 1000 ng/mL in genital tract fluid had a significantly greater protection from HIV infection than those women receiving placebo gel (2.4 vs. 9.1 per 100 person-years; P = 0.01) (Karim et al., 2011).

Cervicovaginal and colorectal explant models have been developed to provide *in vitro* PD data for candidate microbicides (Greenhead et al., 2000; Abner et al., 2005; Fletcher et al., 2006; Fletcher, Wallace, Mesquita, & Shattock, 2006) and can be used to explore the impact of API dosage on viral replication (Figure 2.2). As discussed in Section 1.5.4, explant

challenge experiments can be performed on cervicovaginal (Greenhead et al., 2000), colorectal (Fletcher et al., 2006; Abner et al., 2005), penile (Harman et al., 2012), and foreskin tissue samples (Zhou et al., 2011). Tissue samples can be polarised to try and recapitulate the circumstances of *in vivo* infection (Abner et al., 2005) or non-polarized which may represent a worst case scenario where mucosal integrity is disrupted either through coital trauma or concomitant STIs (Fletcher et al., 2006).

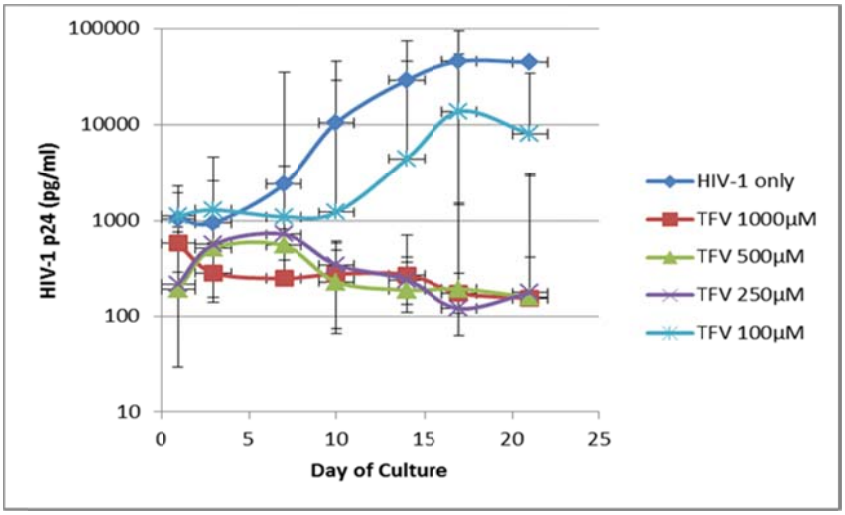


Figure 2-2 Colorectal explant challenge with tenofovir

Example of an *in vitro* colorectal explant challenge experiment. Colorectal biopsies were incubated for 2 hours with HIV-1 ± tenofovir at various concentrations. After the viral exposure period the explants are washed and provided with fresh media. Explant supernatant is collected at varying timepoints out to 21 days to look for evidence of drug-associated viral suppression (Unpublished data provided by Dr. Charlene Dezzutti, University of Pittsburgh, Pennsylvania, USA from the CHARM Program).

In vitro explant challenge has been used extensively to evaluate candidate microbicides and APIs. Richardson-Harman et al. have presented data on their experience in attempting to standardize the explant model across multiple research groups (Richardson-Harman et al., 2009). The study compared viral replication kinetic of multiple HIV clades, the ability of PRO 2000 to inhibit HIV infection in explant tissue, and a range of virological endpoints across the participating centres. The virological endpoints

included; (i) a SOFT p24 endpoint (the last time point when the increase in virus concentration between two consecutive time points is greater than the square root of the sum of sequential changes in virus concentration for the entire assay), (ii) Day 12 or Day 15 p24, (iii) the slope of the virus growth curve, and (iv) p24 AUC defined by the trapezoidal rule. Among the conclusions of the study, the authors suggested that the most important factors in obtaining reproducible results across sites were; (i) use of a common viral stock, (ii) adherence to standardized explant challenge protocols, and (iii) use of the SOFT endpoint or another standardised endpoint in explant studies.

The *in vitro* explant model has now been adapted to explore microbicide efficacy within clinic trials. The *ex vivo / in vitro* explant challenge model has been used in Phase 1 rectal microbicide (Anton et al., 2011; Anton et al., 2012) and intravaginal studies (MTN-013). It will also be used in a subset of the HPTN-069 (Next PrEP) study (http://clinicaltrials.gov/: NCT01505114) in which at risk MSM and women will be randomised to receive oral ARV combination therapy.

The advantage of the *ex vivo / in vitro* explant challenge model is that the target tissue (colorectal or cervicovaginal) is exposed to pharmacologically relevant concentrations of the candidate agent delivered from the formulation that will be used in future clinical trials.

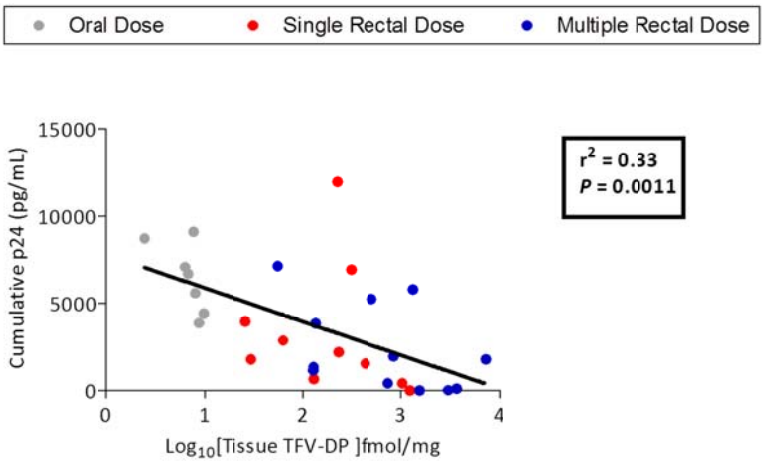


Figure 2-3 PK/PD profile of tenofovir from RMP-02/MTN-006

Example of a PK/PD profile derived from a comparison of explant viral infection (y-axis) and rectal tissue levels of TFV diphosphate (x-axis) showing an inverse correlation between drug concentration and viral replication (Anton et al., 2012)

In the two completed Phase 1 rectal microbicides studies the *ex vivo / in vitro* model demonstrated the efficacy of UC781 and TFV gels in inhibiting HIV viral replication in the explant tissue (see Section 1.5.4 for further details). Combining PK and PD data from these Phase 1 studies has the potential to create PK/PD models that can be used to help dose selection for future studies. Figure 2-3 demonstrates an inverse relationship between rectal tissue TFV-DP levels and the cumulative HIV-1 p24 levels in the rectal explant supernatant at Day 14 seen in the RMP-02/MTN-006 study (Anton et al., 2012). In a second paper, Richardson-Harman *et al.* undertook a retrospective correlational analysis of the PK and PD data from the RMP-01 Phase 1 rectal microbicide study of UC781 gel (Anton et al., 2011; Richardson-Harman, Mauck, McGowan, & Anton, 2012). The study compared multiple virological endpoints in the explant model and concluded that SOFT and cumulative p24 endpoints provided the most precise measurement of *ex vivo* HIV infection and significantly correlated with rectal

tissue UC781 concentrations. Logistic regression analyses showed that the $EC_{50,90,95}$ values were inversely related to p24 levels and provided clinically relevant insights into the tissue drug concentrations that might be necessary to prevent tissue infection.

Ongoing studies are currently looking at less invasive PD models that do not require tissue sampling. One example is the collection of cervicovaginal or rectal fluid at varying times following product exposure. The fluid is incubated with a reporter cell line such as TZM-bl and challenged with virus to see whether the fluid has antiviral efficacy (Keller et al., 2011). Similar studies are planned for the MTN-017 Phase 2 rectal safety study of TFV gel and will also include tissue explant challenge so that the two models can be compared.

2.11 Rectal microbicide advocacy

Drug development does not occur in a vacuum and from the outset advocacy groups have played a critical role in the development of rectal microbicides. The International Rectal Microbicide Advocates group (IRMA; http://www.rectalmicrobicides.org/) has helped focus attention on rectal microbicide development including conducting community/internet based studies on lubricant usage (Javanbakht, Murphy, Gorbach, LeBlanc, & Pickett, 2010; Dezzutti et al., 2012). IRMA has also led efforts to define the need for rectal microbicides for men and women at risk of HIV infection associated with URAI in Africa. IRMA convened a meeting in Addis Ababa, Ethiopia in November 2011 that has helped catalyze community interest in rectal microbicides within Sub-Saharan Africa. Proceedings from this

meeting are available at the IRMA website. Unfortunately, MSM activity is stigmatized, illegal, and even punished by death in many countries across the world (Altman et al., 2012) and conducting rectal microbicides trials or indeed rolling out rectal microbicides as prevention in these communities would currently be difficult if not impossible (Semugoma, Nemande, & Baral, 2012; Kyomya, Todyrs, & Amon, 2012). From a human rights perspective, as well as a drug development perspective, there is much to be done.

2.12 Summary

The field of rectal microbicide development has advanced significantly since the HIVNET-008 study of N9. Although rectal microbicide development has been delayed compared to vaginal microbicide development, this delay has in many ways provided critical guidance for how the rectal microbicide development pathway should proceed. The vaginal microbicide research community spent almost two decades focused on non-ARV microbicide candidates before the first success was seen with the CAPRISA 004 study of vaginal TFV gel. During this period, much was learnt about product acceptability, the potential for microbicide candidates to actually increase the risk of HIV acquisition, the need for careful safety surveillance throughout microbicide development, the problems associated with non-adherence to study products as well as the need to enhance and objectively monitor adherence behaviour in microbicide trials, and the utility of animal models of HIV infection to generate preliminary evidence of product efficacy. Learning from this extensive experience, the rectal microbicide research community has focused on the development of potent ARV candidates from the outset. Systemic and mucosal safety assessment are a critical component of Phase

1 study design, objective measures of product adherence are being evaluated, and PK/PD measurements are being used to provide unique insights into potential product efficacy.

As of October 2013, it is clear that new HIV infections among MSM have not fallen and indeed may be rising in incidence; there is a clear need to develop new prevention options for individuals at risk of HIV infection through URAI. The iPrEx study was a significant milestone in HIV prevention research for MSM but the overall level of effectiveness was modest and non-adherence was a common problem. Groups such as IRMA are advocating vigorously for rectal microbicides, NHP models provide proof of concept that a rectal ARV microbicide might be efficacious, and the PK/PD data emerging from human Phase 1 studies provide preliminary but supportive data to encourage later stage development of rectal microbicides.

The MTN-007 study, that forms the experimental core of this thesis, was a Phase 1 safety and acceptability assessment of a new formulation of TFV gel. The experimental approach, results, and conclusions from this study are provided in Chapters 3-7 of this thesis.

Chapter 3

MTN-007 Overview

3 MTN-007 overview

Rectal microbicides are being developed to prevent or at least significantly reduce the risk of HIV acquisition associated with URAI (McGowan, 2011). Attention is currently focused on the development of TFV 1% gel as a potential rectal microbicide. The vaginal formulation of TFV used in the CAPRISA 004 study (Karim et al., 2011) was evaluated in the RMP-02/MTN-006 study (Anton et al., 2012) and rectal use of the gel was associated with mild to moderate gastrointestinal symptoms including bloating, pain, urgency, and diarrhea. The vaginal formulation of TFV is hyperosmolar (3111 mOsmol/kg) and it is possible that these symptoms were linked to product osmolality (Rohan et al., 2010). Consequently, the TFV gel used in the MTN-007 study was formulated with a lower glycerin concentration (5% w/w mg rather than the 20% w/w in the vaginal formulation) that resulted in a product osmolality of 836 mOsmol/kg (Dezzutti et al., 2012). It was anticipated this formulation would be better tolerated by study participants.

In the two Phase 1 rectal safety studies of ARV rectal microbicides conducted to date (RMP-01 and RMP-02/MTN-006), product use was not associated with any significant change in mucosal safety parameters (Anton et al., 2011; Anton et al., 2011). Whilst this is reassuring, the possibility exists that the range of mucosal safety parameters used in these studies was too narrow and might have missed unanticipated or subtle but important mucosal changes. To address this situation, the MTN-007 study was designed to include a broad range of mucosal safety parameters including the use of microarray assessment of mucosal gene expression. In addition,

an N9 arm was included to help determine the utility of individual mucosal safety assays in detecting mucosal injury. Rectal use of N9 in humans has been associated with transient mild gastrointestinal discomfort as well as minor histological abnormality (Tabet et al., 1999) and has been associated with induction of proinflammatory responses in cervical epithelial cells (Fichorova et al., 2001). It was hoped that these additional assessments would help provide a more comprehensive assessment of mucosal safety.

3.1 The Microbicide Trials Network

The MTN-007 study was conducted by the NIH sponsored Microbicide Trials Network (MTN). The MTN is an NIH/NIAID/DAIDS sponsored clinical trials network that was established in 2006 to identify safe and effective microbicides for HIV prevention. From the outset, the MTN has targeted key populations at risk of HIV including women in Sub-Saharan Africa, adolescents, pregnant and lactating women, and MSM. Over the last six years, the MTN has developed an expansive program in vaginal and rectal microbicide development, with unique and substantial contributions to the field in the understanding of the safety, effectiveness, pharmacokinetics, pharmacodynamics, and the behavioral context associated with the use of topical microbicides (Table 3-1). The MTN Core is based in Pittsburgh, Pennsylvania (PA), USA and provides oversight for the development and execution of all MTN clinical trials.

Table 3-1 Completed, ongoing, or planned MTN clinical trials

Study	Product(s)	Route	Sites	N	Phase	Status
HPTN 035	PRO-2000 BufferGel®	Vaginal	USA, MA, ZI, SA	3,101	2B	Completed
HPTN 059	TFV 1% gel	Vaginal	USA, IN	200	2	Completed
MTN-001	TFV tablet TFV 1% gel	Oral Vaginal	USA, SA, UG	168	2	Completed
MTN-002	TFV 1% gel in pregnancy	Vaginal	USA	21	1	Completed
MTN-003 VOICE study	TFV 1% gel TDF tablet TDF/FTC tablet	Oral Vaginal	SA, UG, ZI	5,029	2B	Completed
MTN-003B VOICE-B	VOICE bone density substudy		UG, ZI	518		Completed
MTN-003C VOICE-C	VOICE community substudy		SA	164		Completed
MTN-003D VOICE-D	VOICE efficacy dilution study		SA, UG, ZI	80		Ongoing
MTN-P01	Wisebag pilot study		SA	50	N/A	Completed
MTN-004	VivaGel®	Vaginal	USA	61	1	Completed
MTN-005	Placebo ring	Vaginal Ring	USA, IN	195		Completed
RMP-02/ MTN-006	TDF tablet TFV 1% gel	Oral Rectal	USA	22	1	Completed
MTN-007	RG-TFV 1% gel	Rectal	USA	65	1	Completed
MTN-008	TFV 1% gel in pregnancy and lactation	Vaginal	USA	90 Pregnant: 15 Lactating:	1	Completed
MTN-009	HIV resistance in the community	N/A	SA	1,074		Completed

MTN-011	TFV 1% gel	Vaginal	USA	40 couples	1	Ongoing
MTN-012 IPM 010	DPV gel	Penile	USA	48	1	Completed
MTN-013 IPM 026	DPV ring MVC ring DPV + MVC ring	Vaginal	USA	48	1	Completed
MTN-014	TFV 1% gel	Vaginal Rectal	USA, SA	28	1	Ongoing
MTN-015	HIV seroconverter protocol	N/A		Not available*	N/A	Ongoing
MTN-016	Pregnancy registry	N/A	USA, SA, UG, ZI	Women: 274 Infants: 213	N/A	Ongoing
MTN-017	RG-TFV 1% gel TDF/FTC tablet	Oral Rectal	USA, SA, PE, TH	186	2	Ongoing
MTN-020 ASPIRE study	DPV ring	Vaginal	SA, UG, ZI, ZA, MA	3,476	3	Ongoing
MTN-023	DPV ring in adolescents	Vaginal	USA	100	1	In development
MTN-024	DPV ring in post-menopausal women	Vaginal	USA	90	1	Ongoing

Data as of February, 2014. *MTN Core blinded to number of seroconverters on MTN-015. Sites: United States of America (USA), India (IN), South Africa (SA), Uganda (UG), Zimbabwe (ZI), Zambia (ZA), Malawi (MA), Peru (PE), and Thailand (TH). N/A: Not applicable; TFV: Tenofovir; RG-TFV: Reduced glycerin tenofovir; TDF: Tenofovir disoproxil fumarate; TDF/FTC: Tenofovir disoproxil fumarate/emtricitabine (Truvada®); DPV: Dapivirine; MVC: Maraviroc

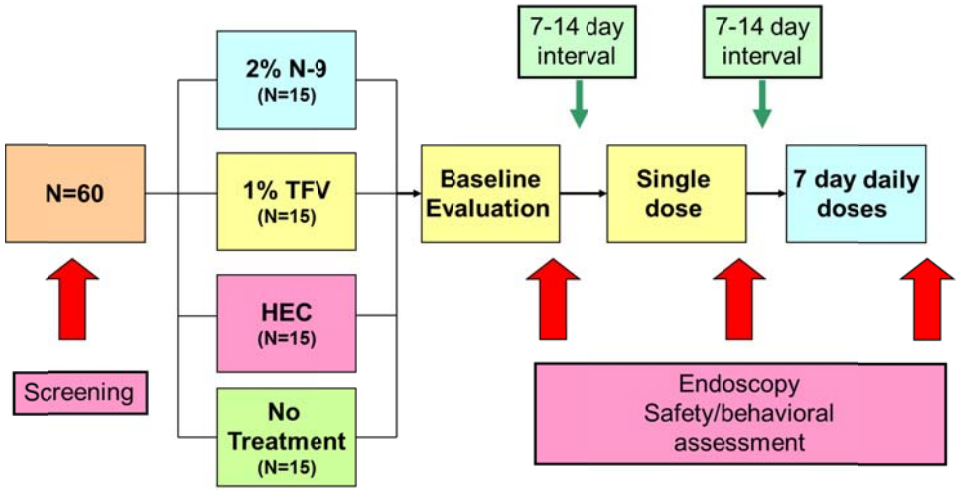
MTN protocol development is a stepwise process that begins with submission of a protocol concept to the MTN Executive Committee (EC). Protocol concepts can be submitted by MTN Core staff, site investigators, or

external investigators. Concepts may arise as part of the developmental pathway for candidate microbicides being evaluated by the MTN, in response to needs within the broader microbicide/PrEP field, or in response to specific requests by other product sponsors. Once a protocol concept has been approved, a protocol team is assembled and a full protocol is developed. Protocol development is coordinated by MTN Core Staff and usually involves a one day face-to-face meeting at which all key stake holders (MTN Core staff, clinical investigators, community representatives, product sponsors, statisticians, and DAIDS Medical Officers) have the opportunity to comment on and contribute to the study protocol. Once the protocol is completed it has to be submitted to the DAIDS Prevention Science Review Committee (PSRC) for review and approval. The DAIDS PSRC is a multidisciplinary committee that includes NIH staff with expertise in clinical trial design, pharmacovigilance, pharmacy affairs, clinical operations, statistics, and ethics. PSRC review usually results in protocol revisions. Once any PSRC required protocol changes are completed and approved by the PSRC or the DAIDS Medical Officer assigned to the protocol, the protocol moves to final regulatory approval and generation of Version 1.0 of the protocol that can be submitted to Institutional Review Boards (IRBs) or Ethics Committees for local approval.

3.2 The design of MTN-007

The MTN-007 study was a Phase 1 randomised, double-blinded, multi-site, placebo controlled rectal safety study in which approximately 60 participants were randomised to one of four arms as illustrated in Figure 3-1.

Figure 3-1 MTN-007 study design



The study participants were characterized by the following inclusion and exclusion criteria.

3.2.1 MTN-007 inclusion criteria

- Age of ≥ 18 years at screening
- Willing and able to provide written informed consent for screening and enrollment
- HIV-1 uninfected
- Willing and able to communicate in English
- Willing and able to provide adequate locator information
- Availability to return for all study visits, barring unforeseen circumstances
- Per participant report at screening, a history of consensual RAI at least once in the prior year
- Willing to abstain from insertion of anything rectally, including sex toys, other than the study gel for the duration of study participation
- Willing to abstain from RAI for the duration of study participation

- Must agree to use study provided condoms for the duration of the study for vaginal and insertive anal intercourse
- Must be in general good health
- Must agree not to participate in other drug trials

In addition to the criteria listed above, female participants met the following criteria:

- Postmenopausal or using (or willing to use) an acceptable form of contraception (e.g., barrier method, intra-uterine device (IUD), hormonal contraception, surgical sterilization, or vasectomization of male partner). If the female participant has female partners only, the method of contraception will be noted as a barrier method in the study documentation.

3.2.2 MTN-007 exclusion criteria

- Abnormalities of the colorectal mucosa, or significant colorectal symptom(s), which in the opinion of the clinician represents a contraindication to biopsy (including but not limited to presence of any unresolved injury, infectious or inflammatory condition of the local mucosa, and presence of symptomatic external hemorrhoids)
- At screening: participant-reported symptoms, and/or clinical or laboratory diagnosis of active rectal or reproductive tract infection requiring treatment per current CDC guidelines or symptomatic urinary tract infection. Infections requiring treatment include symptomatic bacterial vaginosis, symptomatic vaginal candidiasis, other vaginitis,

trichomoniasis, *Chlamydia trachomatis* (CT), *Neisseria gonorrhoea* (GC), syphilis, active herpes simplex virus (HSV) lesions, chancroid, pelvic inflammatory disease, genital sores or ulcers, cervicitis, or symptomatic genital warts requiring treatment. Note that an HSV-1 or HSV-2 seropositive diagnosis with no active lesions is allowed, since treatment is not required

- Anorectal STI within six months prior to the Screening Visit
- At screening:
    a. Positive for hepatitis B surface antigen
    b. Hemoglobin < 10.0 g/dL
    c. Platelet count < 100,000/mm^3
    d. White blood cell count < 2,000 cells/mm^3 or > 15,000 cells/mm^3
    e. *For females:* calculated creatinine clearance < 60 mL/min by the Cockcroft-Gault formula where creatinine clearance in mL/min (140- age in years) x (weight in kg) x (0.85 for female)/72 x (serum creatinine in mg/dL)
    f. *For males:* calculated creatinine clearance < 60 mL/min by the Cockcroft-Gault formula where creatinine clearance in mL/min = (140 - age in years) x (weight in kg) x (1 for male)/72 x (serum creatinine in mg/dL)
    g. Serum creatinine > 1.3× the site laboratory upper limit of normal (ULN)
    h. Alanine transaminase (ALT) and/or aspartate aminotransferase (AST) > 2.5× the site laboratory ULN
    i. +1 glucose or +1 protein on urinalysis (UA)

j. History of bleeding problems

- History of significant gastrointestinal bleeding in the opinion of the investigator
- Allergy to methylparaben, propylparaben, sorbic acid, and components of N9
- Known HIV-infected partners
- By participant report at enrollment, history of excessive daily alcohol use (as defined by the CDC as heavy drinking consisting of an average consumption of more than 2 drinks per day for men, and more than 1 drink per day for women), frequent binge drinking or illicit drug use that includes any injection drugs, methamphetamines (crystal meth), heroin, or cocaine including crack cocaine, within the past 12 months
- Per participant report at screening, anticipated use and/or unwillingness to abstain from the following medications during the period of study participation:
    a. Heparin, including Lovenox®
    b. Warfarin
    c. Plavix® (clopidogrel bisulfate)
    d. Rectally administered medications (including over-the-counter products)
    e. Aspirin
    f. Non-steroidal anti-inflammatory drugs (NSAIDS)
    g. Any other drugs that are associated with increased likelihood of bleeding following mucosal biopsy

- By participant report at screening, use of systemic immunomodulatory medications, rectally administered medications, rectally administered products (including condoms) containing N9, or any investigational products within the 4 weeks prior to the Enrollment/Baseline Evaluation Visit and throughout study participation
- History of recurrent urticaria
- Any other condition or prior therapy that, in the opinion of the investigator, would preclude informed consent, make study participation unsafe, make the individual unsuitable for the study or unable to comply with the study requirements. Such conditions may include, but are not limited to, current or recent history of severe, progressive, or uncontrolled substance abuse, or renal, hepatic, hematological, gastrointestinal, endocrine, pulmonary, neurological, or cerebral disease

In addition to the criteria listed above, female participants were excluded if they met any of the following criteria:

- Pregnant at the Enrollment/Baseline Visit
- Breastfeeding at screening or intend to breastfeed during study participation per participant report.

3.2.3 Primary objectives and endpoints

The primary objective of the MTN-007 study was to evaluate the safety of TFV 1% gel when applied rectally. Safety endpoints included Grade 2 or higher AEs as defined by the Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events, Version 1.0, December 2004

(Clarification dated August 2009) and/or Addenda 1 and 3 (Female Genital and Rectal Grading Tables for Use in Microbicide Studies) to this table.

3.2.4 Secondary objectives and endpoints

The secondary objectives and endpoints explored in the MTN-007 study were as follows:

- To evaluate the acceptability of TFV 1% gel when applied rectally.

The acceptability endpoint was defined as the proportion of participants who at their Final Clinic Visit report via the acceptability questionnaire that they would be very likely to use the candidate microbicide during receptive anal intercourse.

- To evaluate the safety of HEC placebo gel when applied rectally

The safety endpoint for the HEC placebo gel was defined as Grade 2 or higher adverse events, as defined by the Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events, Version 1.0, December 2004 (Clarification dated August 2009) and/or Addenda 1 and 3 (Female Genital and Rectal Grading Tables for Use in Microbicide Studies).

- To determine whether use of TFV 1% gel is associated with rectal mucosal damage
- To determine whether use of 2% N9 gel (Gynol-II®) is associated with rectal mucosal damage

The mucosal damage endpoints for both TFV and N9 gels were defined as changes in the following parameters (and are further discussed in Chapter 5):

- Epithelial sloughing

- Intestinal histopathology
- Intestinal mucosal mononuclear cell phenotype
- Intestinal mucosal cytokine messenger RNA (mRNA)
- Intestinal mucosal gene expression arrays
- Cytokine profile in rectal secretions
- Fecal calprotectin
- Microflora

3.2.5 Exploratory objectives and endpoints

The exploratory objectives and endpoints evaluated in the MTN-007 study were as follows:

- To determine whether regional heterogeneity exists between mucosal endpoints in samples collected at 9 cm and 15 cm for all parameters examined
- To determine whether there is a correlation between histological abnormality and changes in mucosal biomarkers

Both exploratory objectives were evaluated by the analysis of changes in the following mucosal parameters:

- Epithelial sloughing
- Intestinal histopathology
- Intestinal mucosal mononuclear cell phenotype
- Intestinal mucosal cytokine messenger RNA (mRNA)
- Intestinal mucosal gene expression arrays
- Cytokine profile in rectal secretions
- Fecal calprotectin

- Microflora

3.3 Study products

Each participant was assigned a carton of applicators, based on the randomisation number. At the Treatment 1 Visit the participant's first dose of study product was administered by the clinic staff. During the period of daily administration study participants were instructed to insert one dose of gel into the rectum once daily throughout the 7-day period. Rectal administration of study product occurred in the evening or before the longest period of rest. All study products were provided in identical opaque HTI polypropylene pre-filled applicators (HTI Plastics, Lincoln, NE, USA) containing 4 mL of study product.

3.3.1 Reduced glycerin formulation of tenofovir 1% gel

TFV 1% gel (weight/weight) is a gel formulation of TFV (PMPA, 9-[(R)-2-(phosphonomethoxy)propyl]adenine monohydrate), formulated in purified water with edetate disodium, citric acid, glycerin, methylparaben, propylparaben, and hydroxyethyl cellulose, with a pH adjusted to 4-5. TFV 1% gel is a transparent, viscous gel that was filled into applicators to form pre-filled, single-use applicators. Each pre-filled applicator contains a dose of approximately 4.0 mL of TFV 1% gel (equal to 4.4 g). TFV 1% gel was supplied by CONRAD (Arlington, VA, USA). Under direction from CONRAD, Patheon Inc., (Cincinnati, OH USA) a contract manufacturing facility, manufactured the TFV 1% gel and analysed/released the gels under cGMP. Patheon Inc. filled the applicators with TFV 1% gel to create pre-filled applicators and packaged each applicator and plunger in a wrapper.

3.3.2 2% Nonoxynol-9 gel

N9 gel was provided as Gynol II® (Johnson & Johnson, Fort Washington, PA, USA). Gynol II® contains 2% N9 and inactive ingredients including lactic acid, methylparaben, povidone, propylene glycol, purified water, sodium carboxymethyl cellulose, sorbic acid and sorbitol sodium. N9 is a non-ionic surfactant. It is the active ingredient in many commonly used over-the-counter contraceptive preparations (gels, creams, foams, films, sponges and suppositories) in the United States and worldwide. DPT Laboratories Ltd (San Antonio, TX, USA) filled the applicators with 2% N9 to create pre-filled applicators and Patheon, Inc., packaged each applicator and plunger in a wrapper. Each applicator contained approximately 4.0 mL of 2% N9 gel for delivery.

3.3.3 Hydroxyethyl cellulose (HEC) placebo gel

HEC gel, sometimes called the "Universal Placebo Gel" contains hydroxyethyl cellulose as the gel thickener, purified water, sodium chloride, sorbic acid and sodium hydroxide (Tien et al., 2005). The gel is isotonic and formulated at a pH of 4.4 to avoid disrupting the normal vaginal pH and has minimal buffering capacity to avoid the inactivation of sexually transmitted pathogens. HEC, the gelling agent, is used to approximate the viscosity of other microbicide gel candidates. Each pre-filled applicator contained approximately 4 mL of placebo gel for delivery.

HEC gel was supplied by CONRAD (Arlington, VA, USA). Under direction from CONRAD, Patheon Inc., (Cincinnati, OH USA) a contract manufacturing facility, manufactured the HEC gel, and analysed/released

the gels under cGMP. Patheon Inc., filled the applicators with HEC gel to create pre-filled applicators and packaged each applicator and plunger in a wrapper.

3.4 Study procedures

The MTN-007 study included a total of five study visits and two follow-up phone calls. After obtaining informed consent all participants were screened with a thorough medical history, a targeted physical examination, a digital rectal examination, and collection of swabs for CT and GC nucleic acid amplification testing (NAAT). Urine was collected for CT/GC NAAT and for pregnancy testing in the female participants (pregnancy testing was repeated at all subsequent clinical visits). Blood was collected for safety labs (complete blood count, urea nitrogen, creatinine, ALT, and AST) and serology (syphilis, HIV-1, hepatitis B, and HSV-1/2). Participants who met the inclusion and exclusion criteria during the Screening Visit proceeded to an Enrollment Visit. At the Enrollment Visit participants were randomized, a behavioral questionnaire was administered and a rectal examination and a focused physical examination were performed. Swabs were collected for assessment of rectal microflora and quantification of cytokines/chemokines in rectal secretions. Participants then received a 120 mL Normosol-R pH 7.4 enema and effluent was collected for evidence of epithelial sloughing and a sample of feces collected for measurement of fecal calprotectin. A flexible sigmoidoscope was then inserted into the rectum and 7 biopsies were collected at 15 cm from the anal verge. A disposable anoscope was inserted into the anal canal and high resolution anoscopy (HRA) of the anorectum was performed at 16X magnification with collection of 7 rectal biopsies at 9

cm from the anal verge. Biopsies were used for histology, qRT-PCR, microarray analysis, and flow cytometry. At the Treatment 1 Visit (performed within 7-28 days of the Enrollment visit), all participants randomized to receive gel product had a single applicator of study gel inserted into the rectum. Within 30 minutes, swabs were collected for microflora and cytokines. An isotonic enema was administered and the same rectal samples, including biopsies, were collected as occurred during the Enrollment Visit. At the Treatment 2 Visit (performed at least 7 days after Treatment Visit 1) participants randomized to receive gel product were provided with 7 applicators of study product to take home and asked to insert the contents of one applicator daily for 7 days. The Final Clinic Visit occurred no more than 21 days after Treatment Visit 2 and was identical to the Enrollment Visit except that anogenital testing (CT/GC) was only performed if clinically indicated.

3.5 Statistics and data management

The statistical analysis plan for the MTN-007 study was developed in collaboration with the MTN Statistics and Data Management Center (SDMC) located within the Statistical Center for HIV/AIDS Research & Prevention (SCHARP) at the Fred Hutchinson Cancer Research Center, Seattle, WA, USA. The methodological approach was based on previous Phase 1 studies conducted by the MTN and was reviewed and approved by the US Food and Drug Administration (FDA) as the MTN-007 study was conducted under an FDA Investigational New Drug (IND) application (CONRAD IND#: 73,382).

3.5.1 Accrual, randomization, blinding, and sample size

The study planned to recruit a total of 60 RAI abstinent, HIV uninfected men and women from the three study sites (Pittsburgh, PA, USA; Boston, MA, USA; and Birmingham, AL, USA). Within each study site, twenty participants were to be enrolled and randomised to each of four study arms at a 1:1:1:1 ratio. Based on the prior studies with similar eligibility requirements, each site was expected to enroll 4 participants per month. Therefore accrual was anticipated to take approximately 5 months. The target for retention was 95% of enrolled participants over the study period. To preserve the study power in the case of discontinuation/non-adherence, additional participants would be allowed to enroll, at discretion of the protocol team, to replace participants who are discontinued or non-adherent to study product or scheduled study visits. Therefore the total sample size might exceed 60 at the end of the study.

The SDMC provided each study site with a series of numbered, sealed envelopes containing the randomization assignment for each participant. The envelopes were to be assigned sequentially by site staff. Each participant was assigned a product code number. Using a blinded list of product codes and assigned products, the pharmacist at each site supplied the study product. Multiple codes were utilised to conceal and protect the randomisation assignments in this study.

Throughout the period of study implementation and data analysis, neither study staff nor participants were informed of the participants' random

assignments. Study staff and participants were unblinded after all study visits and data analyses were completed. In emergency situations, if a participant experienced a serious AE (SAE) that, in the opinion of the investigator required unblinding to protect participant safety, the investigator was able to request unblinding.

For the proposed study sample size, the statistical properties of this study in assessing the safety of study products are summarized in Table 3-2. With 15 participants in each study arm, the probability of observing zero safety events, at least one safety event, and two or more safety events are listed in Table 3-2 assuming various true event rates. For instance, if the true rate of a safety endpoint is 5%, the probability of observing that endpoint in at least one participant out of 15 participants is 0.54. A higher true event rate will result in a larger probability to observe at least one event.

Table 3-2 Power considerations for MTN-007

Event Rate	Pr(0 event/n=15)	Pr(≥ 1 event/n=15)	Pr(≥ 2 events/n=15)
1%	0.86	0.14	0.01
5%	0.46	0.54	0.17
10%	0.21	0.79	0.45
15%	0.09	0.91	0.68
25%	≤ 0.01	≥ 0.99	0.92

The statistical properties of this study may also be characterized by the width of the confidence intervals (CI) around observed event rate. Table 3-3 presents the exact 95% confidence intervals (Clopper-Pearson method) of

the estimated rate when zero, one, or two endpoints are observed among 15 participants:

Table 3-3 Confidence intervals for safety endpoints

Number of Endpoints	Lower Bound of CI	Upper Bound of CI
0	0.0%	16.1%
1	0.1%	23.8%
2	1.2%	30.4%

3.6 Data analysis plan

Descriptive statistics and graphics were used to summarize the characteristics of endpoints among the three treatment-randomized groups. For categorical variables, the numbers and the proportions were tabulated; for continuous variables, the mean, median, standard deviation, and quartiles were reported. To assess the difference of certain endpoints after a treatment phase across treatment arms, Chi-square tests were used for categorical variables with exact P-values if the expected cell count in some stratum was small; t-test or linear regression were used for continuous variables; nonparametric methods such as Wilcoxon rank-sum test were used if sample size was small and data were non-normal. Generalized linear models were used to regress continuous or categorical response variables on treatment arm, with or without adjusting for important baseline predictors. The longitudinal data combining endpoints measured at two treatment phases were analyzed using generalized estimation equations (GEE) with robust variance estimates. Baseline characteristics were

tabulated for three arms to check any imbalance of randomisation. Due to small sample size, formal comparison was not performed.

3.6.1 Primary and secondary safety analysis

For the primary safety analysis, per-protocol or modified intention to treat analysis based on the participants who have completed the baseline visit and at least one of two treatment visits were used. The rationale was that (i) the primary objective of this study was to evaluate the safety of study products. Adverse effects could only be induced by actual exposure to the study products. (ii) Due to the small sample size being planned, any missing data generated from discontinuation/non-adherence could be a serious threat to the study power. Therefore replacing participants who are discontinued /non-adherent was allowed. The number and the frequency of ≥ Grade 2 adverse events was tabulated by study arm and treatment visit. Additional safety analyses also tabulated the number and type of AEs observed overall, and by severity, site, and study arm. AEs that lead to discontinuation of product use and/or study participation were tabulated separately. At each treatment visit, the rate of safety events was compared to the baseline within the same treatment arms using McNemar's test, and the event rate was also compared across treatment arms by Chi-square test. Logistic regression was used to assess the difference of event rates across arms adjusting for baseline predictors.

3.6.2 Secondary analysis on acceptability

Consistent with the secondary study objective to evaluate the acceptability of TFV 1% gel when applied rectally, the secondary endpoint was to examine

the proportion of participants who at their Final Clinic Visit report in the Product Acceptability Questionnaire stated that they would be very likely to use the candidate microbicide during RAI. The proportion of participants who report high intentionality, operationalized as having a rating in the upper one third of the 10-point Likert scale, to use the product in the future every time they have RAI was calculated and proportions by study arm were compared using a Chi-square test with exact P-values.

Additionally, to address the secondary study objective, non-parametric tests (Kruskal-Wallis non-parametric test), were used if data were non-normal and sample sizes were unequal across the three conditions, to evaluate whether acceptability assessed at the Final Clinic Visit in the Product Acceptability Questionnaire was different by study condition. Because of insufficient statistical power to detect small or medium differences and the need to be aware of any trends, the distributions of each acceptability variable by treatment condition were also assessed. Furthermore, effect sizes were estimated to determine whether how much variance in the measure of acceptability is accounted by the treatment arms.

3.6.3 Secondary analysis on mucosal safety

The association of six sets of mucosal parameters with study products was examined. Epithelial sloughing, histopathology scores, mucosal mononuclear cell phenotype, mucosal cytokine profile, weck cel cytokine levels, and fecal calprotectin were treated as continuous measures. All six parameters were measured at baseline, after the Treatment 1 Visit and at the Final Clinic Visit.

Statistical analyses were first performed to evaluate the potential changes in immunological biomarkers after exposure to the N9. It is known that the N9 exposure induces transient rectal mucosal inflammation. Therefore the mucosal parameters, namely various histology measures, cytokines, cell phenotypes or calprotectins, were compared in biopsies sampled before and after the N9 single-dose or seven-dose administration. The control arm without gel use was included in this analysis to adjust for the within-subject fluctuation of these mucosal parameters across 3 sampling time-points. In particular, two sample t-tests (Wilcoxon rank-sum test if skewed data) was used to compare the differences of each individual parameter before and after gel use to those of the control arm. Longitudinal data modeling (GEE method) combining all three sampling time-points in both the N9 arm and the placebo arm was used to evaluate the differences of biomarkers induced by the N9 application. Despite the randomisation, the imbalance of baseline predictors could occur due to small sample size, additional regression analysis were performed adjusting for other baseline demographic factors. Those markers that were significantly associated with N9 application were considered as candidate mucosal parameters.

For the TFV and HEC placebo gel arms, the mucosal parameters were evaluated before and after the gel application, using similar statistical methods as above. If there were mucosal parameters that were changed upon microbicide gel use, these parameters would be candidate markers for mucosal damage; however if there are no difference detected, we would

determine whether there is no mucosal damage or the parameter is not a good marker of mucosal damage by an inspection of results of this parameter from the N9 arm.

3.6.4 Exploratory analyses on regional heterogeneity

To determine whether regional heterogeneity exists between mucosal endpoints, the mucosal parameters were first compared between samples from 9 cm and 15 cm at each time point, using McNemar's test if the parameter was categorical, paired *t*-test (Wilcoxon signed-rank test if skewed data) if the parameter was continuous. Mixed-effect ANOVA models were used to evaluate the heterogeneity of two sites across time with subject level modeled as a random effect. Additional analyses were performed to evaluate the correlation between mucosal biomarkers and histological abnormality across arms and three time points. At each time point, various cytokines, cell phenotypes or calprotectins were compared between groups with different levels of histological abnormality, defined by sloughing or histopathology. Longitudinal data modeling (GEE method) combining three time points were employed to evaluate the collected association of biomarkers with mucosal damage over the study period.

3.6.5 Behavioral and product adherence questionnaire

Data collected using the Baseline Behavioral Questionnaire was primarily descriptive on demographic variables, such as ethnic background, age, education, income; sexual behavior in the prior three months; behavioral practices, such as lubricant and enema use; frequency of HIV testing; and substance use in the prior three months. Associations between pre-existing

practices (i.e., lubricant use) and willingness to use a microbicidal gel was also explored.

Product adherence data was tallied by the Phone Reporting System was analyzed using repeated measures logistic regression to compare gel-use rates (the proportion of outpatient doses used of the seven, once-daily doses prescribed) between treatment arms. GEE was used to adjust for the within-subject correlations for repeated measures.

3.7 Trial oversight

The development and execution of the MTN-007 study was conducted in observance with the MTN Manual of Operational Procedures (MOP; available at http://www.mtnstopshiv.org/node/187) and guidelines from the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Oversight of the study was provided by multiple parties as illustrated in Figure 3-2 and discussed below.

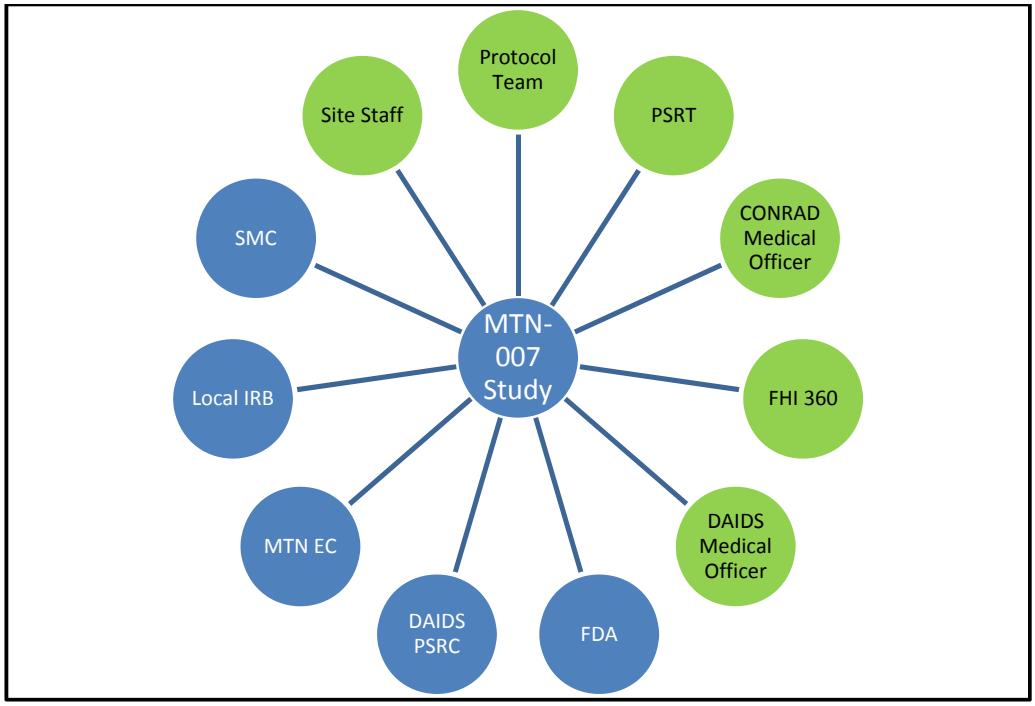


Figure 3-2 Oversight of the MTN-007 study

SMC: Study Monitoring Committee, IRB: Institutional Review Board (Ethics Committee), MTN EC: Microbicide Trials Network Executive Committee, DAIDS PSRC: DAIDS Prevention Science Review Committee, FDA: US Food and Drug Administration, FHI 360: Family Health International 360, CONRAD (www.conrad.org), PSRT: Protocol Safety Review Team

Day-to-day management of the study (illustrated in green) was conducted by the site investigators and their clinical research teams, Medical Officers from DAIDS and CONRAD, the PSRT, and FHI 360. More distant oversight was provided by the groups in blue. Specific responsibilities of the different parties are discussed below.

3.7.1 MTN-007 protocol team

The MTN-007 protocol team included the Protocol Chair (Ian McGowan MB ChB, DPhil, FRCP), the site PIs (Boston, MA, USA: Kenneth Mayer MD; Pittsburgh, PA, USA: Ross Cranston MD, FRCP; Birmingham, AL, USA:

Craig Hoesley MD), MTN Core staff (Pharmacy, Core Laboratory, and safety physicians) and representatives from FHI 360, DAIDS, the National Institute of Mental Health (NIMH), CONRAD, and SCHARP. The protocol team met by teleconference on a monthly basis during the conduct of the study and communicated through email listservs between teleconferences.

3.7.2 Protocol Safety Review Team

A Protocol Safety Review Team (PSRT) was established for the MTN-007 study and included one MTN CORE protocol safety physician (PSP), the DAIDS and CONRAD Medical Officers, the Protocol Chair, and a Clinical Affairs Safety Associate from SCHARP. The PSRT conducted routine reviews (typically by monthly conference call) of the safety-data reports produced by SCHARP. The CORE safety physician received all queries from the site staff, formulated PSRT responses to the queries and circulated these to the rest of the PSRT, issued consensus PSRT responses to the queries, and maintained documentation of the query process.

The CORE safety physician reviewed all safety-data reports and distributed a summary of each report for consideration by PSRT members prior to each PSRT conference call. Based on their review of the safety-data reports, the CORE PSP worked closely with the SCHARP Clinical Affairs Safety Associate to query the study sites for accurate, complete, and consistent AE reporting. The CORE safety physician chaired PSRT calls and led discussions regarding potential safety concerns. In the event that PSRT discussions raised questions about reported safety data, the CORE safety physician coordinated with the SCHARP Clinical Affairs Safety Associate to

query the sites for additional information. Site investigators were responsible for providing additional information to the PSRT when requested. When applicable, the CORE safety physician communicated consensus PSRT opinions or guidance to site investigators related to safety-data reporting, toxicity management, and/or study-product use management.

3.7.3 CONRAD

CONRAD is a not for profit research organization that focuses on the development of safe, acceptable, affordable products and methods that provide contraception and/or prevent the sexual transmission of HIV/AIDS and other infections (http://www.conrad.org/). Originally based at the Eastern Virginia Medical School, Norfolk, VA, USA, CONRAD now has its headquarters in Arlington, VA, USA. In December 2006, Gilead Sciences, Inc. granted rights to the International Partnership for Microbicides (IPM; http://www.ipmglobal.org) and CONRAD to develop, manufacture and, if proven efficacious, arrange for distribution in resource-limited countries of TFV gel as a microbicide to prevent infection with HIV. The MTN-007 study evaluated a reduced glycerin formulation of TFV 1% gel developed by CONRAD and the study was conducted under a CONRAD IND application (#73,382). As a consequence, CONRAD had the regulatory responsibility to keep the US FDA informed about the conduct (including emergent AEs) that occurred in the study and therefore played an active role in the MTN-007 protocol team.

3.7.4 FHI 360

The MTN partners with FHI 360, a not for profit research organization located in Durham, NC, USA that provides operational support for MTN studies conducted in the USA and in international settings (http://www.fhi360.org/). FHI 360's responsibilities include site training, monitoring of study progress, and community engagement at clinical trial sites. FHI 360 designates a Clinical Research Manager (CRM) for each MTN protocol. The CRM works with the Protocol Chair to ensure that the study is conducted to a high standard and is completed within the anticipate timeline.

3.7.5 DAIDS Medical Officer

The MTN-007 study was sponsored by DAIDS and so a DAIDS Medical Officer (MO) formed part of the protocol team. The DAIDS MO helped with development of the protocol, the DAIDS PSRC review and approval process, and assessment of emergent safety events during the study as part of the PSRT team.

3.7.6 US Food and Drug administration

The MTN-007 study was conducted under an FDA IND and so the protocol was reviewed by the FDA as part of the ongoing oversight of the TFV 1% gel IND. At the end of the study the Protocol Chair and MTN Core staff will be required to generate an ICH compliant Clinical Study Report (CSR) that formally describes the conduct and results from the MTN-007 study.

3.7.7 DAIDS Prevention Science Review Committee

The DAIDS PSRC is an NIH multidisciplinary committee that reviews all clinical protocols submitted by DAIDS sponsored investigators for their

scientific and ethical merits. The DAIDS PSRC can request changes to the protocol if they feel that they are required and the review process routinely generates questions and comments that have to be addressed by the protocol team before the protocol can be finalized.

3.7.8 MTN Executive Committee

All MTN studies are reviewed at the monthly MTN EC meetings. This allows the conduct of the study to be monitored by a group of extremely experienced clinical investigators who can provide advice and support to address any emerging study issues.

3.7.9 Local Institutional Review Boards

Local IRBs are required to review and approve MTN protocols before trials can be activated for screening and enrollment. FHI 360 works with the sites to facilitate IRB submission, review, and approval of MTN protocols.

3.7.10 Study Monitoring Committee

The MTN uses a DSMB to provide independent oversight of Phase 2B/3 effectiveness studies. For smaller Phase 1/2 studies such as MTN-007 DSMBs are not routinely used. However, the MTN Core does use a Study Monitoring Committee (SMC) to provide oversight of Phase 1/2 studies. The SMC is comprised of the SMC Chair and staff from the MTN CORE, FHI 360, Network Laboratory, SCHARP, and DAIDS. In addition, external experts can be asked to join the committee to provide additional expertise related to the study if requested by the SMC and Protocol Chair.

The SMC provides peer review of the conduct of most MTN studies, with an emphasis on key performance indicators such as participant accrual and retention, protocol and intervention adherence, data quality, and laboratory quality. For each protocol, requirements for the SMC review are contained within the protocol. For studies not subject to DSMB review, the SMC also reviews participant safety data. Studies are typically reviewed at an interval determined in accordance with the SMC (unless the SMC Chair waives review). The review schedule for each study is established by the SMC Chair, in consultation with other SMC members, based on a number of factors, including the study design, duration of participant accrual and follow-up periods, and prior review findings. SMC reviews that take place via conference call may be conducted in two sessions:

- In a closed session, SMC members and authorized observers discuss the SMC report and other materials submitted for review.
- In an open session, the Protocol Chair(s) join the SMC to clarify issues and answer questions. Other Protocol Team representatives also may be invited to join the open session, if requested by the SMC Chair or Protocol Team.

Some SMC reviews include a closed safety data review. Typically, this type of review is conducted for randomized and/or multi-cohort studies that are not subject to DSMB review. Closed safety data reviews are scheduled by the SDMC to take place immediately preceding full SMC reviews and are restricted to voting SMC members and the Protocol Statistician. The SDMC

distributes the closed safety data report to voting SMC members just prior to the SMC review. No written summary of the safety review is prepared; however, the SMC Chair communicates review findings to Protocol Team representatives during the open session of the full SMC review, and this discussion is summarized in the written summary of the full SMC review. For non-randomized and single cohort studies that are not subject to DSMB review, safety data is included in the main (open) SMC report and is reviewed as part of the full SMC review (with SMC members and authorized observers present).

Chapter 4

Overall Safety and Acceptability Results

4 Overall safety and acceptability results

4.1 Study conduct

The MTN-007 study began screening in October 2010 and completed follow-up visits in July 2011. Specific dates of site activation, first screening, first enrollment, final enrollment, and final follow-up for each site are shown in Table 4-1.

Table 4-1 Key dates for the MTN-007 study

Clinical Research Site	Site Activation Date	First Screening Visit	First Enrollment Date	Final Enrollment Date	Final Follow-up Date
Pittsburgh, PA	October 8, 2010	October 13, 2010	October 28, 2010	April 20, 2011	May 17, 2011
Birmingham, AL	October 14, 2010	October 20, 2010	November 8, 2010	June 20, 2011	July 20, 2011
Boston, MA	October 22, 2010	November 8, 2010	November 30, 2010	May 23, 2011	July 7, 2011

Table 4-2 Screening and enrollment by site

Site	Total Number Screened	Screen Failures	Total Number Enrolled	Total Number Replacements Enrolled	Total Number Completed
Pittsburgh, PA	26	5	21	1	21
Birmingham, AL	33	10	23	3	23
Boston, MA	43	22	21	1	21
Total	**102**	**37**	**65**	**5**	**65**

4.1.1 Screening and enrollment

Screening and enrollment data by site and arm are shown in the Tables 4-2 and 4-3. Sixty-five participants were enrolled including five replacement participants. Three replacement participants were needed at the Birmingham, AL site. Two gel arm participants were replaced since they used less than five doses of gel, and another no-treatment arm participant was replaced since her Final Clinic Visit anoscopy/sigmoidoscopy was not completed. One replacement participant was needed at the Boston, MA site since he used less than five doses of gel. Likewise, one replacement participant was needed at the Pittsburgh, PA site since he used less than five doses of gel. The accrual process was completed in approximately eight months from the start of the study. All 65 participants were accrued and completed study follow-up as expected.

Table 4-3 Screening and enrollment by arm

Arm	Total Number Screened	Screen Failures	Total Number Enrolled	Total Number Replacements Enrolled	Total Number Completed
Tenofovir 1% gel	NA	NA	16	1	16
2% Nonoxynol-9 gel	NA	NA	17	2	17
Placebo gel	NA	NA	16	1	16
No treatment	NA	NA	16	1	16
Total	**102**	**37**	**65**	**5**	**65**

4.2 Baseline demographics

Tables 4-4 and 4-5 show the distribution of age, gender, Hispanic origin and race of the enrolled participants by site and arm, respectively. The average age of participants was 35.7 years, with a median age of 34.0 years. Thirty-

one percent of participants were females, with six (38%), four (24%), four (25%), and six (38%) in the TFV gel, N9 gel, HEC placebo gel, and no gel arms, respectively. Nine percent of participants reported that they were of Hispanic origin, 68% reported they were White, 17% reported they were Black or African American. Asian race accounted for 5%, Native Hawaiian or Other Pacific Islander accounted for 2% and other race accounted for the remaining 9%.

Table 4-4 Baseline demographics by site

	All sites	**Pittsburgh**	**Birmingham**	**Boston**
Age				
• N	**65**	21	23	21
• Mean (STD)	**35.7 (11.0)**	38.9 (11.8)	32.8 (9.1)	35.6 (11.7)
• Median	**34.0**	35.0	32.0	38.0
• Min, Max	**18.0, 61.0**	29.0, 47.0	23.0, 41.0	25.0, 45.0
• 25^{th}, 75^{th} percentile	**27.0, 45.0**	29.0, 47.0	23.0, 41.0	25.0, 45.0
Gender				
• Male	**45 (69%)**	18 (86%)	9 (39%)	18 (86%)
• Female	**20 (31%)**	3 (14%)	14 (61%)	3 (14%)
Latino or of Hispanic origin				
• Yes	**6 (9%)**	1 (5%)	1 (4%)	4 (19%)
• No	**59 (91%)**	20 (95%)	22 (96%)	17 (81%)
Race				
• American Indian or Alaska Native	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• Asian	**3 (5%)**	0 (0%)	1 (4%)	2 (10%)
• Black or African American	**11 (17%)**	4 (19%)	5 (22%)	2 (10%)
• Native Hawaiian or	**1 (2%)**	0 (0%)	1 (4%)	0 (0%)
• other Pacific Islander				
• White	**44 (68%)**	16 (76%)	15 (65%)	13 (62%)
• Other	**6 (9%)**	1 (5%)	1 (4%)	4 (19%)

Table 4-5 Baseline demographics by arm

	All arms	TFV gel	N9 gel	HEC gel	No Rx
Age					
• N	**65**	16	17	16	16
• Mean (STD)	**35.7 (11.0)**	35.3 (8.7)	37.0 (9.9)	36.8 (12.6)	33.5 (13.0)
• Median	**34.0**	35.0	32.0	34.0	32.0
• Min, Max	**18.0, 61.0**	22.0, 48.0	25.0, 57.0	20.0, 57.0	18.0, 61.0
• 25^{th}, 75^{th} percentile	**27.0, 45.0**	28.5, 42.5	29.0, 45.0	27.0, 46.0	22.5, 43.0
Gender					
• Male	**45 (69%)**	10 (63%)	13 (76%)	12 (75%)	10 (63%)
• Female	**20 (31%)**	6 (38%)	4 (24%)	4 (25%)	6 (38%)
Latino or of Hispanic origin					
• Yes	**6 (9%)**	1 (6%)	2 (12%)	2 (13%)	1 (6%)
• No	**59 (91%)**	15 (94%)	15 (88%)	14 (88%)	15 (94%)
Race					
• American Indian or Alaska Native	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Asian	**3 (5%)**	1 (6%)	1 (6%)	0 (0%)	1 (6%)
• Black or African American	**11 (17%)**	3 (19%)	2 (12%)	5 (31%)	1 (6%)
• Native Hawaiian or other Pacific Islander	**1 (2%)**	0 (0%)	0 (0%)	0 (0%)	1 (6%)
• White	**44 (68%)**	10 (63%)	13 (76%)	9 (56%)	12 (75%)
• Other	**6 (9%)**	2 (13%)	1 (6%)	2 (13%)	1 (6%)

4.2.1 Study retention

Tables 4-6 and 4-7 show the retention of participants for each study visits by site and arm, respectively. The proportion retained at a scheduled visit was obtained by dividing the number of participants who completed the visit by the number of participants expected for that visit. The MTN-007 study targeted ≥ 95% retention by site and by arm and this metric was achieved in the study.

Table 4-6 Study retention by site

Site	Treatment 1	Treatment 2	Final Clinic Visit
Pittsburgh, PA	100%	100%	100%
Birmingham, AL	96%	96%	91%
Boston, MA	100%	95%	95%
All sites	**98%**	**97%**	**95%**

Table 4-7 Study retention by arm

Arm	Treatment 1	Treatment 2	Final Clinic Visit
Tenofovir 1% gel	100%	94%	94%
2% Nonoxynol-9 gel	100%	94%	94%
Placebo gel	100%	100%	100%
No Gel	94%	100%	94%
All arms	**98%**	**97%**	**95%**

4.2.2 Study termination

The last study participant was terminated on July 20, 2011 at the Birmingham, AL site. Sixty-one (94%) of participants terminated at their scheduled exit visit. One participant enrolled in the TFV gel arm from the Birmingham, AL site and another participant enrolled in the N9 gel arm from the Boston, MA site refused further participation. One participant enrolled in the No Gel arm from the Birmingham, AL site was terminated since the study staff could not contact her. One participant enrolled in the HEC placebo gel arm from the Boston, MA site was terminated because he was incarcerated.

4.2.3 Visit adherence: completion of procedures

Tables 4-8 and 4-9 summarize visit adherence by completion of required and expected procedures, by arm and site, for participant-visits for the following: (i) rectal exams collected including perianal visual examination and digital rectal examination; (ii) anoscopy and sigmoidoscopy results; (iii) pregnancy tests; (iv) laboratory tests; (v) specimen storage; and (vi) Computer Assisted Self-Interview (CASI) of the Acceptability Questionnaire. Completion of a procedure was defined for post-enrollment follow-up visit as follows:

4.2.3.1 Follow-up visits where rectal exams were required and expected

Rectal exams were required at Treatment 1, Final Clinic and Early Termination visits for all participants. For the TFV gel, N9 gel, HEC placebo gel and no gel arms, staff completed the perianal visual examination at 94%, 97%, 100% and 100% as well as the digital rectal examination at 94%, 97%, 97% and 100% of the participant-visits, respectively. For the Pittsburgh, PA, Birmingham, AL and Boston, MA sites, staff completed the perianal visual examination at 100%, 98% and 95% as well as the digital rectal examination at 100%, 95% and 95% of the participant-visits, respectively.

4.2.3.2 Follow-up visits where anoscopy and sigmoidoscopy were required and expected

Anoscopy and sigmoidoscopy were required at Treatment 1 and Final Clinic visits for all participants. For the TFV gel, N9 gel, HEC placebo gel and no gel arms, staff completed the anoscopy and sigmoidoscopy at 97%, 97%, 100% and 100% of the participant-visits, respectively. For the Pittsburgh,

PA, Birmingham, AL and Boston, MA sites, staff completed the anoscopy and sigmoidoscopy at 100%, 100% and 95% of the participant-visits, respectively.

4.2.3.3 Follow-up visits where pregnancy tests were required and expected

Pregnancy tests were required for females of childbearing potential at Treatment 1, Treatment 2, Final Clinic and Early Termination Visits and during Interim visits (as recorded on the Interim Visit CRF) where the participant visited the clinic for one of the following reasons:

- In-person visit to report new symptoms;
- Participant needed study product; or
- Participant was returning unused study product.

For the Pittsburgh, PA, Birmingham, AL and Boston, MA sites, as well as for each arm, staff completed the pregnancy tests at 100% of the participant-visits.

4.2.3.4 Follow-up visits where laboratory tests were required and expected

Laboratory tests were required at Final Clinic and Early Termination Visits only and were based on the following:

- Full blood count: WBC, hemoglobin, hematocrit, MCV, platelets
- Differential: Neutrophils (both percentage and absolute count), lymphocytes, monocytes, eosinophils, and basophils
- Liver function tests: aspartate aminotransferase (AST) and alanine transaminase (ALT)
- Renal function tests: Creatinine, blood urea nitrogen (BUN)

- Dipstick urinalysis tests: Leukocyte esterase (LE), nitrites, protein, glucose
- HIV EIA test: If positive, the final HIV status must be confirmed on the HIV Test Results Case Report Form (CRF).

For the TFV gel, N9 gel, HEC placebo gel and no gel arms, staff completed laboratory tests at 100%, 94%, 100% and 93% of the participant-visits, respectively. For the Pittsburgh, PA, Birmingham, AL and Boston, MA sites, staff completed laboratory tests at 100%, 100% and 90% of the participant-visits, respectively.

4.2.3.5 Follow-up visits where specimen storage was required and expected

Specimen storage was required at Treatment 1 and Final Clinic Visits only and was based on the following:

- Rectal swabs for microflora
- Rectal sponge specimens
- Rectal lavage fluid
- Anoscopic biopsies for:
    - Histology
    - Cytokine gene expression
    - Phenotyping of mucosal mononuclear cells
    - Gene expression (microarray)
- Sigmoidoscopy biopsies for:
    - Histology
    - Cytokine gene expression
    - Phenotyping of mucosal mononuclear cells

- Gene expression (microarray)

- Fecal sample (calprotectin)

Specimen storage (n = 12) was considered completed when the stored or collected box was marked for each specimen specified above. Specimen storage was considered partially completed when less than 12 of the stored or collected boxes specified above were marked, and not completed if all 12 of the stored or collected boxes specified above were not marked.

For the TFV gel, N9 gel, HEC placebo gel and No Gel arms, staff completed specimen storage at 81%, 67%, 97% and 77% of the participant-visits, respectively. For the Pittsburgh, PA, Birmingham, AL and Boston, MA sites, staff completed specimen storage at 76%, 98% and 66% of the participant-visits, respectively.

4.2.3.6 Follow-up visits where completion of CASI product acceptability questionnaire is required and expected

The CASI Product Acceptability Questionnaire was required at Final Clinic and Early Termination Visits only for participants in any of the gel groups. Ninety-eight percent of participants in the gel groups completed the Questionnaire at their Final Clinic Visit. One participant (2%) who did not complete the Questionnaire was in the TFV gel arm at the Birmingham, AL site.

Table 4-8 Completion of study procedures by site

	All sites	Pittsburgh	Birmingham	Boston
Perianal visual examination	**98%**	100%	98%	95%
Digital rectal examination	**97%**	100%	95%	95%
Anoscopy	**98%**	100%	100%	95%
Sigmoidoscopy	**98%**	100%	100%	95%
Pregnancy tests	**100%**	100%	100%	100%
Laboratory tests	**97%**	100%	100%	90%
Specimen storage	**80%**	76%	98%	66%
Completion of CASI	**98%**	100%	94%	100%

Table 4-9 Completion of study procedures by arm

	All arms	TFV gel	N9 gel	HEC gel	No Rx
Perianal visual examination	**98%**	94%	97%	100%	100%
Digital rectal examination	**97%**	94%	97%	97%	100%
Anoscopy	**98%**	97%	97%	100%	100%
Sigmoidoscopy	**98%**	97%	97%	100%	100%
Pregnancy tests	**100%**	100%	100%	100%	100%
Laboratory tests	**97%**	100%	94%	100%	93%
Specimen storage	**80%**	81%	67%	97%	77%
Completion of CASI	**98%**	94%	100%	100%	0%

4.2.4 Product adherence

Adherence to the study product is shown by site and arm in Tables 4-10 and 4-11, respectively. Of the 46 out of 65 participants who were randomized to the gel arms and dispensed study product, 98% completed at least 80% of the expected product use from the Treatment 2 visit to the Final Clinic Visit.

Table 4-10 Product adherence by site

	All sites	Pittsburgh	Birmingham	Boston
Participants enrolled	**65**	21	23	21
Participants randomized to gel groups	**48**	16	17	15
Participants dispensed gel product	**46**	16	15	15
Participants not dispensed product	**2**	0	2	0
Percentage of study product use				
• 0.0%	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• 0.1-49.9%	**1 (2%)**	1 (6%)	0 (0%)	0 (0%)
• 50.0-79.9%	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• ≥ 80.0%	**45 (98%)**	15 (94%)	15 (100%)	15 (100%)
Number of used applicators returned				
• 0	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• 1	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• 2	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• 3	**1 (2%)**	1 (6%)	0 (0%)	0 (0%)
• 4	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• 5	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• 6	**4 (9%)**	3 (19%)	1 (7%)	0 (0%)
• 7	**37 (80%)**	12 (75%)	13 (87%)	12 (80%)
• 8	**4 (9%)**	0 (0%)	1 (7%)	3 (20%)

Thirty-seven participants (80%) used seven applicators (the expected dose), four participants (9%) used eight applicators, four participants (9%) used six applicators and one participant (2%) used three applicators. The participant who used three applicators was enrolled in the N9 gel arm and from the Pittsburgh, PA site.

Table 4-11 Product adherence by arm

	All arms	TFV gel	N9 gel	HEC gel	No Rx
Participants enrolled	**65**	16	17	16	NA
Participants randomized to gel groups	**48**	16	16	16	NA
Participants dispensed gel product	**46**	15	16	15	NA
Participants not dispensed gel product	**2**	1	0	1	NA
Percentage of study product use					
• 0.0%	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• 0.1-49.9%	**1 (2%)**	0 (0%)	1 (6%)	0 (0%)	NA
• 50.0-79.9%	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• ≥ 80.0%	**45 (98%)**	15 (100%)	15 (94%)	15 (100%)	NA
Number of used applicators returned					
• 0	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• 1	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• 2	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• 3	**1 (2%)**	0 (0%)	1 (6%)	0 (0%)	NA
• 4	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• 5	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• 6	**4 (9%)**	1 (7%)	2 (13%)	1 (7%)	NA
• 7	**37 (80%)**	12 (80%)	12 (75%)	13 (87%)	NA
• 8	**4 (9%)**	2 (13%)	1 (6%)	1 (7%)	NA

4.2.5 Product discontinuation

The number and percentages of participants with product holds, reasons for holds, and whether or not the product holds were resumed are presented in Tables 4-12 and 4-13 by site and arm, respectively. Three participants were placed on product hold. One participant, enrolled in the HEC placebo gel arm and from the Birmingham, AL site, reported a Grade 4 adverse experience consisting of a major depression episode judged not related to study product. This participant was permanently discontinued from product

use. The other two participants resumed product use. They were from the Boston, MA site, with one participant enrolled in the TFV gel arm and the other one enrolled in the N9 gel arm.

Table 4-12 Product hold/discontinuation by site

	All sites	**Pittsburgh**	**Birmingham**	**Boston**
Participants enrolled	**65**	21	23	21
Participants with at least one product hold	**3**	0	1	2
Reasons for product hold				
• Pregnancy	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• HIV infection	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)
• Adverse event	**2 (67%)**	0 (0%)	1 (100%)	1 (50%)
• Other	**1(33%)**	0 (0%)	0 (0%)	1 (50%)
Product resumed				
• Yes	**2 (67%)**	0 (0%)	0 (0%)	2 (100%)
• No	**1 (33%)**	0 (0%)	1 (100%)	0 (0%)

Table 4-13 Product hold/discontinuation by arm

	All arms	**TFV gel**	**N9 gel**	**HEC gel**	**No Rx**
Participants enrolled	**65**	16	17	16	NA
Participants with at least one product hold	**3**	1	1	1	NA
Reasons for product hold					
• Pregnancy	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• HIV infection	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	NA
• Adverse event	**2 (67%)**	0 (0%)	1 (100%)	1 (100%)	NA
• Other	**1 (33%)**	1(100%)	0 (0%)	0 (0%)	NA
Product resumed					
• Yes	**2 (67%)**	1 (100%)	1 (100%)	0 (0%)	NA
• No	**1 (33%)**	0 (0%)	0 (0%)	1 (100%)	NA

4.2.6 Protocol deviations and enrollment violations

There was one protocol deviation identified in this study. This deviation occurred at the Boston, MA site and involved enrollment of an ineligible participant due to site staff misreading a screening urine protein result. The result, originally thought to be “trace”, was discovered to be +1 (see exclusion criterion 4i above) after the participant had been enrolled. Site staff immediately brought the participant in for retesting, at which time the urine protein result was negative. Since this participant was randomized to the “no treatment” arm, the PSRT decided that the participant should remain in follow-up for the duration of the study.

4.2.7 Study Monitoring Committee review history

There was one Study Monitoring Committee (SMC) review for the MTN 007 study that occurred on May 23, 2011. The Committee commented that they would like to see an improvement in the response time for data clarifications from the Boston site. There were no other recommendations to change the conduct of the study.

4.3 General product safety

4.3.1 Adverse events

Table 4-14 shows the incidence of adverse events by severity and by arm.

Table 4-14 Incidence and severity of adverse events by arm

	All arms	TFV gel	N9 gel	HEC gel	No Rx
N	**65**	16	17	16	16
Participants with one or more adverse events					
• Grade 1	**30 (46%)**	7 (44%)	10 (59%)	7 (44%)	6 (38%)
• Grade 2	**18 (28%)**	3 (19%)	7 (41%)	4 (25.0%)	4 (25%)
• Grade 3	**2 (3%)**	0 (0%)	0 (0%)	0 (0%)	2 (13%)
• Grade 4	**1 (2%)**	0 (0%)	0 (0%)	1 (6%)	0 (0%)
• Grade 5	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• **Total**	**51 (79%)**	10 (63%)	17 (100%)	12 (75%)	12 (75%)

Table 4-15 shows the incidence of adverse experiences and relationship to study product by arm.

Table 4-15 Incidence of adverse events and relationship to study product

	All arms	TFV gel	N9 gel	HEC gel	No Rx
N	**65**	16	17	16	16
Participants with one or more adverse					
• Not related	**22 (34%)**	3 (19%)	4 (24%)	3 (19%)	12 (75%)
• Related	**29 (45%)**	7 (44%)	13 (77%)	9 (56%)	0 (0%)
• Total	**51 (79%)**	10 (63%)	17 (100%)	12 (75%)	12 (75%)

Table 4-16 shows the total number and percentages of adverse events by severity and relationship to study product overall.

Table 4-16 Adverse event severity and relationship to study product

	Total	Related	Not Related
Severity Grade			
• Grade 1	**121 (80%)**	41 (34%)	80 (66%)
• Grade 2	**27 (18%)**	6 (22%)	21 (78%)
• Grade 3	**2 (1%)**	0 (0%)	2 (100%)
• Grade 4	**1 (1%)**	0 (0%)	1 (100%)
• Grade 5	**0 (0%)**	0 (0%)	0 (0%)
• **Total**	**151 (100%)**	47 (31%)	104 (69%)

Figure 4-1 Adverse events and relationship to product use

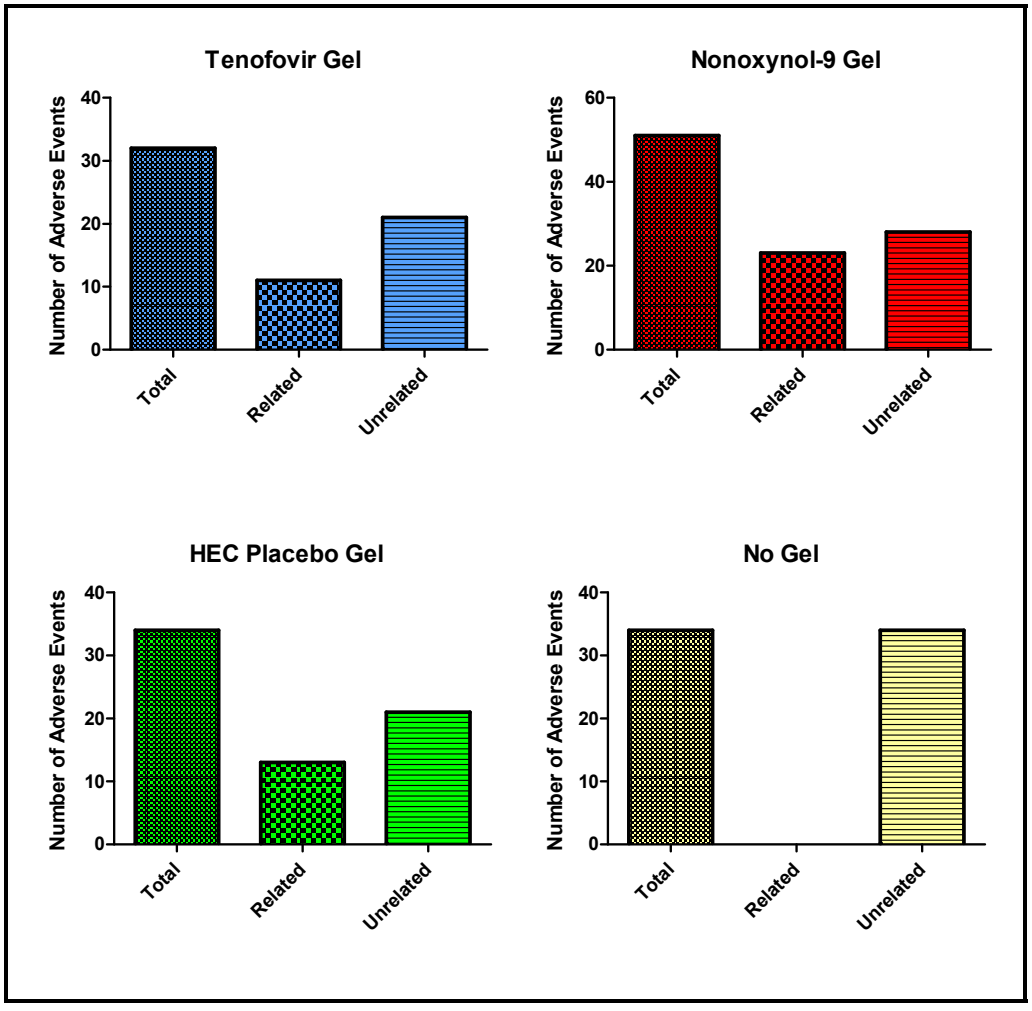


Figure 4-1 shows the number of adverse experiences and their reported relationship to study product for the TFV gel, N9 gel, HEC placebo gel and No Gel arms, respectively.

There was only one SAE in the MTN-007 study. The participant, enrolled in the HEC placebo gel arm and from the Birmingham, AL site, reported a Grade 4 adverse experience consisting of a major depression episode judged not related to study product.

4.3.2 Primary endpoints

Consistent with the primary study objective to assess the safety of TFV 1% gel when applied rectally, the following primary endpoint was assessed:

Grade 2 or higher AEs as defined by the Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events, Version 1.0, December 2004 (Clarification dated August 2009) and/or Addenda 1 and 3 (Female Genital and Rectal Grading Tables for Use in Microbicide Studies) to this table.

Table 4-17 shows the primary endpoint analyses of participants with at least one Grade 2 or higher AE from the full cohort. The full cohort included all participants that were randomized into the study, and any replacement participants. The analysis was completed for three separate time frames as follows: post-randomization up to the Treatment 2 visit, the Treatment 2 visit through the end of the study, and post-randomization through the end of the study. The table displays the total number of Grade 2 AEs out of the total

number of participants who were eligible for assessment within the time frame. Note that there were three participants who missed product dispensation at the Treatment 2 visit and so, therefore, were removed from the number of eligible participants analyzed for the Treatment 2 visit through the end of the study time frame.

Table 4-17 Primary endpoint analysis (All)

	Post-Randomization Up to Treatment Visit 2 (N=65)			**Treatment Visit 2 through end of the Study (N=62)**			**Post-Randomization through End of the Study (N=65)**		
	n/N (%)	**P-value***		**n/N (%)**	**P-value***		**n/N (%)**	**P-value***	
Arm		**HEC**	**No Rx**		**HEC**	**No Rx**		**HEC**	**No Rx**
TFV	1/16 (6.3)	0.60	1.0	2/15 (13.3)	1.0	0.65	3/16 (18.8)	0.69	0.43
N9	2/17 (11.8)	0.66	1.0	5/16 (31.3)	0.69	1.0	7/17 (41.2)	0.72	1.0
HEC	3/16 (18.8)		1.0	3/16 (18.8)		0.69	5/16 (31.3)		1.0
No Rx	2/16 (12.5)			4/15 (26.7)			6/16 (37.5)		

*P-values based on Fisher's Exact Test

Comparisons between randomization arms of TFV gel versus HEC placebo gel and No Rx, N9 gel versus HEC placebo gel and No Gel, and HEC placebo gel versus No Rx showed no statistically significant results based on the nominal p-values found using Fisher's Exact Test.

Table 4-18 shows the primary endpoint analyses of participants with at least one Grade 2 or higher AE from the ITT cohort. The ITT cohort includes only participants that were randomized into the study, excluding any replacement participants. The analysis was completed for three separate time frames as follows: post-randomization up to the Treatment 2 visit, the Treatment 2 visit through the end of the study, and post-randomization through the end of the study. The table displays the total number of Grade 2 AEs out of the total number of participants who were eligible for assessment within the time frame.

Table 4-18 Primary endpoint analysis (Intention to Treat Cohort)

	Post-Randomization Up to Treatment Visit 2 (N=60)			**Treatment Visit 2 through end of the Study (N=57)**			**Post-Randomization through End of the Study (N=60)**		
	n/N (%)	**P-value***		**n/N (%)**	**P-value***		**n/N (%)**	**P-value***	
Arm		**HEC**	**No Rx**		**HEC**	**No Rx**		**HEC**	**No Rx**
TFV	1/15 (6.7)	0.60	1.0	2/14 (14.3)	1.0	0.65	3/15 (20.0)	1.0	0.43
N9	1/15 (6.7)	0.60	1.0	5/14 (35.7)	0.21	1.0	6/15 (40.0)	0.7	1.0
HEC	3/15 (20.0)		1.0	2/15 (13.3)		0.39	4/15 (26.7)		0.7
No Rx	2/15 (13.3)			4/14 (28.6)			6/15 (40.0)		

*P-values based on Fisher's Exact Test

Note that there were three participants who missed product dispensation at the Treatment 2 visit and so, therefore, were removed from the number of eligible participants analyzed for the Treatment 2 visit through the end of the study time frame. Comparisons between randomization arms of tenofovir gel versus HEC placebo gel and no gel, N9 gel versus HEC placebo gel and no gel, and HEC placebo gel versus no gel showed no statistically significant results based on the nominal p-values found using Fisher's Exact Test.

Table 4-19 below shows primary endpoint analyses of participants with at least one Grade 2 or higher AE adjusting for the number of returned applicators. This analysis was conducted on the Treatment 2 through the end of the study time frame only for both the full and ITT cohorts.

Table 4-19 Primary endpoint analysis based on returned applicators

	Treatment Visit 2 through End of the Study			
Randomization Arm	**HEC Placebo Gel**			
	Full Cohort		**ITT Cohort**	
	OR (95% CI)	**P-value**	**OR (95% CI)**	**P-value**
Tenofovir gel	0.35 (0.03, 4.0)	0.40	0.61 (0.05, 8.3)	0.71
N9 gel	1.9 (0.36, 10.1)	0.45	3.4 (0.53, 22.3)	0.20

The HEC placebo arm was used as the reference group and was compared to TFV gel and N9 gel groups. Although not statistically significant, odds ratios showed that there was a trend toward protection with respect to Grade 2 AEs of TFV gel compared to HEC placebo gel in the full and ITT cohorts. Conversely, but also not statistically significant, odds ratios showed that

there was a trend toward harm from N9 with respect to Grade 2 AEs compared to HEC placebo gel.

Table 4-20 Rectal examination findings at the enrollment visit by arm

	All arms	TFV gel	N9 gel	HEC gel	No Rx
N	**65**	16	17	16	16
Findings from the enrollment perianal examination					
Normal	**57 (88%)**	15 (94%)	15 (88%)	13 (81%)	14 (88%)
Abnormal	**8 (12%)**	1 (6%)	2 (12%)	3 (19%)	2 (13%)
• Warts	**2 (3%)**	0 (0%)	0 (0%)	0 (0%)	2 (13%)
• Fissure	**1 (2%)**	0 (0%)	0 (0%)	1 (6%)	0 (0%)
• Ulceration	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Pigmentation	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Haemorrhoids	**1 (2%)**	0 (0%)	1 (6%)	0 (0%)	0 (0%)
• Skin tags	**4 (6%)**	1 (6%)	1 (6%)	1 (6%)	1 (6%)
• Leukoplakia	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Fistula	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Petechiae	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Purpura	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Ecchymosis	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Other abnormal findings	**1 (2%)**	0 (0%)	0 (0%)	1 (6%)	0 (0%)
Findings from the enrollment digital rectal exam					
Normal	**62 (95%)**	13 (81%)	17 (100%)	16 (100%)	16 (100%)
Abnormal	**3 (5%)**	3 (19%)	0 (0%)	0 (0%)	0 (0%)
• Palpable warts	**1 (33%)**	1 (33%)	0 (0%)	0 (0%)	0 (0%)
• Sphincter defect	**1 (33%)**	1 (33%)	0 (0%)	0 (0%)	0 (0%)
• Anal canal mass	**1 (33%)**	1 (33%)	0 (0%)	0 (0%)	0 (0%)

4.3.3 Rectal examination

Table 4-20 shows the number and percentages of rectal exam findings reported by arm at the enrollment visit. Table 4-21 shows the number and percentages of new rectal exam findings reported after enrollment by arm.

Table 4-21 New rectal examination findings after enrollment

	All arms	TFV gel	N9 gel	HEC gel	No Rx
N	**64**	16	17	16	15
New perianal findings after the enrollment examination					
New findings	**2 (3%)**	0 (0%)	0 (0%)	2 (13%)	0 (0%)
• Warts	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Fissure	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Ulceration	**1 (2%)**	0 (0%)	0 (0%)	1 (6%)	0 (0%)
• Pigmentation	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Haemorrhoids	**1 (2%)**	0 (0%)	0 (0%)	1 (6%)	0 (0%)
• Skin tags	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Leukoplakia	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Fistula	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Petechiae	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Purpura	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Ecchymosis	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Other abnormal findings	**0 (0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
New digital examination findings after the enrollment examination					
Normal	**62 (95%)**	13 (81%)	17 (100%)	16 (100%)	16 (100%)
Abnormal	**3 (5%)**	0 (0%)	2 (12%)	1 (6%)	0 (0%)
• Increased mucosal granularity	**1 (2%)**	0 (0%)	1 (6%)	0 (0%)	0 (0%)
• Haemorrhoids	**1 (2%)**	0 (0%)	1 (6%)	0 (0%)	0 (0%)
• Circumferential discomfort	**1 (2%)**	0 (0%)	0 (0%)	1 (6%)	0 (0%)

Two participants enrolled in the HEC placebo gel arm from the Pittsburgh, PA site had new rectal exam findings from the perianal examination with one having ulceration and the other one having hemorrhoids. Three participants had new rectal exam findings from the digital rectal examination.

The first participant was enrolled in the N9 gel arm from the Boston, MA site and was reported to have increased granularity to fingertip at right lateral

and left lateral area of the anorectal canal. The second participant was enrolled in the N9 gel arm from the Pittsburgh, PA site and had hemorrhoids felt in the anal canal. The third participant was enrolled in the HEC placebo gel arm from the Pittsburgh, PA site and had circumferential discomfort.

Gastrointestinal adverse events occurring in more than 10% of participants (approximately equal or greater than 2 participants per arm) are summarized in Table 4-22.

Table 4-22 Incidence of gastrointestinal adverse events by arm

	TFV gel	N9 gel	HEC gel	No Rx	All Arms
N	16	17	16	16	65
Abdominal pain lower					
• Grade 1	2 (12.5%)	0 (0%)	1 (6.3%)	0 (0%)	3 (4.6%)
• Grade 2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Defecation urgency					
• Grade 1	0 (0%)	5 (29.4%)	1 (6.3%)	0 (0%)	6 (9.2%)
• Grade 2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Diarrhoea					
• Grade 1	1 (6.3%)	3 (17.6%)	1 (6.3%)	2 (12.5%)	7 (10.8%)
• Grade 2	0 (0%)	1 (5.9%)	0 (0%)	0 (0%)	1 (1.5%)
• Grade 3	0 (0%)	0 (0%)	0 (0%)	1 (6.3%)	1 (1.5%)
• Grade 4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Flatulence					
• Grade 1	6 (37.5%)	2 (11.8%)	1 (6.3%)	3 (18.8%)	12 (18.5%)
• Grade 2	0 (0%)	0 (0%)	1 (6.3%)	0 (0%)	1 (1.5%)
• Grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Painful defecation					
• Grade 1	0 (0%)	2 (11.8%)	0 (0%)	0 (0%)	2 (3.1%)
• Grade 2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Proctalgia					
• Grade 1	1 (6.3%)	2 (11.8%)	0 (0%)	0 (0%)	3 (4.6%)
• Grade 2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Post procedural diarrhoea					
• Grade 1	2 (12.5%)	0 (0%)	0 (0%)	0 (0%)	2 (3.1%)
• Grade 2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Grade 4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

The majority of events were either Grade 1 or 2 with only one Grade 3 event (diarrhoea) seen in a No Rx participant. Most events were more common in

the N9 gel arm with the exception of flatulence which was more common in the TFV gel recipients.

4.4 Product acceptability

There were 47 participants who completed the CASI, and 46 with valid responses to the secondary endpoint question as follows: If a rectal microbicide were available that provided some protection against HIV, and it looked like the gel you have used in this study, how likely would you be to use it (a microbicidal gel) every time you have RAI? In the TFV gel group 13/15 (86.7%), N9 gel group 10/16 (62.5%), and HEC placebo gel group 14/15 (93.3%) reported high intentionality, operationalized as having a rating in the upper one third of a 10-point Likert scale (values of 7-10), to use the product in the future every time they have RAI. The overall comparison across groups using Fisher's Exact Test showed a p-value of 0.11. Pairwise comparisons between the TFV gel group versus the HEC placebo gel group, and the N9 gel group versus the HEC placebo gel group showed nominal p-values of 1.0 and 0.08, respectively.

4.5 Discussion

In the HIVNET-008 trial of N9, Tabet et al. enrolled a seroconcordant population of MSM who underwent a complex dose escalation evaluation of an N9 gel. Couples were also allowed to be sexually active (Tabet et al., 1999). Subsequent Phase 1 studies of antiretroviral rectal microbicides (RMP-01 and RMP-02/MTN-006) have adopted a more conservative study design in which participants are sexually abstinent, have a baseline evaluation, receive a single dose of study product followed by a recovery

period, and then seven daily doses. Comprehensive evaluation of safety and acceptability occurs after the single and repeated dosing periods. The MTN-007 followed this study design but, in contrast to the RMP-01 and RMP-02/MTN-006 studies, did not include PK or PD assessments as it was felt these data had already been collected in the RMP-02/MTN-006 study of TFV gel.

Similar to the RMP-01 and RMP-02/MTN-006 studies, the MTN-007 study recruited a population with a mean age of 35.7 years of whom 69% were men and 31% were women. All participants had a history of receptive anal intercourse. The fact that almost one third of the participants recruited in to the MTN-007 study were women highlights the need to develop rectal microbicides for both men and women. The majority of participants in the MTN-007 study were white (68%). Racial disparity in study recruitment is a common feature of US clinical trials (Colon-Otero et al., 2008) but particularly unfortunate in the context of domestic HIV prevention trials as the current US HIV epidemic is focused on the African American community. Project Gel (described in Section 2.8) is one attempt to conduct a rectal microbicide safety and acceptability study in young ethnic minority MSM.

Study retention was excellent (>94%) as is usual for Phase 1 studies. Data quality, judged by the number of quality control (QC) notes sent to the site, was good and equivalent across all three sites (range 4.6 to 7.1 per 100 records). All QCs were resolved prior to the database lock. The original target of 60 participants (15 per study arm) was expanded to 65 to ensure

that there were 60 participants who had evaluable mucosal safety samples. The primary reason for participants being dropped from the study was because they had used less than 5 doses of the study product (4/5). One participant failed to have the final mucosal sampling studies and was therefore replaced.

Product adherence was measured using a phone reporting system (PRS). Participants were asked to use the PRS after each episode of gel use Adherence was calculated using PRS data (Leu et al., 2013). Based on these data, 98% of study participants reported using at least 80% of the expected product doses. Recent failures in HIV prevention trials have documented a significant gap between reported accounts of adherence and actual levels of product adherence based on objective measures such plasma PK. In the FEM-PrEP study of Truvada, 95% of the participants reported that they had usually or always taken the assigned drug. However, among women who seroconverted in the study, PK assessment suggested that only 26% of the women had taken the drug in the last 48 hours (Van Damme et al., 2012). Similar results have been seen in other PrEP studies such as VOICE study (van der Straten et al., 2012). One limitation of the MTN-007 study is that product adherence was based on self-report, albeit using a sophisticated PRS and PK data are not available to substantiate, or refute, reported adherence. The HIV prevention field is rapidly moving to develop novel objective markers of behavior, including adherence. Studies are beginning to incorporate “real time” PK monitoring into study design. This provides the opportunity, at least in open label studies, to provide

participants with feedback on how adherent they have been but also raises ethical and operational issues about what you do with non-adherent participants. Another approach, that will be used in a future MTN Phase 1 PK study of vaginal and rectal TFV gel (MTN-014), is for the participants to administer study product under supervision (directly observed therapy or DOT). With regards to topical PrEP, this is really only feasible for Phase 1 studies, but DOT has been used in a recently completed study of oral PrEP in injection drug users in Thailand (Choopanya K et al., Lancet 2013).

A primary goal of the MTN-007 study was to determine whether a reduced glycerin formulation (RG)-TFV gel had an improved safety and acceptability profile compared to the TFV gel formulation used in the RMP-02/MTN-006 study. Based upon the primary safety endpoint (the number participants with a Grade 2 or higher AE), there was no significant difference seen across the four arms of the study. However, there did seem to be more frequent gastrointestinal AEs in the N9 arm (see Table 4-22). The one exception was flatulence which was more common in the TFV gel arm. Interestingly, there was a significant increase in diarrhea among women receiving TFV gel in the CAPRISA 004 study (Sokal et al., 2013). Since TFV is known to cross from vaginal to rectal compartments (Nuttall et al., 2012), it is possible that this gastrointestinal AE is TFV-related. Overall, the prevalence of gastrointestinal symptoms with the RG-TFV gel appeared lower than the levels seen in the RMP-02/MTN-006 study (Table 4-23).

Table 4-23 GI adverse events in MTN-007 and RMP-002/MTN-006

Symptom (%)	RG-TFV gel used in MTN-007	TFV gel used in RMP-02/MTN-006
Abdominal pain	16%	50%
Rectal urgency	0%	42%
Bloating	0%	42%
Nausea	0%	33%
Diarrhea	6%	58%
Flatulence	38%	25%
Proctalgia	6%	0%

It is presumed that the improved safety profile seen with the RG-TFV gel relates to its lower osmolality compared to the TFV gel used in the RMP-02/MTN-006 study (836 vs. 3,111 mOsmol/kg). The safety and acceptability data seen in the MTN-007 study have allowed the RG-TFV gel to advance to Phase 2 testing in the MTN-017 study. However, an even lower osmolar formulation of TFV gel (479 mOsmol/kg) will be evaluated in the CHARM-01 study (Section 2.9).

One concern raised about the design of Phase 1 vaginal microbicide trials is that they enroll too few participants who are then followed for an insufficient period of time to fully evaluate the safety and acceptability profile of novel candidate microbicides (Poynten et al., 2009). However, in the context of rectal microbicide development, the sample size on active product in the RMP-02/MTN-006 study (N = 12) was sufficient to recognise that the formulation was not optimal for rectal use. Whether this sample size is sufficient to evaluate changes in mucosal safety biomarkers, where multiple

comparisons are routinely undertaken, is discussed in Chapter 6 of this thesis.

4.6 Summary

The Phase 1 MTN-007 study enrolled 65 participants and demonstrated that a RG-TFV gel, in comparison to a placebo gel and a no treatment arm, was both safe and acceptable for rectal use. It is possible that conventional parameters such as reported adverse events or acceptability are insufficient to exclude product-associated perturbation of mucosal tissue that might, over an extended period of time, result in changes that could increase the risk of HIV infection in participants using these products. As a consequence, the MTN-007 study was designed to include a portfolio of exploratory mucosal biomarker studies (described in Chapters 5 and 6) that might provide preliminary evidence of product associated changes in mucosal biology. These changes could be further evaluated in Phase 2 studies where the study sample size and duration of product exposure might provide more robust data on the interactions between antiretroviral microbicides and the intestinal mucosa.

Chapter 5

Mucosal Toxicity Assays Used in

MTN-007

5 Mucosal safety assays used in MTN-007

5.1 Introduction

The rectal compartment is highly vulnerable to HIV transmission. A single layer of columnar epithelium separates the intestinal lumen from the lamina propria. The lamina propria is populated with a broad range of HIV target cells including macrophages, dendritic cells, and activated CD4+ T lymphocytes expressing the CCR5 and CXCR4 HIV-1 coreceptors (Poles et al., 2001; Grivel et al., 2007). It is likely that the immune composition of the rectal mucosa is at least partially responsible for the 10-20 fold increased risk of HIV transmission associated with URAI compared to unprotected vaginal intercourse (UVI) (Baggaley et al., 2010; Boily et al., 2009). Any product or process, including STIs, that induces local inflammation is likely to further increase this risk by recruiting and/or enhancing activation of immune target cells in the rectal mucosa. It has been shown that HSV infection, a significant cause of anorectal mucosal ulceration / inflammation, increases the risk of HIV infection in MSM. Lama et al. demonstrated that HSV-2 seroprevalence in HIV seropositive Peruvian MSM was 80.5% compared to 40.8% in HIV seronegative MSM (Lama et al., 2006). This effect may be mediated through the persistence and enrichment of HIV-receptor positive inflammatory cells in the anorectum following HSV infection (Zhu et al., 2009) and/or the presence of HSV-associated $\alpha_4\beta_7^{hi}$ CD4 positive T cells in the rectal mucosa (Martinelli et al., 2011). Another possible example of mucosal inflammation was seen in the STEP trial that evaluated an adenovirus vector HIV vaccine in high risk men and women (Buchbinder et al., 2008). An unanticipated outcome of the study was the finding that

uncircumcised, adenovirus antibody positive, MSM who received the HIV vaccine had an increased risk of HIV acquisition compared to the placebo recipients. Although the mechanism underlying this phenomenon was unclear, the study authors postulated that one possible explanation was that vaccine recipients with previous adenovirus exposure had an amnestic vaccine response that resulted in accumulation of HIV target cells in the foreskin and/or rectal mucosa (Duerr et al., 2012). Collectively, these observations suggest that an STI, vaccine, or microbicide that induces mucosal inflammation and/or recruitment of target cells to the rectal mucosa is likely to increase vulnerability to HIV infection. It is therefore critical that all candidate rectal microbicides are carefully evaluated for potential mucosal toxicity.

Methods to assess microbicide induced toxicity in the rectal compartment are in a state of evolution. Mucosal changes may be subtle and require new modes of detection above and beyond the collection of adverse event data and the histological assessment of rectal biopsies following exposure to microbicide candidates. Based on experience from the vaginal microbicide development pathway, possible new approaches have included (i) collection of rectal swabs for the quantification of locally released cytokines and chemokines, (ii) extraction of RNA from biopsies for the characterization of the expression of selected genes associated with mucosal inflammation, and (iii) the isolation of mucosal mononuclear cells (MMCs) from rectal biopsies for flow cytometric assessment of GALT T cells. Prior to integrating these types of assays into rectal microbicide trials, the HIV Prevention Trial

Network (HPTN) sponsored the HPTN-056 study "Characterization of baseline mucosal indices of injury and inflammation in men for use in rectal microbicide trials" to explore the biological variability of selected mucosal parameters that might be relevant for rectal microbicide development (McGowan et al., 2007). The HPTN-056 study enrolled 16 men (8 HIV-seronegative (4 with a history of RAI and 4 who did not practice RAI) and 8 HIV-1 seropositive men (4 with a plasma HIV-1 RNA viral load of ≥ 10,000 copies/mL and 4 with a plasma HIV-1 RNA viral load < 50 copies/mL)) who underwent flexible sigmoidoscopy on three occasions separated by 2 week intervals. Rectal biopsies were collected at 10 and 30 cm from the anal verge. Swabs were also collected to quantify mucosal immunoglobulins. The range of assays conducted in HPTN-056 study is summarized in Table 5-1.

Table 5-1 HPTN-056 mucosal assays

Assay	Analytes
Histology	Qualitative and quantitative
RT-PCR for mucosal cytokine mRNA	RANTES, IFN-γ, IL-10
Mucosal immunoglobulins	IgG total and IgA total
MMC flow cytometry	CD3+, CD4+, CD8+, CD19+, CD16+, CD56+, CD4+/CCR5+, CD4+/CXCR4+, CD4+/CCR5+/CXCR4+, CD4+/CD38+, CD8+/CD38+, CD4+/HLA-DR+, CD8+/HLA-DR+, HLA-DR+/DCSIGN-

RT-PCR: reverse transcription polymerase chain reaction; RANTES: regulated and normal T cell expressed and secreted

Mixed linear models were used to characterize the stability of these parameters. Parameters with an intraclass correlation (ICC) of > 0.7 (strong stability over time) included T cell phenotypic markers and cytokine mRNA.

In contrast, quantitative histology and mucosal immunoglobulins (IgG) had ICC scores < 0.35 and have not been included in future rectal microbicide trials.

5.2 Histological assessment of rectal biopsies

5.2.1 Introduction

Histopathological assessment of intestinal tissue is a routine method of demonstrating mucosal abnormality associated with gastrointestinal diseases such as ulcerative colitis, Crohn's disease, and gluten enteropathy (coeliac disease). In general, mucosal change in these diseases can be quite dramatic whereas microbicide-induced changes may be quite subtle.

5.2.2 Previous studies

The HIVNET 008 rectal safety study of N9 employed a simple scoring system of normal, slightly abnormal (focal, mild acute inflammation), or abnormal (an intense inflammatory infiltrate, reactive changes (increased mitotic activity and enlarged nuclei)) (Tabet et al., 1999). Using this histological system, 69% of the placebo recipients and 89% of the N9 recipients had slightly abnormal or abnormal rectal biopsies. In contrast, there were no significant differences in rectal histology (using a qualitative scoring system discussed in Section 5.2.3) noted in the RMP-01 study of UC781 and the RMP-02/MTN-006 study of TFV gel (Anton et al., 2011; Anton et al., 2012).

5.2.3 Methods

A qualitative scoring system developed by the inflammatory bowel disease community (Geboes et al., 2000) and adapted for use in the HPTN 056 study (McGowan et al., 2007) was used to assess potential microbicide induced mucosal injury in MTN-007 (summarised in Table 5.2 and illustrated in Figure 5.1). It was hoped that the scoring system developed for the HPTN 056 study might provide better discrimination between abnormal and normal histology. Intestinal biopsies collected at 9 cm and 15 cm from the anal verge were placed in 10% formalin and stored at room temperature prior to being orientated and embedded in paraffin. 7 μm sections of rectal tissue were then cut from the block and two ribbons placed on each slide, deparaffinised, and stained with haematoxylin and eosin (H & E).

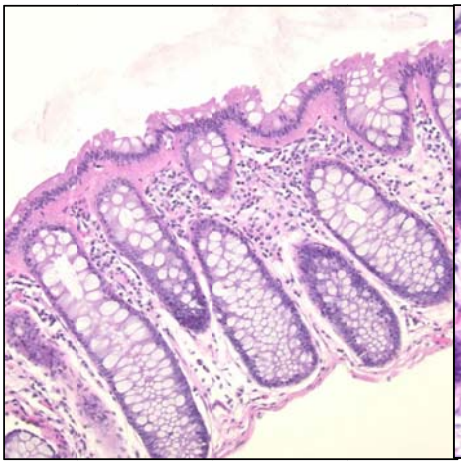
A pathologist with specialist training in gastrointestinal pathology then reviewed the H & E slides. Four slides (up to 32 tissue sections) from the rectal biopsies were graded at low power (40-100X magnification) with the inflammatory cells examined at higher power (200 or 400X magnification). A score was assigned based upon the highest degree of abnormality seen in the samples.

Table 5-2 MTN-007 histopathology scoring system

Histology Grade
Grade 0 No abnormality
Grade 1 Mononuclear cell infiltrate
Grade 2 Neutrophilic infiltrate in the lamina propria
Grade 3 Neutrophilic infiltrate in the epithelium
Grade 4 Crypt destruction
Grade 5 Erosion or ulceration

Figure 5-1 Histological scoring system

Grade 0



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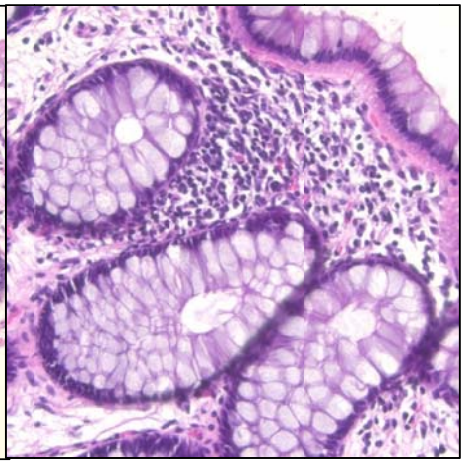

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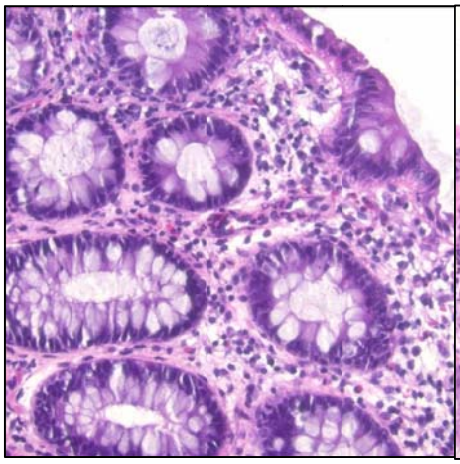

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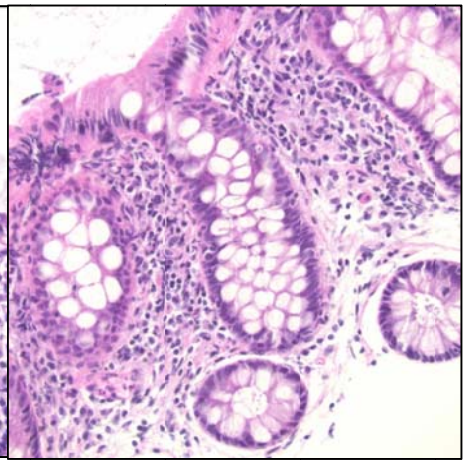

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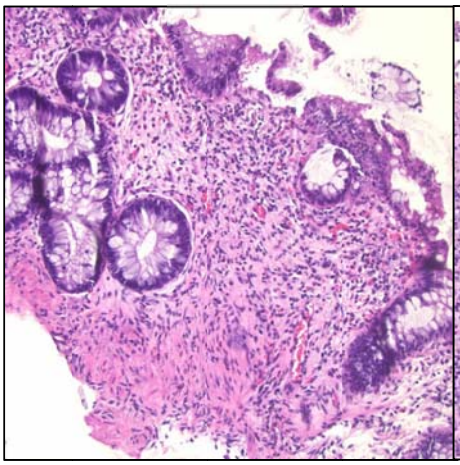

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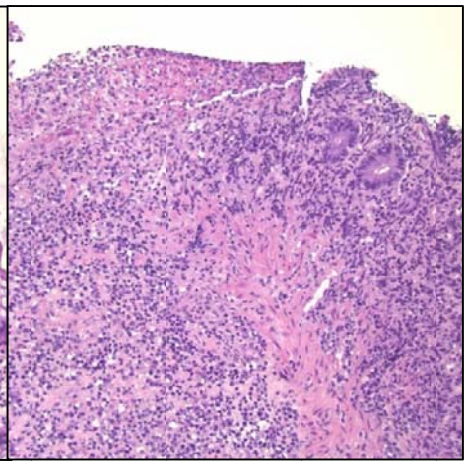


5.3 Epithelial sloughing in rectal lavage fluid

5.3.1 Introduction

Rectal lavage and examination of effluent for shedding of epithelial cells has been used to characterize the rectal safety profile of sexual lubricants and microbicide candidates in murine (Sudol & Phillips, 2004), non-human primate (Patton, Sweeney, & Paul, 2009) and human studies (Phillips, Taylor, Zacharopoulos, & Maguire, 2000; Phillips et al., 2004).

5.3.2 Previous studies

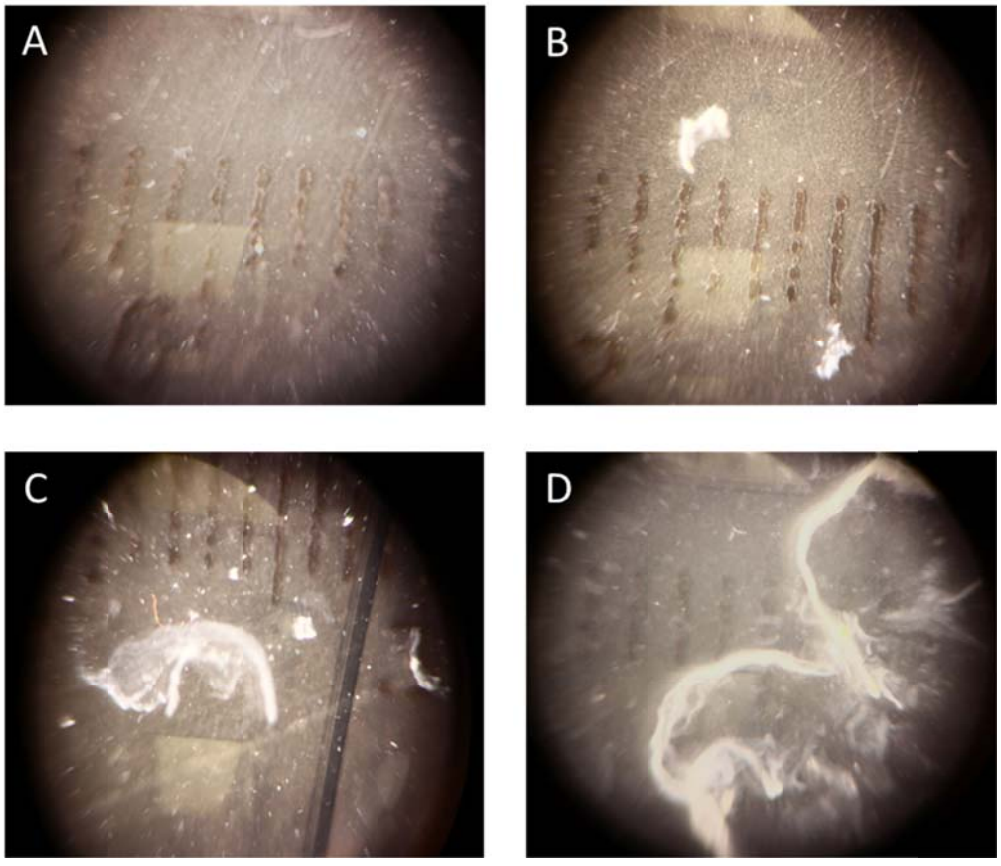
In two human studies, examination of rectal lavage fluid has demonstrated that exposure to N9 is associated with transient rectal epithelial disruption (Phillips et al., 2000; Phillips et al., 2004). Substantial reversal of these mucosal changes occurs after 2 hours and microscopically normal epithelium was seen after 24 hours. In both the RMP-01 and RMP-02/MTN-006 studies, no increase in epithelial sloughing was noted after exposure to the active product or placebo (Anton et al., 2011; Anton et al., 2012).

5.3.3 Methods

With the participant resting in the left lateral position, approximately 120 mL of Normosol®-R isotonic solution (Hospira Inc., Lake Forest, IL, USA) is delivered into the rectum. The participants were asked to hold the fluid for 3-5 minutes before expelling it into a collection hat. Approximately 30 mL of rectal lavage fluid was collected and sent to the laboratory for analysis. The lavage fluid was transferred to a 50 mL centrifuge tube and spun at 1,000 rpm for 5 minutes. The supernatant was removed to a second 50 mL centrifuge tube, leaving a cell pellet in the first tube, and then spun for 1,000

rpm for 5 minutes. The supernatant from the second tube is discarded leaving one pellet in each tube. 1 mL of 2% paraformaldehyde was gently added to resuspend each pellet. The cellular suspension was then added to a 2 cm x 2 cm petri dish with 10 mL of PBS. The petri dish that was then scanned at 40X using a dissecting microscope and the number of epithelial sheets >2 mm counted in each quadrant (Figure 5-2). The total number of epithelial sheets in all four quadrants was then submitted as the final score for the epithelial sloughing assay.

Figure 5-2 Examples of rectal lavage fluid from study participants



A: Rectal lavage fluid with no epithelial sloughing, **B:** 2 mm sheets of epithelium, **C:** 7 mm sheets of epithelium, and **D:** 10 mm sheets of epithelium

5.4 Fecal calprotectin

5.4.1 Introduction

Calprotectin accounts for 60% of the cytoplasmic protein fraction of polymorphonuclear granulocytes and is also found in monocytes, macrophages, and eosinophils (Poullis, Foster, Mendall, & Fagerhol, 2003). Calprotectin plays an important role in innate immunity and has antibacterial, antifungal, and immunomodulatory effects *in vivo*. Because intestinal granulocytes end their lifespan by migrating through the intestinal wall and since granulocyte-derived calprotectin can be found in feces, calprotectin is felt to be a useful indirect index of mucosal inflammation (Poullis et al., 2003; Abraham & Kane, 2012). Fecal calprotectin levels are elevated in inflammatory bowel disease and correlate well with disease activity in Crohn's disease and ulcerative colitis (Gaya et al., 2005; Roseth, Aadland, Jahnsen, & Raknerud, 1997; Komraus, Wos, Wiecek, Kajor, & Grzybowska-Chlebowczyk, 2012; D'Haens et al., 2012). In addition, fecal calprotectin levels have been found to be significantly elevated in first-degree relatives of patients with Crohn's disease even though all the relatives were clinically asymptomatic (Tamboli, Richard, & Colombel, 2003). Elevated levels of fecal calprotectin are also seen in patients with enteric infections such as *Campylobacter jejuni* (Nielsen, Engberg, Ejlertsen, & Nielsen, 2013; Shastri et al., 2008). Fecal calprotectin levels associated with various gastrointestinal conditions are summarized in Figure 5-3.

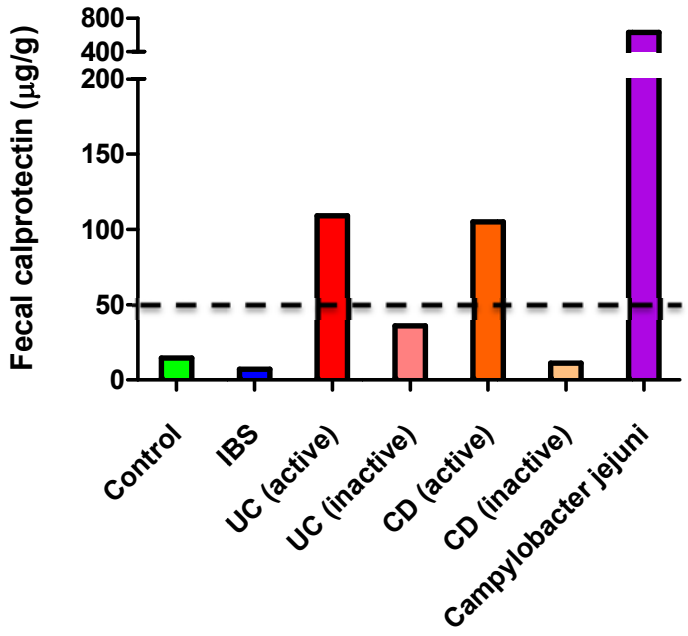


Figure 5-3 Fecal calprotectin levels in specific gastrointestinal conditions

Median fecal calprotectin levels in a variety of gastrointestinal diseases. Data derived from published literature (Komraus et al., 2012; Langhorst et al., 2008; Nielsen et al., 2013). The black dotted line illustrates the upper limit of normal for the Genova Diagnostics® fecal calprotectin assay (50µg/g) used in the MTN-007 study (IBS; irritable bowel syndrome, UC; ulcerative colitis, CD; Crohn's disease).

5.4.2 Previous studies

Fecal calprotectin was measured in both the RMP-01 and RMP-02/MTN-006 rectal safety studies of UC781 and TFV gels (Anton et al., 2011; Anton et al., 2012). No significant elevations in fecal calprotectin were observed in any arm or at any stage of product exposure.

5.4.3 Methods

Following the Normosol®-R enema described in Section 5.3.3, samples of stool were collected, stored in 50 mL conicals, and transported on ice, in real time, to a commercial laboratory (Genova Diagnostics®, Ashville, NC, USA) where calprotectin levels were quantified using an enzyme linked

immunosorbent assay (ELISA). The Genova Diagnostics laboratory states that fecal calprotectin levels of < 50 μg/g of stool are considered to be normal whereas values between 50 and 100 μg/g of stool suggests moderate inflammation of the mucosal tissue.

5.5 Assessment of rectal microflora

5.5.1 Introduction

Characterization of colonic microbiota and their impact on health and disease has become an area of intense scientific research (Shanahan, 2013). The gut flora is composed of approximately 10^{14} bacterial cells, a figure that is estimated to be 10-fold higher than the total number of human cells in the body. Predominant bacterial phyla include Firmicutes, Bacteroidetes, and Proteobacteria although more interindividual diversity occurs at bacterial species and strain level (Hong, Croix, Greenberg, Gaskins, & Mackie, 2011; Shanahan, 2012). A critical step in the evolution of our understanding about colonic microbiota has been the development of culture-independent techniques for the characterization and quantification of gut bacteria. These techniques include extraction of bacterial DNA and amplification of 16S ribosomal RNA (Fraher, O'Toole, & Quigley, 2012). It is now clear that there is an intimate relationship between the colonic microbiota, the mucosal immune system, and the metabolic environment of the gut. Acquired or inherited perturbations of any of these parameters can lead to gastrointestinal and systemic disease. Studies are currently ongoing to determine whether HIV infection is associated with significant changes in colonic microbiota (Saxena et al., 2012).

In the context of microbicide development, there has been significant concern that repetitive exposure to a microbicide might be associated with changes in vaginal or rectal flora that could increase the risk of HIV infection. It is known that women with bacterial vaginosis have an increased risk of acquisition (Atashili, Poole, Ndumbe, Adimora, & Smith, 2008) and transmission (Cohen et al., 2012) of HIV infection. Consequently, the majority of vaginal microbicide studies have evaluated vaginal microflora using conventional culture techniques (McGowan et al., 2011) and more recently with pyrosequencing of 16s RNA (Ravel et al., 2012). Studies evaluating cellulose sulphate, N9, and VivaGel® have demonstrated shifts in vaginal flora with reductions in *Lactobacillus* spp. and increases in anaerobic spp. but no significant increase in the prevalence of bacterial vaginosis (Ravel et al., 2012; McGowan et al., 2011).

5.5.2 Previous studies

Patton et al. have previously characterized the rectal microflora of the *Macaca nemestrina* (pig-tailed macaque) and healthy women using semi-quantitative culture and biochemical techniques (Patton, Cosgrove-Sweeney, Rabe, & Hillier, 2001; Patton, Sweeney, Rabe, & Hillier, 1996). They showed that rectal colonisation in both species was similar although the prevalence of H_2O_2 producing lactobacilli was higher in the macaques compared to the women (80% versus 48%) and women had a higher carriage rate of anaerobic gram-positive cocci compared to the macaques (98% versus 70%). Subsequently, the group evaluated the impact of a broad range of microbicide candidates (pH buffers, membrane disrupters,

entry/fusion inhibitors, and reverse transcriptase inhibitors) on rectal microflora (Patton et al., 2009). Only C31G (Patton, Sweeney, Balkus, & Hillier, 2006) and a combination of SPL7013 and BufferGel® (Patton et al., 2009) resulted in transient changes in rectal microflora. The techniques developed by Patton and Hillier were subsequently used to characterize the impact of UC781 and TFV gels on human rectal microflora (Anton et al., 2011; Anton et al., 2012). No significant changes in rectal microflora were noted at any time point in these studies.

5.5.3 Methods

Rectal specimens obtained per rectum with a sterile cotton swab were placed in an anaerobic transport tube (Port-a-Cul; Becton-Dickinson Corp., Cockeysville, MD, USA) and shipped by overnight mail to Magee-Womens Research Institute, Pittsburgh, PA, USA. The swab was removed, placed into 0.9 mL of buffered salt solution and vortexed to release fluid. Plates were incubated in an anaerobic chamber for 72-96 hours for detection of anaerobes; agar plates for aerobic bacteria were evaluated after 48 hours of incubation at 37 °C in 6% CO_2. Given the enormous variety of normally-present bacteria in the colon and the unknown variations related to diet and time, the bacterial groups selected for monitoring before and after product exposure was based on an FDA defined panel. Changes in bacterial frequencies and concentrations within exposed individuals as well as between study groups were examined between baseline visit and following the 7-day exposure (which included any changes occurring after the single dose exposure). Results were quantified by colonization growth on a scale from 0-4 as follows: 0 = no growth; 1 = 10^3cfu/mL; 2 = 10^5cfu/mL; 3 =

10^6cfu/mL; and 4 = 10^7cfu/mL. Raw means and SDs were computed by time (before or/after gel use), using McNemar's test for evaluating paired changes in colonization status.

Table 5-3 Rectal microflora evaluated in the RMP-01 study

Bacteria	Baseline prevalence
Lactobacillus (H_2O_2-producing)	30%
Lactobacillus (H_2O_2-nonproducing)	17%
Gardnerella vaginalis	8%
Escherichia coli	89%
Other gram-negative rods	22%
Anaerobic gram-positive cocci	92%
Anaerobic gram-positive rods (*Clostridium*)	81%
Anaerobic gram-positive rods (other)	72%
Anaerobic gram-negative rods	100%
Black-pigmented anaerobic gram-negative rods	83%

*Based on samples from 36 participants (Anton et al., 2011)

5.6 Rectal secretion of cytokines and chemokines

5.6.1 Introduction

In 2001, Fichorova et al. demonstrated that vaginal exposure to an N9 gel by healthy women was associated with increased release of pro-inflammatory cytokines and chemokines into vaginal fluid (Fichorova et al., 2001). As N9 was later found to be harmful in the COL-1492 trial (Van Damme et al., 2002) monitoring levels of both pro-inflammatory and anti-inflammatory molecules has become a routine component of vaginal microbicide trials. Levels of cytokines and chemokines can be highly variable in healthy women and are likely to be influenced by factors including the stage of the menstrual cycle, contraceptive use, vaginal flora, and recent sexual activity

(Kyongo et al., 2012). Levels are also likely to be influenced by collection methods and the period of sample storage before analysis (Dezzutti et al., 2011; de, Bourcier, Rijkers, Prakken, & Seyfert-Margolis, 2009). A final consideration is that microbicide exposure may also be associated with decreases in these molecules (Keller et al., 2012). Since intestinal inflammation, irrespective of cause, is commonly associated with increased production of cytokines and chemokines (MacDonald & Monteleone, 2005), these molecules have also been measured in Phase 1 rectal microbicide studies.

5.6.2 Previous studies

In both the RMP-01 and RMP-02/MTN-006 trials, rectal fluid was collected with cellulose sponges and quantified for RANTES, MIP-1α, TNF-α, IFN-γ, IL-12p40, IL-6, and IL-1β using the BioRad Luminex® platform. Baseline values are presented in Figure 5-4. In the RMP-01 study, after single rectal dose exposure, there was a significant difference in RANTES between the placebo gel (71.79 ± 54.38 pg/mL) and the 0.1% UC781 gel (31.06 ± 17.2 pg/mL) (Anton et al., 2011).

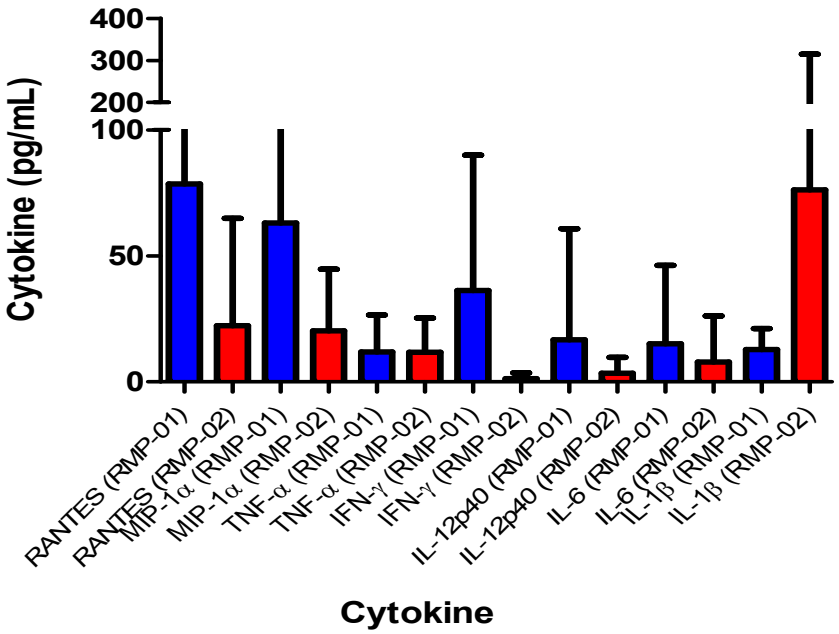


Figure 5-4 Baseline rectal fluid cytokine concentrations

Baseline cytokine concentrations in rectal fluid from the RMP-01(blue) and RMP-02/MTN-006 (red) Phase 1 rectal safety studies of UC781 and TFV gel. Cytokine concentrations were quantified using Luminex® and presented as mean ± SD values.

In the RMP-02/MTN-006 study, after 7 days of dosing, there were significant cytokine reductions seen in the TFV gel compared to the HEC placebo gel: IL-1β (1.55 ± 1.64 *vs.* 5.43 ± 3.71 pg/mL), MIP-1α (4.69 ± 10.78 *vs.* 11.76 ± 7.88 pg/mL), IL-6 (1.29 ± 1.48 *vs.* 4.13 ± 3.25 pg/mL), and TNF-α (4.57 ± 9.18 *vs.* 8.15 ± 9.49 pg/mL) (Anton et al., 2012).

5.6.3 Choice of cytokines and chemokines

A key concern in microbicide development is whether repetitive application of a microbicide gel might induce mucosal inflammation and potentially increase the risk of HIV infection. The MTN-007 study evaluated a broad range of cytokines and chemokines that were chosen either because they were known to be increased in previous preclinical/clinical microbicide

studies or are thought to be involved in the pathogenesis of mucosal inflammation such as inflammatory bowel disease.

5.6.3.1 IL-1β

IL-1β is produced by activated macrophages and is an important mediator of the inflammatory response. It is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.

5.6.3.2 IFN-γ

IFN-γ, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. IFN-γ is an important activator of macrophages. Aberrant IFN-γ expression is associated with a number of inflammatory and autoimmune diseases. IFN-γ plays an important role in the immune system stems due to its immunostimulatory and immunomodulatory effects. IFN-γ is produced predominantly by natural killer (NK) T cells as part of the innate immune response, and CD4+ and CD8+ T lymphocytes during antigen specific responses.

5.6.3.3 TNF-α

TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells and neurons. The primary role of TNF-α is in the regulation of immune cells. TNF-α, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, sepsis,

cachexia, and inflammation. It can also inhibit tumorigenesis and viral replication. Dysregulation of TNF-α production has been implicated in a variety of human conditions including Alzheimer's disease, cancer, major depression, and inflammatory bowel disease.

5.6.3.4 IL-6

IL-6 is an important mediator of fever and of the acute phase response. It is capable of crossing the blood brain barrier and initiating synthesis of PGE_2 in the hypothalamus. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen associated molecular patterns (PAMPs). PAMPs bind to highly important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). These are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to inflammatory cytokine production.

5.6.3.5 IL-8

IL-8 has two primary functions. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward sites of infection. IL-8 also induces phagocytosis once they have arrived. IL-8 can be secreted by any cells with TLR that are involved in the innate immune response. Macrophages are often the first cells to engage with antigens and are therefore commonly the first cells to release IL-8 to recruit other cells.

5.6.3.6 IL-12

IL-12 is involved in the differentiation of naive T cells into Th1 cells. It is known as a T cell-stimulating factor, which can stimulate the growth and function of T cells. It stimulates the production of IFN-γ and TNF-α from T and NK cells, and reduces IL-4 mediated suppression of IFN-γ. IL-12 plays an important role in the activities of NK cells and T lymphocytes. IL-12 mediates enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes.

5.6.3.7 IL-17

IL-17 is a cytokine that acts as a potent mediator in delayed-type reactions by increasing chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation, similar to IFN-γ. IL-17 is produced by T-helper cells and is induced by IL–23 which results in destructive tissue damage in delayed-type reactions. Interleukin 17 as a family functions as a proinflammatory cytokine that responds to the invasion of the immune system by extracellular pathogens and induces destruction of the pathogen's cellular matrix. Interleukin 17 acts synergistically with TNF-α and IL-1β. IL-17 has been linked to many immune/autoimmune related diseases including rheumatoid arthritis, asthma, lupus, allograft rejection, anti-tumour immunity and psoriasis.

5.6.3.8 IL-23

IL-23 is an important part of the inflammatory response against infection. It promotes upregulation of the matrix metalloprotease MMP9, increases angiogenesis, and reduces CD8+ T-cell infiltration. IL-23 can stimulate naive

CD4+ T cells to differentiate into a novel subset of cells called Th17 cells, which are distinct from the classical Th1 and Th2 cells. Th17 cells produce IL-17, a proinflammatory cytokine that enhances T cell priming and stimulates the production of other proinflammatory molecules such as IL-1β, IL-6, and TNF-α resulting in inflammation.

5.6.3.9 MIP-1α / MIP-1β

Macrophage Inflammatory Proteins (MIP) belong to the family of chemotactic cytokines known as chemokines. In humans, there are two major forms, MIP-1α (CCL3) and MIP-1β (CCL4). They are produced by macrophages following stimulation with bacterial endotoxins and are important in generating immune responses towards infection and inflammation. They activate human granulocytes (neutrophils, eosinophils and basophils) which can lead to acute neutrophilic inflammation. They also induce the synthesis and release of other pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α from fibroblasts and macrophages.

5.6.3.10 RANTES

RANTES (**R**egulated on **A**ctivation, **N**ormal **T** cell **E**xpressed and **S**ecreted) or CCL5 is an 8kDa protein classified as a chemotactic cytokine or chemokine. RANTES is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. With the help of particular cytokines such as IL-2 and IFN-γ that are released by T cells, RANTES also induces the proliferation and activation of NK cells. RANTES, along with the related chemokines MIP-1α and MIP-1β, has been

identified as a natural HIV-suppressive factor secreted by activated CD8+ T cells and other immune cells.

5.6.4 Methods

A BioRad Luminex® platform was used to measure IFN-γ, IL-1β, IL-6, IL-8, IL-12 (p40), IL-17, MIP-1α, MIP-1β, RANTES, and TNF-α in rectal secretions.

5.6.4.1 Elution of rectal fluid from Merocel sponges

After collection of rectal fluid samples, the Merocel polyvinyl acetate sponges (Beaver-Visitec International Waltham, MA, USA) were placed immediately into pre-labeled 5mL Nalgene cryovials (Thermo Scientific, Rochester, NY, USA) containing 400µL of D-PBS and once received by the laboratory were stored at -80°C until processed. Batched samples were thawed at room temperature and then centrifuged at 12,000 rpm for 20 minutes at 2-8°C using Corning® Costar® Spin-X® polypropylene centrifuge tubes with a 0.45µm cellulose acetate filter (Sigma-Aldrich, St. Louis, MO, USA). Aliquots (100µL) of the filtered solution were then stored at -80°C until performing the Luminex® assays.

5.6.4.2 Quantification of cytokines/chemokines in rectal fluid

Aliquots of filtered rectal fluid were processed following the Millipore MILLIPLEX human cytokine / chemokine kit assay protocol (EMD Millipore, Billerica, MA, USA). The cytokine / chemokine assays were performed in duplicate on a Luminex® 100 system (Luminex, Austin, TX, USA). A standard curve was constructed using the Millipore Human Cytokine Standard (10,000 pg/mL to 3.2 pg/mL) with the assay buffer used as the 0

pg/mL standard. The minimum detectable concentrations of the study analytes are summarized in Table 5-4.

Table 5-4 Luminex assay sensitivity

Cytokine / Chemokine	Mean Minimum Detectable (pg/mL)*
IFN-γ	0.1
IL-1β	0.4
IL-6	0.3
IL-8	0.2
IL-12 (p40)	10.5
IL-17	0.2
MIP-1α	3.5
MIP-1β	4.5
RANTES	1.0
TNF-α	0.1

*Data derived from the Millipore Human Cytokine / Chemokine Assay protocol

5.7 Cytokine and chemokine gene expression in mucosal tissue

5.7.1 Introduction

The development of polymerase chain reaction (PCR) technology by Kary Mullis and his colleagues in 1986 revolutionised molecular biology as it provided a sensitive and specific technique to quantify gene expression in a broad range of biological systems (Mullis et al., 1986). In essence, PCR relies upon the ability of specific oligonucleotide primers to bind to denatured DNA and initiate DNA synthesis of the relevant gene of interest. A series of 20-40 denaturing, annealing, and extension phases allows gene amplification. Theoretically, a single gene copy undergoing 20 rounds of PCR amplification will result in generation of approximately one million copies of the original target. PCR amplification has three main phases. The

early phase is when primers are binding to the template DNA, the mid phase is when amplification is underway with exponential accumulation of the product fragment, and a late phase, often referred to the plateau phase, when amplification is suboptimal either due to exhaustion of the reagents or inhibition of the PCR reaction. For semi-quantitative PCR, it is important that product accumulation is measured in the exponential phase of the PCR reaction (Giulietti et al., 2001).

Since the initial description of PCR, numerous applications have been developed that attempt to provide absolute quantification of gene expression using “real-time” (RT) PCR techniques that do not require post-amplification processing to yield quantitative data. RT-PCR employs the use of specific primers together with either DNA-binding dyes (SYBR® or EvaGreen® technologies) that fluoresce when bound to double-stranded (ds) DNA or to specific oligonucleotide probes with a reporter dye bound to the 5’ end of the probe and a quencher dye at the 3’ end of the probe. When the probe is intact the quencher dye absorbs the fluorescence from the reporter dye. When the probe is hydrolyzed by 5’ exonuclease activity of the *Taq* polymerase during PCR amplification, the reporter dye is separated from the quencher dye resulting in fluorescence emission. RT-PCR is performed in sealed tubes thus limiting the likelihood of DNA contamination. In addition, with the incorporation of DNA standards, the system allows absolute quantification of gene expression in biological samples.

Although RT-PCR can accurately quantify gene expression, it is important for studies in which the input RNA is derived from tissue biopsies, especially when samples are being compared across arms of a study, that the levels of specific gene expression are standardized to a housekeeping gene. Ideally, expression of a housekeeping gene should not vary in the tissues under study and should not be influenced by experimental treatment such as exposure to candidate microbicides (Vandesompele et al., 2002). The stability of housekeeping genes may vary by tissue or experimental design and so in MTN-007 we explored the stability of three housekeeping genes (β-Actin, β-2 Microglobulin (β2M), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) in the study samples.

5.7.2 Previous studies

Rectal mucosal cytokine gene expression has been quantified in two mucosal pathogenesis studies and two rectal microbicide studies. The cytokines / chemokines evaluated have varied by study and are summarized in Table 5-5. Baseline data for RANTES, IFN-γ, and IL-10 are presented in Figure 5-5.

Table 5-5 Mucosal gene expression studies

Study	Cytokine / Chemokine	Reference
McGowan et al. JAIDS 2004	RANTES, TNF-α, IL-6, IL-1β, IL-10, IL-2, IFN-γ, IL-12	(McGowan et al., 2004)
HPTN-056	RANTES, IL-10, IFN-γ	(McGowan et al., 2007)
RMP-01	IFN-γ	(Anton et al., 2011)
RMP-02/MTN-006	IFN-γ	(Anton et al., 2012)

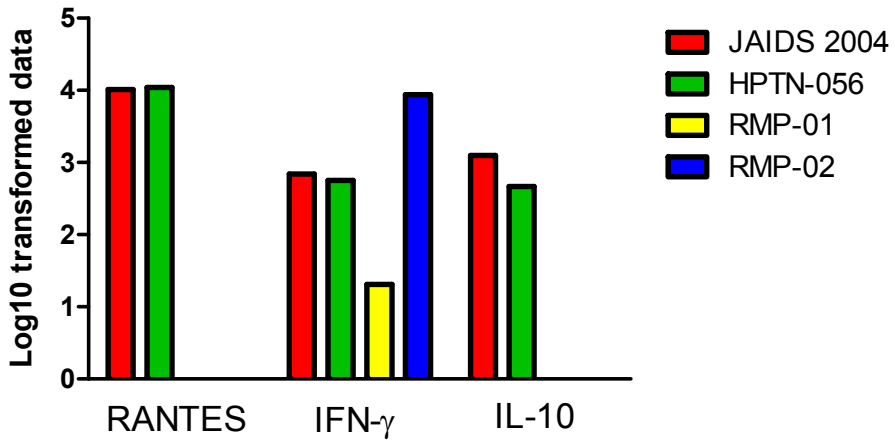


Figure 5-5 Cytokine mRNA levels from previous studies

RNA was isolated from endoscopic rectal biopsies, reverse-transcribed, and amplified using specific cytokine primers and by measuring the increase in SYBR Green associated fluorescence. Cytokine copy number was standardised to 10^6 β-Actin copies per sample.

5.7.3 Methods

Real-Time quantitative reverse transcription PCR (qRT-PCR) was used to quantify mucosal mRNA expression of the following proinflammatory cytokines, chemokines, and chemokine receptors: IL-1β, IFN-γ, TNF-α, IL-6, IL-8, IL-12, IL-17, IL-23, MIP-1α, MIP-1β, RANTES, and CCR5.

5.7.3.1 RNA extraction

Tissue was collected as pinch biopsy specimens harvested via anoscopy (ARB) or via flexible sigmoidoscopy (FSB). Following collection, tissue biopsies were immediately placed in RNA*later*™ (Applied Biosystems/Ambion, Austin, TX, USA) solution and kept at -4°C for at least 4 hours prior to final storage at -80°C. In preparation for extraction of total RNA, the samples were thawed on ice. Biopsies were removed from the RNA*later*™ and placed in guanidinium lysis buffer supplied with the RNAqueous®-4PCR Kit (Applied Biosystems/Ambion, Foster City, CA,

USA). Samples were homogenized in the presence of 0.5 mm RNase-free zirconium beads with the aid of the Bullet Blender homogenizer (Next Advance Inc., Averil Park, NY). RNA was then purified on columns according to the RNAqueous®-4PCR Kit instructions (Applied Biosystems/Ambion, Foster City, CA, USA). The extracted total RNA was eluted in a volume of 100 µL and DNase-1 treated. DNase-1 was inactivated before RNA was used in cDNA preparation.

5.7.3.2 Assessing RNA quantity and quality

All DNAse-1- treated RNA samples were assessed for quantity and quality on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA, USA). Quantitative measurements were determined and recorded in ng/µl. Quality measurement were assessed by the 18S/28S ribosomal RNA peak ratios (Figure 5-6).

Based on these measurements, RNA Integrity Numbers (RIN) were calculated automatically by the Agilent 2100 Bioanalyzer software. The absence of genomic DNA contamination was assessed by visualization of low baselines between the 18S and 28S ribosomal RNA peaks from the electropherogram image. RNA quality control limits were set within the laboratory as nucleic acid concentrations > 25 ng/µL, 18S/28S ratios of 2.0 $\pm$ 0.5, and RIN $\geq$ 6.0.

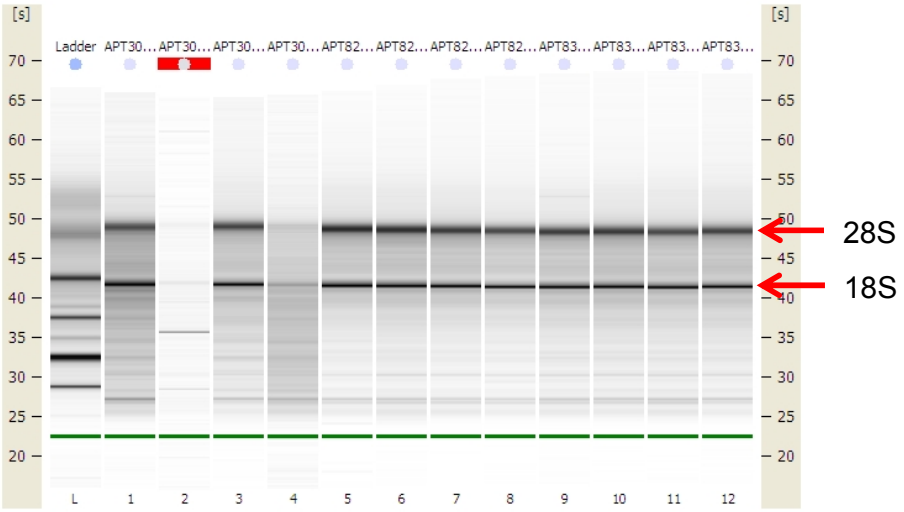


Figure 5-6 Agilent electropherogram

Lanes 1, 3, and 5-12 have sharp 18S/28S RNA bands indicating high quality RNA. Lanes 2 and 4 demonstrate degraded RNA

5.7.3.3 Reverse transcription of total RNA into complementary DNA (cDNA)

Equal quantities (1000 ng) of total RNA from biopsy samples were converted to cDNA using MultiScribe™ reverse transcriptase and TaqMan® Reverse Transcription reagents (Applied Biosystems, Roche Molecular Systems, Inc., Branchburg, NJ, USA). Oligo(dT)$_{20}$ (Invitrogen, Grand Island, NY, USA) was used to prime the reverse transcription (RT) reaction and reactions were run on the Applied Biosystems Veriti™ Dx Thermal Cycler (Life Technologies Corporation, Carlsbad, CA, USA). Identical quantities of RNA and reagents were used in the no reverse transcriptase (NRT) reaction which contained all components with the exception of reverse transcriptase.

5.7.3.4 Quantitative real time PCR for reference gene expression

Rectal cDNA was used as a template for the PCR amplification. The three reference genes used to normalize cytokine gene expression were GAPDH,

β-Actin and β2 Microglobulin (β2M). All PCR reactions were performed using the Bio-Rad CFX96 RT-PCR System (Bio-Rad, Hercules, CA, USA). Probes and/or primers were designed to span the intron-exon boundaries to ensure amplification from cDNA rather than from genomic DNA. The master mix and primer/probe mixes for the reference genes (GAPDH, β-Actin, and β2M) were obtained from Solaris QPCR Gene Expression Assays (Thermo Fisher Scientific Inc., Waltham, MA, USA). One µL of the cDNA was added to 5 µL of Solaris qPCR Master Mix (2X), 0.5 µL of Primer/Probe Set (20X), and 3.5 µL of nuclease free water for each reaction replicate. Solaris master mix assays utilized standard TaqMan cycling conditions of 95°C denaturation for 15 minutes followed by 40 cycles of 95°C denaturation for 15 seconds and 60°C annealing for 1 minute. Serial 1:10 dilutions of plasmid DNA were used to construct standard curves for quantitatively assessing reference gene expression.

Table 5-6 Gene sequences for PCR primers

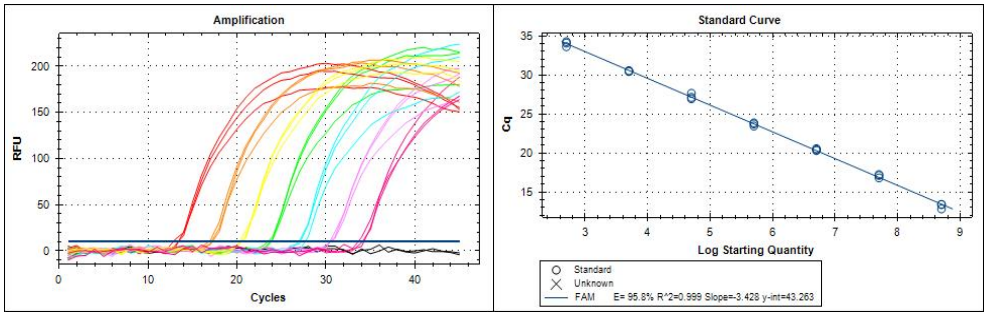
Gene Name	Primers	Sequence 5' to 3'	Length
GAPDH	Forward	GCCTCAAGATCATCAGCAATG	
	Reverse	CTTCCACGATACCAAAGTTGTC	
	Probe	GCCAAGGTCATCCATGA	89bp
β-Actin	Forward	TGGAGAAAATCTGGCACCAC	
	Reverse	GGTCTCAAACATGATCTGG	
	Probe	ACCGCGAGAAGATGACC	106bp
B2M	Forward	CTTTGTCACAGCCCAAGATAG	
	Reverse	ATCCAAATGCGGCATCTTC	
	Probe	CAGCATCATGGAGGTTTG	80bp
CD45	Forward	GGAAGTGCTGCAATGTGTCATT	
	Reverse	CTTGACATGCATACTATTATCTGATCTCA	
	Probe	ACAACTAAAAGTGCTCCTCCAAGCCAGGTCT	101bp
IL-1β	Forward	ACAGATGAAGTGCTCCTTCCA	
	Reverse	ATCCAGCTACGAATCTCCGAC	
	Probe	CTCTGCCCTCTGGATGGCGG	73bp
IL-6	Forward	GGTACATCCTCGACGGCATCT	
	Reverse	GTGAAAGCAGCAAAGAGGCACT	
	Probe	AGCCCTGAGAAAGGAGACATGTAACAAGAGT AACA	81bp
IL-12p40	Forward	TGGAGTGCCAGGAGGACAGT	
	Reverse	CAAACCTGACCCACCCAAGA	
	Probe	ATGGTGGATGCCGTTCACAAGCTCAA	147bp
IFN-γ	Forward	TCAGCTCTGCATCGTTTTGG	
	Reverse	TTCAGATGTAGCGGATAATGGAAC	
	Probe	TTGGCTGTTACTGCCAGGACCCATATGT	120bp
TNF-α	Forward	CCAGGCAGTCAGATCATCTTCTC	
	Reverse	AAGCTGAGGGGCAGCTCC	
	Probe	AGCCTGTAGCCCATGTTGTAGCAAACCC	86bp
IL-8	Forward	CCACACTGCGCCAACA	
	Reverse	ATCCAAGAATCAGTGAAGATGC	
	Probe	CTGGGTGCAGAGGGTTGTGG	168bp
MIP-1α	Forward	GAGACGAGCAGCCAGTGCTC	
	Reverse	GCCGGCAGGTCTGTGC	
	Probe	CGGTGTCATCTTCCTAACCAAGCGA	64bp
MIP-1β	Forward	TCTCAGCACCAATGGGCTC	
	Reverse	TTCTTACACCGCGAGGAAGC	

	Probe	CCCTCCCACCGCCTGCTGCT	63bp
RANTES	Forward	CTACACCAGTGGCAACTGCT	
	Reverse	AGAAGAAATGGGTTCGGGA	
	Probe	TCACCCGAAAGAACCGCCAAGTGT	95bp

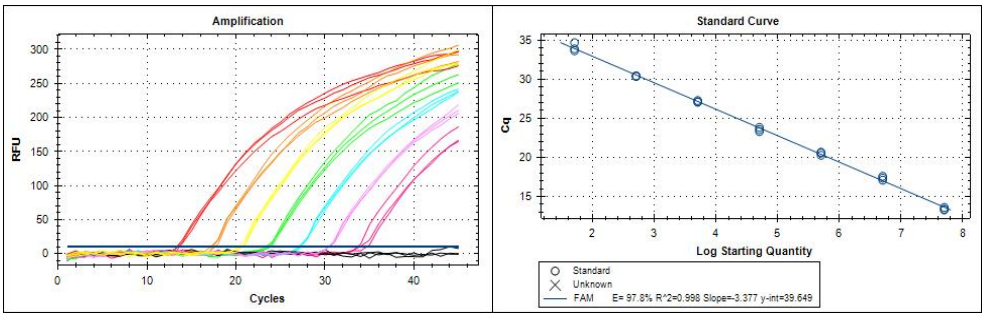
5.7.3.5 Quantitative real time PCR for chemokine/cytokine gene expression

CD45 gene expression by real-time PCR was used in addition to the expression of GAPDH, β-Actin and β2M as a reference gene (Pennington, et al., 2001) and was a means of assessing the presence of lymphoid cells in the biopsy specimen. Real-time PCR assays were also used to measure the expression of cytokine/chemokine genes which included IL-1β, IL-6, IL-12p40, IL-8, IL-17, IFN-γ, MIP-1α, MIP-1β, TNF-α, IL-23, CCR5 and RANTES (Table 5-6). All PCR experimentation was performed using the Bio-Rad CFX96™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Probes and or primers were designed to span the intron-exon boundary to ensure the amplification of cDNA rather than genomic DNA. For each of the chemokine/cytokine gene targets and CD45, 1 µL of cDNA was added to the reaction mixture which consisted of 200 nM forward primer, 200 nM reverse primer, 200 nM probe, 200 nM dNTP, 0.5 units AmpliTaq Gold DNA Polymerase, in1X reaction buffer containing 3.5mM $MgCl_2$ (Applied Biosystems, Carlsbad, CA, USA). Cycling conditions were: 50°C for 2 minutes followed by a 95°C for 10 minutes. This was followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute. Serial dilutions of plasmid DNA were used to construct standard curves for quantitatively assessing copy numbers of target gene expression (Figures 5-7 to 5-12).

GAPDH



β-actin



β2-microglobulin

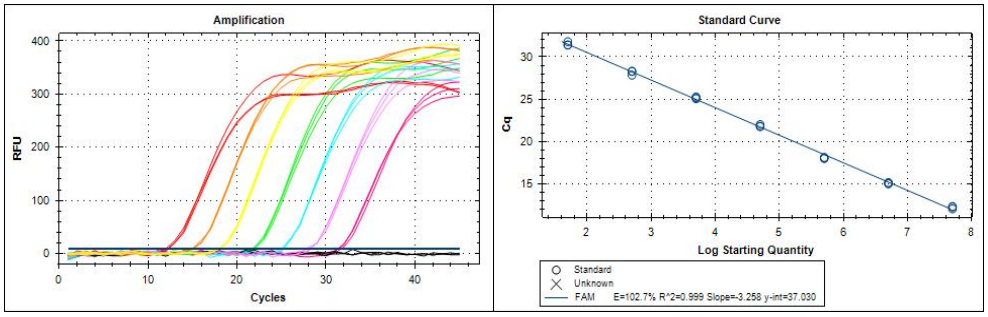
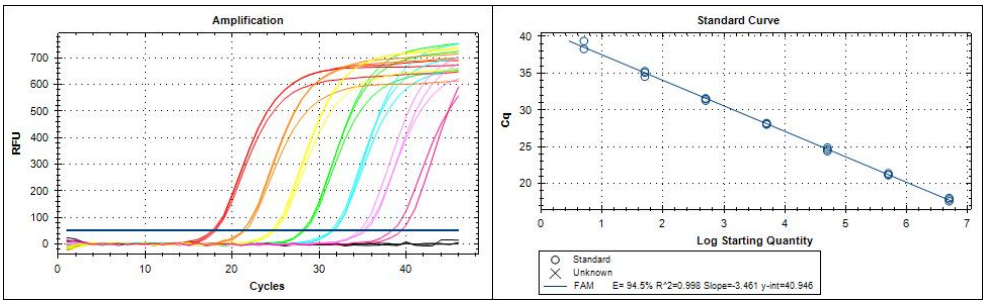
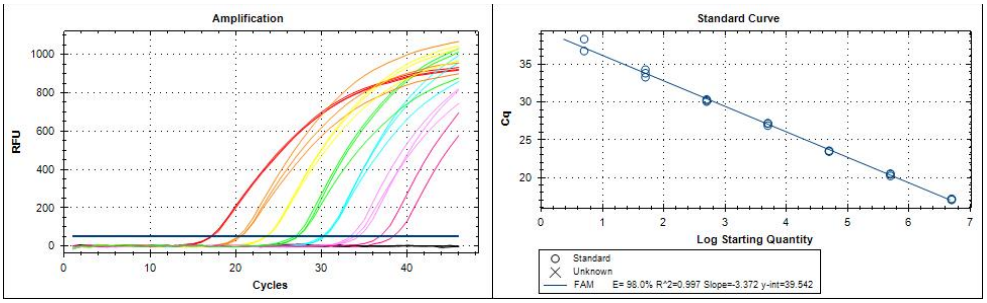


Figure 5-7 RT-PCR standard curves (I)

CD45



CCR5



IL-1β

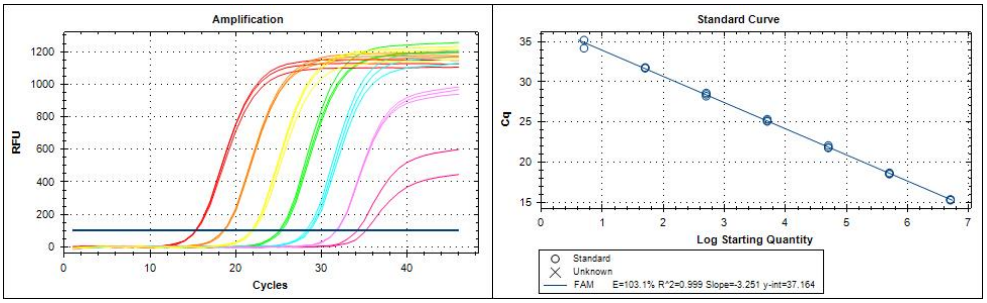
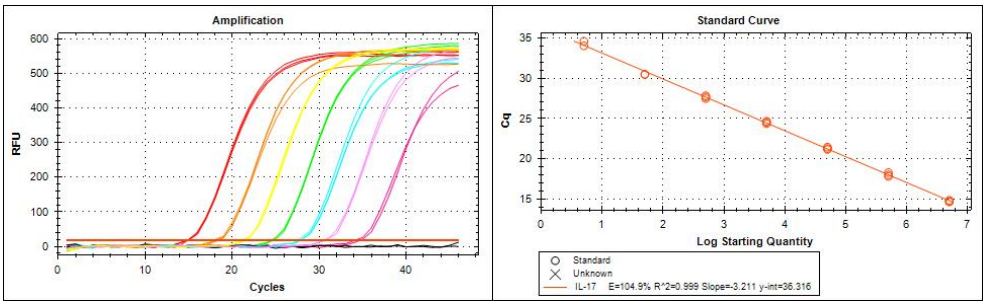
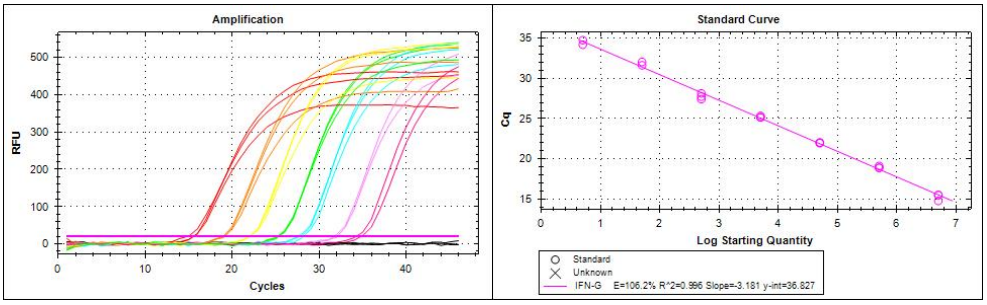


Figure 5-8 RT-PCR standard curves (II)

IL-17



Interferon-γ



TNF-α

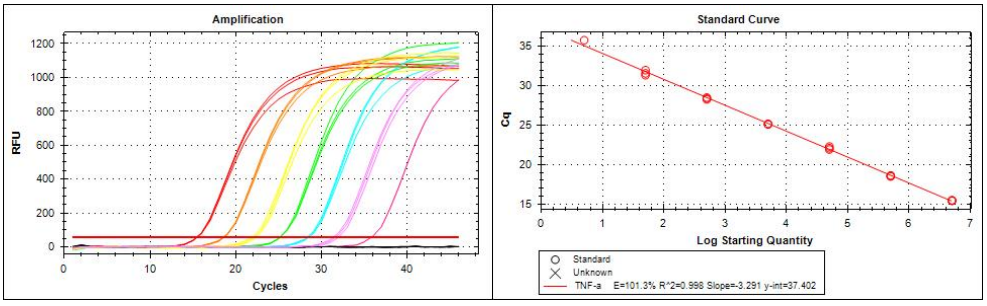
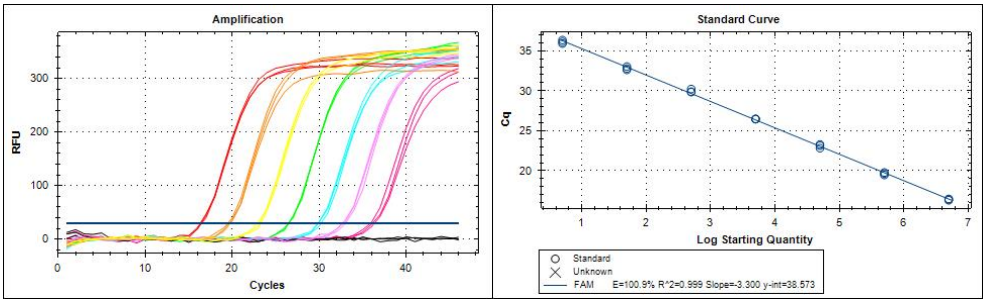
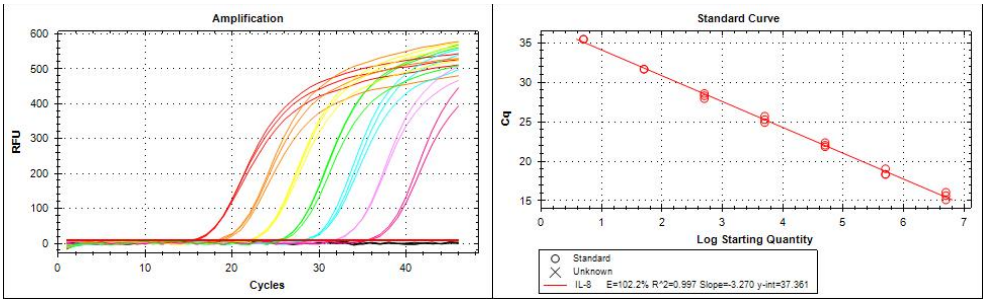


Figure 5-9 RT-PCR standard curves (III)

IL-6



IL-8



IL-12

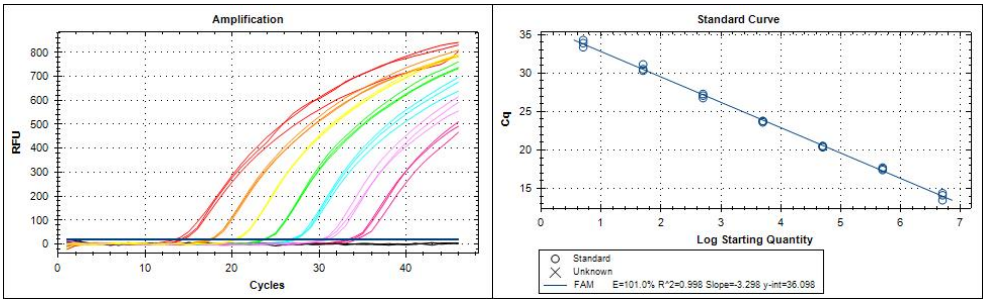
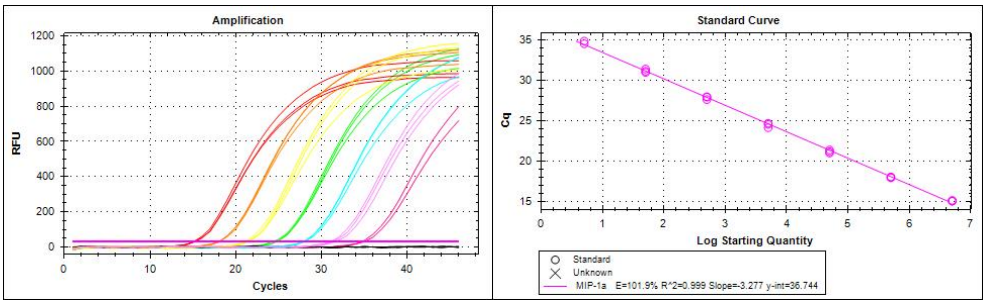
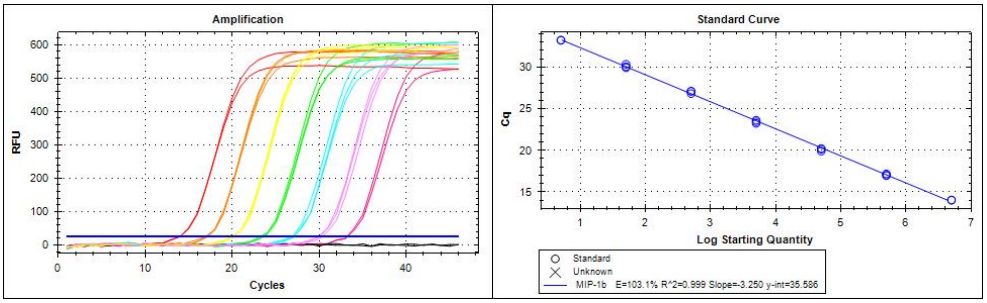


Figure 5-10 RT-PCR standard curves (IV)

MIP-1α



MIP-1β



RANTES

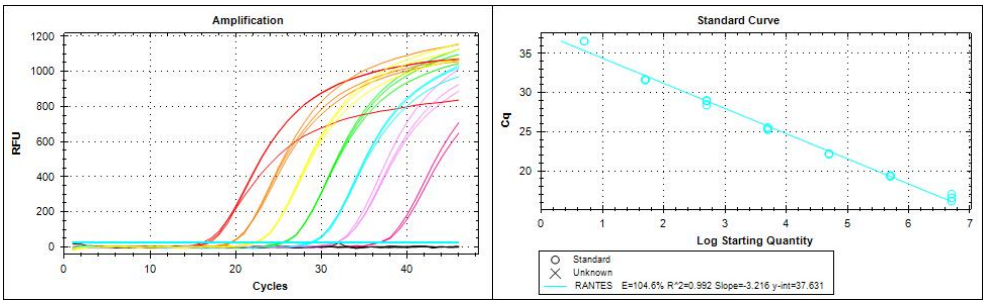


Figure 5-11 RT-PCR standard curves (V)

IL-23

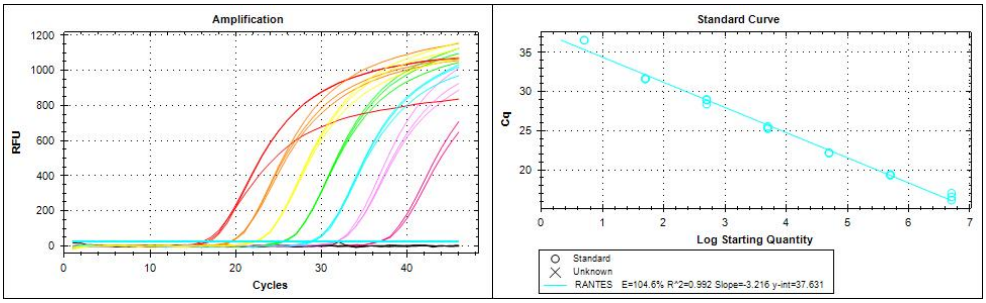


Figure 5-12 RT-PCR standard curves (VI)

5.8 Flow cytometric analysis of mucosal T cells

5.8.1 Introduction

The gastrointestinal tract is a rich source of HIV-1 target cells. Isolated lymphoid follicles, which serve as inductive sites for immune responses, are found throughout the colon (Koboziev et al., 2010). The number of follicles generally increases toward the anus with the greatest numbers found in the rectum (Langman & Rowland, 1986; Langman & Rowland, 1992). Antigen presenting cells (macrophage and dendritic cells) along with effector and regulatory T cells are found within the follicles. These cells are generally activated and express HIV-1 co-receptors, CCR5 and CXCR4, as well as soluble immune mediators (Anton et al., 2000; Poles et al., 2001; Zhang et al., 1998; McGowan et al., 2004) thus creating the perfect environment for HIV-1 infection.

A primary concern in rectal microbicide development is that repeated application of a candidate microbicide to the rectal mucosa might result in changes in the composition of the GALT that could increase the risk of HIV infection. Changes could include enhanced recruitment of HIV target cells to the mucosa, increased expression of HIV co-receptors (CCR5 or CXCR4) on resident cells and/or increased T cell activation. This possibility is not entirely theoretical. Two recent studies have demonstrated significant changes in T cell phenotype associated with the use of maraviroc in HIV-infected patients. These changes included an expansion of CCR5+/CD4+ T cells in peripheral blood (Cossarini et al., 2012), and an increase in both T cell activation and CCR5 expression in both peripheral blood and GALT (Hunt et al., 2013). The

combination of increased T cell activation and CCR5 expansion, especially in GALT, would clearly be an undesirable feature of an oral or topical PrEP regimen. As a consequence all rectal microbicide studies conducted to date have evaluated GALT T cell phenotype.

5.8.2 Previous studies

GALT T cell phenotype data have been generated using flow cytometry in the HPTN-056, RMP-01, and RMP-02 studies. HPTN-056 enrolled 16 participants who underwent flexible sigmoidoscopy at baseline, + 2 weeks, and + 4 weeks. The purpose of the study was to generate pilot data on the biological variability within and between HIV negative and HIV positive male participants. GALT phenotypic data are also available from the RMP-01 and RMP-02/MTN-006 studies.

Table 5-7 GALT T cell phenotype from previous studies

GALT T Cell Phenotype Mean %	**HPTN-056 (N = 4)**		**RMP-01 (N = 36)**		**RMP-02/MTN-006 (N = 18)**
Distance from anal verge	**10 cm**	**30 cm**	**10 cm**	**30 cm**	**15 cm**
CD3+	62.7	73.3	-	-	58.6
CD3+/CD4+	38.1	45.0	42.8	47.2	34.4
CD3+/CD8+	27.1	30.1	-	-	22.4
CD3+/CD4+/CCR5+	65.1	65.5	70.2	75.5	52.1
CD3+/CD4+/CXCR4+	76.2	77.5	81.6	82.5	-
CD3+/CD4+/CCR5+/CXCR4+	45.3	48.7	57.6	62.9	-
CD3+/CD4+/HLA-DR+	9.8	11.7	16.5	20.4	10.3
CD3+/CD4+/CD38+	46.0	39.4	12.1	10.0	44.1
CD3+/CD4+/HLA-DR+/CD38+	-	-	2.28	2.44	5.65

GALT phenotypic data for the subgroup of HIV negative participants (who practiced RAI) from the HPTN-056 study and Baseline data from participants in the RMP-01 and RMP-02/MTN-006 rectal microbicide studies are presented in Table 5-7.

5.8.3 Methods

5.8.3.1 Isolation of mononuclear cells from intestinal biopsies

Rectal biopsies were collected via anoscopy (ARB) and flexible sigmoidoscopy (FSB) from study participants. The biopsies were placed in complete RPMI 1640 transport medium which contained 7.5% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic (Gibco® Life Sciences, Carlsbad, CA, USA) and transported to the laboratory. The biopsies were scissor-minced in 2 mL of digest solution consisting of complete RPMI transport medium as described above, with the addition of 0.5 mg/mL Collagenase II (Sigma, St. Louis, MO, USA) and 0.83 units/mL DNAse 1 (New England BioLabs, Ipswich, MA, USA). Using a sterile pipette, the minced tissue was transferred into a 50 mL conical polystyrene tube containing 20 mL of pre-warmed digest solution. The tube was secured horizontally in a 37°C shaker incubator for 30 minutes and shaken at 250 revolutions per minute (rpm). The minced tissue and cell suspension were filtered through a 40 μM cell strainer (BD Falcon, Franklin Lakes, NJ, USA) into a sterile 50 mL conical tube. Cells were washed from the strainer into the 50 mL conical tube with D-PBS containing 2 mM EDTA (Gibco/Life Sciences, Carlsbad, CA, USA) and kept on ice. The remaining undigested tissue on the strainer was dislodged with 3 mL of digest solution and

transferred to a 50 mL conical tube with 20 mL of fresh digest solution. The 50 mL conical tube was secured onto the shaker incubator as described above and incubated for an additional 30 minutes with shaking at 250 rpm. Following the second digestion, the tissue and cells were again filtered through the 40 μM cell strainer into the 50 mL conical tube with wash buffer. If a third digestion was required, the tissue digestion and cell collection procedures were repeated. Cells obtained from all of the digestions were collected by centrifugation at 1000 x g. The supernatant fluid was removed and the cell pellet was resuspended in complete RPMI 1640 medium. Cell counts were obtained using a haemacytometer.

5.8.3.2 Flow cytometry

The steps used included the following: i) staining cells for viability, ii) setting up compensation tubes, iii) staining cells with monoclonal antibody and iv) establishing “fluorescence minus one” or FMO staining controls.

Cell viability was assessed using the LIVE/DEAD® fixable dead cell stain kit (Invitrogen, Grand Island, NY, USA). Aqua fluorescent dye is reactive with cellular amines. In dead cells with compromised cell membranes, the dye reacts with the free amines in the interior of the cell resulting in a 50-fold greater fluorescence in dead cells compared to live cells.

In multi-color flow cytometry, compensation is a process by which mathematical corrections are made for spectral overlap. If uncorrected, spectral overlap can lead to fluorochrome emission in an inappropriate detector. Lack of compensation could therefore result in misinterpretation of

data leading to false positive populations or artifacts on multi-color plots. By using controls for compensation, unwanted signals from spectral overlap can be removed electronically and accurate flow cytometric analysis of multi-color stained cells can be achieved. Fluorescence compensation settings were optimized using BD™ CompBead particles (BD Biosciences, San Jose, CA, USA). The bead set provides two populations of microparticles; the BD™ CompBeads Anti-Mouse IGg, κ particles which bind any mouse kappa light chain-bearing immunoglobulin and the BD™ CompBeads negative control labeled with fetal bovine serum (FBS) which have no binding capacity.

Compensation for the Aqua Blue fluorescent dye in the LIVE/DEAD® cell viability assay (Invitrogen, Grand Island, NY, USA) was performed separately using the ArC™ (Amine Reactive Compensation) Bead Kit (Invitrogen, Grand Island, NY, USA). The ArC™ kit contains two types of modified polystyrene microspheres to allow compensation with the LIVE/DEAD® kit. The ArC™ reactive microspheres (Component A) bind any amine-reactive dyes while the ArC™ negative microspheres (Component B) have no reactivity.

MMCs were stained with monoclonal antibody/fluorochrome conjugates for eight cell surface markers (Table 5-8). Staining with monoclonal antibodies for the CD45 marker is used to select all cells of hematopoietic origin except for erythrocytes. This common leukocyte marker reacts with all lymphoid cells. Monoclonal antibodies to CD3 bind to the transducing elements of the

T cell receptor complex and are thus used to identify T cells. Cells were stained for CD4, which is expressed on most thymocytes and about two thirds of peripheral blood T cells. The other third of all peripheral blood T cells are CD8 positive. CD69, a marker of T cell early activation, is widely expressed and is present on both CD4 and CD8-positive hematopoietic cells. It is not expressed in resting lymphocytes but is rapidly induced upon activation of B, T and natural killer cells. CCR5 and CXCR4 are co-receptors for HIV, and are expressed on CD4-positive lymphocytes.

"Fluorescence minus one" (FMO) controls contain all antibodies in the panel except one. They are used to define the negative population gates for dim markers. FMO tubes were used to define the difference between positive and negative populations for CD69, CXCR4, and CCR5.

Table 5-8 Flow cytometry monoclonal antibody reagents

T cell phenotype	Purpose	Fluorochrome	Supplier	Catalogue number
CD45	Leucocyte common antigen	PerCP	BD	340665
CD3	T-cell co-receptor	Pacific Blue	BD	558117
CD4	T-cell co-receptor	PE-Cy7	BD	348789
CD8	T-cell co-receptor	APC-H7	BD	557834
CCR5	HIV co-receptor	FITC	BD	555530
CXCR4	HIV co-receptor	APC	BD	555976
CD69	Early marker of T cell activation	PE	BD	555993

BD; BD Biosciences

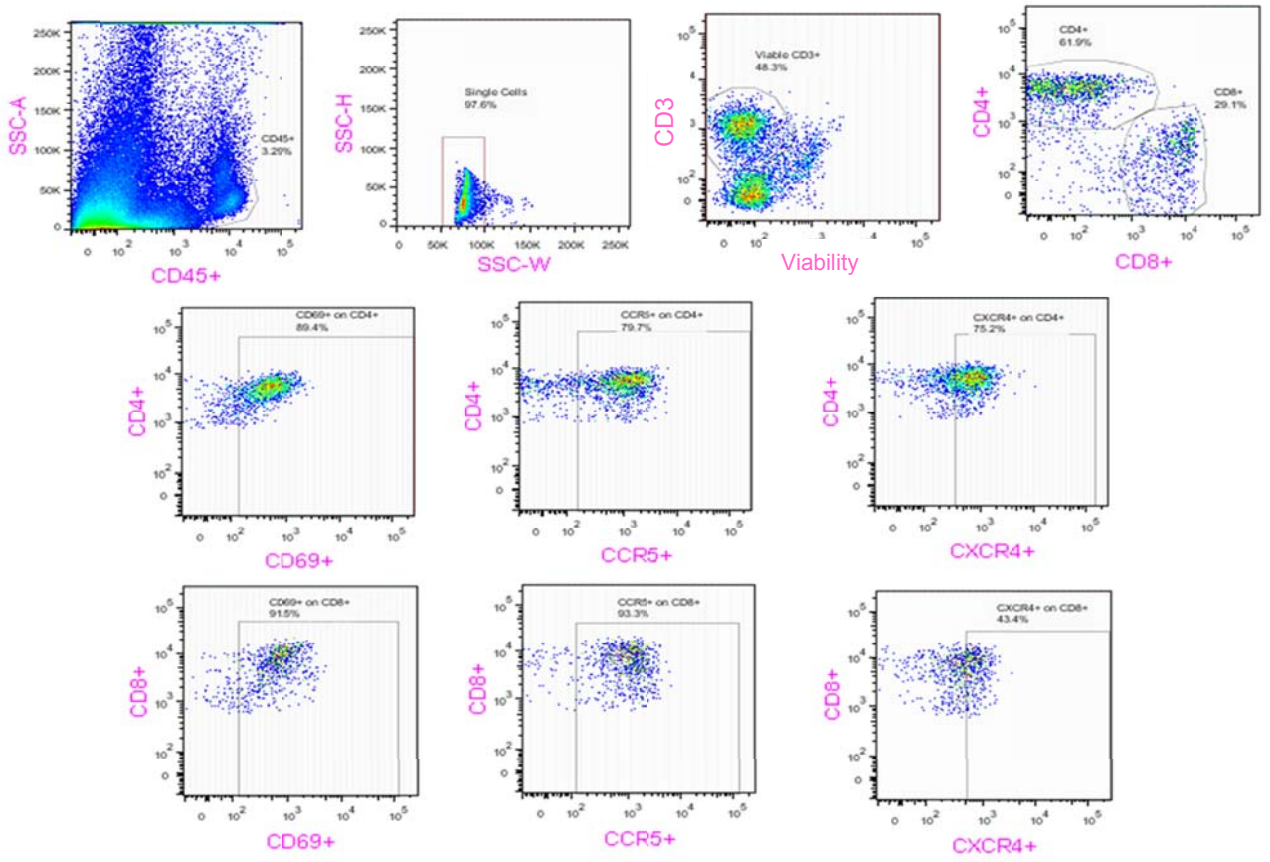
5.8.3.3 Flow cytometry protocol

Flow cytometric analysis was performed on a BD™ LSRFortessa cytometer (BD Biosciences, San Jose, CA, USA). All data were stored in list mode and analyzed with BD™ FACSDIVA operating system and Flow Jo (Tree Star, Inc., Ashland, OR, USA).

5.8.3.4 Gating strategy

Gating was undertaken to characterize a population of single lymphocytes based on morphological criteria and CD45+ staining (Figure 5-13). Subsequent gates defined viable CD3+ lymphocytes, CD4+ lymphocytes, CD8+ lymphocytes, and CCR5+, CXCR4+, CCR5+/CXCR4+, and CD69+ subsets of CD4+ and CD8+ lymphocytes.

Figure 5-13 Gating strategy for MTN-007



5.9 Statistical analysis

Data were used from the 60 evaluable participants including the replacement participants. The association between the mucosal parameters and randomisation arm was explored for samples collected at 9 cm and 15 cm. Mucosal parameters were measured at the Baseline visit, the Treatment 1 Visit, and the Final Clinic Visit. Differences in histology, fecal calprotectin, epithelial sloughing, rectal microflora and mucosal biomarkers were evaluated among participants who completed at least 5 doses of gels during the 7-day product use period and completed the Final Clinic Visit. Pairwise group comparisons were conducted across groups after adjustment for baseline variability for biomarkers measured after the single-dose use and for the 7-day use, separately. Cytokine/chemokine gene expression (RT-PCR) data were transformed by log base 10. Microflora data were dichotomized by treating Grade 0 as “absence” and Grades 1-4 as “presence”.

Chapter 6

Mucosal Safety Results

6 Mucosal safety results

6.1 Histological assessment of rectal biopsies

Rectal biopsies were collected via anoscopy (9 cm from the anal verge) and flexible sigmoidoscopy (15 cm from the anal verge). Biopsies were formalin fixed; paraffin embedded, sectioned, and stained with haematoxylin and eosin and scored using a semi quantitative score of mucosal damage in a blinded fashion by a single gastrointestinal pathologist (Section 5.2).

6.1.1 Results

Within the anorectal biopsy (ARB) samples, following seven exposures to study product, the histology score was significantly increased in the N9 arm (1.8) compared to the TFV (0.7, P = 0.01) and the HEC (1.1, P = 0.03) arms (Figure 6-1). Within the flexible sigmoidoscopy biopsy (FSB) samples, there were no significant differences between the study arms (Figure 6-2).

6.1.2 Discussion

Previous human studies have reported that rectal exposure to N9 is associated with increased rates of epithelial sloughing (Phillips et al., 2000; Phillips et al., 2004) and histological abnormalities including separation of the epithelium from the underlying mucosa (Phillips et al., 2004). In the HIVNET 008 study of N9 gel, 69% of the placebo recipients had slightly abnormal or abnormal histology compared to 89% of the N9 recipients (Tabet et al., 1999). Consequently, it is not surprising that N9 use in the MTN-007 study was associated with an increase in the histology damage score. However, it was interesting that the mucosal changes were only seen in the ARB samples suggesting that the exposure to N9 at the FSB site (15

cm from the anal margin) was insufficient to result in mucosal damage. No changes were seen in the TFV, HEC, or No Rx arms of the study although an exposure period of 7 days is still relatively brief for a product that could be used over extended periods of time.

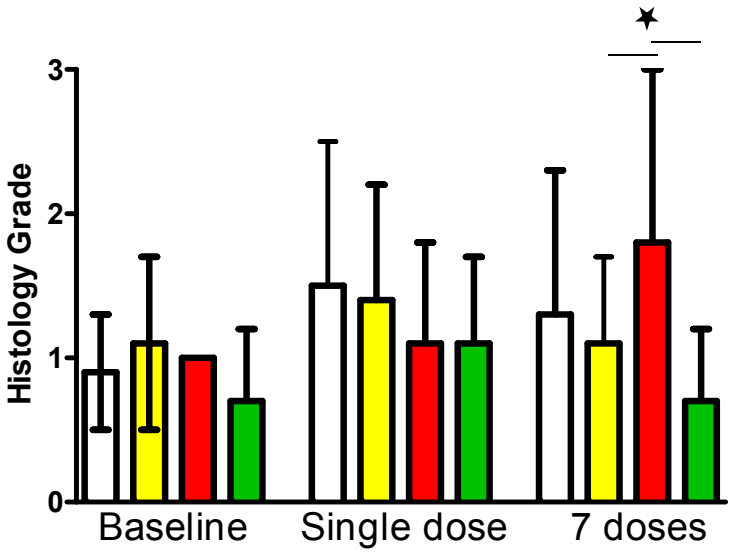


Figure 6-1 Anorectal biopsy histological scores

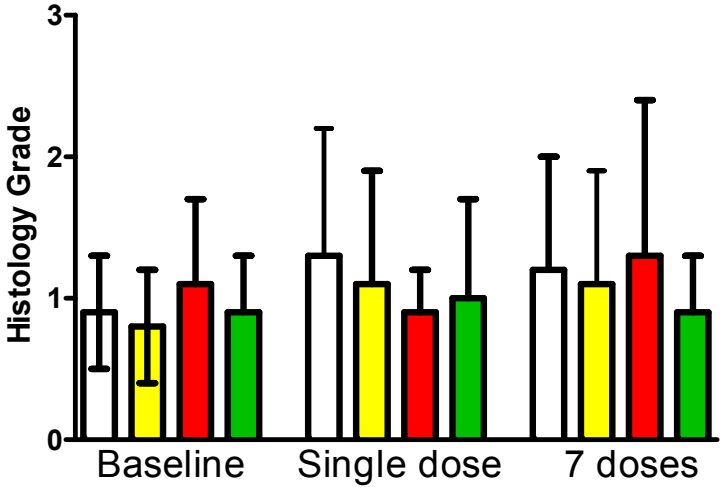


Figure 6-2 Histology grade for the flexible sigmoidoscopy biopsies

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

6.2 Epithelial sloughing in rectal lavage fluid

Cell pellets were isolated from lavage fluid obtained from participants following an isotonic enema. 2% paraformaldehyde (1 mL) was gently added to resuspend each pellet. The cellular suspension was then added to a petri dish with 10 mL of PBS. The petri dish that was then scanned at using a dissecting microscope and the number of epithelial sheets >2 mm counted in each quadrant. The total number of epithelial sheets in all four quadrants was then submitted as the final score for the epithelial sloughing assay (Section 5.3).

6.2.1 Results

There were no significance differences in the epithelial sloughing score between study arms at any time point during the study (Figure 6-3).

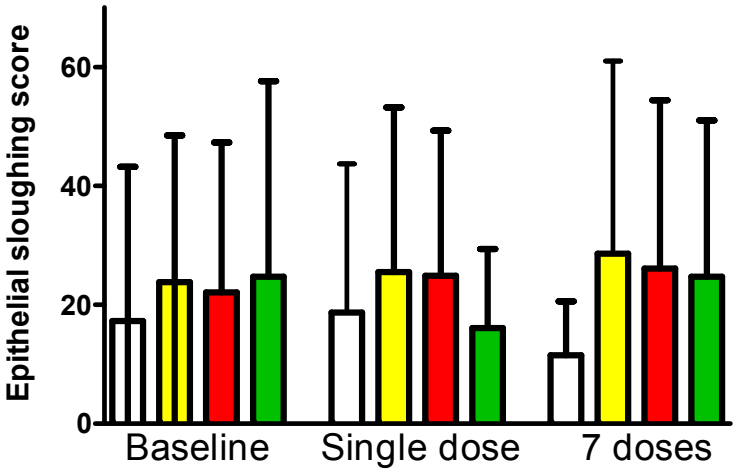


Figure 6-3 Epithelial sloughing scores

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

6.2.2 Discussion

Increases in epithelial sloughing following exposure to N9 have been previously reported in murine (Phillips & Zacharopoulos, 1998), NHP (Patton, Cosgrove Sweeney, Rabe, & Hillier, 2002) and human studies (Phillips et al., 2000; Phillips et al., 2004). We did not see significant changes in epithelial sloughing across any of the study arms. Indeed, we saw evidence of sloughing in 15-20% of baseline samples, a finding not documented in the studies by Phillips et al. (Phillips et al., 2000; Phillips et al., 2004). It is possible that the preparatory isotonic enema used in the MTN-007 study contributed to these divergent results. In the Phillips study, participants administered a 10 mL saline lavage (Phillips et al., 2000) whereas the MTN-007 preparatory enema was 120 mL of Normosol. The significantly larger volume in the MTN-007 study may have induced baseline epithelial sloughing. It is also possible that the processing of the lavage fluid in MTN-007 increased the sensitivity of the assay and created more background noise. Both the RMP-01 and RMP-02/MTN-006 Phase 1 rectal safety studies included epithelial sloughing assays and in both cases exposure to antiretroviral rectal microbicides was not associated with significant increases in epithelial sloughing (Anton et al., 2011; Anton et al., 2012).

6.3 Fecal calprotectin

Stool samples were collected from study participants at each clinic visit and sent to a commercial laboratory for quantification of fecal calprotectin levels (Section 5.4).

6.3.1 Results

There were no significance differences in the faecal calprotectin levels between study arms at any time point during the study (Figure 6-4).

6.3.2 Discussion

Quantification of fecal calprotectin is widely used in gastroenterology to help differentiate between patients with inflammatory or infectious colitis and patients with functional bowel disease in order to avoid unnecessary invasive diagnostic procedures such as colonoscopy (Abraham & Kane, 2012). Fecal calprotectin has been used in previous rectal microbicide trials but has not been shown to be increased after exposure to UC781 or TFV gels (Anton et al., 2011; Anton et al., 2012). There were no significant increases in fecal calprotectin in the MTN-007 trial. One participant in the HEC gel arm had an unusually high fecal calprotectin level after a single dose of HEC (1,347 μg/mL) but the values at the Baseline visit and after 7 doses of HEC gel were both < 16 μg/mL). Consequently, it is unlikely that this elevation was product related. In addition there were no gastrointestinal adverse events reported at this clinic visit and so it is most likely that this represents a laboratory error.

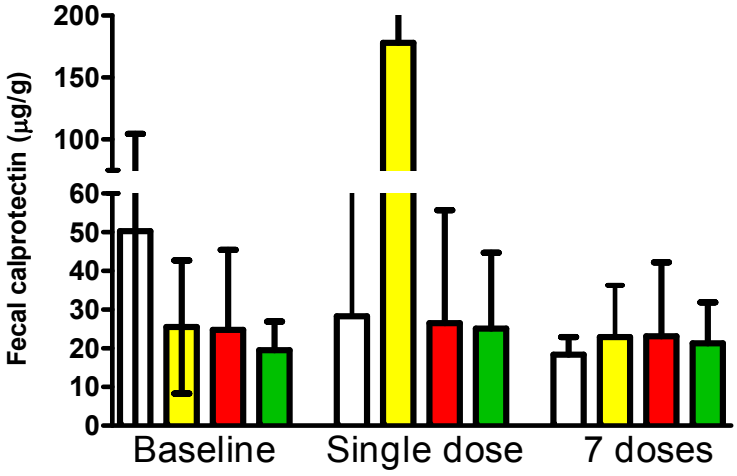


Figure 6-4 Fecal calprotectin levels

6.4 Assessment of rectal microflora

Rectal swabs were collected at each study visit and rectal microflora were characterized using semi-quantitative techniques (Section 5.5).

6.4.1 Results

There were relatively few significant changes in the rectal microflora following seven daily doses of study product. The prevalence of *Streptococcus viridans* (H_2O_2 producing) was significantly reduced in the TFV arm (20%) compared to the HEC (73%) and N9 (73%) arms (P = 0.006). The prevalence of other gram positive cocci was increased in the N9 arm (67%) compared to the no treatment arm (27%) (P = 0.03). Anaerobic gram positive rods were increased in the HEC arm (60%) compared to the TFV arm (13%) (Table 6-1).

Table 6-1 Rectal microflora culture data

Bacteria	No Rx		HEC gel		N9 gel		TFV gel	
	BL	D7	BL	D7	BL	D7	BL	D7
Lactobacillus (H_2O_2+)	33%	27%	27%	13%	33%	47%	67%	53%
Lactobacillus (H_2O_2-)	27%	27%	7%	13%	20%	20%	27%	13%
Gardnerella vaginalis	20%	13%	27%	27%	20%	13%	13%	7%
Diptheroids	93%	93%	80%	87%	100%	80%	93%	93%
Bacillus species	0%	7%	0%	7%	0%	0%	0%	0%
Gram-positive rods, other	60%	53%	53%	73%	60%	47%	60%	80%
Group B Streptococcus	40%	20%	27%	13%	33%	33%	47%	47%
Enterococcus species	13%	40%	27%	33%	0%	33%	13%	20%
Staphylococcus aureus	0%	0%	0%	0%	7%	0%	0%	0%
Staphylococcus species	60%	40%	67%	27%	40%	53%	33%	40%
Micrococcus species	0%	0%	13%	0%	7%	7%	0%	0%
Streptococcus viridans (H_2O_2+)	33%	47%	53%	**73%**	47%	**73%**	47%	**20%**
Streptococcus viridans (H_2O_2-)	40%	47%	53%	53%	60%	47%	53%	53%
Gram positive cocci*, other*	27%	**27%**	27%	60%	53%	**67%**	53%	40%
Escherichia coli	87%	87%	100%	93%	73%	80%	73%	80%
Proteus species	7%	7%	0%	0%	0%	0%	0%	0%
Gram negative rod, other	7%	0%	0%	7%	7%	20%	27%	20%
Bacteroides fragilis	100%	93%	100%	100%	100%	87%	100%	100%
Non-pigmented anaerobic GNR	87%	80%	73%	73%	87%	80%	67%	47%
Pigmented anaerobic GNR	87%	87%	87%	87%	87%	87%	93%	87%
Anaerobic gram-positive cocci	47%	93%	73%	80%	100%	73%	73%	80%
Anaerobic gram-positive rods, clostridium like	53%	67%	67%	53%	40%	60%	67%	60%
Anaerobic gram-positive rods, other	60%	33%	47%	**60%**	67%	47%	73%	**13%**
Yeast	0%	0%	0%	0%	0%	0%	0%	0%

Statistically significant differences between rectal microflora across arms after seven daily doses are highlighted in red and described further in Section 6.4.1.

6.4.2 Discussion

It is recognized that perturbations in vaginal microflora, such as those associated with bacterial vaginosis, may increase the risk of acquiring or transmitting HIV infection (Atashili et al., 2008; Cohen et al., 2012). As a consequence, semi-quantitative characterization of changes in vaginal microflora is an important component of the preclinical (Patton, Cosgrove Sweeney, & Paul, 2008) and clinical (Rosenstein et al., 1998) evaluation of candidate vaginal microbicides. A similar approach has been incorporated into the design of preclinical (Patton et al., 2009) and clinical (Anton et al., 2011; Anton et al., 2012) rectal microbicide studies.

In the MTN-007 study we demonstrated a significant decrease in *Streptococcus viridans* in the TFV gel arm (20%) compared to 73% in both the HEC and N9 gel arms although none of these arms were significantly different to the No Rx arm. Gram positive cocci were increased in the N9 gel arm (67%) compared to the No Rx arm (27%). Anaerobic gram positive rods were reduced in the TFV gel arm (13%) compared to the HEC gel arm. The biological or clinical significance of these findings is unclear as there were no significant differences in the prevalence of Grade 2 or higher gastrointestinal AEs across the arms of the study. There was a trend towards higher prevalence of flatulence in the TFV gel arm and other gastrointestinal AEs in the N9 gel arm. It is not clear how use of TFV gel might lead to a reduction in the prevalence of *Streptococcus viridans* and anaerobic gram positive rods as TFV gel does not have intrinsic antibacterial activity. The increase in gram positive cocci associated with N9 gel has previously been documented

in women receiving vaginal N9 (Rosenstein et al., 1998). MTN-007 did not collect rectal samples following a wash-out period and so it is unclear whether these perturbations persisted once product administration was stopped.

There has been an increasing focus on the interactions between the gut microbiome and GALT in health and disease (Shanahan, 2013; Saxena et al., 2012). This expansion of research has been driven by the development of culture-independent techniques to characterize gut microbiota. Examples of these techniques include terminal restriction fragment length polymorphism (T-RFLP), fluorescence in situ hybridisation (FISH), DNA microarrays, and next-generation sequencing of the 16S ribosomal (r) RNA (Fraher et al., 2012). These techniques have demonstrated the microbial diversity of the gut microbiota and have shown that changes in microbial composition and function can be associated with disease states such as obesity and inflammatory bowel disease (Shanahan, 2012). Culture-independent techniques have recently been used in Phase 1 vaginal microbicide studies to characterize the vaginal microbiota (Ravel et al., 2012). This approach was able to demonstrate a shift towards a microbial pattern seen in bacterial vaginosis. Use of similar techniques in Phase 1 rectal microbicide studies may provide further insights into the gut microbial response to candidate microbicides.

6.5 Rectal secretion of cytokines and chemokines

Rectal fluid samples were collected at each study visit and cytokines/chemokines of interest were quantified using Luminex® technology (Section 5.6)

6.5.1 Results

Rectal fluid TNF-α levels following seven daily doses of study product were significantly higher in the HEC arm (2.7 pg/mL) compared to the no treatment arm (1.4 pg/mL; P = 0.04). Day 7 IL-8 levels were also significantly higher in the N9 arm (537.3 pg/mL) compared to the TFV arm (164.3; P = 0.02). Following a single dose of study product MIP-1α levels were significantly lower in the TFV arm (2.6 pg/mL) compared to the No Rx arm (7.2 pg/mL; P = 0.03). RANTES levels were increased following a single dose of N9 gel (335.9 pg/mL) compared to the no treatment (33.1 pg/mL; P = 0.01), HEC (12.1 pg/mL; P = 0.0013), and TFV (8.3 pg/mL; P = 0.0036) arms of the study (Figures 6-5 and 6-6).

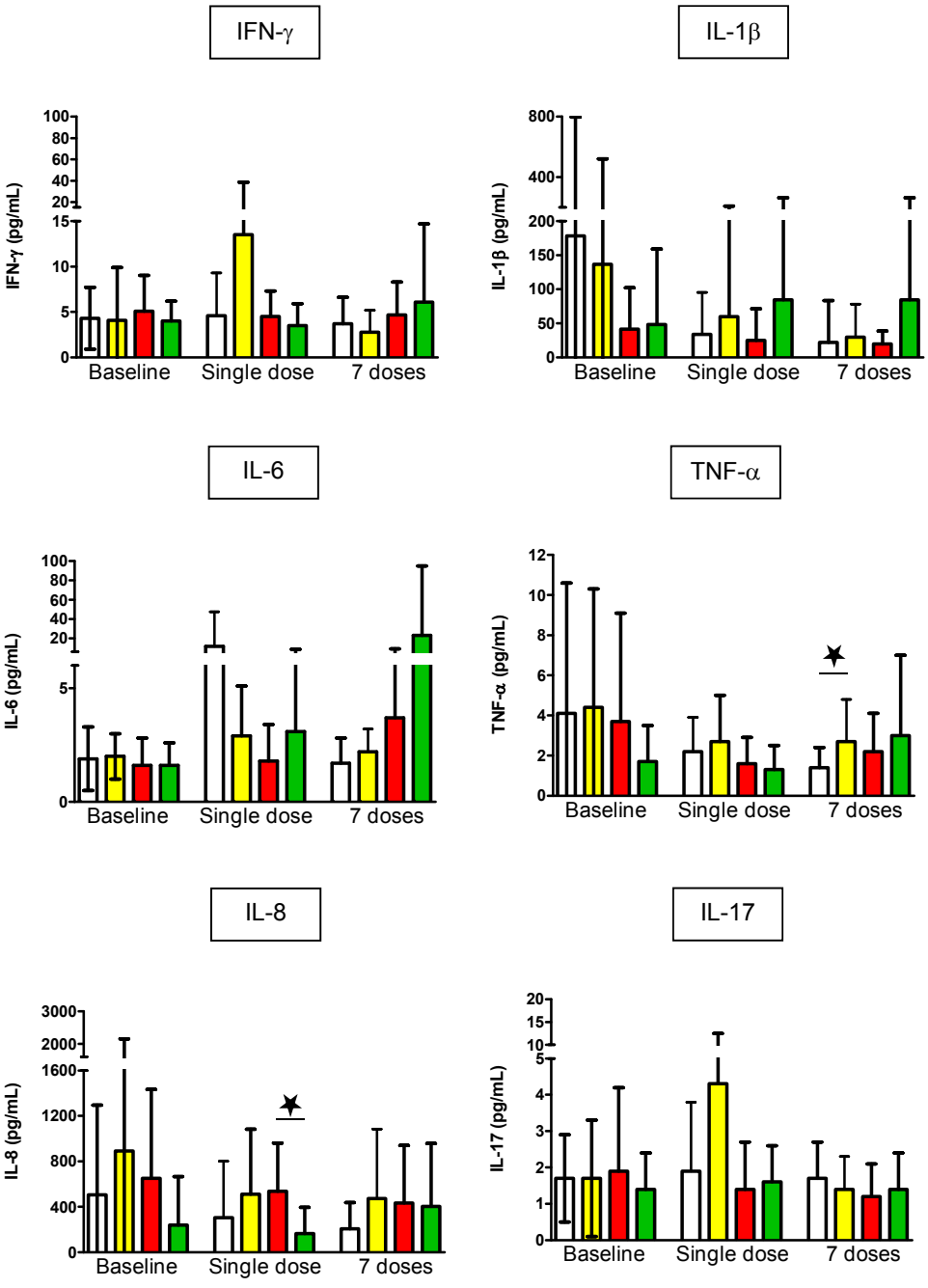


Figure 6-5 Luminex data (I)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

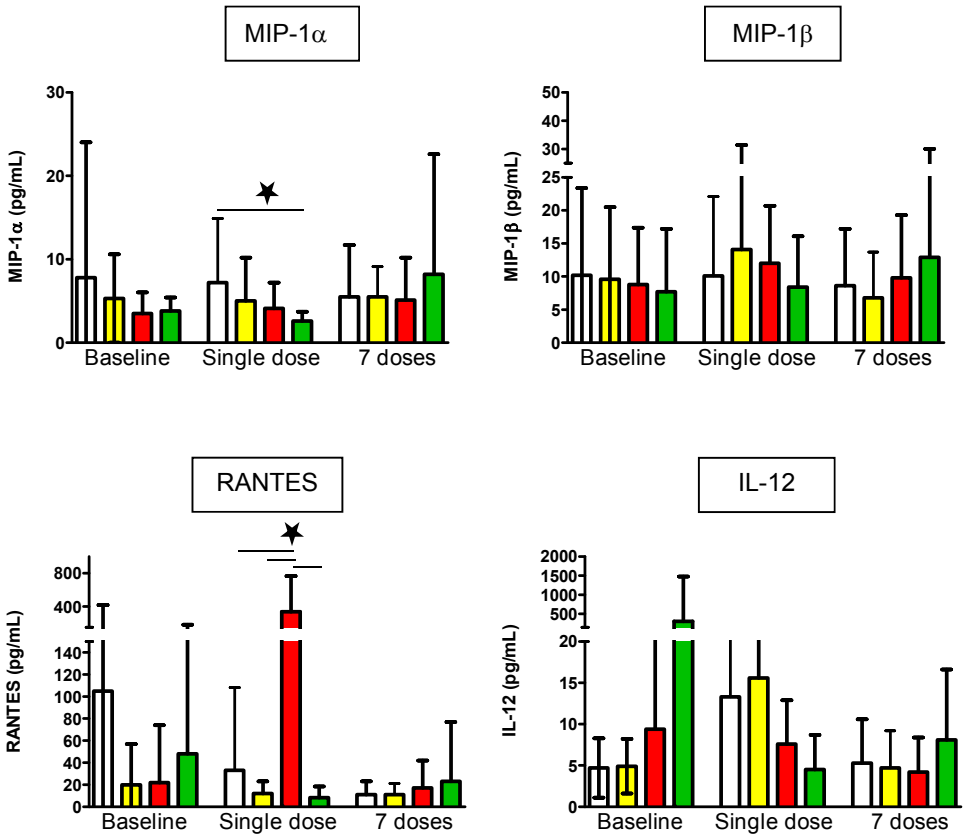


Figure 6-6 Luminex data (II)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

6.5.2 Discussion

The Luminex data from the MTN-007 study demonstrated a significant increase in TNF-α secretion associated with exposure to HEC gel, an increase in IL-8 and RANTES associated with exposure to N9 gel, and a decrease in MI-1β associated with exposure to TFV gel. The HEC data are somewhat surprising as the HEC gel is generally regarded as inert and not associated with induction of mucosal responses (Tien et al., 2005; Schwartz

et al., 2007; Cone et al., 2006; Richardson et al., 2013). However, N9 is known to be associated with induction of mucosal inflammation and release of inflammatory mediators such as IL-1β and IL-8 (Fichorova et al., 2001; Hillier et al., 2005). Reduced levels of cytokine/chemokine secretion following exposure to ARV gels have been seen in previous rectal microbicide trials. In the RMP-01 study, after single rectal dose exposure, there was a significant reduction in RANTES in the 0.1% UC781 gel arm (31.06 ± 17.2 pg/mL) compared to the placebo gel (71.79 ± 54.38 pg/mL) (Anton et al., 2011). In the RMP-02/MTN-006 study, after 7 days of dosing, there were significant cytokine reductions seen in the TFV gel compared to the HEC placebo gel: IL-1β (1.55 ± 1.64 *vs.* 5.43 ± 3.71 pg/mL), MIP-1α (4.69 ± 10.78 *vs.* 11.76 ± 7.88 pg/mL), IL-6 (1.29 ± 1.48 *vs.* 4.13 ± 3.25 pg/mL), and TNF-α (4.57 ± 9.18 *vs.* 8.15 ± 9.49 pg/mL) (Anton et al., 2012). The mechanism of action by which a topically applied microbicide might modulate secretion of cytokines/chemokines is uncertain. However, Melchjorsen et al. have demonstrated the ability of TFV to reduce lipopolysaccharide (LPS) induced cellular secretion of IL-8 and MIP-1α in U937 monocyte cells (Melchjorsen et al., 2011).

Collection of biological samples for quantification of cytokines/chemokines has been widely used in vaginal microbicides studies (Fichorova, 2004) and has been adopted as a potential biomarker in rectal microbicide studies. However, some of the preliminary studies undertaken prior to implementation of quantification of cytokine/chemokines in vaginal studies have not been conducted in the rectal compartment. Fichorova et al. have

conducted multi-centre studies to determine the biological and technical variable associated with quantification of cytokine/chemokine levels in vaginal fluids (Fichorova, 2004). Dezzutti et al. have undertaken comparative assessment of different vaginal collection modalities (endocervical swabs, vaginal swabs, and cervicovaginal lavage fluid) and their influence on the quantity of cytokine/chemokine recovery (Dezzutti et al., 2011). Studies have also been undertaken to characterize the baseline variation in vaginal cytokine/chemokine secretion in different populations (Fichorova et al., 2011). Unfortunately, none of these studies have been conducted for studies involving collection of anorectal samples. A further complication is that the stability of plasma and serum cytokines/chemokines during prolonged storage is highly variable as is their response to freeze/thaw cycles (de et al., 2009) and there is a need to conduct similar studies for vaginal and rectal samples.

6.6 Cytokine and chemokine gene expression in mucosal tissue

RNA was isolated from rectal biopsies and reverse transcribed into cDNA. Quantitative real time PCR was then performed using standard curves generated from plasmid DNA for the targets of interest. All the gene expression data were transformed to log base 10 and normalised to the house keeping gene β2-microglobulin (β2M) by calculating the ratio of the gene expression levels of the cytokine/chemokine of interest to β2M gene expression. In this analysis higher ratio numbers are associated with lower levels of gene expression. As one example, a ratio figure of -2.2 represents a one log higher level of gene expression than -3.2 (Section 5.7).

6.6.1 Results

IL-1β gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-2.2) compared to the other study arms; No Rx (-3.2; P = 0.001), HEC (-2.9; P = 0.0007), and TFV (-2.9; P = 0.0006). No significant differences were seen in the FSB samples (Figure 6-7).

IL-6 gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-3.1) compared to the other study arms; No Rx (-4.3; P = 0.004), HEC (-4.0; P = 0.003), and TFV (-4.2; P = 0.00001). IL-6 expression in the FSB samples, following seven product exposures, was significantly increased in HEC arm (-4.0) compared to the N9 arm (-4.3; P = 0.02) (Figure 6-7).

There were no significant changes in IFN-γ gene expression in the ARB samples, following single or multiple dose product exposure. IFN-γ gene expression in the FSB samples, following single dose exposure, was significantly increased in the HEC arm (-4.3) compared to the No Rx (-4.7; P = 0.04) and N9 (-4.4; P = 0.04) arms (Figure 6-7).

TNF-α gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-2.8) compared to the No Rx (-3.8; P = 0.003) and HEC (-3.5; P = 0.01) arms. There were no significant changes in TNF-α gene expression in the FSB samples (Figure 6-8).

IL-8 gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-2.1) compared to the other study arms; No Rx (-3.2; P = 0.005), HEC (-3.0; P = 0.0003), and TFV (-3.0; P = 0.00004). There were no significant changes in IL-8 gene expression in the FSB samples (Figure 6-8).

MIP-1α gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-2.8) compared to the other study arms; No Rx (-3.7; P = 0.003), HEC (-3.5; P = 0.003), and TFV (-3.4; P = 0.003). There were no significant changes in MIP-1α gene expression in the FSB samples (Figure 6-8).

MIP-1β gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-2.5) compared to the other study arms; No Rx (-3.3; P = 0.008), HEC (-3.0; P = 0.007), and TFV (-2.9; P = 0.002). MIP-1β gene expression in the FSB samples, following single dose exposure, was significantly increased in the TFV arm (-3.0) compared to the N9 (-3.2; P = 0.02) arm (Figure 6-9).

There were no significant changes in RANTES gene expression in the ARB samples, following single dose exposure. Variable changes were seen after seven product exposures. RANTES gene expression was increased in the N9 arm (-2.5) compared to the No Rx (-2.6; P = 0.02) and the TFV (-2.7; P = 0.02) arms. However RANTES gene expression was increased in the HEC

arm (-2.4) compared to the N9 arm (-2.5; P = 0.03). In the FSB samples, following single dose exposure, there was a significant reduction in RANTES gene expression between the TFV arm (-2.5) and the No Rx arm (-2.5; P = 0.01). After seven exposures, there was a significant increase in RANTES gene expression in the N9 arm (-2.2) compared to the TFV arm (-2.5; P = 0.01) (Figure 6-9).

IL-12p40 gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-4.4) compared to the No Rx arm (-5.2; P = 0.002). IL-12p40 gene expression in the FSB samples, following seven dose exposures, was significantly increased in the N9 arm (-4.8 compared to the HEC arm (-4.9; P = 0.04). IL-12p40 gene expression was also significantly increased in the HEC arm (-4.9) compared to the TFV arm (-5.2; P = 0.006) (Figure 6-9).

There were no significant changes in IL-17 gene expression in the ARB samples, following single dose exposure. After seven product exposures, IL-17 gene expression was increased in the No Rx arm (-4.9) compared to the TFV (-4.9; P = 0.02) arm. In the FSB samples, following single dose exposure, there was a significant increase in IL-17 gene expression in the HEC arm (-4.4) compared to the No Rx arm (-5.2; P = 0.02) (Figure 6-10).

IL-23 gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-3.6) compared to the No Rx (-4.6; P = 0.01) and HEC (-3.9; P = 0.02) arms. IL-23 gene expression in the FSB

samples, after seven product exposures, was significantly increased in the HEC arm (-3.9) compared to the No Rx arm (-4.7; P = 0.008) (Figure 6-10).

CCR5 gene expression in the ARB samples, following single dose exposure, was significantly increased in the N9 arm (-3.9) compared to the No Rx (-4.6; P = 0.006) and HEC (-4.1; P = 0.03) arms. There were no significant changes in CCR5 gene expression in the FSB samples (Figure 6-10).

6.6.2 Discussion

Significant changes in gene expression were noted in 36 of 144 potential comparisons (25%). Changes were more common in the ARB samples (26/72; 36%) compared to the FSB samples (10/72; 14%) suggesting the possibility of a dose response effect. The majority of changes (30/36; 83%) resulted in increased gene expression and were associated with exposure to N9 and were more common in the ARB samples (25/26; 96%) compared to the FSB samples (5/10; 50%). N9 exposure was associated with increases in all of the cytokines/chemokines evaluated apart from IFN-γ. N9 exposure also resulted in increased expression of the HIV co-receptor CCR5. In contrast, changes associated with TFV were much more limited and included increased expression of RANTES compared to the HEC arm (FSB samples), reduced expression of IL-12 (FSB) compared to HEC, and reduced expression of IL-17 (ARB) compared to No Rx. HEC exposure resulted in increased expression of IFN-γ (FSB), IL-17 (FSB) and IL-23 (FSB) compared to No Rx. The changes in gene expression associated with exposure to N9 are in keeping with previous studies that have demonstrated mucosal injury and increased secretion of proinflammatory cytokines (Hillier

et al., 2005). Dose dependent increased expression of RANTES has previously been documented in murine peritoneal macrophages exposed to TFV (Zidek, Frankova, & Holy, 2001) and it appears that TFV can inhibit inflammatory cytokine expression in cell lines (Melchjorsen et al., 2011). The changes associated with HEC are more surprising as HEC gel is regarded as an inert universal placebo (Schwartz et al., 2007). The significance of the changes associated with HEC is unclear. A recent large Phase 2B trial (HPTN-035) confirmed that there were no significant differences in the clinical safety profile of women randomised to receive HEC gel (N= 771) compared to No Rx (N = 772) (Richardson et al., 2013). However, it is possible that the columnar epithelium of the distal colon is more susceptible to product related damage than the stratified squamous epithelium of the vaginal compartment. It was interesting that increased gene expression was more common in the ARB samples compared to the FSB samples suggesting product dilution by rectal fluid and or fecal matter might result in lower exposure to the more proximal sampling site.

Previous rectal microbicide studies have included limited testing of mucosal gene expression (discussed in Section 5.7.2) and so the MTN-007 data set provides a more comprehensive assessment of changes in gene expression following product exposure. Another advantage of the qRT-PCR method used in the study is that gene expression data were normalised to a house keeping gene (β2M). Prior to final data analysis, preliminary assessment was conducted for three other housekeeping genes (β-actin, GAPDH, and CD45). Intra-class correlation (ICC) was calculated based on data from all

three visits for the four house-keeping genes (Table 6-2). Based on ICC, β2-microglobulin was found to be the most stable housekeeping gene and was therefore used to normalise the cytokine/chemokine gene expression data.

Table 6-2 Intraclass correlation for house-keeping genes

	Log GAPDH	Log β-actin	Log β2-microglobulin	Log CD45
ARB samples	0.69	0.87	0.95	0.78
FSB samples	0.79	0.84	0.92	0.70
Combined	0.72	0.85	0.93	0.70

In comparing the Luminex and qRT-PCR data there were more significant changes noted with qRT-PCR than with Luminex. This is not surprising as the qRT-PCR was able to detect down to 5 copies of the majority of the cytokines/chemokines (Figures 5-6 to 5-11). Neverthertheless, increases in expression of TNF-α, IL-8, and RANTES noted by qRT-PCR were also seen in the Luminex data.

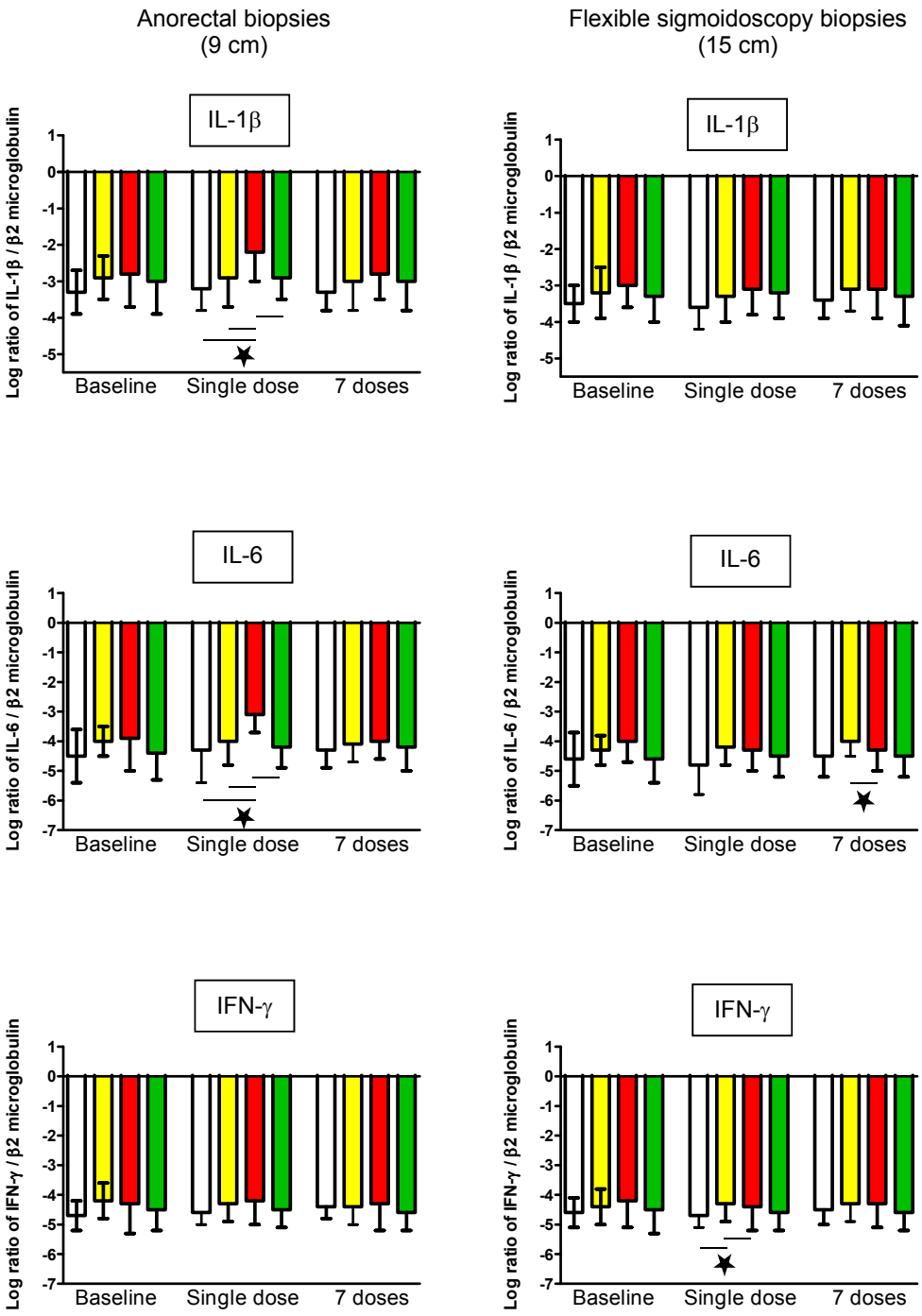


Figure 6-7 RT-PCR data (I)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

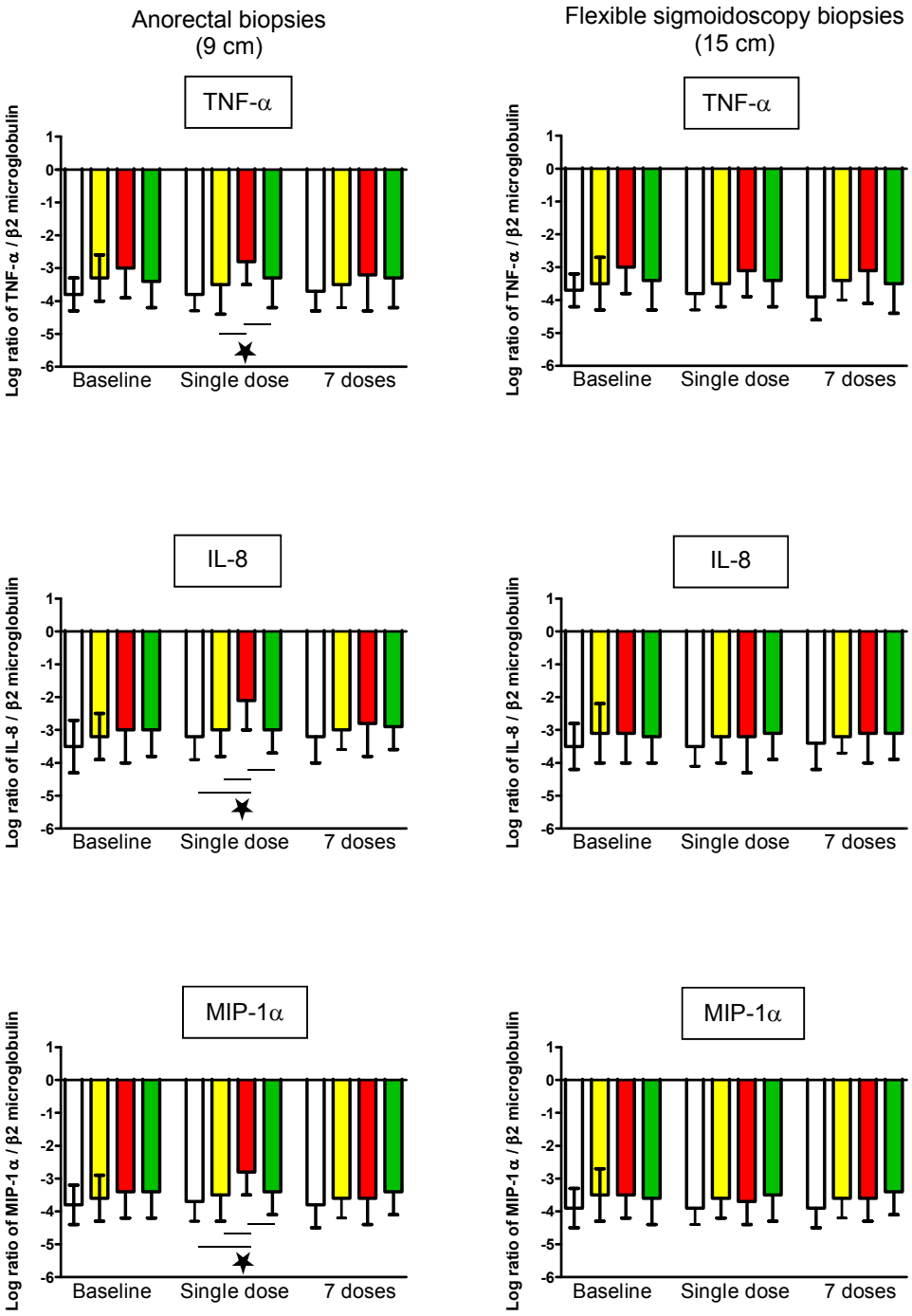


Figure 6-8 RT-PCR (II)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

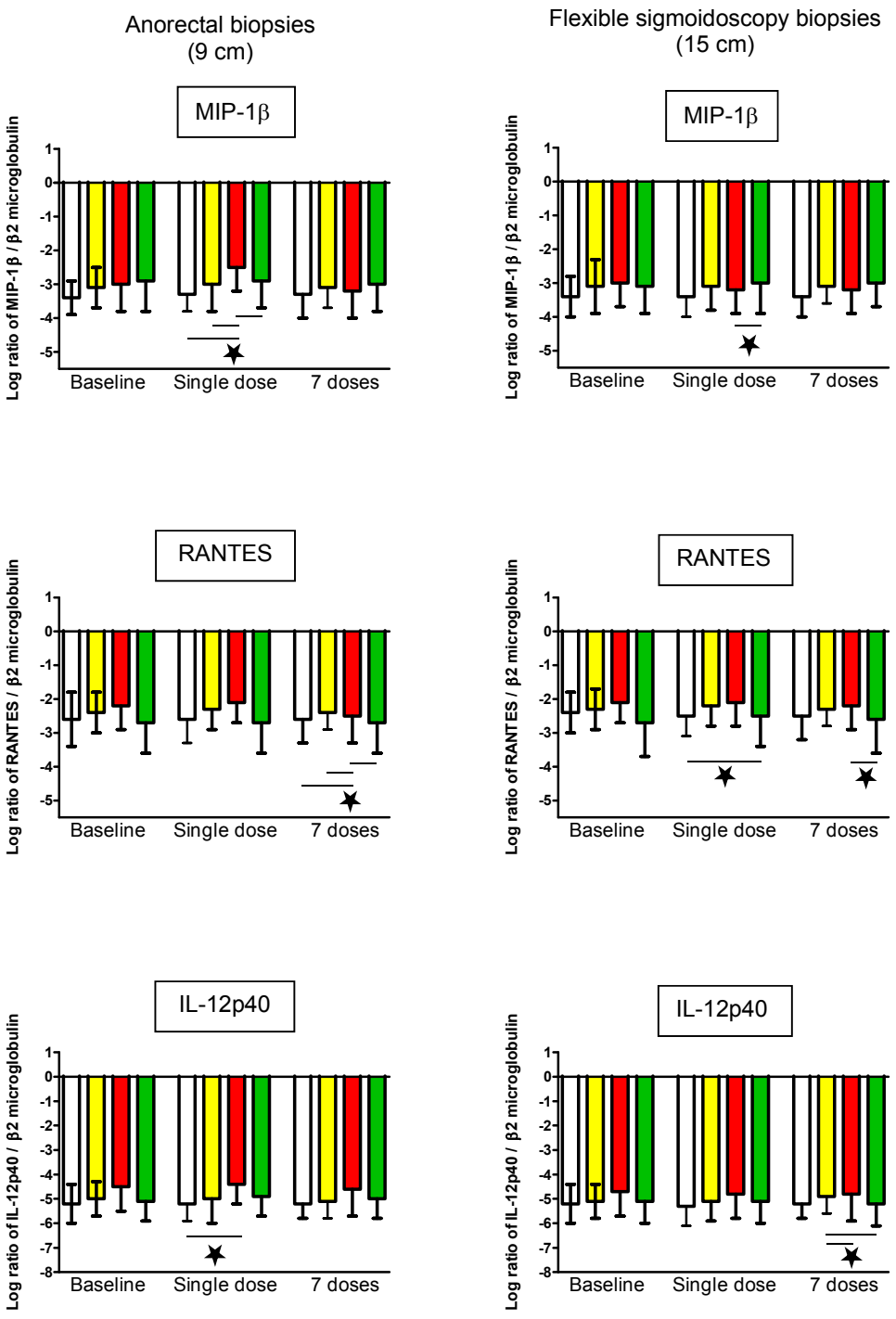


Figure 6-9 RT-PCR data (III)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

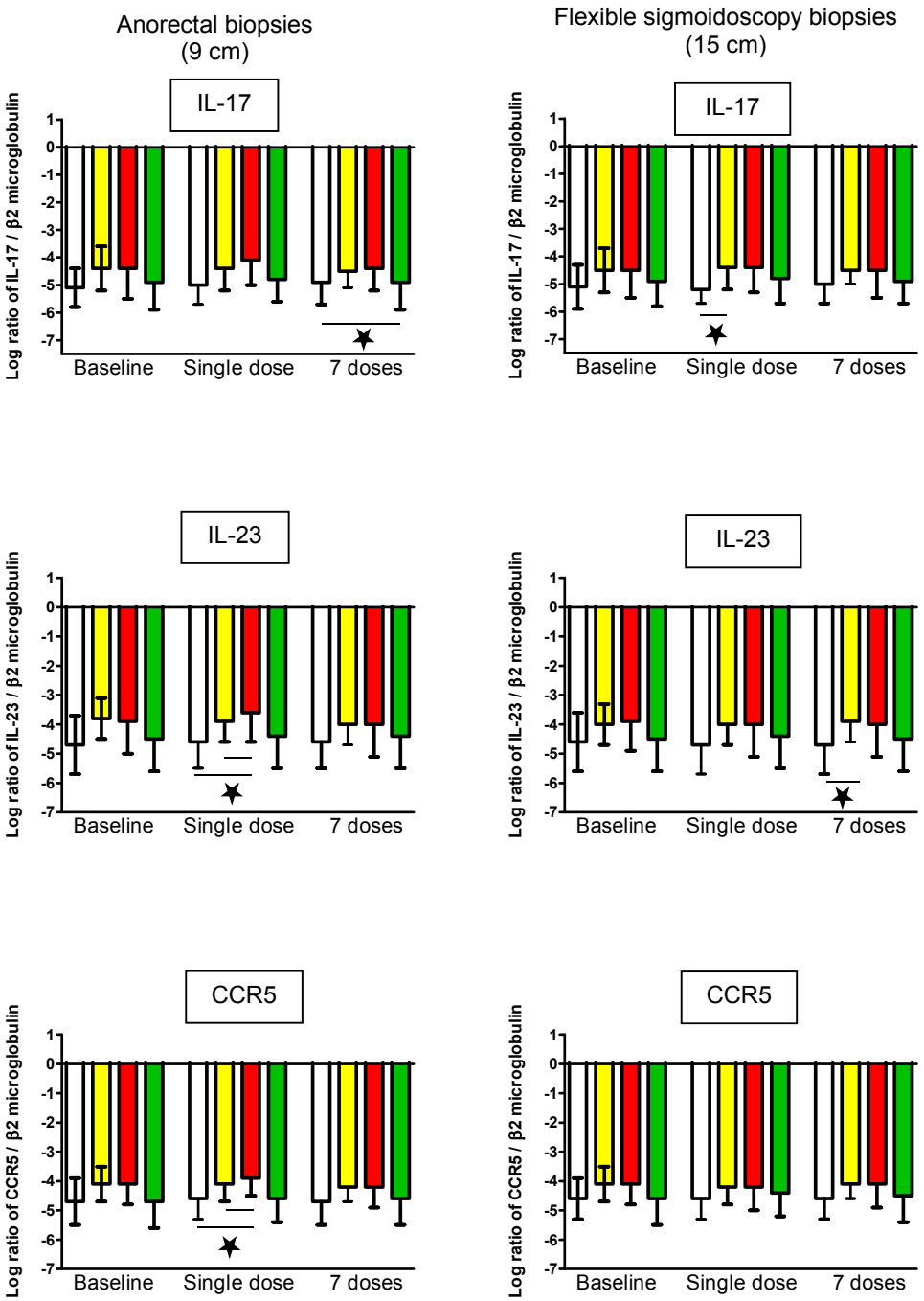


Figure 6-10 RT-PCR data (IV)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

6.7 Flow cytometric analysis of mucosal T cells

Mucosal mononuclear cells were liberated from intestinal biopsies by enzymatic digestion. They were then stained with monoclonal antibodies to a range of T cell markers. Flow cytometry was undertaken to quantify the prevalence of various GALT T cell populations before and after exposure to study products. Data are presented on the prevalence of the following T cell subsets; CD45+/CD3+, CD3+/CD4+, CD3+/CD8+, CD3+/CD4+/CCR5+, CD3+/CD4+/CXCR4+, CD3+/CD4+/CCR5+/CXCR4+, CD3+/ CD4+/CD69+, and CD3+/ CD4+/CD69+ T cells (Section 5.8).

6.7.1 Results

The prevalence of CD45+/CD3+ T cells in the ARB samples, following seven doses of study product, was significantly reduced in the HEC arm (41.0%) compared to the No Rx arm (57.7%; P = 0.006). There was a significant increase in CD45+/CD3+ T cells in the FSB samples, following seven doses of study product in the TFV arm (57.3%) compared to the HEC arm (42.8%; P = 0.04)(Figure 6-11).

The prevalence of CD3+/CD4+ T cells in the ARB samples, following seven doses of study product, was significantly increased in the N9 arm (60.5%) compared to the No Rx (50.1%; P = 0.01) or HEC arms (53.8%; P = 0.04). There was no significant increase in CD3+/CD4+ T cells in the FSB samples (Figure 6-11).

The prevalence of CD3+/CD8+ T cells in the ARB samples, following seven doses of study product, was significantly reduced in the N9 arm (27.5%)

compared to the No Rx arm (37.3%; P = 0.002). The prevalence of CD3+/CD8+ T cells in the FSB samples, following seven doses of study product, was also significantly reduced in the N9 arm (32.8%) compared to the No Rx arm (36.5%; P = 0.04) (Figure 6-11).

There were no significant changes in CD3+/CD4+/CCR5+ T cells in the ARB or FSB samples in any of the study arms (Figure 6-12).

The prevalence of CD3+/CD4+/CXCR4+ T cells in the ARB samples, following seven doses of study product, was significantly increased in the N9 arm (59.6%) compared to the No Rx arm (38.2%; P = 0.02). There was no significant increase in CD3+/CD4+/CXCR4+ T cells in the FSB samples (Figure 6-12).

There were no significant changes in CD3+/CD4+/CCR5+/CXCR4+ T cells in the ARB or FSB samples in any of the study arms (Figure 6-12).

There were no significant changes in CD3+/CD4+/CD69+ T cells in the ARB or FSB samples in any of the study arms (Figure 6-13).

The prevalence of CD3+/CD8+/CD69+ T cells in the ARB samples, following seven doses of study product, was significantly decreased in the N9 arm (69.5%) compared to the No Rx arm (83.4%; P = 0.02). The prevalence of CD3+/CD8+/CD69+ T cells in the FSB samples, following a single dose of

study product, was significantly decreased in the TFV arm (81.8%) compared to the No Rx arm (89.0%; P = 0.02) (Figure 6-13).

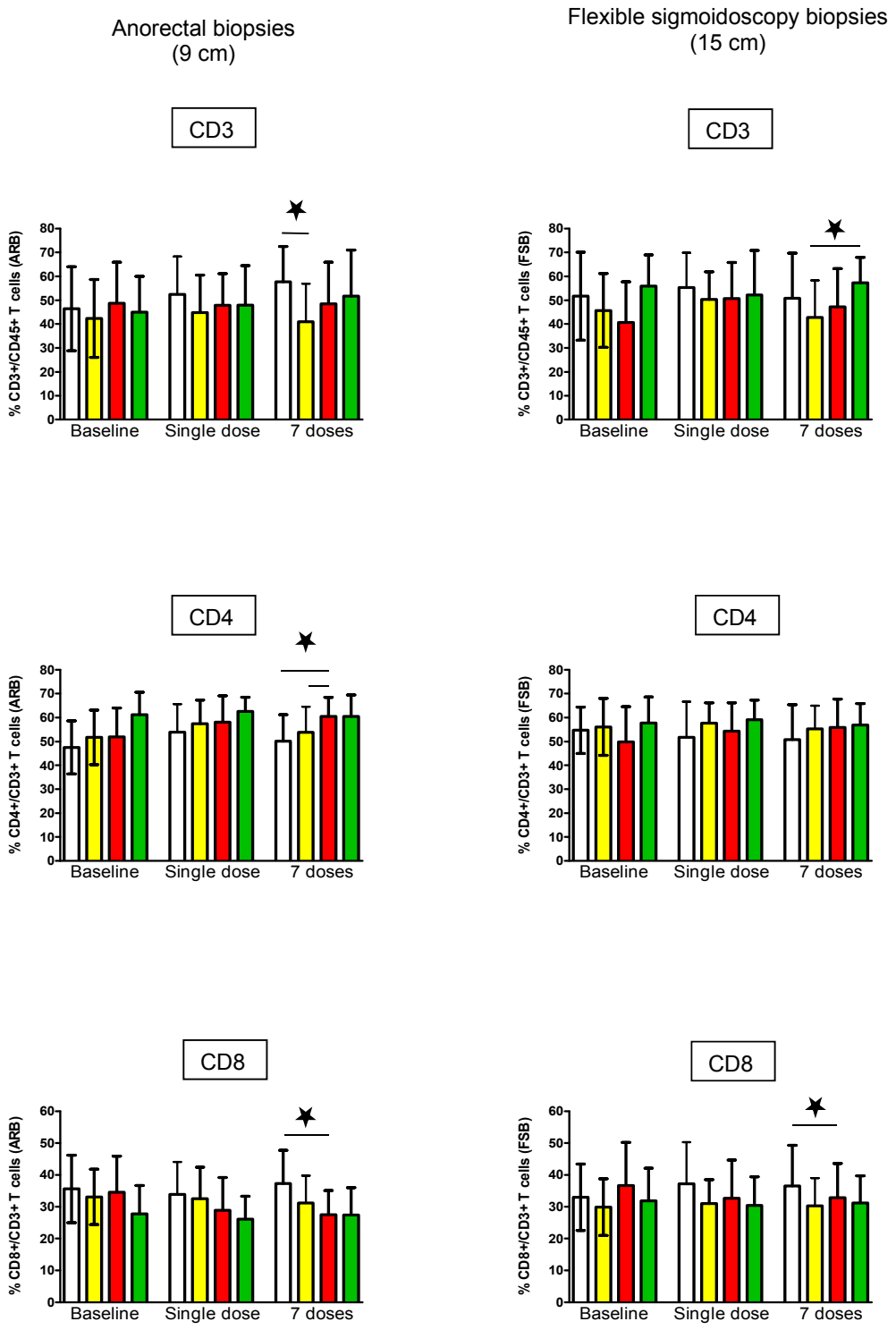


Figure 6-11 Flow cytometry data (I)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

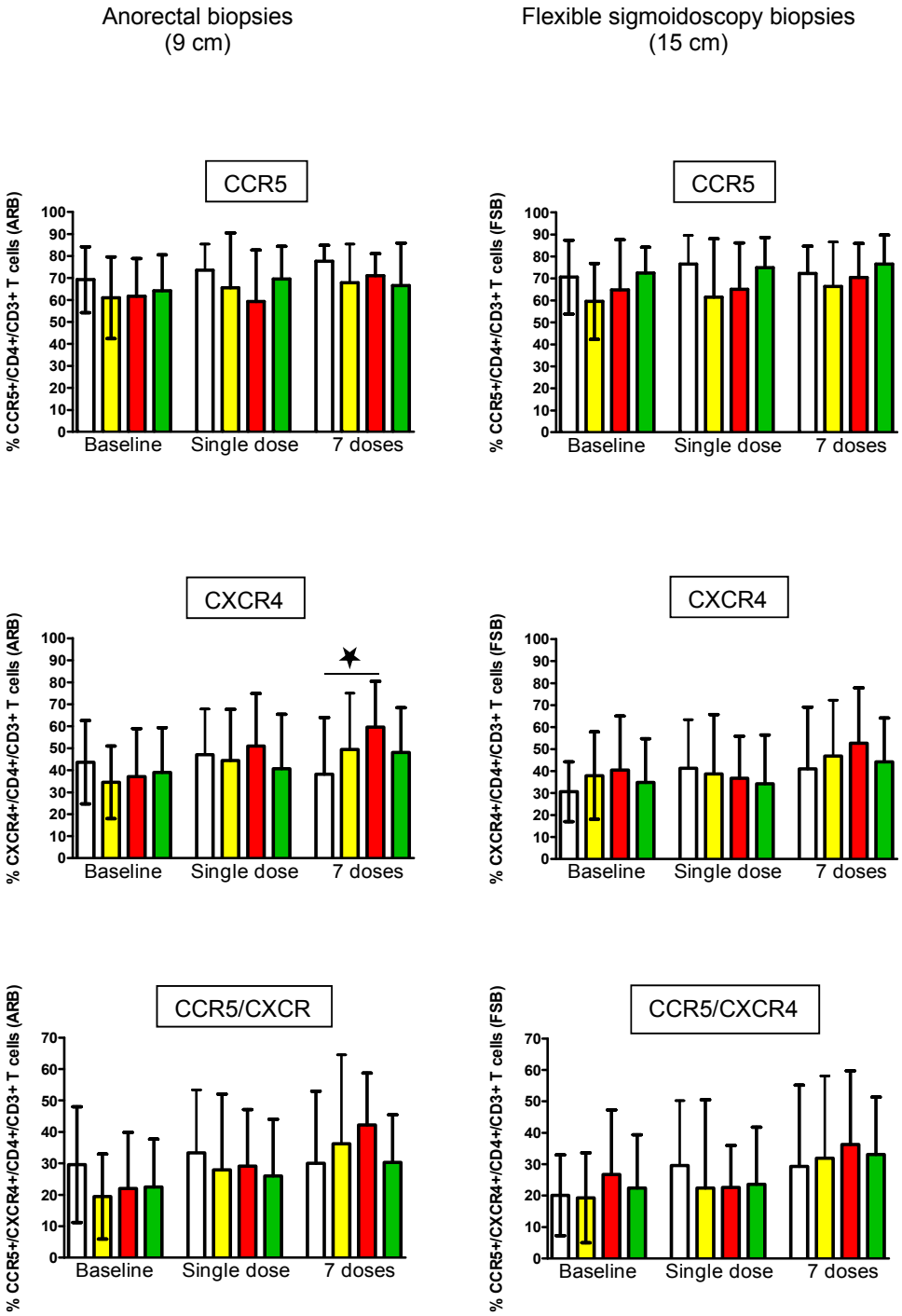


Figure 6-12 Flow cytometry data (II)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

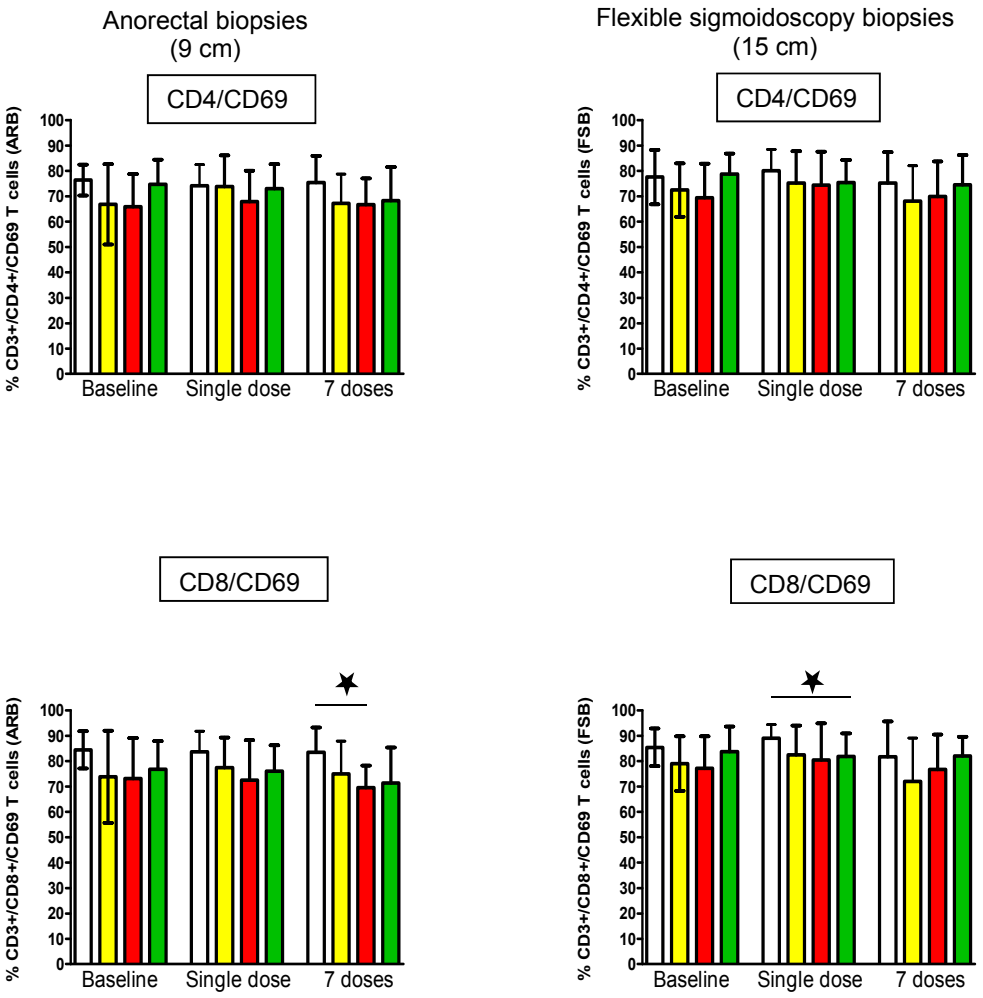


Figure 6-13 Flow cytometry data (III)

No treatment, HEC gel, Nonoxynol-9 gel, Tenofovir gel

P< 0.05

6.7.2 Discussion

The primary rationale for including flow cytometric assessment in the MTN-007 study was the concern that repetitive exposure to rectal microbicides might be associated with changes in GALT that could lead to increased risk of HIV acquisition. Changes in T cell phenotype might include increased prevalence of CD4+ HIV target cells in the intestinal mucosa, increased prevalence of CD4+ T cells expressing CCR5 or CXCR4, and increased activation phenotype (CD69+) of those target cells.

In the MTN-007 study we saw a reduction in CD45+/CD3+ cells in the HEC arm (ARB) after seven exposures compared to No Rx and conversely an increase in CD45+/CD3+ cells in the TFV arm (FSB) compared to the HEC arm after seven exposures. Exposure to N9 was associated with an increase in CD3+/CD4+ cells, a decrease in CD3+/CD8+ cells, an increase in CD3+/CD4+/CXCR4+ cells, and a decrease in CD3+/CD8+/CD69+ cells in both TFV (FSB) and N9 (ARB) arms compared to the No Rx arm.

Flow cytometry did not identify any significant increases in CD3+/CD4+ T cells following exposure to TFV gel. In contrast, exposure to N9 did result in an increase in CD3+/CD4+ T cells. In a previous Phase 3 vaginal microbicide study (COL1492), repetitive exposure to an N9 gel did increase the rate of HIV acquisition in study participants (Van Damme et al., 2002). These women, who used 3-5 applicators per day, also had a high incidence of vaginal lesions with epithelial disruption. A possible increase in CD4+ T

cells could have also contributed to the risk of HIV acquisition in these women.

There are a number of limitations associated with the flow cytometry approach used in MTN-007. The flow panels used in the study focused on T cell populations. Other mucosal cell populations such as macrophages, dendritic cells, and B cells all play important roles in the transmission of HIV infection (Haase, 2001) and were not characterized in this study. In addition we explored changes in the prevalence of various T cell populations but did not attempt to quantify these changes in absolute terms as is routinely undertaken in flow cytometric assessment of peripheral blood samples. Another issue that is often raised is the degree of sampling heterogeneity associated with random biopsies of the colon. As one example, a biopsy that samples a lymphoid aggregate in the mucosa may reveal a different population of cells compared to a biopsy that only samples lamina propria. The MTN-007 flow panels were derived from three individual rectal biopsies and so this would have reduced any bias associated with the sampling process. We were unable to demonstrate an increase in T cell activation defined by expression of the CD69 phenotype. Indeed, exposure to N9 and TFV was associated with a reduction in CD69 expression. CD69 is expressed early during T cell activation and was highly expressed at baseline in gut T cells (66-84%). Future studies should explore the differential expression of other activation markers such as CD38 and HLA-DR. Recent studies of maraviroc have demonstrated changes in GALT phenotype including enhanced CCR5 expression and markers of T cell

activation (Hunt et al., 2013). These are not desirable features for a PrEP agent and so it will be important to continue to monitor GALT phenotype in future rectal microbicide studies involving maraviroc as well as other antiretroviral agents.

6.8 Additional analyses of mucosal safety data

6.8.1 Gender

The MTN-007 study enrolled 45 men and 20 women. Data were evaluated to determine whether gender-specific significant differences across the range of mucosal biomarkers. Table 6-3 summarises mucosal parameters that reached significance.

Table 6-3 Gender specific differences in mucosal biomarkers

Biomarker	Female vs. male difference	SE	P value
Rectal microflora			
Anaerobic gram positive cocci	0.68	0.19	0.004
Anaerobic gram positive rods	-0.62	0.27	0.02
Flow cytometry			
CD3+/CD8+/CD69+	4.79	2.13	0.02
Cytokine/chemokine RT-PCR			
IL-1β (ARB)	0.41	0.19	0.03
IL-1β (FSB)	0.35	0.17	0.04
IL-23 (ARB)	0.53	0.26	0.04
IL-23 (FSB)	0.69	0.26	0.008
CCR5 (ARB)	0.44	0.20	0.03
Luminex			
TNF-α	-1.81	0.45	7e-5
RANTES	-37.54	17.56	0.03

The analysis suggests potential differences between men and women with regards to rectal flora, T cell activation, and expression of

cytokines/chemokines. One limitation of the study is that, although women had to be sterilized or on effective contraception, we did not attempt to synchronize sample collection with a specific phase of the menstrual cycle or control for use of exogenous hormones. The MTN-007 study lacks adequate power to provide definitive evidence of such changes but provides hypothesis generating ideas that can be explored in future studies. There is increasing interest in how changes in hormonal levels associated with the menstrual cycle may influence women's vulnerability to HIV infection through changes in innate or adaptive anti-HIV immune responses in the reproductive tract (Rodriguez-Garcia, Patel, & Wira, 2013). Vishwanathan et al. have also demonstrated increased risk of SHIV acquisition in NHP during the late luteal phase of the menstrual cycle when progesterone levels are high and local immunity may be low (Vishwanathan et al., 2011). It is unclear whether similar changes may affect the gastrointestinal mucosa. However, estrogen receptors can be found in the colon (Campbell-Thompson, Lynch, & Bhardwaj, 2001) and exogenous ethynyl estradiol has been to improve gut inflammation in transgenic HLA-B27 model of inflammatory bowel disease (Harnish et al., 2004) suggesting that the relationship between reproductive hormones and intestinal vulnerability to HIV infection should be further explored.

6.8.2 Regional variation in mucosal parameters

Histology, flow cytometry, and qRT-PCR data were evaluated to determine whether there were significant differences in mucosal parameters stratified by the site of sample collection. Parameters with significant differences are summarised in Table 6-4.

Table 6-4 Regional differences in mucosal parameters

Biomarker	ARB vs. FSB difference	SE	P value
Flow cytometry			
CD3+/CD4+/CD69+	-3.34	0.98	7e-4
CD3+/CD8+/CD69+	-4.52	1.02	1e-5
Cytokine/chemokine RT-PCR			
IL-6	0.30	0.04	2e-10
IL-8	0.23	0.05	7e-7
MIP-1α	0.14	0.03	7e-5
MIP-1β	0.11	0.03	7e-5
RANTES	-0.10	0.02	2e-6
IL-12p40	0.13	0.03	3e-4
IL-17	0.10	0.04	7e-3
IL-23	0.09	0.02	1e-4

T cell activation and cytokine/chemokine gene expression were found to vary by site. CD69 expression on both CD4+ and CD8+ T cells was reduced in the ARB compared to the FSB samples. In contrast, a broad range of cytokines/chemokines were increased in the ARB compared to the FSB samples. One of the goals of the MTN-007 study was to determine whether anoscopic sampling could provide adequate tissue for microbicide studies compared to the use of flexible sigmoidoscopy to collect the same samples. The practical limitations of this approach are that the maximum number of biopsies collected anoscopically (N = 5) is significantly lower than the number available through flexible sigmoidoscopy (N = 20 to 30). This has implications in terms of the breadth of mucosal assays that can be incorporated into studies. Another issue is whether tissue samples collected at 9 cm (ARB) and 15 cm (FSB) are equivalent. The data presented in Table 6-4 suggest there are regional differences in a number of parameters. This phenomenon was seen in the HPTN-056 study where samples from 10 cm

and 30 cm were compared. In this study, cells expressing CD3, CD4, CD8, CD4+/CCR5+, and HLA-DR+/ DC-SIGN lineage-negative cells were more common in the 30 cm samples. CD19+ cells (a marker of B cells) were more common in the 10 cm samples (McGowan et al., 2007). McElrath et al. have recently reported a 3-fold increase in CD68+ macrophages expressing CCR5 in rectal tissue (4 cm from the anal verge) compared to colon tissue (30 cm from the anal verge) (McElrath et al., 2013). Collectively, these studies suggest that there is significant regional heterogeneity in composition of GALT and that care must be taken in interpreting the results of studies where tissue samples have been collected at different anatomical sites within the colon.

The optimal site for tissue sampling in rectal microbicide studies remains uncertain. Imaging studies by Louissaint et al. suggest that, for most participants, the highest colorectal concentration of semen and microbicide surrogates is found 10-20 cm above the anal verge (Louissaint et al., 2012). These data would appear to offer a rationale for flexible sigmoidoscopic collection of samples at 15 cm from the anal verge. This approach also provides the ability to safely collect the greatest number of tissue biopsies during one procedure.

6.9 Summary

In 1992, Joan Kreiss and her colleagues reported the results of a study evaluating the efficacy of an N9 contraceptive sponge in preventing heterosexual acquisition of HIV in a group of Nairobi prostitutes (Kreiss et al., 1992). Unfortunately, despite positive *in vitro* efficacy data, the Kreiss

study found that use of the N9 sponge increased the risk of HIV infection in this population with a hazard ratio of 1.7. Use of the N9 sponge was also associated with increased incidence of genital ulcers. Since this time, a critical concern in microbicide research has been whether candidate microbicides have the potential to increase, rather than decrease, the risk of HIV infection. The COL1492 study also demonstrated an increased risk of HIV infection associated with N9 use and again there was increased incidence of epithelial disruption associated with N9 use (Van Damme et al., 2002). Not surprisingly, the key components of safety monitoring in vaginal microbicide trials include assessment of clinical symptoms and signs associated with product use (Poynten et al., 2009).

In January 2007 a DSMB stopped a Phase 3 study of a cellulose sulphate (CS) gel study due to concerns of an increased risk of HIV infection in the active arm of the study. The interim hazard ratio was 2.23 ($p = 0.02$) and the trial was stopped (Van Damme et al., 2008). There was no obvious increase in genital signs or symptoms in participants receiving the CS gel compared to the placebo gel participants. However, subsequent *in vitro* studies demonstrated that CS had the ability to disrupt epithelial tight junctions and potentially increase the risk of HIV acquisition (Mesquita et al., 2009). Unfortunately, the CS study describes a scenario wherein significant product associated mucosal changes occurred in the absence of clinical signs or symptoms. This observation has increased the need to develop sensitive and specific mucosal safety biomarkers to identify and exclude harmful microbicide candidates at an early stage of clinical development. This

strategy obviously applies to the development of both vaginal and rectal microbicides.

The design of MTN-007 reflects this concern about the need for mucosal biomarkers. Multiple candidate biomarkers were included in MTN-007. They were chosen because of their utility in previous microbicide trials or because they had proven useful in identifying inflammation in other settings e.g. the use of fecal calprotectin in IBD. This process has two major limitations. The first limitation is the selectivity applied to the choice of biomarkers. One example might be the decision to choose to characterize IL-17 gene expression rather than expression of IL-4. IL-17 may not be involved in the biological pathway of mucosal damage caused by candidate “X” whereas up or down regulation of IL-4 gene expression may be critical. Failure to see any change in IL-17 expression may provide the investigators with a false sense of security that candidate “X” is safe.

A second and perhaps more important problem is that Phase 1 microbicide safety studies are usually powered based on the ability to discriminate differential rates of ≥ Grade 2 AEs across the arms of the studies. The sample size is routinely 15 participants per study arm. However, with the proliferation of biomarkers used in Phase 1 studies, the number of comparisons across study arms increases significantly. As one example, in the MTN-007 study there were a total of 84 biomarkers evaluated with 18 statistical comparisons conducted for each biomarker. Using a Bonferroni correction to control for multiple comparisons the convention level of

significance (P = < 0.05) falls to 0.05/672 = < 0.00007. Few biomarkers will withstand this level of correction and so an argument can be made that, in the context of small trials, use of approaches such as the Bonferroni correction is unhelpful (Perneger, 1998). For the purpose of the MTN-007 study we described changes in mucosal biomarkers with an unadjusted p value of < 0.05 as providing suggestive evidence of a difference in biomarker expression.

In the absence of definitive safety biomarkers it is likely that microbicide trials will continue to evaluate novel safety biomarkers and, depending on the performance characteristics of these assays, decide whether to include them in future microbicide studies. Based upon recent experience in the RMP-01, RMP-02/MTN-006, and MTN-007 studies, investigators in this field have chosen to discard epithelial sloughing and fecal calprotectin from future studies. In addition, there is growing interest in adopting a more systems biology approach in characterising mucosal safety in microbicide trials. Rather than characterising specific microbes as part of a semi-quantitative assessment of vaginal microflora, Ravel et al. chose to use pyrosequencing to evaluate microbicide-induced changes in vaginal microbiota (Ravel et al., 2012). MTN-007 is the first rectal microbicide study to incorporate microarray analysis of the entire mucosal transcriptome. This approach has generated provocative data on the impact of TFV gel on mucosal mitochondrial and immune function that are described in Chapter 7 of this thesis.

Chapter 7

MTN-007 Microarray Analysis

7 MTN-007 microarray analysis

7.1 Introduction

Currently, no validated biomarkers that reliably measure the potential mucosal toxicity of microbicides are available. Certain cytokines and chemokines may in principle be suitable as mucosal biomarkers for microbicide-induced toxicity or inflammation. The concentration of inflammatory cytokines in mucosal secretions has therefore been evaluated in prior microbicide studies. However, these studies have their limitations. First, only a few genes/proteins can be studied from one sample. Second, it is unclear what constitutes a meaningful change in cytokine concentration.

Because of the described limitations when measuring individual biomarkers, it would be useful to develop assays that evaluate the cumulative impact of candidate microbicides on mucosal immune function as a whole. To evaluate the global impact of microbicides on the mucosa, gene expression microarrays were performed on RNA isolated from mucosal biopsies taken before and after application of TFV, N9, or HEC placebo gels. HumanHT12 v4 Expression BeadChips (Illumina Inc., San Diego, CA, USA) permit the measurement of 48,000 mRNAs simultaneously on a single high-density oligonucleotide microarray. This offers the opportunity to: (i) identify signature expression patterns of dozens or even hundreds of genes that correlate with microbicide side effects on the mucosa; (ii) interpret expression changes of a particular gene group, such as inflammatory cytokines, in relationship to other genes; and (iii) cross-validate the array results with measurements of mucosal gene expression by qRT-PCR and of

other techniques such as the measurement of cytokine/chemokine proteins in mucosal secretions by the Luminex® technique.

An expected outcome of the array studies in MTN-007 was the identification of groups of genes, in particular apoptotic, pro-inflammatory and/or innate immunity-related genes, which were modulated significantly from baseline in response to topical N9 application. In comparison, at the outset of the MTN-007 study it was not clear whether such changes might be expected after exposure to TFV or HEC placebo gel application. Another expected outcome of the array studies was the validation of any significant cytokine/chemokine/chemokine receptor changes found by qRT-PCR and Luminex measurements. Similar results in all three assays (expression array, qRT-PCR, and Luminex) would underscore the potential usefulness of a biomarker to predict toxicity of a candidate microbicide. Moreover, interpreting such a promising marker in the light of other gene expression changes in the microarray would provide an opportunity to better understand its biological significance.

7.2 Methods

7.2.1 Mucosal biopsy procedures

Rectal biopsies for the microarray studies were obtained before treatment at enrollment (time point “0”), 30-60 minutes following application of the single gel dose (time point “I”), and again on the day following the last dose of the seven once-daily gel applications (time point “VII”). Following an enema with Normosol-R pH 7.4, a flexible sigmoidoscope was inserted into the rectum

and biopsies were collected at 15 cm from the anal margin (FSB). Following the sigmoidoscopy, a disposable anoscope was inserted into the anal canal with collection of rectal biopsies at 9 cm from the anal margin (ARB). Immediately after harvest, biopsies were immersed in RNAlater (Qiagen, Valencia, CA, USA), stored at 4°C overnight and transferred to a -80°C freezer for long-term storage prior to sample processing.

7.2.2 RNA isolation and cRNA preparation

RNA was isolated from the biopsies using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions, treated with 27 Kunitz units of DNAse (Qiagen, Valencia, CA, USA) to remove genomic DNA contamination, and evaluated for integrity using the Agilent RNA 6000 Nano Kit (Agilent, Palo Alto, CA, USA) on an Agilent 2100 Bioanalyzer. All samples had a RIN score of 7 or greater. 200 ng of total RNA was amplified and labeled using the Illumina TotalPrep RNA Amplification kit (Ambion, Grand Island, NY, USA). cRNA from a total of 192 biopsy samples (8 men per study arm, 4 study arms, 3 time points and 2 biopsy sites (9 and 15cm)) was hybridized to HumanHT12 v4 Expression BeadChips (Illumina, San Diego, CA, USA) according to the manufacturer's protocols. Each chip contained 47,323 probes, corresponding to 30,557 genes.

7.2.3 Quality control and processing of microarray data

All chips used in the analysis passed standard quality control metrics assessed by GenomeStudio (Illumina, Inc., San Diego, CA, USA) as well as visual inspection for anomalies and artifacts. Using GenomeStudio, a

detection P value was calculated for each probe, representing the confidence that a given transcript is expressed above background defined by negative control probes. Further processing and statistical analysis of data was done using R/Bioconductor (Dudoit, Gentleman, & Quackenbush, 2003). Data were pre-processed by robust spline normalization and variance stabilizing transformation using lumi (open source software available from www.bioconductor.org) (Baldi & Long, 2001; Long et al., 2001). The pre-processed data were filtered to include only probes with detection threshold P values of < 0.05 in 100% of biopsies in at least one of the four study arms, and to remove probes that had a low standard deviation (≤ 0.5) across all arrays, using genefilter (open source software available from www.bioconductor.org). Finally, probes without an Entrez ID were removed, leaving 1928 probes for further statistical analysis.

7.3 Statistical analysis

A Bayesian statistical framework, Cyber-T, was used to test for the effect of gel treatment on gene expression in paired comparisons between either time points I or VII and time point 0 (Long et al., 2001). Each study arm was considered separately. The Benjamini Hochberg method estimating false discovery rates (FDR) was used to control for multiple comparisons (Benjamini & Hochberg, 1995). Criteria for significance and relevance were an estimated FDR ≤ 0.05 and a $\log_2$ fold expression change of ≥ 0.5 (up-regulation) or ≤ 0.5 (down-regulation), respectively. For confirmation, the pre-processed but unfiltered data (all 47,323 probes) were statistically re-analyzed for significant treatment effects using a double subtraction strategy, where the paired differences between time point 0 and time points I or VII

within each treatment arm were first calculated for each probe and study subject. In a second step, the average paired differences for each probe within each of the three treatment arms were subtracted from the average paired differences for each probe within the no-treatment arm. Analysis for significance was then performed using a linear fit model, Limma (Smyth, 2005), with criteria for significance and relevance set at an estimated Benjamini Hochberg FDR ≤ 0.05 and a $\log_2$ fold difference to no-treatment of ≥ 0.5 (up-regulation) or ≤ 0.5 (down-regulation), respectively. Confirmatory Limma analysis greatly overlapped with the Cyber-T results. The numbers of up- and down-regulated genes are based on the Cyber-T analysis. Heat maps of differentially regulated genes were generated using MeV 4.8 within the TM4 Microarray Software Suite (Saeed et al., 2006), and hierarchically clustered according to selected gene ontologies found in the databases DAVID 6.7 (Huang et al., 2009; Huang, Sherman, & Lempicki, 2009), and InnateDB (Lynn et al., 2008).

7.3.1 General statistics

Correlation of $\log_2$ fold gene expression changes between ARB and FSB samples was tested by Spearman's rank correlation coefficient. Ratios of stimulated to suppressed genes were tested for a difference between cellular compartments using Chi-square statistics. Differences in gene expression between study groups or between time points were tested by paired or unpaired, parametric or non-parametric statistical tests, and adjusted for multiple testing, as appropriate.

7.3.2 Pathway and network analysis of microarray data

Entrez ID designations, assigned to the array probes by Illumina, were uploaded to the Innate DB database and a Gene Ontology (GO) over-representation analysis was performed for gene groups signifying a particular molecular function or biological process, or occurring in specific cellular compartments (Lynn et al., 2008). The following strategy was used to determine which gene groups were enriched in the data set: separately for down- and up-regulated genes, ratios of the number of genes in a particular GO group to the total number of genes detected in the data set were compared to the ratios in the same GO group reported for the complete human genome using Fisher's exact test. Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) was used to visualize direct and indirect relationships between individual gene products and map their cellular localizations.

7.3.3 Confirmation of microarray results

A two-step RT droplet digital PCR (RT-ddPCR) was used to confirm microarray transcriptome data for selected genes of interest (Hindson et al., 2011; Pinheiro et al., 2012). In a ddPCR assay, each sample is partitioned into approximately 20,000 droplets representing as many individual PCR reactions. The number of target DNA copies present per sample can be quantified based on Poisson distribution statistics, because each individual droplet is categorized as positive or negative for a given gene. cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA). ddPCR was carried out using the QX100 droplet digital PCR system (BioRad, Hercules, CA, USA) with cDNA

amplified in a duplex reaction using Primetime (Integrated DNA Technologies, Coralville, IA, USA) and Taqman (Life Technologies, Carlsbad, CA, USA) qPCR assays. Reactions were set up using the 2X ddPCR Supermix for Probes (BioRad, Hercules, CA, USA), 20X 6-carboxyfluorescein (FAM)-labeled target gene-specific qPCR assay (Integrated DNA Technologies or Life Technologies) and 20X VIC-labeled housekeeping haemoglobin B (HBB) gene-specific Taqman gene expression assay (Life Technologies, Carlsbad, CA, USA). Each assembled ddPCR reaction mixture was loaded in duplicate into the sample wells of an eight-channel disposable droplet generator cartridge (BioRad, Hercules, CA, USA) and droplet generation oil (BioRad, Hercules, CA, USA) was added. After droplet generation, the samples were amplified to the endpoint in 96-well PCR plates on a conventional thermal cycler using the following conditions: denaturation/enzyme activation for 10 min at 95°C, 40 cycles of 30 sec denaturation at 94°C and 60 sec annealing/amplification at 60°C, followed by a final 10 min incubation step at 98°C. After PCR, the droplets were read on the QXT100 droplet Reader (BioRad, Hercules, CA, USA). Analysis of the ddPCR data was performed with QuantaSoft analysis software version 1.3.1.0 (B BioRad, Hercules, CA, USA).

7.3.4 Electron microscopy

Formalin-fixed paraffin-embedded rectal biopsies were de-paraffinized and fixed overnight in half-strength Karnovsky's fixative. Staining, embedding, cutting and viewing on a JEOL 1400 SX transmission electron microscope were performed as previously described (Hladik et al., 2007; Hladik et al., 1999). Twenty images per sample were acquired at 5,000X magnification.

Using ImageJ (available at http://rsbweb.nih.gov/ij/), the 2-dimensional sizes in μm^2 of all individual mitochondria with a circularity index of ≥ 0.9 were calculated (for standardisation purposes, only mitochondria cut near perfectly along their minor axis were evaluated). Ten images per sample were also acquired at 2,000X magnification, always including the epithelial cell brush border. Using ImageJ, a grid of 1.32 μm^2 squares of defined size was overlaid onto each image. All mitochondria in the images were counted, except those in the first row of squares falling on the brush border and in squares along the image rims which only partially covered tissue), and the mean numbers of mitochondria per were calculated for each of the acquired 2,000X images (range of counted squares per image: 23 to 50). Mitochondrial sizes and mean numbers per μm^2were compared between time point 0 and time point VII using unpaired two-sided t tests.

7.4 Results

7.4.1 Microarray data

A surprisingly large number of genes were altered by topical treatment with TFV gel, in particular after 7 days of treatment and in the biopsies closer to the anal margin (Figure 7-1). TFV gel suppressed 505 genes and induced 137 genes after 7 days of treatment, whereas N9 suppressed only 56 genes and induced 60 genes (Figure 7.2). Changes induced by HEC gel were much lower (4 genes decreased and 12 genes increased) and comparable to the number of genes fluctuating between baseline and final biopsy in the No Rx arm (6 genes decreased and 17 genes increased). Thus, TFV gel

had a profound effect on gene expression in the rectum, modulating many more genes than N9 gel.

There was little overlap between the expression changes caused by TFV and N9. After 7 days of treatment, only 15 genes decreased and 4 genes increased simultaneously in both arms, less than 5% of the genes affected by TFV (Figure 7-2), indicating that the effects of the TFV gel were quite specific and not caused by general irritation as is known to occur in response to N9. Not unexpectedly, the effects of TFV gel were greater after 7 days of treatment than after a single application (Figure 7.2). This was more evident for suppression, with only 8 genes decreasing in expression after a single application. In contrast, 70 genes increased in expression after a single tenofovir application, with 44 of these genes remaining up-regulated after 7 treatment days. Three kinetic patterns typified the genes that were up-regulated after 7 days of tenofovir treatment (Figure 7-3): no change or decrease after a single application followed by an increase after 7 days; slight increase followed by further increase; and strong increase followed by sustained high expression. Thus, by microarray analysis, TFV gel's pronounced inhibitory effect on gene expression was mostly evident after seven applications, while some up-regulation could be observed immediately following the first application.

The effects of TFV gel were less apparent in the 15 cm (FSB) than the 9 cm (ARB) biopsies. 277 genes were suppressed and 89 genes induced in the 15 cm biopsies after seven daily applications (Figure 7-2). While these were

markedly fewer genes than were impacted at 9 cm, the general pattern of expression changes was similar, in particular for suppressed genes. 237 of the 277 genes suppressed at 15 cm were also suppressed at 9 cm, and 32 of the 89 genes induced at 15 cm were also induced at 9 cm. The similarity of 9 and 15 cm expression changes of genes suppressed by TFV gel is underscored by the strong correlation between the expression changes of the 505 genes suppressed at 9 cm and the expression changes in these same genes at 15 cm (Spearman $r = 0.4775$; $p < 0.0001$) (Figure 7-4).

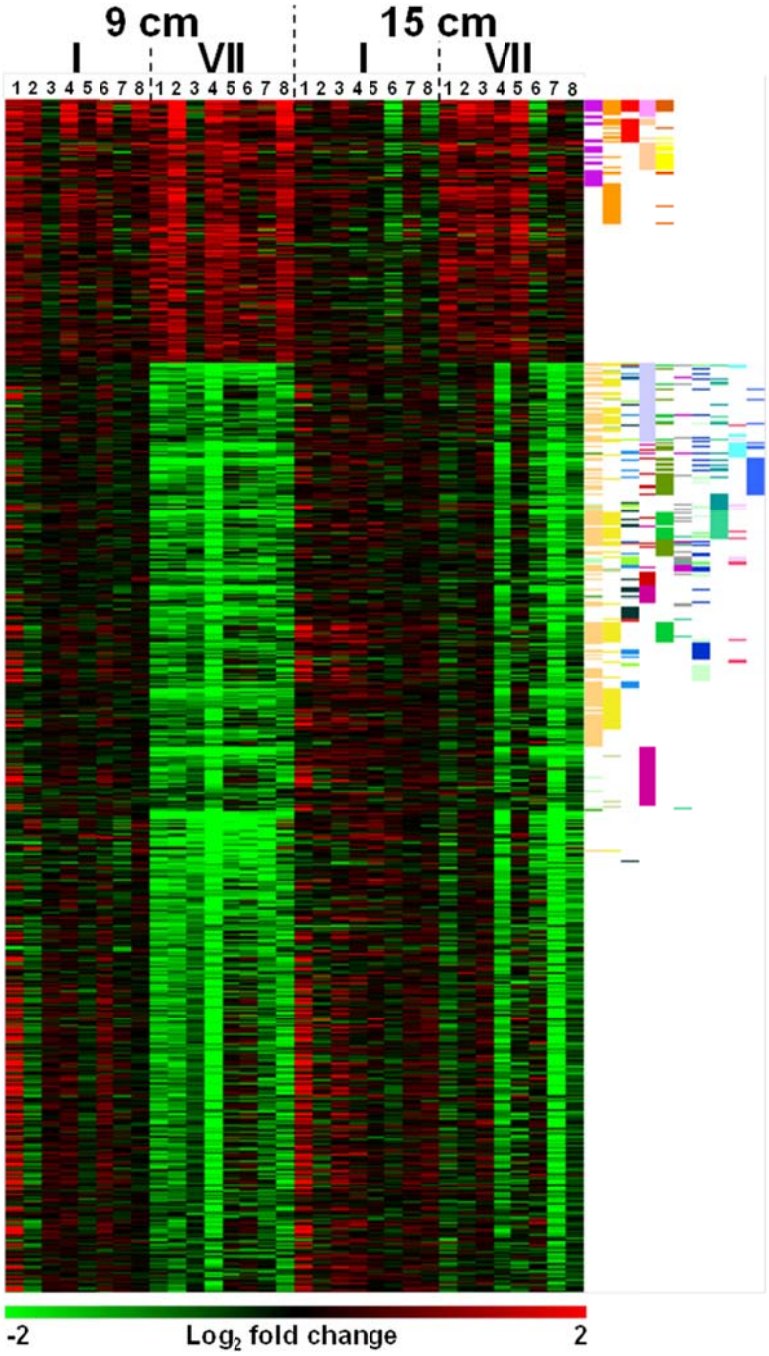


Figure 7-1 Heat map of mucosal gene expression

Heat map of differentially expressed genes in 8 subjects after a single (I) and after 7 consecutive once-daily (VII) rectal applications of tenofovir (TFV) gel in biopsies taken at 9 cm and 15 cm above the anal margin. Intensities of the red and green bars signify strength of gene up- and down-regulation, respectively. 642 genes are shown, all of which exhibited an estimated false discovery rate (FDR) ≤ 0.05 and a $\log_2$ fold expression change of ≥ 0.5 (up-regulation) or ≤ 0.5 (down-regulation) when evaluated jointly for all 8 subjects at time point VII in the 9 cm biopsies. Colored bars to the right of the heat map mark genes falling within particular biological processes defined in the InnateDB database. Only processes containing at least 8 genes in the heat map are depicted.

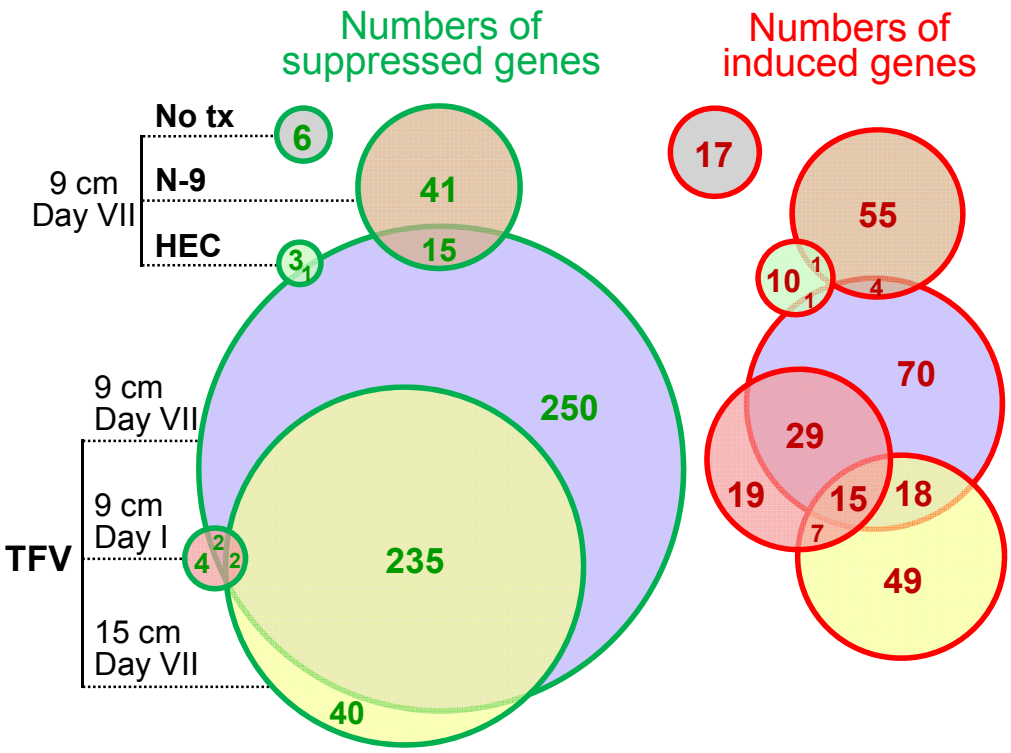


Figure 7-2 Venn diagram of changes in mucosal gene expression

Numbers of significantly down- (circles in green hues) and up-regulated (red/brown hues) genes after tenofovir (TFV) gel treatment and their overlap with the other three study arms, Nonoxynol-9 (N-9), hydroxyethyl cellulose (HEC), and No Treatment (No tx). When not otherwise labeled, circles depict TFV-induced changes. All circles depict changes on Day VII at 9 cm, except where labeled as “Day I” and “15 cm”. Circle area symbolizes the number of affected genes; size of overlapping area symbolizes the number of genes simultaneously affected by two or three conditions.

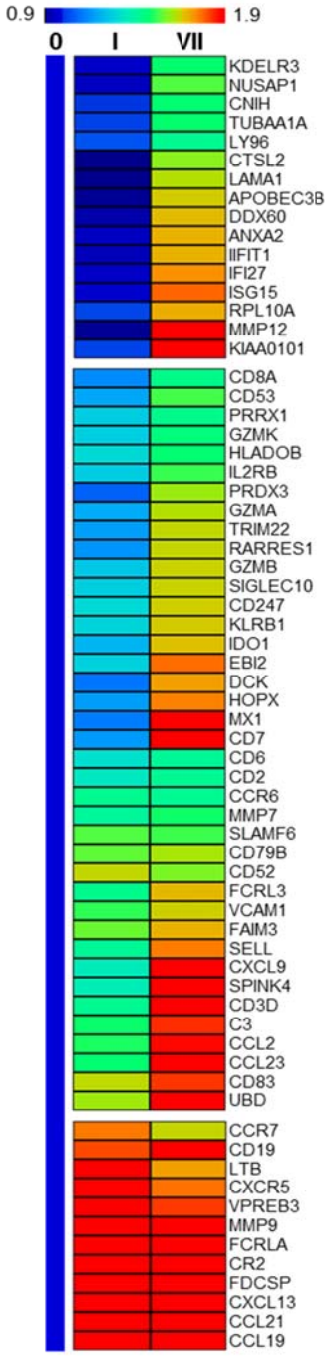


Figure 7-3 Gene up-regulation associated with exposure to tenofovir gel

Average strength of gene up-regulation by TFV gel across all 8 study subjects. Heat map colors depict fold-change at Day I and Day VII over baseline. Baseline is depicted as the vertical bar labeled “0” filled with the shade of blue corresponding to a fold-change of 1. Genes included in the list exhibited ≥1.6 fold average up-regulation on Day VII or were ≥1.1 fold up-regulated in at least 6 of 8 subjects, and some knowledge about the gene products exists in the literature.

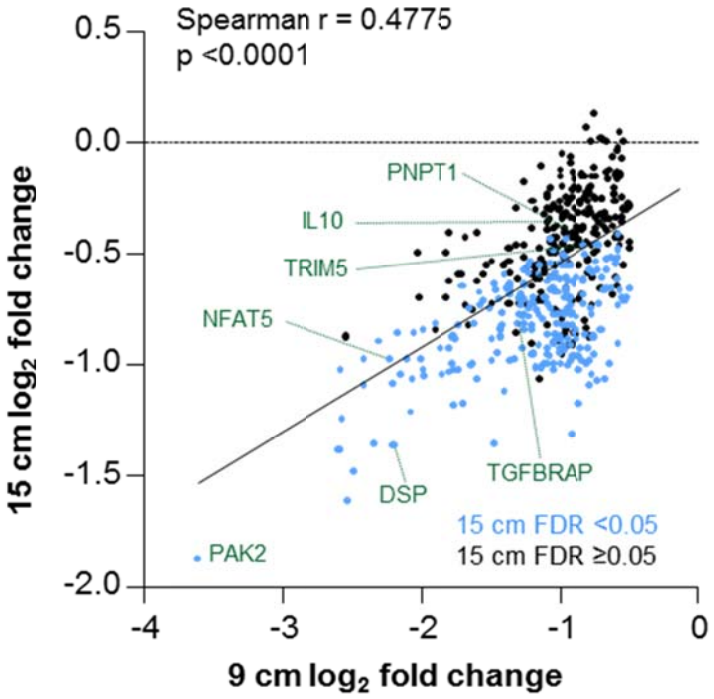


Figure 7-4 Correlation of mucosal gene expression by site

Correlation of log_2 fold gene expression changes from baseline to Day VII between 9 and 15 cm biopsies. All 505 genes significantly down-regulated at 9 cm are included (9 cm false discovery rate (FDR) <0.05). Genes depicted as blue dots were significantly down-regulated in both 9 and 15 cm biopsies (15 cm FDR <0.05), genes depicted as black dots were only significant in 9 cm biopsies (15 cm FDR ≥0.05).

Although many of the 505 genes were not identified as suppressed at 15 cm (FDR ≤ 0.05 and log2 fold expression change of ≤ 0.5), the direction of change at 15 cm for all of these genes was almost always downward. Thus, the gene expression changes caused by TFV gel exhibited similar patterns in 9 and 15 cm rectal biopsies, with fewer genes affected at 15 cm, likely reflecting dilution of the gel away from the application site and/or signifying higher sensitivity of the distal rectum to the drug.

7.4.2 Expression patterns and functional pathways

The surprisingly broad impact of TFV gel on rectal gene expression prompted an exploration of the patterns of expression changes and their predicted functional consequences. Interestingly, while the overall effect of TFV gel was more suppressive than stimulatory, genes encoding for proteins known to be secreted from the cell were more often stimulated than suppressed (Figure 7-5). After seven days of treatment, 50 genes encoding products that are secreted were differentially expressed in the 9 cm tissue samples. Of these, 35 genes were up-regulated and 15 were down-regulated). This contrasted significantly to the ratio of up-regulated to down-regulated genes encoding for products known to localize to the cell nucleus. Of 163 such genes, 17 were up-regulated and 146 were down-regulated; χ^2 P <0.0001). Particularly noteworthy among upregulated genes for secreted products were a number of chemokines, specifically CCL2, CCL19, CCL21, CCL23, CXCL9 and CXL13 (Figure 7-5). Correspondingly, transcripts of a number of genes encoding for leukocyte-specific cell surface markers increased, specifically CD2, SLAMF6 (belonging to CD2 family), CD3D,

CD7, CD8A, CD19, CD52, CD53, CD196 (CCR6) and CD197 (CCR7). Consistent with increases in chemokines and immune cell presence, TFV gel suppressed IL-10, an essential secreted immunoregulator with anti-inflammatory activity.

Among suppressed genes with products localizing to the cell nucleus, we identified a large number of known or putative transcription factors, for example CREBBP (CREB-binding protein), a co-activator for IL-10 transcription (Woodgett & Ohashi, 2005; Martin, Rehani, Jope, & Michalek, 2005; Alvarez, Municio, Alonso, Sanchez, & Fernandez, 2009), (nuclear factor of activated T cells 5) (Neuhofer, 2010; Buxade et al., 2012; Trama, Go, & Ho, 2002) and many zinc finger proteins whose specific functions are still unknown (Figure 7-5).

Figure 7-5 Expression pattern and functional pathway analysis

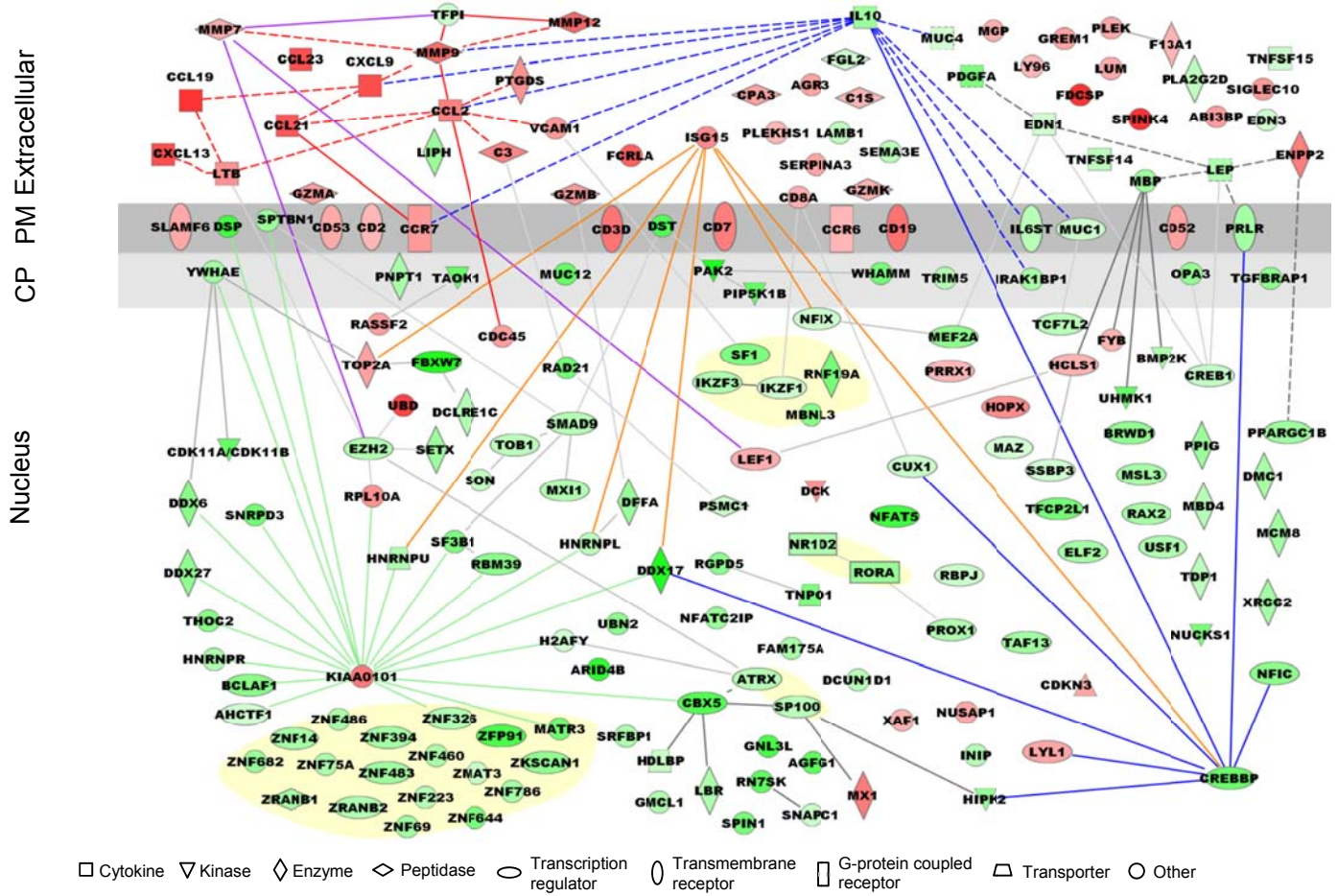


Figure 7-5: Ingenuity pathways analysis showing cellular localizations of and relationships between individual gene products. Red symbols indicate up-regulation and green symbols down-regulation, at Day VII relative to baseline in 9 cm biopsies. The diagram includes all significant genes in our data set identified by Ingenuity as primarily located in the extracellular space and the cell nucleus. A few selected significant genes with products localizing to the plasma membrane (PM) or cytoplasm (CP) are also shown based on their putative functional roles. Direct (solid lines) and indirect (dashed) interactions between gene products are indicated. Line color is arbitrary.

Of note, TFV gel not only suppressed transcription of a large number of genes, but this suppression was also relatively strong, as the comparison with N9 demonstrates ($P < 0.0001$) (Figure 7-6). Thus, through suppression of the transcription of many genes, TFV gel appears to initiate a process whereby biological loops such as mediated through CREBBP and IL-10 suppression lead to up-regulation of leukocyte-attracting chemokines.

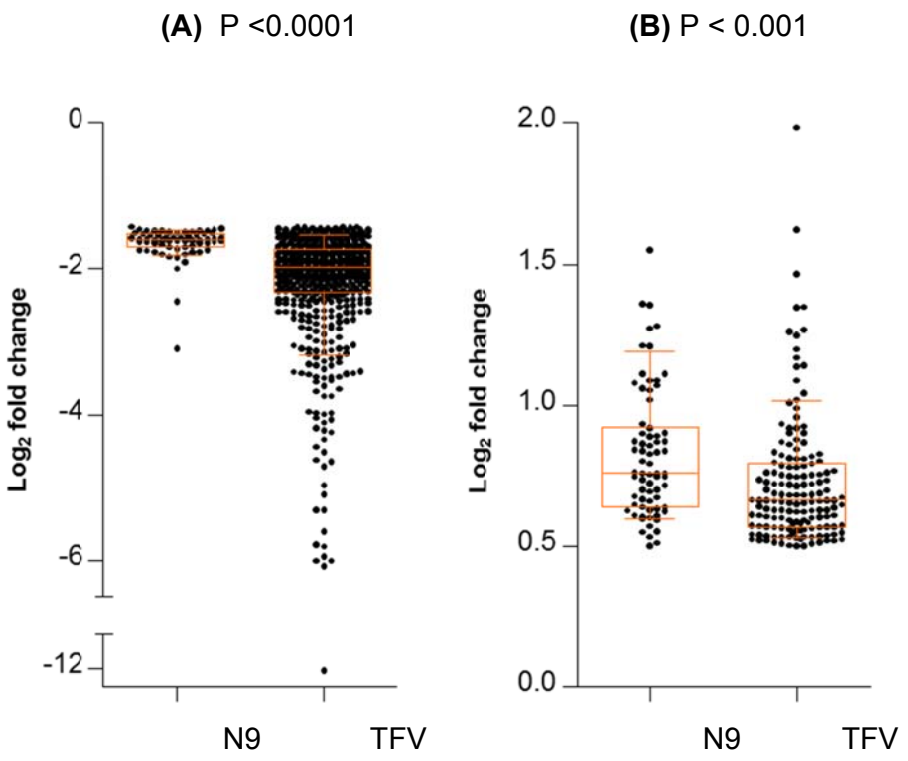


Figure 7-6 Overall gene regulation from baseline to Day VII

(A) All genes with an estimated FDR ≤ 0.05 and a $\log_2$ fold expression change of ≤ 0.5 are included (N-9: 56 genes; tenofovir: 505 genes). **(B)** All genes with an estimated FDR ≤ 0.05 and a $\log_2$ fold expression change of ≥ 0.5 are included (N-9: 60 genes; tenofovir: 137 genes). Differences in magnitude of gene expression changes were statistically compared between N-9 and tenofovir by unpaired Mann-Whitney tests. The box plots indicate medians and interquartile ranges and the whiskers indicate 10^{th} to 90^{th} percentiles.

A scenario of transcriptional inhibition and increased immune chemotaxis also emerges from an analysis of the biological processes categorized in the InnateDB database (Figure 7-7) (Lynn et al., 2008). Chemotaxis-related genes and, more broadly, immune response genes were among the top groups induced by TFV, and processes regulating DNA transcription were among the groups most significantly inhibited by TFV gel. TFV gel also suppressed genes important for RNA processing, as well as pathways involving transforming growth factor beta (TGFβ) and MAP3K signaling, cytoskeleton organization, and apoptosis.

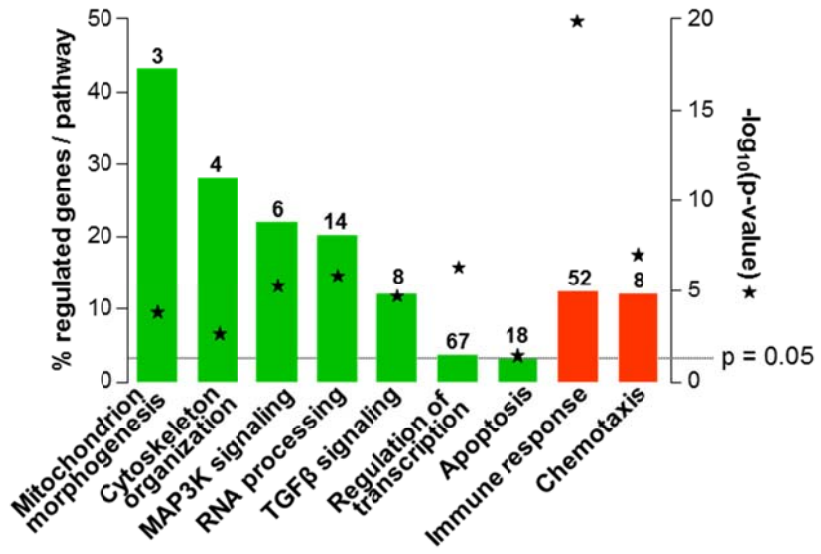


Figure 7-7 InnateDB biological pathway analysis

Selected InnateDB biological processes with significant enrichment of genes up- or down-regulated by tenofovir at Day VII in 9 cm biopsies. Green bars depict the percentage of genes identified as down-regulated in a particular process out of the total number of genes included by InnateDB in that process. Red bars depict corresponding percentages for up-regulation. Numbers of down- and up-regulated genes are indicated above the bars. Gene enrichment in each biological process was tested for statistical significance as described in Methods and the computed p values are depicted by the stars. Not all processes with significant gene enrichment are shown.

Lastly, TFV gel suppressed genes regulating mitochondrion morphogenesis, including genes such as polyribonucleotide nucleotidyltransferase 1 (PNPT1) (Wang et al., 2010; Wang, Shimada, Koehler, & Teitell, 2012) and optic atrophy 3 (OPA3) (Misaka, Miyashita, & Kubo, 2002; Misaka, Murate, Fujimoto, & Kubo, 2006) raising the possibility of mitochondrial toxicity.

7.4.3 RT-ddPCR quantification of mRNA

Given the intriguing results obtained from the microarray analysis of eight study subjects in each arm, it was decided to independently confirm some of the most pertinent findings by RT-ddPCR assays in the remaining 7

individuals who were also enrolled in the TFV gel arm. For this purpose, nine up-regulated and six down-regulated genes were selected based on a combined assessment of magnitude of change in the microarrays and/or their potential functional significance (Figure 7.6). The up-regulated genes were matrix metallopeptidase 12 (MMP12), serine protease inhibitor of the Kazal type 4 (SPINK4), the chemokines CCL19, CCL21, CCL23 and CXCL9, and the leukocyte cell surface receptors CD7, CD19 and CCR7 (Figure 7-8A). The down-regulated genes were IL-10, NFAT5, p21-activated kinase (PAK2), desmoplakin (DSP), TGF-β receptor associated protein 1 (TGFBRAP1) and tripartite motif-containing protein 5 (TRIM5) (Figure 7-8B). Because expression changes measured in the microarray assays were in general stronger in the 9 cm than in the 15 cm biopsies, RT-ddPCR confirmations were focused on the 9 cm biopsies.

mRNA copy numbers of all genes tested by RT-ddPCR increased or decreased as predicted from the microarray data between baseline and following 7 days of TFV gel treatment. For up-regulated genes, similar kinetic patterns were observed as seen in the microarray data, with one gene (CD7) being mostly flat across the seven subjects tested after a single TFV application and increasing thereafter, two genes (CXCL9 and MMP12) increasing after a single application and then staying mostly flat, and the remaining six genes increasing after a single application and then increasing further during seven days of treatment (Figure 7.3A). For down-regulated genes, five of the six genes tested were also already inhibited after a single TFV PAK2 was mostly flat after a single application, before decreasing

strongly thereafter. Of note, none of these six genes were identified as down-regulated after a single TFV gel application by microarray screening, which is mostly reflective of the stringent microarray analysis criteria, but also underscores the sensitivity and merit of the RT-ddPCR assays. To evaluate the impact of TFV gel across all 15 study subjects in the TFV gel arm, microarray (8 subjects) and RT-ddPCR (7 subjects) expression data were combined and statistically tested for the fold-change after seven days of treatment compared to the normalized baseline (Figure 7-8). Expression changes for all fifteen genes tested by microarray and RT-ddPCR assay were highly significant (Bonferroni-adjusted $P < 0.01$ for all genes except TRIM5 [$P = 0.02$]). Thus, the effect of TFV gel treatment on expression of these fifteen genes at 9 cm in the rectal mucosa was overall extremely consistent across all 15 study subjects.

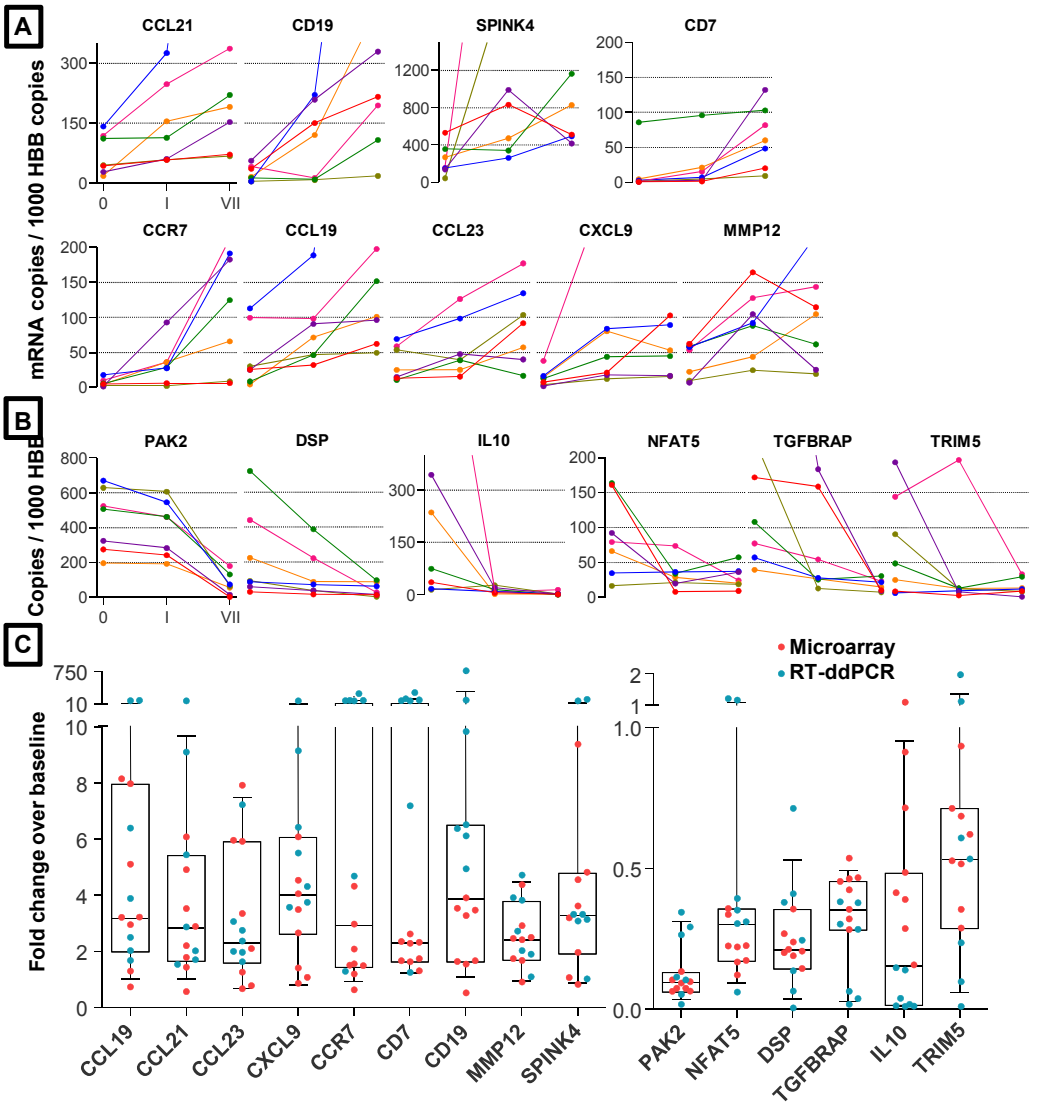


Figure 7-8 Quantification of mRNA by ddPCR

(A) mRNA copy numbers measured in 9 cm biopsies by reverse transcription (RT) droplet digital PCR (ddPCR) relative to hemoglobin beta (HBB) copy numbers for nine selected genes identified as up-regulated in the microarray data set. Copy numbers at baseline (0), after a single TFV gel application (I) and after seven consecutive once-daily applications (VII) are shown. Line colors signify each of the 7 subjects. **(B)** mRNA copy numbers in 9 cm biopsies for six select genes identified as down-regulated in the microarray data set. **(C)** Normalized fold changes of gene expression at Day VII over baseline in all 15 individuals enrolled in the TFV gel arm of the study. Red dots depict fold changes measured by microarray assay in 8 subjects, blue dots depict fold changes measured by RT-ddPCR in the 7 remaining subjects. The horizontal line in each box denotes the median, the ends of the box denote the 25th and 75th percentiles, and the whiskers indicate 10^{th} to 90^{th} percentile. Expression changes were tested for statistical significance using one-sided Wilcoxon tests and adjusted for multiple testing ($p < 0.01$ for all genes except TRIM5 [$P = 0.02$]).

7.4.4 Signs of mitochondrial toxicity

The analysis of biological processes indicated an effect of TFV gel on mitochondria (Figure 7-9). Because mitochondrial toxicity has been reported after systemic treatment with TFV (Herlitz et al., 2010; Lewis, Day, & Copeland, 2003; Perazella, 2010), this finding was further explored. PNPT1 was clearly inhibited in the microarray assays and has recently been characterized as a master regulator of RNA import into mitochondria, leading to visible changes of mitochondrial morphology when experimentally deleted (Wang et al., 2010; Wang et al., 2012). TFV-induced inhibition of PNPT1 across all 15 subjects was therefore first confirmed by RT-ddPCR assays in the 9 cm and 15 cm rectal biopsies (Figure 7-9A). Before treatment, the mean PNPT1 mRNA copy numbers were 140/1000 hemoglobin B copies (SD ± 78) at 9 cm and 76 (SD ± 42) at 15 cm (ANOVA Bonferroni post-test $P < 0.01$). After a single application of TFV gel, copy numbers decreased to 106 (SD ± 106) at 9 cm (P = NS) and 50 (SD ± 43) at 15 cm ($P < 0.01$). After 7 days of treatment, copy numbers fell further to 10 (SD ± 19) at 9 cm (VII versus 0; $P < 0.001$) and 40 (SD ± 41) at 15 cm (VII versus 0; $P < 0.001$). Thus, the RT-ddPCR assays confirmed a strong inhibition of rectal PNPT1 transcription by TFV gel.

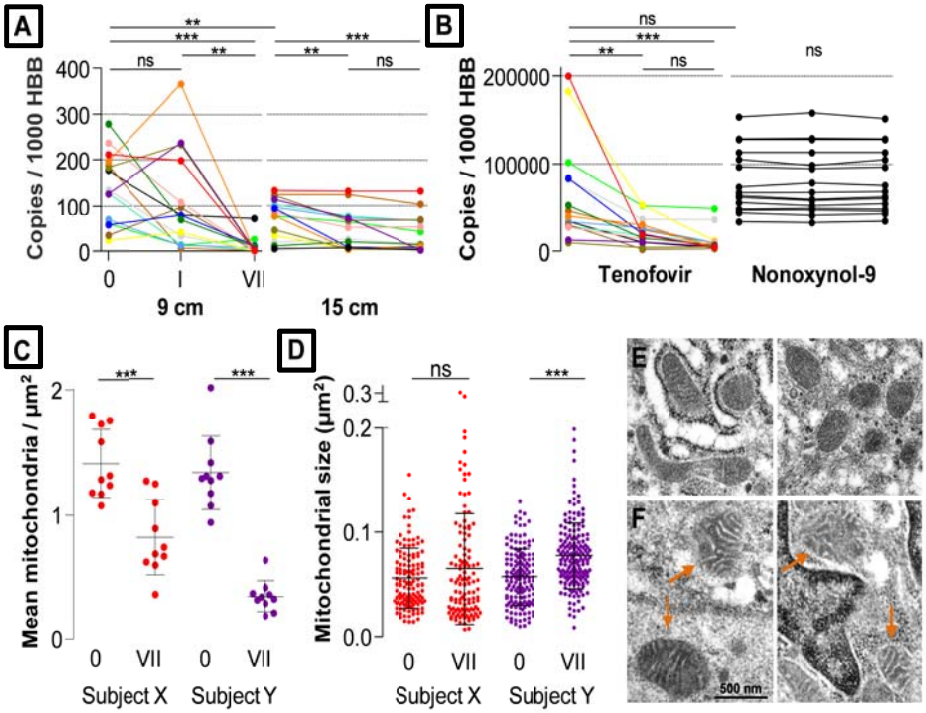


Figure 7-9 Mucosal mitochondrial structure and function

(A) PNPT1 mRNA copy numbers measured in 9 and 15 cm biopsies at baseline (0), after a single TFV gel application (I) and after seven consecutive once-daily applications (VII). **(B)** Mitochondrial ATP6 mRNA copy numbers measured in 9 cm biopsies at baseline (0), after a single TFV or N9 gel application (I) and after seven consecutive once-daily applications of TFV or N9 (VII). Line colors in **(A)** and **(B)** signify each of the 15 subjects in the TFV arm. Black lines signify the 15 subjects in the N9 arm. Baseline values were compared between 9 and 15 cm biopsies by paired t-test, between TFV and N9 by unpaired t-test. Expression changes over time were tested for statistical significance by ANOVA and Bonferroni adjusted post-tests. Two asterisks indicates P <0.01, three asterisks P <0.001, and "ns" not significant. **(C)** Assessment of mitochondrial density by electron microscopy of 9 cm biopsies in 2 subjects. Each dot indicates the mean number of mitochondria per μm^2 in a separate 2,000X image. Ten images were evaluated per time point and subject. **(D)** Assessment of mitochondrial sizes by electron microscopy in the same biopsies. Each dot depicts the size in μm^2 of an individual mitochondrion measured at 5,000X. Dot colors in (C) and (D) correspond to the line colors of the same 2 subjects in (A) and (B). Density and size changes were tested for statistical significance by unpaired t-tests. Horizontal lines and error bars depict means and standard deviations. **(E)** Representative electron microscopy images of inconspicuous mitochondria at baseline in 9 cm biopsies of Subject Y. Fine structural detail is limited due to formalin fixation of biopsies. **(F)** Images of enlarged and dysmorphic mitochondria in 9 cm biopsies of Subject Y after seven days of once-daily rectal tenofovir gel application.

To assess whether TFV's inhibition of PNPT1 and/or other genes leads to impairment of mitochondrial function, the transcription of ATP synthase F0 subunit 6 (ATP6), a key component of the proton channel (Houstek et al., 2006), was measured by RT-ddPCR assays across all 15 subjects in the 9 cm biopsies (Figure 7-9B). Because mitochondrial genes are not included on the microarray chips, there was no preexisting information on its expression. Before treatment, the mean ATP6 mRNA copy numbers were 64,446/1000 hemoglobin B copies (SD ± 57,731). After a single application of TFV gel, copy numbers decreased to 22,500 (SD ± 15,390) ($P < 0.01$). After 7 days of treatment, copy numbers fell further to 11,701 (SD ± 13,030) (VII versus 0; $P < 0.001$). In contrast, ATP6 copy numbers were stable in all 15 subjects treated with 2% N9 gel ($P = 0.4911$) (Figure 7-6B). These results indicate that TFV gel impaired mitochondrial function whereas N9 gel did not.

Impairment of mitochondrial function can be accompanied by morphological changes that are visible by electron microscopy (Wang et al., 2010; Perazella, 2010). Although the only tissue samples available were formalin-fixed biopsies, which do not optimally preserve ultrastructural details, mitochondrial number and size changes between baseline and after 7 days of topical TFV treatment were evaluated in two subjects. In the first subject, the number of mitochondria / μm^2 decreased significantly ($P <0.001$) whereas mitochondrial size did not change markedly ($P = 0.098$). In the second subject, significant changes were seen for both parameters: the number of mitochondria decreased ($P < 0.0001$) and their size increased (P

< 0.0001) (Figures 7-9C & 7-9D). In this subject, there were disorderly convoluted mitochondria, which were never seen at baseline or in the first subject (Figures 7-9E & 7-9F). Taken together, these studies related to mitochondria suggest that TFV gel inhibits genes such as PNPT1 that are important for mitochondrial function and consequently leads to functional impairment as evidenced by a decrease in mitochondrial gene transcription and ultrastructural alterations.

7.5 Discussion

When the MTN-007 study was planned, it was anticipated that N9 gel would impact gene expression more strongly and across a larger number of genes than TFV gel. Past clinical trials found TFV gel to be well tolerated and without overt toxicities (Mayer et al., 2006). It was therefore surprising to see the profound impact of TFV gel on the rectal transcriptome. Indeed, TFV changed the expression of a much larger number of genes than N9 after one week of once-daily use. Its broadly inhibitory effect was particularly striking, with approximately ten times as many rectal genes inhibited by TFV as by N9 and the magnitude of inhibition by TFV on average more pronounced than by N9. The effects of TFV gel were also drug-specific, because few of the changes in rectal gene expression were shared between N9 and TFV.

The specific effects of TFV gel on the rectal transcriptome affected several pathways that are likely relevant for gut immune and self-repair homeostasis, HIV transmission and stimulation of local immune memory responses by infection or vaccination. As such, the data raise three principal questions. Firstly, could topical TFV lead to changes that decrease the

mucosa's natural resistance to HIV infection over time? Secondly, could prolonged daily use of topical TFV cause irreversible side effects to the mucosa? And thirdly, as preventative combination approaches of microbicides and vaccines are currently being considered, could topical tenofovir alter the mucosa's response to an HIV vaccine?

These findings indicate that TFV gel interferes on multiple levels with mucosal stress responses and tissue remodeling, which may in turn compromise the mucosa's resistance to infection. Two well-studied genes regulating these functions, NFAT5 and PAK2, were strongly inhibited by TFV. Both genes also play important roles in development, as knocking out either is lethal during mouse embryogenesis (Mak et al., 2011; Hofmann, Shepelev, & Chernoff, 2004). It is well established that the same genes and pathways that mediate essential functions during embryogenesis often regulate and control self-renewing organs, such as the gut in adult organisms (Radtke, Clevers, & Riccio, 2006). NFAT5 has been identified as a central factor in cellular osmoregulation and appears necessary for Toll-like receptor (TLR)-mediated signaling (Neuhofer, 2010; Buxade et al., 2012; Trama et al., 2002). PAK2 is a central effector molecule downstream of Cdc42 and Rac, which are ancient, highly conserved Rho GTPases mediating extracellular signals that trigger reorganization of the actin cytoskeleton (Cotteret & Chernoff, 2002). TFV gel also inhibited other effector molecules mediating downstream effects of Cdc42 and Rac on regulation of actin polymerization, specifically WHAMM (Wiskott-Aldrich syndrome homolog associated with actin, golgi and microtubules)

(Campellone, Webb, Znameroski, & Welch, 2008) and Pi5K1B (phosphatidylinositol 4-phosphate-5 kinase type I beta) (Cotteret & Chernoff, 2002). PAK2 also links Rho GTPases to MAP kinase pathways (Cotteret & Chernoff, 2002). MAP kinases can translocate to the nucleus where they phosphorylate transcription factors such as c-Fos, c-Jun and STAT-3 to regulate their activities (Pearson et al., 2001), and they direct cellular responses to stress such as hyperosmosis, heat shock and radiation. Besides PAK2, a second activator of MAP kinase pathways, TAOK1 (TAO kinase 1) was also significantly inhibited by TFV gel. Thus, via suppressive effects on NFAT5, PAK2 and other related genes, TFV gel likely impairs the response of the rectal mucosa to stress stimuli, including the hyperosmotic formulation of the gel, as well as its capacity to remodel actin following alterations in cellular shape and cell matrix adhesion or during cell migration and cell-cell synapse formation.

TFV gel also impacted genes that are likely important for maintaining mucosal integrity, potentially worsening problems arising from capacity constraints to mucosal remodeling and stress responses resulting from NFAT5 and PAK2 inhibition. DSP (desmoplakin) and DST (dystonin or bullous pemphigoid antigen 1), which participate in hemi-desmosomal adhesive junctions in epithelia (Lai Cheong, Wessagowit, & McGrath, 2005; Hatsell & Cowin, 2001; Sonnenberg & Liem, 2007) were strongly inhibited. Additionally, expression of MMP12, an elastase involved in the break-down of extracellular matrix (Feinberg et al., 2000; Shipley, Wesselschmidt, Kobayashi, Ley, & Shapiro, 1996; Belaaouaj et al., 1995), was induced. Taken together, the above described effects of topical TFV may lead to an

overall decrease in the rectal mucosa's repair ability and a higher likelihood of loss of epithelial integrity. It is conceivable that these effects could facilitate HIV virion penetration into the mucosa. Once suitable target cells are reached by HIV, their permissiveness to infection might be increased by the observed inhibition of the HIV restriction factor TRIM5 by TFV (Stremlau et al., 2004; Nisole, Stoye, & Saib, 2005).

Another category of genes inhibited by TFV gel is involved in signaling initiated by TGF-β, a central anti-inflammatory mediator in the gut that by itself was not altered by TFV (Konkel & Chen, 2011; Surh & Sprent, 2012). This group included the TGFBRAP1 (Wurthner et al., 2001), spectrin (Baek et al., 2008), and 14-3-3 epsilon genes (McGonigle, Beall, Feeney, & Pearce, 2001). Other inhibited anti-inflammatory factors were IL-10 (interleukin 10), mucin 12 and IRAK1BP1 (interleukin-1 receptor-associated kinase 1 binding protein 1) (Conner, Smirnova, Moseman, & Poltorak, 2010). This blockade of immuno-dampening pathways/effectors does not necessarily signify that TFV gel directly induces inflammation in the rectal mucosa. Indeed, up-regulation of classic inflammatory markers such as IL-1, IL-6, IL-8, IL-12, TNF-α, or MIP-1 was not detected. However, TFV gel did increase the expression of a number of chemokine genes, most prominently among them CXCL13, which is selectively chemotactic for B lymphocytes (Ansel, Harris, & Cyster, 2002), and CCL19 and CCL21, both ligands of CCR7 on T lymphocytes and dendritic cells (Forster, Davalos-Misslitz, & Rot, 2008). Correspondingly, CCR7 was strongly up-regulated, confirming the chemotactic effects of CCL19 and CCL21. The B cell marker CD19 and the T cell markers CD2, CD3D, and CD7 were also up-regulated,

indicating that chemokine induction by TFV indeed enhanced lymphocytic infiltration or retention in the rectal mucosa.

In concert, the changes to the expression patterns of gut immune homeostasis genes suggest that TFV creates a state of potential hyper-responsiveness to external pro-inflammatory stimuli rather than directly causing inflammation. Particularly in populations with a high incidence of mucosal infections and associated immune activation, this could negate the protective effect of topical TFV prophylaxis over time (Naranbhai et al., 2012). Intriguingly, recent findings in CAPRISA 004 participants support this notion: while pericoital vaginal TFV gel was overall protective (Abdool et al., 2010), when inflammation did occur within the TFV arm it dramatically increased the risk of HIV infection, much more so than within the placebo arm (Jo-Ann Passmore; personal communication). It is tempting to speculate that daily use of the gel in VOICE created an even more pro-inflammatory state than the pericoital use in CAPRISA 004, and ultimately enhanced HIV infection and contributed to the observed futility of the gel in VOICE (Celum & Baeten, 2012; van der Straten et al., 2012).

A third category of genes inhibited by TFV gel was associated with mitochondrial function. Most prominently among these, TFV strongly and consistently inhibited expression of the PNPT1 gene, which encodes what is more commonly named polynucleotide phosphorylase or PNPASE. PNPASE is a master regulator of RNA import into mitochondria, including RNAs that control the transcription and translation of electron chain transport proteins encoded by the mitochondrial genome (Wang et al., 2010). In PNPT1 knock-out mice, mitochondrial morphology and respiratory capacity

is disrupted (Wang et al., 2010; Wang et al., 2012) in a manner quite similar to what has been observed in renal proximal tubular cells in patients with TFV-induced nephrotoxicity (Perazella, 2010), which is thought to be a disease of mitochondrial dysfunction (Fernandez-Fernandez et al., 2011). Strikingly, in the MTN-007 study, just one week of once-daily application of TFV gel strongly impaired mitochondrial function as measured by transcription of two mitochondrial genes encoding electron chain transport proteins, ATP6 (ATP synthase F0 subunit 6) and COX1 (cytochrome c oxidase subunit I), and by morphological swelling of mitochondria. These findings suggest that the negative effect of TFV on PNPT1 expression may underlie its reported, but heretofore unexplained, mitochondrial toxicity.

Mitochondrial respiratory capacity has been identified as a critical regulator of CD8+ T cell memory development and maintenance (van der Windt et al., 2012; Pearce et al., 2009). Thus, TFV could negatively affect mucosal memory T cell generation after vaccination. Moreover, the observed suppression of immune-dampening factors such as IL-10 and signaling effectors downstream of TGF-β could intensify the reactivity of the mucosa to antigenic stimuli and increase the likelihood that vaccine vector-specific responses paradoxically increase HIV susceptibility, as has been observed in individuals with pre-existing Ad5-specific immunity in the Step Study (Buchbinder et al., 2008; Moodie et al., 2005). Local immune homeostasis could also be altered by the stimulatory effect of TFV gel on the expression of CCL19 and CCL21, leading to retention of $CCR7^{+}$ naive and central memory T cells (T_{CM}) and CCR7+ dendritic cells in the mucosa rather than

allowing their migration to mucosal lymph nodes (Debes et al., 2005; Bromley, Thomas, & Luster, 2005). This could alter the kinetics or impede the generation of primary antigen-specific T cell responses to vaccination, or change the secondary response of T_{CM} cells to HIV exposure following vaccination (Pepper & Jenkins, 2011). Thus vaccination/topical tenofovir combination studies need to be designed in light of the possibility that TFV interferes with vaccination outcomes.

Finally, some of the genes impacted by TFV have been implicated in carcinogenesis and tumor metastasis, although a clear pattern predicting either lower or higher risk could not be discerned. Inhibition of PAK2 and TAOK1 also interferes with cellular apoptosis in response to DNA damage, potentially resulting in a tumourigenic effect (Cotteret & Chernoff, 2002). Additional tumourigenic pressure could arise from up-regulation of UBD (ubiquitin D), which has been reported to increase mitotic non-disjunction and chromosome instability and which appears highly upregulated in gastrointestinal cancers (Lee et al., 2003; Ren et al., 2006; Ren, Wang, Gao, Mehta, & Lee, 2011).

One uncertainty in our study was the discrepancy of gel vehicle osmolarities between the slightly hyperosmolar TFV gel (836 mOsmol/kg) and the isotonic HEC control gel (304 mOsmol/kg). Thus, it is not certain that all the effects observed in response to TFV were drug-related. In particular, some of the immediate effects observed after a single gel application could have been caused by the hyperosmolarity of the gel. However, the N9 gel was also hyperosmolar (1406 mOsmol/kg) and therefore effects occurring due to

gel hyperosmolarity should have been observed in both the N9 and the TFV gel arms. Since only minimal overlap existed between the N9 and TFV arms, we conclude that the active drugs rather than the gel vehicles were mostly causing the gene expression changes. Furthermore, the powerful blocking effect of TFV gel on a much larger number of genes than were inhibited by the N9 gel is consistent with the known off-target inhibition of cellular DNA polymerase by nucleoside reverse transcriptase inhibitors (De & Holy, 2005). Perhaps the strongest indicator that many of the effects observed in the TFV gel arm of the study were due to the active drug and not the gel vehicle is the pronounced inhibitory effect of TFV gel on mitochondrial function, whereas no such inhibition was observed with N9 gel. Mitochondrial toxicity is a well-documented side effect of TFV and other nucleoside reverse transcriptase inhibitors (Lewis et al., 2003; Perazella, 2010; Gallant & Deresinski, 2003), strongly suggesting that the mitochondrial dysfunction in our study was directly caused by TFV.

While the observed impact of seven once-daily applications of TFV gel on the rectal transcriptome was strong and broad, and allowed for predictions of its functional consequences, many questions remain. Would the gut regain its homeostasis after longer periods of gel use or become even more imbalanced? Does the cervicovaginal mucosa react to the gel in a similar way to the rectal mucosa? Does TFV actually cause the functional changes predicted by the transcriptome data, such as, in broad categories, hyper-responsiveness to inflammatory stimuli, impaired aerobic respiration in mitochondria, aberrant mucosal self-renewal, and perhaps even

carcinogenesis? The first two questions can be answered in currently planned clinical trials. MTN-014 will compare vaginal and rectal TFV gel in the same women in a crossover format. MTN-017 will apply rectal TFV gel daily or pericoitally over a period of 8 weeks. Extending the current findings by similar investigations in these trials is important. Addressing the last question requires a step back to basic science, studying the short- and longer-term functional impact of TFV on mucosal cells and tissues *in vitro*, *ex vivo*, and in animal models. While TFV has been proven safe as an orally administered systemic drug, this does not necessarily imply its safety as a topical drug applied repeatedly at high concentrations to relatively small, circumscribed mucosal areas.

Chapter 8

Discussion and Conclusions

8 Discussion and conclusions

8.1 The need for rectal microbicides

Epidemiological studies have confirmed that RAI is a common practice among MSM in the Global North (Wolitski & Fenton, 2011; Hart & Williamson, 2005). Unfortunately, a sizeable proportion of RAI among MSM is unprotected (Chen, Gibson, Weide, & McFarland, 2003; Begley, Chan, Jeganathan, Batterham, & Smith, 2008) and contributes to the growing number of new infections among MSM (Beyrer et al., 2010). More recent data have clearly identified at risk MSM in the Global South (Beyrer et al., 2012; Baral et al., 2012). Unfortunately this prevalence of URAI also appears, at least in MSM, to be associated with high prevalence and incidence of HIV infection as well as other STIs (Beyrer et al., 2012). These observations emphasize the need to develop new prevention strategies, including rectal microbicides, in populations at risk of HIV infection through URAI who are unable or unwilling to use a condom. The prevalence of HIV infection in transgender women (i.e. male to female transgender persons) is equal or greater than that seen in other high-risk groups such as MSM (Herbst et al., 2008). In a systematic review of 29 U.S. studies of transgender women, the average laboratory confirmed HIV prevalence was 28% (Herbst et al., 2008). This high prevalence of HIV infection was associated with an equally high prevalence of URAI with multiple male partners, psychological health problems, and alcohol/substance use (Brennan et al., 2012). Similar patterns emerge in recent studies from

Thailand and Peru (Newman, Lee, Roungprakhon, & Tepjan, 2012; Silva-Santisteban et al., 2012) and provide a clear rationale for the inclusion of transgender women in microbicide studies. MTN-017 as well as future MTN rectal microbicide studies will include transgender women.

8.2 Alternatives to rectal microbicides for HIV prevention

Rectal microbicide development is occurring within a broader field of HIV prevention research. Other strategies may offer alternative approaches to HIV prevention for individuals at risk of HIV infection associated with URAI.

8.2.1 Treatment as prevention

It is clear that the risk of HIV transmission is directly correlated to an infected individual's plasma viral load; the higher the viral load the greater the greater risk of HIV transmission (Quinn et al., 2000). Reducing an individual's viral load through the use of antiretroviral therapy should reduce their infectiousness and in 2011, Cohen et al. demonstrated that this strategy did indeed dramatically reduce rates of HIV transmission among serodiscordant couples enrolled in the HPTN-052 study (Cohen et al., 2011). Inevitably, this observation has led to a vigorous debate about the public health benefits of an HIV treatment strategy versus a HIV prevention strategy (Cohen et al., 2012). Proponents of the "treatment as prevention" strategy argue that treating HIV infected individuals has both personal and public health benefits whereas prevention efforts only shift limited resources away from treatment - an argument that is particularly relevant in the developing world. Key limitations of a treatment strategy are (i) the fact that roll out of antiretroviral therapy cannot, and may never, keep pace with new HIV infections, (ii) identification of early HIV infections is challenging and account for a

significant number of new infections (Hurt et al., 2012), and (iii) once HIV infected individuals are identified they may not be able to access or sustain antiretroviral treatment. In the United States it is estimated that the treatment "cascade" results in only 19-25% of the HIV infected population actually achieving virological suppression (Mugavero, Amico, Horn, & Thompson, 2013). Rather than debating for treatment or prevention, the debate should probably focus on how resources can be found to adequately fund comprehensive treatment and prevention strategies - especially in the developing world setting.

8.2.2 Oral pre-exposure prophylaxis

The iPrEx study demonstrated that MSM and transgender women receiving PrEP with Truvada were significantly protected from HIV infection (Grant et al., 2010). These data together with data from the Partners PrEP study (Baeten et al., 2012) led to the FDA approving use of Truvada for the prevention of HIV infection (http://www.truvadapreprems.com/#). Although PrEP is now available in the US, uptake by physicians and individuals at risk of HIV infection has been quite limited (Mimiaga, White, Krakower, Biello, & Mayer, 2013). Moreover, the iPrEx study, like many other PrEP studies had quite poor adherence to antiretrovirals. Anderson et al. demonstrated in a case control study nested within the iPrEx study that only 8% of the HIV seroconverters and 44% of the case controls had detectable levels of drug (Anderson et al., 2012). PrEP will only work when taken and it is clear that there is a need for alternative strategies that increase the likelihood of product adherence.

8.2.3 Injectable PrEP

Ironically, some of the first studies demonstrating the efficacy of antiretroviral PrEP in macaques used injectable formulations of TFV (Tsai et al., 1995). This approach clearly has the potential to circumvent adherence issues associated with oral or topical PrEP. Almost two decades later the HIV prevention field has returned to investigate the safety and acceptability of long acting antiretroviral agents including rilpivirine and S/GSK1265744. These formulations are designed to be given every 2-3 months. In the MWRI-01 study (http://clinicaltrials.gov/: NCT01656018) currently being conducted at the University of Pittsburgh we are enrolling healthy volunteers who receive an intramuscular injection of either 600 or 1200 mg of rilpivirine (Janssen Research & Development, Beerse, Belgium).

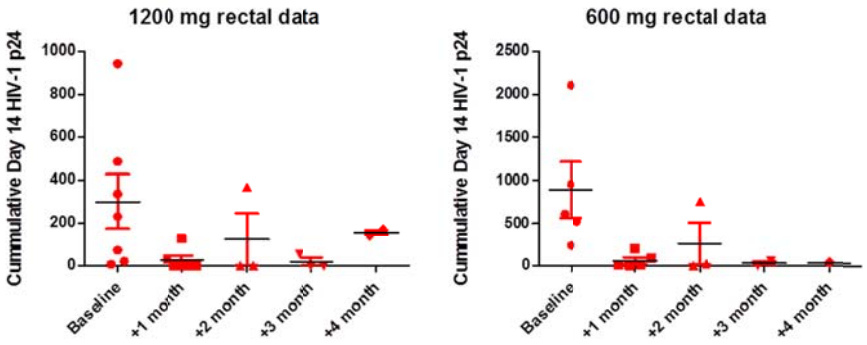


Figure 8-1 Colorectal explant infection following IM rilpivirine

Colorectal tissue was collected from healthy HIV negative volunteers before and after administration of either 600 or 1200 mg of a long acting rilpivirine nanosuspension. Explants were challenged with 10^5TCID$_{50}$ of HIV-1$_{BaL}$ and supernatant cumulative HIV-1 p24 was quantified after 14 days of culture. Significant viral suppression was seen at Month 1 post injection and remained below baseline level up to the final tissue collection time point at Month 4 (Ian McGowan unpublished data from the MWRI-01 trial).

Rectal and cervicovaginal tissue samples are collected prior to the injection and at one month intervals thereafter. The tissue explants are challenged in the laboratory with HIV-1$_{BaL}$ to determine whether exposure to rilpivirine is

associated with protection from explant infection. Preliminary data from this study suggest that rilpivirine does indeed provide significant protection from colorectal explant infection (Figure 8-1) and provides a rationale for further evaluation of this HIV prevention strategy in MSM and transgender women.

An integrase inhibitor (GSK1265744) (Spreen et al., 2013) is also being evaluated as a possible long acting injectable PrEP agent (Spreen, Margolis, & Pottage, Jr., 2013). A nanoparticle suspension of GSK1265744 (GSK744LAP) has been evaluated in an NHP rectal challenge model (Andrews et al., 2013). A total of 16 macaques received either GSK744LAP (50mg/kg; N = 8) or no treatment (N = 8). The animals were challenged with 50 $TCID_{50}$ of SHIV162p3 weekly for eight weeks. All of the GSK744LAP were protected from SHIV infection whereas all the macaques who did not receive GSK744LAP became infected over a 1-7 week period.

In a more recent study the same group have conducted a study to determine the plasma levels of GSK744LAP at which protection is maintained and lost during repeated low-dose rectal SHIV challenges in male rhesus macaques (Andrews et al., 2014). Of the twelve GSK744LA-treated RM, none had detectable systemic viremia following the first 4 weekly challenges confirming that monthly administration of GSK744LA protects an additional twelve male rhesus macaques against repeated rectal low-dose SHIV challenges. The GSK744LA-treated animals became infected after six to seventeen challenges compared with one to seven challenges for the twelve placebo controls (four current and eight historical). None of the SHIV

challenges resulted in infection when plasma levels of GSK744LAP were >3X protein-adjusted (PA) IC_{90}, compared with one of twenty two challenges when plasma levels were between 1X to 3X $PAIC_{90}$ and eleven of forty challenges when plasma levels were <1X $PAIC_{90}$.These TMC278LA and GSK744LAP data suggest that injectable PrEP should be further evaluated as a means of preventing rectally acquired HIV infection.

8.3 The results of the MTN-007 study

The MTN-007 study demonstrated that a reduced glycerin formulation of TFV gel was safe and acceptable to men and women with a history of RAI. Importantly, use of this formulation was not associated with the frequent gastrointestinal adverse events seen with the hyperosmolar formulation of TFV 1% gel used in the RMP-02/MTN-006 study (Anton et al., 2012) Adverse events that occurred in the MTN-007 study were uncommon, predominately graded as mild or modest, and occurred mainly in the N9 arm. The study included a comprehensive array of mucosal safety assays. Changes were seen in a number of mucosal parameters but again these changes were mainly confined to the N9 arm of the study.

One of the most important findings in the MTN-007 study was the demonstration of altered mucosal gene expression associated with exposure to TFV gel. The incorporation of microarray assessment of gene expression in rectal biopsies obtained from MTN-007 participants resulted in the generation of novel data suggesting that TFV gel has the potential to induce significant changes in mucosal biology including activation of innate

immunity pathways, suppression of mitochondrial function, and modulation of gene expression associated with carcinogenesis.

The MTN-007 microarray data have important ramifications for the manner in which mucosal safety is assessed in microbicide trials. *A priori* selection of mucosal biomarkers such as T cell phenotypic markers or cytokines/chemokines to characterize mucosal safety may miss alternative or unanticipated pathways of mucosal injury. As a consequence, future rectal microbicide studies are increasingly adopting a more holistic / systems biology approach to the assessment of mucosal safety. Transcriptomic and proteomic assays have been included in rectal microbicide trials being conducted within Project Gel (Section 2.8) and the CHARM Program (Section 2.9). Data from the Project Gel and CHARM-01 studies, in which participants also received 7 daily rectal doses of TFV gel, may confirm or refute the results from the MTN-007 study.

At this point in time, the clinical significance of these changes in the mucosal transcriptome is uncertain as there were no overt safety signals seen in participants receiving TFV gel in the MTN-007 study. Future studies such as MTN-017 where participants will receive 8 weeks rather than 1 week of TFV gel may provide further insights into these changes. A subset of 36/186 participants in the MTN-017 study will undergo intensive tissue evaluation (18 participants in Pittsburgh, Pennsylvania, USA and 18 participants in Bangkok, Thailand) including mucosal safety and compartmental PK. These studies will allow the exploration of the relationship between PK and potential changes in the mucosal transcriptome/proteome.

In addition, proteomic and immunohistochemical studies are currently being conducted on residual tissue samples from the MTN-007 study. Preliminary proteomic data derived from rectal fluid samples have also demonstrated significant changes associated with exposure to TFV gel (Figure 8-2).

Limitations of the MTN-007 study included the fact that the participants were sexually abstinent during the study and so it was not possible to determine product safety and acceptability in the context of RAI. Product adherence was based upon self-report which often overestimates actual product use. In addition the sample size and period of product exposure were both relatively small. During the development of the MTN-007 protocol, a decision was made not to include multicompartmental PK and PD assays as these data had already been collected in the RMP-02/MTN-006 study (Anton et al., 2012). In retrospect, this decision was unfortunate as it is now not possible to confirm product use based on PK parameters and to correlate the microarray data with tissue specific levels of TFV. Preliminary evidence of a dose-response relationship between tissue levels of TFV and outcomes such as mitochondrial dysfunction would increase the significance of this observation. However, these data will be available from the CHARM-01 study in which participants received exposure to three different formulations of TFV 1% gel (the CAPRISA 004 formulation, the formulation used in the MTN-007 study, and a new rectal-specific formulation) in a cross-over fashion.

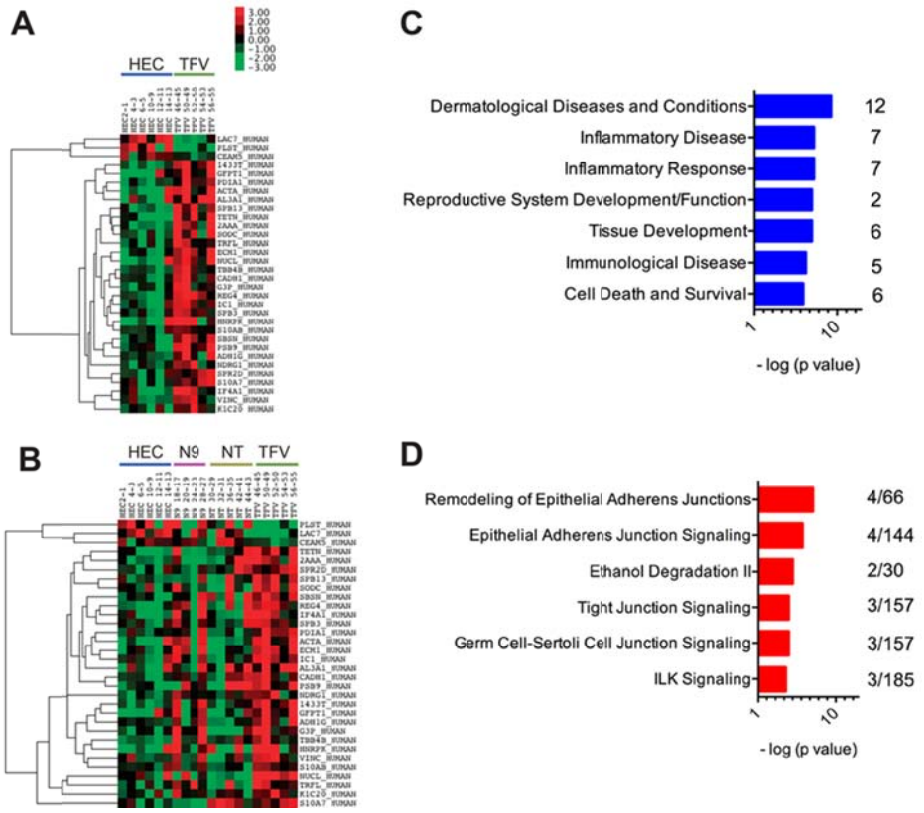


Figure 8-2 TFV exposure associated changes in rectal fluid proteins

Rectal sponge samples were collected from 7 individuals pre (Visit 1) and after 7 consecutive days (Visit 3) of once-daily doses of Tenofovir (TFV) gel or placebo HEC gel controls. Sponge eluates were processed and analyzed by tandem mass spectrometry as described previously (Burgener et al., 2013) identifying 488 unique protein factors. **(A)** This heatmap illustrates proteins which were differentially expressed ($p<0.05$, 33 proteins), between TFV and HEC controls, after 7 days of exposure (normalized to baseline). Clustering of proteins and tissues were generated by unsupervised average linkage hierarchical clustering using Pearson correlation coefficient as the distance metric. A general overexpression of proteins is noted in the TFV treatment arm compared to HEC controls. **(B)** This heatmap shows the expression of differentially expressed proteins across all study arms (HEC, N9, No Rx, and TFV), after 7 days of exposure (normalized to baseline). **(C)** The top biological functional pathways identified by the Ingenuity Pathway Analysis tool (IPA) as significantly correlated ($P<0.05$, B-H corrected) with the dataset. Numbers denote proteins identified in each categorical pathway. These involved many functions related to dermatological diseases, inflammation, and tissue development. **(D)** Top canonical pathways associated with TFV-induced protein expression changes. Numbers denote proteins identified out of total proteins in pathway. A vast majority of these pathways are involved with tissue integrity (Remodeling of epithelial adherence junctions; tight junction signaling, ILK signaling) indicating a potential effect of TFV on tissue integrity functions. For example, the ILK (Integrin-linked kinase) pathway is involved with linking integrins to the cytoskeleton. Furthermore, the adherens are sub-epithelial structures that function as principle mediators of cell-cell adhesion.

8.4 Further development of TFV gel as a rectal microbicide

Successful completion of the RMP-02/MTN-006 and MTN-007 studies of TFV gel has positioned the MTN to initiate MTN-017, an expanded safety study of the RG-TFV gel. The MTN-017 study will enroll 186 participants from a total of eight sites in the US (Pittsburgh, Boston, San Francisco, and San Juan), South Africa (Cape Town), Peru (Lima), and Thailand (Chiang Mai and Bangkok). Each participant will progress through a randomised sequence of eight week blocks of oral Truvada and rectal TFV gel (daily and associated with RAI). The study will assess the safety and acceptability of Truvada and TFV gel and PK/PD/mucosal safety in a subset of 36 participants at the Pittsburgh and Bangkok sites. It is anticipated that MTN-017 will complete follow-up in Q1 2015 allowing the possible initiation of a Phase 3 study in 2016. Given the recent approval of an HIV PrEP indication by the US FDA, the design of the Phase 3 TFV gel study will have to allow access to Truvada.

In addition to completing MTN-017 and potentially initiating a Phase 3 TFV gel study, the portfolio of Phase 1 TFV rectal studies will be expanded. Studies that explore the influence of sexual activity on product delivery and compartmental PK exposure will be developed. In previous Phase 1 rectal microbicide studies the microbicide was delivered directly into the rectal compartment using an applicator. In non-clinical settings it is more likely that individuals will apply microbicide product to the receptive partner's anorectal area as well as the insertive partner's penis. Whether this form of product application can deliver adequate TFV concentrations to the rectal

compartment is an important scientific question. Although this type of mixed behavioral/product assessment study is logistically challenging, the MTN has previously obtained approval for, and is currently undertaking, a similar study in heterosexual HIV negative/HSV-2 seroconcordant couples (MTN-011; http://clinicaltrials.gov/: NCT01687205) which explores the impact of coitus on the PK of TFV gel in the female genital tract. It is anticipated that this study will be completed in advance of initiating a Phase 3 evaluation of TFV gel as this will allow more nuanced discussions and advice to participants about product use in the study.

8.5 Moving forward with rectal microbicide research

In order to move beyond TFV gel, rectal microbicide development will require new active compounds, formulations, and clinical trials. The dichotomous separation between vaginal and rectal microbicide products and development pathways is neither rationale nor scientific and there is a clear need to develop products that can be used in either compartment. The discussion of the microbicide pipeline (Section 8.5.1 and Table 8-1) is therefore not broken out by anatomical site. Another fundamental issue is whether rectal microbicides will need to be given daily, irrespective of sexual activity, or whether they could be given in a peri-coital fashion analogous to the regimen used in the CAPRISA 004 study. Daily versus peri-coital delivery of TFV gel will be explored in the MTN-017 study. However, it is likely that the PK/PD profile needed for an effective peri-coital dosing regimen (rapid accumulation and persistent of active drug in the rectal tissue) may differ from a product that is effective when given on a daily

basis. It may be that a coformulation of two distinct APIs with a rapid and a more delayed PK release profile may be needed to achieve this goal.

8.5.1 The rectal microbicide pipeline

The current microbicide pipeline is presented below starting with products that are already in clinical development followed by products that are still in preclinical development, but are poised to move into clinical trials, and ends with a brief discussion of products that are in the very earliest stages of development but which provide insight into new mechanisms of action that could be exploited to develop microbicides.

8.5.1.1 Clinical candidates

Dapivirine (DPV) is a di-amino-pyrimidine NNRTI with potent activity against wild type and mutant HIV (Van et al., 2004). Prevention of vaginal transmission of HIV has been demonstrated in a humanized severe combined immunodeficient mouse model using DPV gel (Di Fabio et al., 2001; Di Fabio et al., 2003). IPM and the MTN are currently developing DPV as a microbicide. Initial Phase 1 studies evaluated a gel formulation (Nel et al., 2010; Nel, Smythe, Habibi, Kaptur, & Romano, 2010) but efforts are now focused on the development of an intravaginal ring, which offers the possibility of once monthly application (Malcolm et al., 2005; Woolfson et al., 2006). Following completion of Phase 1/2 studies of a matrix vaginal ring (IPM 024, 013, and 015), development has now advanced to Phase 3 (IPM 027 and MTN-020). A penile tolerance study (MTN-012/IPM 010) and a combination DPV/MVC vaginal ring study (MTN-013/IPM 026) have recently been completed. Protocols for additional safety and PK studies in

adolescents (MTN-023) and post-menopausal women (MTN-024) are in development.

Maraviroc (MVC) is a CCR5 co-receptor antagonist that prevents the interaction of HIV gp120 and CCR5 necessary for CCR5-tropic HIV to enter cells. It is also currently licensed for the treatment of chronic HIV infection (Selzentry®, ViiV Healthcare). Since CCR5 is known to be the primary co-receptor involved in sexual transmission of HIV, MVC has good potential as a topical microbicide and both gel and ring formulations have been shown to be efficacious in vaginal NHP models (Veazey et al., 2010; Malcolm et al., 2012). In addition, MVC is not widely used in the treatment of HIV-1 infection enhancing its potential use as a microbicide. MTN-013 is the first study to evaluate intravaginal delivery of MVC alone and in combination with DPV. An initial NHP study evaluating oral MVC for prevention of SHIV infection following rectal challenge did not demonstrate any protection despite high tissue levels of MVC (Massud et al., 2013). More encouragingly, a second NHP study, that evaluated rectal administration of MVC prior to SHIV challenge, did demonstrate significant protection (Dobard et al., 2013). Rectal specific formulations of MVC have been developed and will be evaluated in the CHARM-03 Phase 1 study.

8.5.1.2 Preclinical and discovery candidates

MIV-150 is an NNRTI initially developed as a therapeutic that is currently being developed by the Population Council as a potential microbicide. After initial demonstration of *in vitro* efficacy (Fernandez-Romero et al., 2007), MIV-150 was shown to be active in NHP rectal and vaginal challenge studies

(Singer et al., 2011; Kenney et al., 2011; Kenney et al., 2012). The Population Council is currently developing gel and ring formulations of MIV-150 in combination with zinc, carageenan, and hormonal contraceptives.

IQP-0528 is an antiretroviral pyrimidinedione NNRTI with nanomolar activity against HIV (Watson, Yang, & Buckheit, Jr., 2011) being developed by ImQuest Biosciences Inc. with support from the NIAID/DAIDS IPCP Program. Vaginal gels, films, and intravaginal rings have been manufactured (Mahalingam et al., 2011; Ham et al., 2012; Ham et al., 2012; Dezzutti et al., 2012) and preliminary supportive safety data have been generated for the intravaginal ring in the NHP model (Johnson et al., 2012).

PSC-RANTES is a synthetic CCR5 antagonist. As these molecules target the host co-receptor and not the HIV envelope, it is hoped that they will retain activity against all HIV clades, and *in vitro* studies using peripheral blood mononuclear cell targets confirm this (Torre et al., 2000). Recently, studies in the rhesus vaginal challenge model demonstrated that blocking CCR5 by PSC-RANTES provided high level protection against vaginal challenge with the $SHIV_{162P4}$ isolate (Lederman et al., 2004).

Concern that manufacturing costs might limit the utility of PSC-RANTES in the developing world has led to the production of two PSC-RANTES derivatives (5P12-RANTES and 6P4-RANTES) that appear stable, lack the ability to induce cell proliferation (Cerini et al., 2008), and are active in the macaque challenge model (Veazey et al., 2009).

Griffithsin (GRFT) is a product discovered by scientists at the National Cancer Institute that targets HIV envelope glycoproteins. A lectin derived from red algae, GRFT has extremely potent inhibitory activity against HIV with an EC_{50} of 40 pM, activity in the cervical explant model, and against multiple viral clades. Importantly, GRFT does not appear to induce cell activation or proliferation (Kouokam et al., 2011). Similar to cyanovirin-N, GRFT has been manufactured using transgenic plant technology (O'keefe et al., 2009). GRFT was safe in a rabbit vaginal irritation study (O'keefe et al., 2009) and in a 28-day rectal safety study in rabbits (Personal communication; Ian McGowan). Another attractive feature of GRFT is its activity against hepatitis C virus (HCV) infection (Meuleman et al., 2011). HCV is emerging as an important public health issue among HIV positive MSM (Larsen et al., 2011). A rectal microbicide with activity against HIV, HSV, and HCV would be of great value for MSM unwilling or unable to use condoms.

A range of other candidates, summarized in Table 8-1, are moving through early preclinical evaluation. They may emerge as viable candidates for Phase 1 studies during the next funding period of the MTN.

The majority have only been evaluated in *in vitro* systems but some, such as glycerol monolaurate, retrocyclin, and the S-acyl-2-mercaptobenzamide thioester have demonstrated antiviral efficacy in a NHP model (Li et al., 2009; Cole et al., 2010; Cheng-Mayer et al., 2011).

A key challenge for these products is finding the resources to conduct IND enabling studies and to manufacture adequate amounts of GMP API to support Phase 1 studies.

Table 8-1 Summary of preclinical candidates

Product	Mechanism of Action	Reference
Raltegravir	Integrase inhibitor	(Dobard et al., 2011)
4'E-2FdA	Nucleoside reverse transcriptase inhibitor	(Murphey-Corb et al., 2012)
BMS793	Entry inhibitor	
S-acyl-2-mercaptobenzamide thioester	Zinc finger inhibitor	(Cheng-Mayer et al., 2011)
Glycerol monolaurate	Anti-inflammatory	(Li et al., 2009)
4E10 and VRC01	Anti-HIV monoclonal antibodies	(Veselinovic, Preston, Mulder, & Akkina, 2012)
Retrocyclin (RC101)	gp41 binding	(Cole et al., 2010)
PD 404, 182	Virion disruption	(Chamoun et al., 2012)
PIE12 trimer	Pocket specific entry inhibitor	(Welch, VanDemark, Heroux, Hill, & Kay, 2007)
C5A Peptide triazoles	Virion disruption Entry inhibitor	(Maskiewicz et al., 2012) (McFadden et al., 2012)
DARPins	CD4 binding	(Pugach et al., 2010)
Bioengineered microbicides	Dependent on peptide	(Lagenaur & Berger, 2005)
Toll like receptor inhibitors (TLR7 & 9)	Inhibition of TLR induced inflammation	(Fraietta et al., 2010)
siRNA	Gene silencing	(Katakowski & Palliser, 2011)

8.5.2 The need for alternative formulations

The first two Phase 1 rectal safety studies of antiretroviral microbicides evaluated gel formulations developed for vaginal use (Anton et al., 2011; Anton et al., 2012). While UC781 vaginal gel was well tolerated in the rectum, usage of the vaginal formulation of TFV gel was associated with a significant increase in GI adverse events. This observation led to development of rectal specific formulations (Wang et al., 2011) that are currently being evaluated in the CHARM IPCP grant (Section 2.8). It remains to be seen whether rectal specific formulations that are well tolerated will also be acceptable for vaginal use. The reduced glycerin (RG)-TFV gel was included in the MTN-007 study after use of the original TFV gel by participants in RMP-02/MTN-006 was associated with gastrointestinal adverse events. While still a vaginal formulation, due to the low pH, it was acceptable to persons using it rectally. The first vaginal use of the RG-TFV gel will be in the MTN-014 study that is anticipated to start in Q4 2013.

Qualitative and clinical studies have suggested that acceptable rectal formulations could include gels, suppositories, or douches (Carballo-Dieguez et al., 2008; Carballo-Dieguez et al., 2008; Leyva et al., 2013). Gels were considered more acceptable than suppositories (Carballo-Dieguez et al., 2008) and a volume escalation of a placebo gel demonstrated that up to 35 mL of gel, with the physical properties of Femglide® (transparent and odorless), was acceptable to the majority of participants (Carballo-Dieguez et al., 2007).

The current generation of rectal microbicides is delivered into the rectum using a vaginal applicator. It is not clear whether this strategy will be acceptable and or effective in longer term studies. An alternative approach might be to use rectal suppositories or rapid dissolving tablets. There is an urgent need to evaluate novel placebo formulations in Phase 1 safety and acceptability trials. Mahan et al. have described innovative and quantitative techniques to capture product preferences for vaginal products and this approach could be adapted for rectal microbicides (Mahan, Morrow, & Hayes, 2011).

8.5.3 Ongoing rectal microbicide studies

As of September 2013, there are active clinical trials related to Project Gel (Section 2.8) and the CHARM Program (Section 2.9). Recruitment and follow up is complete for Project Gel apart from a cohort of male sex workers and data analysis is ongoing. The CHARM-01 and CHARM-02 studies have completed enrolling participants. CHARM-03, a Phase 1 evaluation of oral and rectal-specific formulations of MVC is in protocol development with an estimated start date of Q4 20l4. Building upon experience from previous studies the design of these studies will incorporate safety, acceptability, PK, and PD components. The mucosal safety component will be expanded to include transcriptomic and proteomic assessments in a subset of study participants.

8.5.4 Rectal microbicide studies to be conducted by the MTN

The portfolio of rectal microbicide studies planned to be conducted by the MTN over the next seven years is summarised in Table 8-2 and discussed below. As with all drug development not all proposed studies will be conducted but the studies outlined in Table 8.2 reflect the state of the science as proposed by the MTN in their network recompetition submitted to the NIH in October 2012.

8.5.4.1 MTN-027

It is unclear how rectal microbicides will be used if they are licensed. To date, rectal microbicide trials have required product to be inserted directly into the rectum with an applicator. However, it is possible that individuals may wish to use the rectal microbicide as a sexual lubricant and apply it to the insertive partner's penis and the receptive partner's perianal area (Kinsler et al., 2010). The MTN-027 will explore the PK correlates of different routes of product application. It is anticipated that these data will be important in informing the choice of dosing strategies, and associated counseling, in future studies.

8.5.4.2 MTN-026, MTN-029, MTN-033, and MTN-044

The MTN plans to conduct Phase 1 evaluation of novel compounds that will characterize the rectal safety, acceptability, and PK/PD profile of dapivirine, MIV-150, 5P12 RANTES, and GRFT.

8.5.4.3 MTN-035/HVTN-117

It is likely that a Phase 2B/3 rectal microbicide effectiveness study will be conducted at the same time that effectiveness studies of HIV vaccines will be ongoing. Building upon the experience of working with the HVTN on the

MTN-022/HVTN-095 study exploring interactions between oral/vaginal PrEP and HIV vaccines, the MTN proposes to conduct a similar study in approximately 120 MSM and transgender women in the US and Peru. This study will evaluate the safety and potential impact or oral/rectal TFV on HIV vaccine immunogenicity. The proposed HIV vaccine is the Geovax Clade B HIV vaccine.

Table 8-2 Summary of proposed MTN rectal microbicide studies

Study	Phase	Population	Product(s)	N	Reference
MTN-026	1	Men/Women	Dapivirine	24	
MTN-027	1	Men/Women	TFV gel	45	
MTN-029	1	Men/Women	MIV-150 gel	45	(Singer et al., 2011)
MTN-030	2B/3	MSM and Transgender women	TFV gel	5,000	
MTN-033	1	Men/Women	5P12-RANTES	45	(Hartley et al., 2004; Lederman et al., 2004)
MTN-035/ HVTN 117	1	MSM and Transgender women	Oral TDF/FTC TFV gel HIV vaccine	120	
MTN-044	1	Men/Women	Griffithsin	45	(Emau et al., 2007)

8.5.5 Preparing for rectal microbicide efficacy studies

Contingent upon appropriate safety, acceptability, and PK/PD from MTN-017, the MTN is proposing to conduct a randomised, multi-site, double-blinded, placebo-controlled trial of rectal TFV gel. The main challenge in the design of this efficacy trial is the potential use of oral TFV/FTC (Truvada) or

TFV (Viread) as PrEP. Truvada is licensed for a PrEP indication in the US and we assume that oral PrEP will be available in other communities or potentially provided as part of the standard prevention package. As a preliminary exploration of the sample size requirements, the number of events needed to detect and rule out various levels of effectiveness with 90% power and $\alpha = 0.05$ was calculated (Table 8-3). These calculations were based on a log rank test and show the number of events needed to detect the effectiveness given by the columns while ruling out the level of effectiveness indicated in the rows, where effectiveness is defined as 1 minus the relative risk (product infection rate/placebo infection rate). For example, 113 infections would be required to detect a minimum 55% reduction in HIV infections and rule out a 15% or smaller reduction.

Table 8-3 HIV endpoint analysis for a Phase 3 study

Effectiveness		**Detect**				
		50%	**55%**	**60%**	**65%**	**70%**
Rule Out	0%	93	72	56	44	35
	5%	109	82	63	49	39
	10%	130	95	72	55	42
	15%	160	113	83	62	47
	20%	204	139	98	72	53
	25%	275	177	120	85	61

In all HIV prevention studies, the required number of participants is influenced by a number of factors that are challenging to predict, particularly

the incidence rate of new HIV infections. The introduction of an approved oral PrEP agent and expanded treatment makes this even more challenging as observed incidence rates at participating sites may decrease. To estimate the sample size, a background infection rate of 5% in the placebo arm is assumed.

A trial designed to detect a 55% reduction in the infection rate and rule out a 15% reduction would require 3,997 person-years of follow-up. Assuming the uniform accrual of 3500 participants over one year, it would take approximately 17 months from the start of randomization to reach 113 endpoints.

Assuming that a 64% effective PrEP product becomes available to half the study population, a 3.6% incidence rate per year is expected which would require 4329 person-years of follow-up or 21 months assuming 3500 participants are enrolled over one year. Given the uncertainty about how provision of oral PrEP will evolve and whether participants will be able or want to use oral PrEP a 5,000 participant study would probably be necessary.

8.5.6 Social marketing and microbicide development

The FEM-PrEP and VOICE studies demonstrated that despite counseling, the majority of participants were unwilling or unable to use the study product (Van Damme et al., 2012; Marrazzo et al., 2013). Moreover, based on PK data, the majority of these women were also unwilling or unable to give an accurate account of their pattern of non-adherence. Other studies such as

iPrEx and CAPRISA 004 have also demonstrated significant levels of non-adherence (Grant et al., 2010; Abdool et al., 2010). For an HIV prevention strategy (including rectal microbicides) to succeed it will be necessary to market the concept so that the best (adherent) participants are enrolled into clinical trials and that the licensed product / behaviour is attractive to potential consumers. Social marketing strategies set out to change behaviour by influencing the real and perceived benefits and costs that may result from a specific behaviour such as use of a rectal microbicide. The marketing approach focuses on four key domains (often known as the 4Ps); product, price, place, and promotion. Key issues include how attractive is the product, how expensive is the product, is it easily available (place), and how is the behaviour being promoted. This approach has been used successfully to increase STI and HIV knowledge and testing behaviour (Pedrana et al., 2012). The NIH is planning to convene a meeting in May 2014 to explore whether social marketing could be used to help create desire for microbicides, improve patterns of adherence in clinical trials, and enhance the likelihood of operational success in rolling out a licensed product.

8.5.7 Implementation of rectal microbicides for HIV prevention

The current generation of rectal microbicides under development are all antiretroviral gels. They could only be used by HIV negative individuals and would have to be distributed through a health care system. Unintended exposure to an antiretroviral rectal microbicide by an individual with untreated HIV infection would likely result in the development of HIV resistance. This might generate subsequent challenges in providing an effective ARV regimen for treatment of HIV infection and also present

broader public health issues in terms of dissemination of resistant virus throughout at-risk populations. Consequently, provision of ARV rectal microbicides will require extensive voluntary counseling and testing for HIV infection as well as ongoing surveillance of individuals using these products. These issues may limit the social desirability of these products unless there is a carefully orchestrated public health campaign that targets rectal microbicides to those individuals who could gain the most to benefit from their use. Characterizing this population will be challenging but necessary to focus limited prevention resources to individuals who really need rectal microbicides. Parameters may include a history of frequent unprotected RAI and perhaps anorectal STIs. Such individuals may also benefit from using both oral and topical PrEP. Ongoing Phase 1 rectal microbicides studies will hopefully provide data on the pharmacological consequences of using both oral and topical PrEP as well as preliminary data on the relative efficacy of single or dual therapy using *ex vivo* explant challenge studies (Elliott et al., 2009). Successful roll out of rectal microbicides will also probably require focused marketing and the development of more user friendly delivery devices.

8.5.8 Advocacy for rectal microbicides

The increasing momentum of rectal microbicide development is encouraging and has been driven to a large extent by community advocacy as well as by the reality of the US HIV epidemic that is now clearly concentrated in young ethnic minority MSM (Sifakis et al., 2007; Sifakis et al., 2010). The efforts of groups such as the International Rectal Microbicide Advocates (IRMA) have played a key role in educating the community about advances in RM

development. IRMA is composed of a diverse group of community advocates, clinicians, sponsors, and scientists working on rectal microbicides. Through their website (http://www.rectalmicrobicides.org), frequent interactive teleconferences, and satellite conferences, IRMA plays a critical role in maintaining momentum in rectal microbicide research. Marc-André LeBlanc and Jim Pickett, both members of IRMA, have recently published a chapter on community engagement in rectal microbicide development which provides a comprehensive account of the productive engagement between community activists and research scientists in the development of rectal microbicides (André LeBlanc and Jim Pickett, 2014).

8.6 Conclusions

HIV infection associated with URAI remains a significant public health issue in both the developed and developing world. Recent studies have demonstrated the effectiveness of oral PrEP in serodiscordant couples, MSM, heterosexuals at high risk of HIV infection, and most recently in injecting drug users. In the majority of studies adherence levels were less than 100% and in many cases significantly less than 100% suggesting that there is still a need to develop alternative HIV prevention strategies including rectal microbicides.

Proof of concept that rectal microbicides are a viable strategy comes from NHP studies documenting that ARV gels can provide high levels of protection in the SIV/SHIV challenge model as well as *in vitro* and *ex vivo* colorectal explant challenge studies that have also demonstrated significant inhibition of HIV infection in explant tissue. TFV gel has advanced from

preclinical evaluation to Phase 2 assessment and is poised to enter a Phase 3 effectiveness study.

The design of Phase 1 rectal microbicide studies has evolved to allow integration of safety, acceptability, PK, and PD assessments in parallel which provides the most efficient mechanism to undertake early clinical evaluation of candidate microbicides. The microarray data from MTN-007 are intriguing and will require further evaluation in future studies. However, it is clear that mucosal safety assessments in Phase 1 microbicide studies need to adopt a more holistic approach and include transcriptomic and proteomic evaluation of tissue samples.

Despite these advances, significant challenges remain. The VOICE study was unable to demonstrate effectiveness of daily TFV gel in protecting women from HIV infection. The FACTS-01 study of a peri-coital TFV regimen is currently ongoing. If this study also fails to demonstrate the effectiveness of TFV gel it is uncertain whether funders will be willing to undertake further studies of a TFV gel. It is unclear whether participants will be willing to use TFV gel on a daily basis, whether this is necessary, and whether daily use of a TFV gel may lead to significant changes in mucosal function. Many of these questions will be addressed in the MTN-017 Phase 2 study but the outcome of this study is far from certain.

Building upon the experience of the VOICE study, the MTN-017 will now use “real-time” PK monitoring to try and improve participant adherence to study

products. Participants will be counseled that the study is monitoring drug levels in their blood and they will receive PK data every four weeks as part of a scheduled interview. Hopefully this approach will lead to better product adherence and generation of data that will support progression to a Phase 3 effectiveness study.

To quote the late Senator Edward Kennedy, “The work goes on, the cause endures, the hope still lives, and the dream shall never die.” Certainly, the need for effective interventions to prevent HIV infection, including rectal microbicides, is greater than ever.

Appendices

9 Appendices

9.1 Appendix 1: DAIDS rectal toxicity table

Addendum 3 Rectal Grading Table for Use in Microbicide Studies

Clarification dated May 2012.

PARAMETER	GRADE 1 Mild	GRADE 2 Moderate	GRADE 3 Severe	GRADE 4 Potentially Life-threatening
Anal				
Bruising (in absence of thrombocytopenia)	≤ 25% of perianal tissue	> 25% of perianal tissue	NA	NA
Hemorrhoids	No symptoms or symptoms not requiring medical intervention	Symptomatic with medical intervention indicated	Symptoms causing inability to perform usual social & functional activities with surgical intervention indicated	NA
Anorectal pruritus	Itching localized AND relieved spontaneously or with ≤ 48 hours treatment	Itching requiring > 48 hours treatment	Symptoms causing inability to perform usual social & functional activities	NA
Anal edema	≤25% of perianal tissue with edema	>25% of perianal tissue w/ edema	NA	NA
Anal erythema	≤25% of perianal tissue with erythema	>25% of perianal tissue erythema	NA	NA
Epithelial disruption, NOS (e.g. abrasions)	≤25% of circumference small superficial disruptions	>25% of circumference small superficial disruptions	Symptoms causing inability to perform usual social & functional activities	NA
Anal fissure	Superficial fissure	Deep fissure	Symptoms causing inability to perform usual social & functional activities	NA

PARAMETER	GRADE 1	GRADE 2	GRADE 3	GRADE 4
Incontinence	NA	Incontinence with or without the need for medical treatment or use of a pad	Symptoms causing inability to perform usual social & functional activities, operative intervention indicated	Bowel diversion indicated
External anal condyloma (new onset or increased size/extent)	New lesions covering ≤25% of perianal tissue	New lesions covering >25 of perianal tissue	Lesions causing obstruction with surgical intervention indicated	NA
Internal anal condyloma (new onset or increased size/extent)	New lesions covering ≤25% of circumference	New lesions covering >25% of circumference	Lesions causing obstruction with surgical intervention indicated	NA
Anal intraepithelial neoplasia (by biopsy)	AIN1	AIN2	AIN3/CIS	Anal squamous carcinoma
Anal cytology (when anal biopsy not available)	ASCUS/LSIL	HSIL	CIS	Anal squamous carcinoma
Colorectal				
Colitis, confirmed	No symptoms, regardless of pathologic or radiographic evidence of inflammation	Abdominal pain; mucus or blood in stool	Abdominal pain, fever, change in bowel habits with ileus; peritoneal signs	Life threatening consequences (e.g., perforation, bleeding, ischemia, necrosis, toxic megacolon)
Constipation	Occasional or intermittent symptoms, occasional use of stool softeners, laxatives, dietary modifications, or enema	Persistent symptoms with regular use of laxatives or enemas indicated	Symptoms causing inability to perform usual social & functional activities	Life threatening consequences (e.g., obstruction, toxic megacolon)
Diarrhea	Transient or intermittent episodes of unformed stools OR Increase of ≤ 3 stools over baseline per 24-hour period	Persistent episodes of unformed to watery stools OR Increase of 4 – 6 stools over baseline per 24-hour period	Bloody diarrhea OR Increase of ≥ 7 stools per 24-hour period OR IV fluid replacement indicated	Life-threatening consequences (e.g., hypotensive shock)
PARAMETER	**GRADE 1**	**GRADE 2**	**GRADE 3**	**GRADE 4**

Discharge	Visible discharge	Discharge requiring the use of pads	NA	NA
Distension or bloating	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	NA
Flatulence	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	NA	NA
Urgency +/- incontinence	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	NA
Hematochezia (rectal bleeding)	Mild-intermittent without transfusion	Persistent without transfusion	Requiring transfusion	Life-threatening consequences
Endoscopic colorectal mucosal abnormality	Minimal small superficial disruptions of the mucosa	Moderate superficial and/or scattered deep disruption(s) of the mucosa	Severe extensive deep disruption(s) of the mucosa	NA
Tenesmus	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	NA

PARAMETER	GRADE 1	GRADE 2	GRADE 3	GRADE 4
Proctalgia	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities and/or fecal incontinence	NA
Painful defecation	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	NA
Perforation, colon/rectum	NA	NA	Medical/surgical intervention indicated	Life-threatening consequences
Other GI Events				
GI Bleed	Mild not requiring intervention other than iron supplement	Moderate requiring endoscopic intervention	Requiring transfusion	Life-threatening consequences
Melena, confirmed	NA	No transfusion required	Requiring transfusion	Life-threatening consequences
Abscess, indicate site	Perianal abscess with fluctuance	Abscess that is draining or needs incision and drainage	Abscess that requires more severe surgical intervention	Necrotizing fasciitis from abscess
Fistulae, indicate site	Dry fistulae	Draining fistulae	Fistulae requiring medical/surgical intervention	Life-threatening consequences
Abdominal pain	Pain causing no or minimal interference with usual social & or functional activities	Pain causing greater than minimal interference with usual social & or functional activities	Pain causing inability to perform usual social & or functional activities	Disabling pain causing inability to perform basic self-care OR Hospitalization (other than emergency room visit) indicated

PARAMETER	GRADE 1	GRADE 2	GRADE 3	GRADE 4
STIs				
HSV	Present but no treatment required	Treatment required	Complicated disease requiring medical/surgical intervention	NA
Chlamydia	NA	Treatment required	Complicated disease requiring medical/surgical intervention	NA
Syphilis	NA	Treatment required	Complicated disease requiring medical/surgical intervention	NA
Gonorrhea	NA	Treatment required	Complicated disease requiring medical/surgical intervention	NA
Lymphogranuloma venereum	NA	Treatment required	Complicated disease requiring medical/surgical intervention	NA

9.2 Appendix 2: MTN-007 primary manuscript

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PLOS ONE

A Phase 1 Randomized, Double Blind, Placebo Controlled Rectal Safety and Acceptability Study of Tenofovir 1% Gel (MTN-007)

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Abstract

Objective: Rectal microbicides are needed to reduce the risk of HIV acquisition associated with unprotected receptive anal intercourse. The MTN-007 study was designed to assess the safety (general and mucosal), adherence, and acceptability of a new reduced glycerin formulation of tenofovir 1% gel.

Methods: Participants were randomized 1:1:1:1 to receive the reduced glycerin formulation of tenofovir 1% gel, a hydroxyethyl cellulose placebo gel, a 2% nonoxynol-9 gel, or no treatment. Each gel was administered as a single dose followed by 7 daily doses. Mucosal safety evaluation included histology, fecal calprotectin, epithelial sloughing, cytokine expression (mRNA and protein), microarrays, flow cytometry of mucosal T cell phenotype, and rectal microflora. Acceptability and adherence were determined by computer-administered questionnaires and interactive telephone response, respectively.

Results: Sixty-five participants (45 men and 20 women) were recruited into the study. There were no significant differences between the numbers of $\geq$ Grade 2 adverse events across the arms of the study. Likelihood of future product use (acceptability) was 87% (reduced glycerin formulation of tenofovir 1% gel), 93% (hydroxyethyl cellulose placebo gel), and 63% (nonoxynol-9 gel). Fecal calprotectin, rectal microflora, and epithelial sloughing did not differ by treatment arms during the study. Suggestive evidence of differences was seen in histology, mucosal gene expression, protein expression, and T cell phenotype. These changes were mostly confined to comparisons between the nonoxynol-9 gel and other study arms.

Conclusions: The reduced glycerin formulation of tenofovir 1% gel was safe and well tolerated rectally and should be advanced to Phase 2 development.

Trial Registration: ClinicalTrials.gov NCT01232803.

Citation: Mcgowan I, Hoesley C, Cranston RD, Andrew P, Janocko L, et al. (2013) A Phase 1 Randomized, Double Blind, Placebo Controlled Rectal Safety and Acceptability Study of Tenofovir 1% Gel (MTN-007). PLoS ONE 8(4): e60147. doi:10.1371/journal.pone.0060147

Editor: Willem Daniel Francois Venter, Wits Reproductive Health and HIV Institute, South Africa

Received October 15, 2012; **Accepted** February 21, 2013; **Published** April 3, 2013

Funding: This study was funded by the Microbicide Trials Network (MTN) grant, an HIV/AIDS clinical trials network funded by the National Institute of Allergy and Infectious Diseases with co-funding from the Eunice Kennedy Shriver National Institute of Child Health and Human Development and the National Institute of Mental Health, all components of the U.S. National Institutes of Health (5UM1AI068633). This cooperative agreement involved the active collaboration of the sponsor, NIH, with the grant recipients within the MTN. SCHARP received funding under a separate NIH cooperative grant (UM1AI068615). NIH and the Statistical Center for HIV/AIDS Research & Prevention (SCHARP) employees are authors on this paper. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: CONRAD provided regulatory support for the tenofovir gel IND given their ongoing management of tenofovir gel development and held the IND for the tenofovir gel in this study. However, CONRAD did not provide financial support for the study. MTN paid for the tenofovir gel. Finally, there are no restrictions to data from MTN-007 which is controlled by CONRAD. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Rectal microbicides (RM) are currently being developed to prevent or at least significantly reduce the risk of HIV acquisition associated with unprotected receptive anal intercourse (RAI) [1]. RAI is a common sexual practice among men who have sex with men (MSM) [2]. Recent epidemiological data have suggested that RAI is also common among men and women in the developed and developing world [3–6]. As a consequence there is an urgent need to develop a safe and effective RM. Attention is currently focused on the development of tenofovir (TFV) gel as a potential RM. The

vaginal formulation of TFV that was used in the CAPRISA 004 study [7] has been evaluated in a Phase 1 rectal safety study (RMP-02/MTN-006) [8]. Use of the gel was associated with mild to moderate gastrointestinal symptoms including bloating, pain, urgency, and diarrhea. The vaginal formulation of TFV is hyperosmolar (3111 mOsmol/kg) and it is possible that these symptoms were linked to product osmolality [9]. Consequently, the TFV used in the MTN-007 study was formulated with a lower glycerin concentration (5% w/w mg rather than the 20% w/w in the vaginal formulation) that results in a product osmolality of 836 mOsmol/kg [10]. It was anticipated this formulation would be better tolerated by study participants.

In the two phase 1 trials of antiretroviral rectal microbicides conducted to date, product use was not associated with any significant change in mucosal safety parameters [8,11]. Whilst this is reassuring, the possibility exists that the range of parameters used in these studies was too narrow and might have missed unanticipated or subtle but important mucosal changes. To mitigate this situation, the MTN-007 study included microarray assessment of mucosal gene expression. In addition, a nonoxynol-9 (N-9) arm was included to help determine the utility of individual mucosal safety assays in detecting mucosal injury. Rectal use of N-9 in humans has been associated with transient mild gastrointestinal discomfort as well as minor histological abnormality [12] and has been associated with induction of proinflammatory responses in cervical epithelial cells [13]. It was hoped that these additional assessments would help provide a more comprehensive assessment of mucosal safety.

Materials and Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Ethics Statement

The study was designed by the investigators with collaborative input from CONRAD and the NIAID/DAIDS/Prevention Sciences Integrated Preclinical-Clinical Program (IPCP) for HIV Topical Microbicides, as stipulated in the award notice and reviewed by the U.S. Food and Drug Administration (FDA). The study was approved by the University of Pittsburgh Institutional Review Board (IRB) as well as the University of Alabama IRB and the Fenway Health IRB. All subjects provided written informed consent. The trial is registered at ClinicalTrials.gov, number #NCT01232803 and is in compliance with the CONSORT 2010 recommendations for reporting of trial results (www.consort-statement.org) [14,15].

Study Schema

The primary objective of MTN-007 was to evaluate the safety of TFV gel when applied rectally. Secondary objectives included evaluation of the acceptability of TFV gel, the safety of the hydroxyethyl cellulose (HEC) placebo gel, and determining whether the use of either TFV or N-9 gels was associated with rectal mucosal damage. MTN-007 was a Phase 1, double blind, placebo-controlled trial in which participants were randomized to receive rectal TFV, N-9, HEC gels or No Rx (1:1:1:1) at three clinical sites (Pittsburgh, PA; Birmingham, AL; and Boston, MA). The study protocol was approved by IRBs at all three sites and informed consent was received from all participants. Enrollment began in October 2010 and the last participant completed the study in July 2011. The target sample size is 60, equally split to 15 in each arm, which ensures a 79% probability to observe at least one Grade 2 or higher adverse event in an arm when the true event rate is 10%. Five additional enrollees were recruited to preserve the study power in the case of participants using less than 5 doses in the 7-day dosing period or fail to complete the final clinical visit. A blinded statistician from The Statistical Center for HIV/AIDS Research & Prevention (SCHARP), University of Washington, Seattle, WAS, USA created lists containing randomly generated unique three-digit codes for study product randomization for each clinical site. Participants, study staff, pharmacists, clinicians and statisticians were blinded to study assignments.

Study Population

The study population consisted of healthy, RAI-abstinent, HIV-uninfected, adults (male and female) aged 18 or older at time of screening. Female participants were required to be using an acceptable form of contraception (e.g., barrier method, intra uterine device, hormonal contraception, surgical sterilization, or vasectomization of the male partner). Individuals with abnormalities of the colorectal mucosa, significant gastrointestinal symptoms (such as a history of rectal bleeding), evidence of anorectal *Chlamydia trachomatis* (CT) or *Neisseria gonorrhea* (GC) infection, chronic hepatitis B infection, or a requirement to use drugs that were likely to increase the risk of bleeding following mucosal biopsy were excluded from the study.

Study Products

Reduced glycerin (RG)-TFV 1% gel was supplied by CONRAD (Arlington, VA, USA). 2% N-9 was provided as Gynol II® (Johnson & Johnson, Fort Washington, PA). HEC gel, known as the "Universal Placebo Gel" [16], and used in a previous Phase 1 rectal safety study of the UC781 gel [11], was also supplied by CONRAD (Arlington, VA, USA). Each participant was assigned a carton of applicators, based on the randomization number. At the Treatment 1 Visit the participant's first dose of study product was administered by the clinic staff. During the period of daily administration study participants were instructed to insert one dose of gel into the rectum once daily throughout the 7-day period. Rectal administration of study product occurred in the evening or before the longest period of rest. All study products were provided in identical opaque HTI polypropylene pre-filled applicators (HTI Plastics, Lincoln, NE) containing 4 mL of study product.

Study Procedures

There were a total of five study visits and two follow-up phone calls. After obtaining informed consent all participants were screened with a thorough medical history, a targeted physical examination, a digital rectal examination, and collection of swabs for CT/GC nucleic acid amplification testing (NAAT). Urine was also collected for CT/GC NAAT and for pregnancy testing in the female participants (pregnancy testing was repeated at all subsequent clinical visits). Blood was collected for safety labs (complete blood count, urea nitrogen, creatinine, alanine aminotransferase, and aspartate aminotransferase) and serology (syphilis, HIV-1, hepatitis B, and herpes simplex 1 and 2). Participants who met the inclusion and exclusion criteria during the Screening Visit proceeded to an Enrollment Visit. At the Enrollment Visit participants were randomized, a behavioral questionnaire was administered and a rectal examination and a focused physical examination were performed. Swabs were collected for assessment of rectal microflora and quantification of cytokines/chemokines in rectal secretions. Participants then received a Normosol-R pH 7.4 enema and effluent was collected for evidence of epithelial sloughing and a sample of feces collected for measurement of fecal calprotectin. A flexible sigmoidoscope was then inserted into

the rectum and 7 biopsies were collected at 15 cm from the anal margin. A disposable anoscope was inserted into the anal canal and high resolution anoscopy (HRA) of the anorectum was performed at 16× magnification with collection of 7 rectal biopsies at 9 cm from the anal margin. Biopsies were used for histology, qRT-PCR, microarray analysis, and flow cytometry. At the Treatment 1 Visit (performed within 7–28 days of the Enrollment visit), all participants randomized to receive gel product had a single applicator of study gel inserted into the rectum. Within 30 minutes, swabs were collected for microflora and cytokines. An enema was administered and the same rectal samples, including biopsies, were collected as occurred during the Enrollment Visit. At the Treatment 2 Visit (performed at least 7 days after Treatment Visit 1) participants randomized to receive gel product were provided with 7 applicators of study product to take home and asked to insert the contents of one applicator daily for 7 days. The Final Clinic Visit occurred no more than 21 days after Treatment Visit 2 and was identical to the Enrollment Visit except that anogenital testing (CT/GC) was only performed if clinically indicated.

Clinical Safety and Laboratory Assessments

Emergent adverse events (AEs) were graded using the Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events, Version 1.0, December 2004 as well as Addendum 1 and 3 (*Female Genital and Rectal Grading Table for Use in Microbicide Studies* (http://rsc.tech-res.com/safetyandpharmacovigilance/). In cases where an AE was covered in both tables, the *Female Genital or Rectal Grading Table for Use in Microbicide Studies* was the grading scale utilized.

Product Acceptability

Overall product like (or dislike) and likelihood of gel use in the future were assessed using an internet based computer assisted self-interview (CASI). To monitor adherence, participants were asked to use a phone reporting system after each episode of gel use.

Mucosal Safety

Histology. A qualitative scoring system developed for inflammatory bowel disease (IBD) research [17] and adapted for use in rectal microbicide trials [18] was used to characterize potential product associated injury with a scale of 1 (normal) to 5 (mucosal erosion or ulceration).

Fecal calprotectin. Fecal calprotectin was measured using a commercial assay (Genova Diagnostics, Asheville, NC, USA) [19].

Epithelial sloughing. Epithelial sloughing was evaluated using a modification of a previously described technique [20]. Briefly, lavage fluid, collected following the Normosol enema was spun at 1000 rpm for 5 minutes. The cell pellet was resuspended in 1 mL of 2% paraformaldehyde. The suspension was placed in a petri dish that had previously been marked with quadrants. Each quadrant of the petri dish was scanned with a dissecting microscope at a magnification of 40×. The total number of >2 mm epithelial sheets in each quadrant of the petri dish were recorded.

Rectal microflora. Rectal microflora were characterized using previously described semi-quantitative culture analysis techniques [21,22] that have been used in other rectal microbicide Phase 1 studies [8,11].

Mucosal T cell phenotype. Mucosal mononuclear cells were isolated from rectal biopsies using a combination of mechanical and enzyme digestion as previously described [11]. Flow cytometric analysis was performed on a BD™ LSRFortessa cytometer (BD Biosciences, San Jose, CA). All data were stored in list mode and analyzed with BD™ FACSDIVA operating system and Flow Jo (Tree Star, Inc., Ashland, OR). All antibodies were purchased from BD Biosciences, San Jose, CA (PerCP-CD45, Clone 2D1; Pacific Blue-CD3, Clone UCHT1; PE-Cy7-CD4, Clone SK3; APC-H7-CD8, Clone SK1; FITC-CD69, Clone FN50; APC-CD184 (CXCR4), Clone 12G5 and PE-CD195 (CCR5)) and titrated under assay conditions to determine an optimum saturating dilution. Cells were first stained with LIVE/DEAD® Fixable Aqua stain fluorescence (Life Technologies, Eugene, OR).

Cytokine and chemokine mRNA expression. Rectal biopsies were homogenized in the presence of 0.5 mm RNase-free zirconium beads with the aid of a Bullet Blender homogenizer (Next Advance Inc., Averil Park, NY). RNA was then purified on columns using an RNAqueous®-4PCR kit (Applied Biosystems/Ambion, Foster City, CA). The extracted total RNA was eluted in a volume of 100 µl and DNase 1- treated. 1000 ng of total RNA from biopsy samples were converted to cDNA using MultiScribe™ reverse transcriptase and TaqMan® Reverse Transcription reagents (Applied Biosystems, Roche Molecular Systems, Inc., Branchburg, NJ). Oligo dT(20) was used to prime the reverse transcription (RT) reaction and reactions were run on the Applied Biosystems Veriti™ Dx Thermal Cycler (Life Technologies Corporation, Carlsbad, CA). Three reference genes (GAPDH, β-actin, and β2 Microglobulin β2M) were used to normalize mucosal gene expression of CD45, IL-1β, IL-6, IL-12p40, IL-8, IL-17, IFN-γ, MIP-1α, MIP-1β, TNF-α, IL23, CCR5 and RANTES (Table S1). All qRT-PCR experiments were performed on a Bio-Rad CFX96 Real Time PCR System (Bio-Rad, Hercules, CA).

Luminex analysis of rectal secretions. Luminex® was used to measure IFN-γ, IL-1β, IL-6, IL-8, IL-12 (p40), IL-17, MIP-1α, MIP-1β, RANTES and TNF-α in rectal secretions (MILLIPLEX MAP kit; Millipore, Billerica, MA).

Microarray analysis. RNA was extracted from colorectal biopsies taken from 32 male study participants (8 men per study arm, including the no-treatment arm) using the RNeasy Mini Kit (Qiagen, Valencia, CA). 200 ng of total RNA was amplified and labeled using the Illumina TotalPrep RNA Amplification kit (Ambion, Grand Island NY). cRNA was hybridized to HumanHT12 v4 Expression BeadChips (Illumina Inc., San Diego, CA).

Analysis of Outcomes

Adverse events were evaluated for the full study cohort that included all participants that were randomized into the study. All adverse events were classified using the MedDRA organ system class/preferred term. The proportion of participants having at least one AE event was compared across arms using the Chi Square test or Fisher's exact test when counts are small. Product acceptability was determined through a CASI interview and was operationalized as intentionality of product use with RAI having a rating in the upper one third of a 10-point Likert scale (values of 7–10). Histology, fecal calprotectin, epithelial sloughing, rectal microflora, and mucosal biomarkers were summarized for each arm by mean and standard deviation among participants who completed at least 5 doses of gels during the 7-day product use period and completed the Final Clinic Visit. Using linear regression, pairwise group comparisons were conducted across groups after adjustment for baseline variability for biomarkers measured after the single-dose use and for the 7-day use, separately. Multiple testing in mucosal biomarkers was adjusted for by using Bonferroni correction to control family wise error rate, as well as using the Benjamini-Hochberg procedure to

control false discovery rate (FDR) [23]. Microarray data were pre-processed using robust spline normalization and variance stabilizing transformation. Unexpressed or low variability probes were filtered out, leaving 1928 probes for analysis of treatment effects versus control using a Bayesian statistical framework Cyber-T and Benjamini-Hochberg false discovery rate (FDR) significance adjustment [23]. Criteria for significance and relevance were an FDR ≤ 0.05 and a $\log_2$ fold expression change of ≥ 0.5 (up-regulation) or ≤ 0.5 (down-regulation), respectively.

Results

Enrollment, Retention, and Participant Disposition

A total of 65 participants were enrolled and randomized in the study (TFV, $n = 16$; N-9, $n = 17$; HEC, $n = 16$; and No Rx, $n = 16$) (Figure 1). The majority of participants were white males (Table 1). There is no statistical difference between arms in age distribution (p-value 0.79, ANOVA test), in gender composition (p-value 0.75, Fisher's exact test), nor in the proportion of white participants (p-value 0.54, Chi-square test). One participant in the N-9 arm withdrew from further participation after the enrollment visit (before receiving any study product), due to an unrelated adverse event. After completing their single-dose exposure, two participants did not receive products for the 7-day exposure. One participant in the HEC group had major depression, was placed on product hold, and was subsequently removed from the study. The other participant in the TFV arm missed the Treatment Visit 2. Averaged across all study visits, the proportion of participants who completed expected visits was 95% or above for all four study arms.

Adherence and Acceptability

Among 46 participants who received products for the 7-day use of gel, two participants had at least one product hold but later resumed product use; one was due to moderate elbow cellulitis after the Treatment visit 1 (N-9) and the other was due to prohibited medication. Accounting for these product holds, only 1 participant in the N-9 arm reported using less than 80% of the assigned doses between the Treatment Visit 2 and Final visit (Table S2). Among 46 participants who were assigned to use gel and completed the acceptability question, reported likelihood of future product use (acceptability) was 93% (HEC), 87% (TFV, Fisher exact test p-value = 1.0 when compared to the HEC group), and 63% (N-9, Fisher exact test p-value = 0.08 when compared to the HEC group).

Adverse Events

Adverse events were generally mild (Grade 1, N = 121, 80% of all AEs) or moderate (Grade 2, N = 27, 18% of all AEs). Two Grade 3 events occurred in the No Rx arm and one Grade 4 psychiatric event (the major depression episode mentioned above) occurred in the HEC arm prior to 7-day product use; all Grade 3 and 4 AEs were unrelated to product. Gastrointestinal adverse events were common but the vast majority were mild (Table 2). There were no significant differences in the proportion of participants with $\geq$ Grade 2 or higher adverse events across the arms of the study: 3/16 (19%) in the TFV arm, 7/17 (41%) in the N-9 arm, 5/16 (31%) in the HEC arm, and 6/16 (38%) in the No Rx arm; when compared to the No Rx arm, one sided Fisher exact test yielded p-value 0.94 for the TFV arm, 0.56 for the N-9 arm, and 0.77 for the HEC arm.

Mucosal Safety

Histology, fecal calprotectin, and epithelial sloughing. There was suggestive evidence of increase in the histology scores for the N-9 and HEC arms compared to the TFV arm at the 9 cm site at the Final Visit (Table 3). In contrast, there were no significant differences between the fecal calprotectin levels or epithelial sloughing scores (data not shown).

Rectal microflora. There were no significant changes in rectal microflora between the Baseline and Final Visits in any of the gel arms (data not shown).

Mucosal T cell phenotype, cytokine and chemokine mRNA expression, and Luminex analysis of rectal secretions. Forty-five participants who completed more than 5 doses during the 7-day product use period as well as fifteen

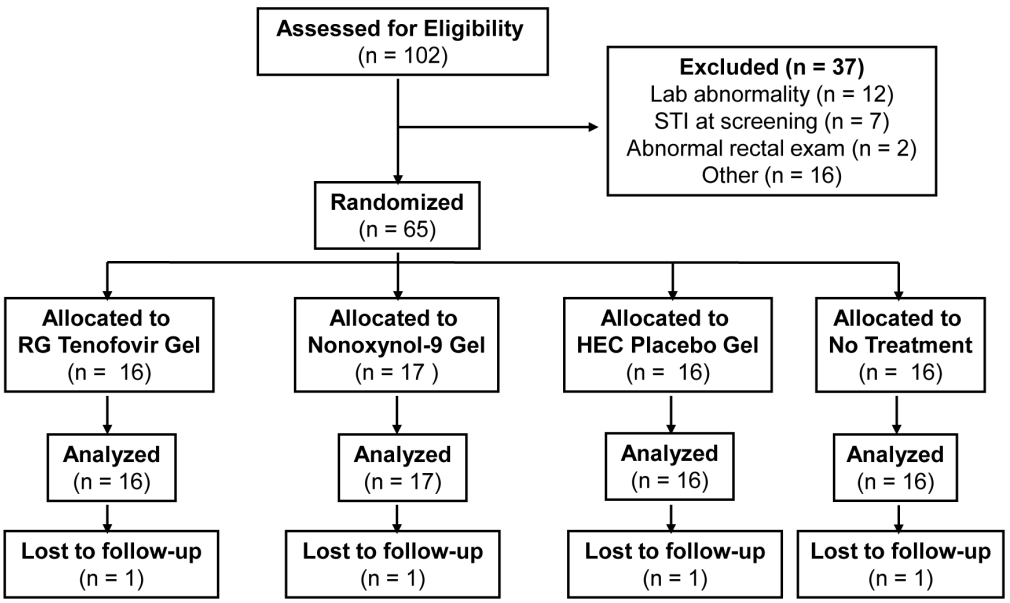

Figure 1. Flow diagram of participant progress through the MTN-007 study.
doi:10.1371/journal.pone.0060147.g001

Table 1. Baseline demographics of each treatment group.

		All Arms	Tenofovir Gel	N-9 Gel	HEC Placebo Gel	No Treatment
Participants Enrolled		65	16	17	16	16
Age (Years)	**Mean (STD)**	35.7 (11.0)	35.3 (8.7)	37.0 (9.9)	36.8 (12.6)	33.5 (13.0)
	p-value	0.79				
Gender	**Male**	45 (69%)	10 (63%)	13 (76%)	12 (75%)	10 (63%)
	Female	20 (31%)	6 (38%)	4 (24%)	4 (25%)	6 (38%)
	p-value	0.75				
Race	**White**	44 (68%)	10 (63%)	13 (76%)	9 (56%)	12 (75%)
	Non-white	21 (32%)	6 (37%)	4 (24%)	7 (44%)	4 (25%)
	p-value	0.54				

HEC, hydroxyethyl cellulose; N-9, Nonoxynol-9.
doi:10.1371/journal.pone.0060147.t001

participants in the No Rx arm who completed the Final Clinic Visit were included in these biomarker analyses. Table 3 shows biomarkers that have pairwise comparison nominal p-values less than 0.05. Only two tests remain significant after Bonferroni multiple testing correction (the comparisons of N-9 versus TFV for IL-6 and IL-8 gene expression after the single-dose treatment). Eight more comparisons meet the more lenient false discovery rate cut-off 0.1 using the Benjamini-Hochberg procedure. The

Table 2. Participants reporting adverse events by study arm.

	All Arms	Tenofovir Gel	N-9 Gel	HEC Placebo Gel	No Treatment
Participants enrolled	65	16	17	16	16
Total number of AEs	151	32	51	34	34
Grade 1 (Mild)	121 (80.1%)	29 (90.6%)	44 (86.3%)	26 (76.5%)	22 (64.7%)
Grade 2 (Moderate)	27 (17.9%)	3 (9.4%)	7 (13.7%)	7 (20.6%)	10 (29.4%)
Grade 3 (Severe)	2 (1.3%)	0	0	0	2 (5.9%)
Grade 4 (Potentially life-threatening)*	1 (0.7%)	0	0	1 (2.9%)	0
Grade 5 (Death)	0	0	0	0	0
Participants with one or more AEs					
Grade 1 (Mild)	30 (46.2%)	7 (43.8%)	10 (58.8%)	7 (43.8%)	6 (37.5%)
Grade 2 (Moderate)	18 (27.7%)	3 (18.8%)	7 (41.2%)	4 (25.0%)	4 (25.0%)
Grade 3 (Severe)	2 (3.1%)	0	0	0	2 (12.5%)
Grade 4 (Potentially life-threatening)	1 (1.5%)	0	0	1 (6.3%)	0
Grade 5 (Death)	0	0	0	0	0
Participants with gastrointestinal AEs					
Abdominal distension (G1/G2)	5 (7.7%)	1 (6.3%)	2 (11.8%)	2 (12.5%)	0
Abdominal pain (G1)	3 (4.6%)	1 (6.3%)	0	1 (6.3%)	1 (6.3%)
Anal pruritus (G1)	3 (4.6%)	1 (6.3%)	1 (5.9%)	1 (6.3%)	0
Defecation urgency (G1)	6 (9.2%)	0	5 (29.4%)	1 (6.3%)	0
Diarrhea (G1/G2/G3)**	9 (13.8%)	1 (6.3%)	4 (23.5%)	1 (6.3%)	3 (18.8%)
Flatulence (G1/G2)	13 (20%)	6 (37.5%)	2 (11.8%)	2 (12.5%)	3 (18.8%)
Haematochezia (G1)	1 (1.5%)	0	0	1 (6.3%)	0
Haemorrhoids (G1)	2 (3.1%)	0	1 (5.9%)	1 (6.3%)	0
Intestinal polyp (G1)	1 (1.5%)	0	0	0	1 (6.3%)
Painful defecation (G1)	2 (3.1%)	0	2 (11.8%)	0	0
Proctalgia (G1)	3 (4.6%)	1 (6.3%)	2 (11.8%)	0	0
Proctitis (G1)	1 (1.5%)	0	1 (5.9%)	0	0
Rectal polyp (G1)	3 (4.6%)	1 (6.3%)	1 (5.9%)	0	1 (6.3%)

HEC, hydroxyethyl cellulose; N-9, Nonoxynol-9; AEs, adverse events, *Grade 4 event was a participant who experienced a psychiatric AE (major depression) that occurred prior to 7-day product use **Grade 3 diarrhea occurred in 1 participant in the No Treatment arm.
doi:10.1371/journal.pone.0060147.t002

Table 3. Mucosal immunology assays with suggestive evidence of changes with nominal pairwise comparison p-value less than 0.05.

		Tenofovir Gel (T)	N-9 Gel (N9)	HEC Placebo Gel (H)	No Treatment (N)	p-Value
Participants analyzed		15	15	15	15	
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
Histology score at 9 cm	**Enrollment**	0.7 (0.5)	1.0 (0.0)	1.1 (0.6)	0.9 (0.4)	
	Treatment 1	1.1 (0.6)	1.1 (0.7)	1.4 (0.8)	1.5 (1.0)	
	Final visit	0.7 (0.5)	1.8 (1.2)	1.1 (0.6)	1.4 (0.6)	T:N9, 0.01, N9:H, 0.03
Flow cytometry at 9 cm						
	Final Visit					
	CD45/CD3 (%)	51.7 (19.3)	48.5 (17.4)	41.0 (15.9)	57.7 (14.8)	N9:H, 5.7^{-3}
	CD3/CD4 (%)	60.5 (9.0)	60.5 (7.9)	53.8 (10.8)	50.1 (11.1)	N9:H, 0.04; N9:N, 0.01
	CD4/CXCR4 (%)	48.0 (20.4)	59.6 (20.8)	49.4 (25.6)	38.2 (25.7)	N9:N, 0.02
	CD3/CD8 (%)	27.4 (8.6)	27.5 (7.6)	31.2 (8.6)	37.3 (10.4)	N9:N, 1.6^{-3}
	CD8/CD69 (%)	71.3 (14.1)	69.5 (8.8)	75.0 (12.8)	83.4 (9.8)	N9:N, 0.02
Flow cytometry at 15 cm						
	Treatment 1					
	CD8/CD69 (%)	81.8 (9.1)	80.5 (14.3)	82.5 (11.5)	89.0 (5.4)	T:N, 0.02
	Final visit					
	CD45/CD3 (%)	57.3 (10.6)	47.2 (16.0)	42.8 (15.5)	50.8 (18.9)	T:H, 0.04
	CD3/CD8 (%)	31.2 (8.5)	32.8 (10.8)	30.3 (8.7)	36.5 (12.8)	N9:N, 0.04
	CD8/CCR5 (%)	90.0 (6.0)	84.7 (8.7)	80.3 (15.4)	80.8 (10.7)	T:N, 0.01
qRT-PCR$^+$ at 9 cm						
	Treatment 1					
	IL-1β	−2.9 (0.6)	−2.2 (0.8)	−2.9 (0.8)	−3.2 (0.6)	N9:H, 6.8^{-4}; N9:N,1.3^{-3}
	IL-6	−4.2 (0.7)	−3.1 (0.6)	−4.0 (0.8)	−4.3 (1.1)	N9:T, 1.3^{-5}*; N9:H, 3.0^{-4}; N9:N, 4.3^{-3}
	TNF-α	−3.3 (0.9)	−2.8 (0.7)	−3.5 (0.9)	−3.8 (0.5)	N9:H, 0.01; N9:N, 2.8^{-3}
	IL-8	−3.0 (0.7)	−2.1 (0.9)	−3.0 (0.8)	−3.2 (0.7)	N9:T, 4.2^{-5}*; N9:H, 2.8^{-4}; N9:N, 4.9^{-3}
	MIP-1α	−3.4 (0.7)	−2.8 (0.7)	−3.5 (0.8)	−3.7 (0.6)	N9:T, 2.7^{-4}; N9:H, 2.5^{-3}; N9:N, 2.6^{-3}
	MIP-1β	−2.9 (0.8)	−2.5 (0.7)	−3.0 (0.8)	−3.3 (0.5)	N9:T, 1.8^{-3}; N9:H, 7.4^{-3}; N9:N, 8.0^{-3}
	IL-12p40	−4.9 (0.8)	−4.4 (0.8)	−5.0 (1.0)	−5.2 (0.7)	N9:N, 0.02
	IL-23	−4.4 (1.1)	−3.6 (1.0)	−3.9 (0.7)	−4.6 (0.9)	N9:H, 0.02; N9:N, 0.01
	CCR5	−4.6 (0.8)	−3.9 (0.6)	−4.1 (0.6)	−4.6 (0.7)	N9:H, 0.03; N9:N, 6.0^{-3}
	Final visit					
	RANTES	−2.7 (0.9)	−2.5 (0.8)	−2.4 (0.5)	−2.6 (0.7)	N9:T, 0.02; N9:H, 0.03; N9:N, 0.02
	IL-17	−4.9 (1.0)	−4.4 (0.8)	−4.5 (0.6)	−4.9 (0.8)	T:N, 0.02
qRT-PCR$^+$ at 15 cm						
	Treatment 1					
	IFN-γ	−4.6 (0.6)	−4.4 (0.8)	−4.3 (0.6)	−4.7 (0.4)	N9:H, 0.04; N9:N, 0.04
	RANTES	−2.5 (0.9)	−2.1 (0.7)	−2.2 (0.6)	−2.5 (0.6)	T:N, 0.01
	IL-17	−4.8 (0.9)	−4.4 (0.9)	−4.4 (0.8)	−5.2 (0.5)	H:N, 0.02
	Final visit					
	IL-6	−4.5 (0.7)	−4.3 (0.7)	−4.0 (0.5)	−4.5 (0.7)	N9:H, 0.02
	MIP-1α	−3.4 (0.7)	−3.6 (0.7)	−3.6 (0.6)	−3.9 (0.6)	T:N, 0.04
	RANTES	−2.6 (1.0)	−2.2 (0.7)	−2.3 (0.5)	−2.5 (0.7)	N9:T, 0.01
	IL-12p40	−5.2 (0.9)	−4.8 (1.1)	−4.9 (0.7)	−5.2 (0.6)	T:H, 6.3^{-3}; N9:H, 0.04

Table 3. Cont.

		Tenofovir Gel (T)	N-9 Gel (N9)	HEC Placebo Gel (H)	No Treatment (N)	p-Value
	IL-23	−4.5 (1.1)	−4.0 (1.1)	−3.9 (0.7)	−4.7 (1.0)	H:N, 7.5^{-3}
Luminex assay						
	Treatment 1					
	IL-8	164.3 (229.9)	537.3 (421.9)	510.9 (570.9)	303.5 (498.4)	N9:T, 0.02
	MIP-1α	2.6 (1.1)	4.1 (3.1)	5.0 (5.2)	7.2 (7.7)	T:N, 0.03
	RANTES	8.3 (10.1)	335.9 (429.1)	12.1 (10.6)	33.1 (74.6)	N9:T, 3.6^{-3}; N9:H, 1.3^{-3}; N9:N, 0.01
	Final visit					
	TNF-α	3.0 (4.0)	2.2 (1.9)	2.7 (2.1)	1.4 (1.0)	H:N, 0.04

HEC, hydroxyethyl cellulose; N-9, Nonoxynol-9, qRT-PCR, quantitative reverse transcriptase PCR, RANTES; Regulated upon activation normal T cell expressed.
[+]Data expressed as logarithm (base 10) transformation of the number of copies of gene of interest/the number of copies of β2 microglobulin in the same sample.
*p-values that are significant after controlling for family-wise error rate at 0.05 using Bonferroni correction.
†p-values that are significant after controlling for false discovery rate at 0.1 using the Benjamini-Hochberg procedure.
doi:10.1371/journal.pone.0060147.t003

majority of these changes involved upregulation of cytokine/chemokine expression in the N-9 arm.

Microarray analysis. A single application of N-9 or TFV up-regulated 24 genes and 70 genes, and down-regulated 30 and 8 genes, respectively. At the Final Visit, N-9 up-regulated 60 and down-regulated 56 genes, whereas TFV up-regulated 137 and down-regulated 505 genes (Figure 2). Background fluctuations in the HEC and no-treatment arms were negligible (manuscript in preparation).

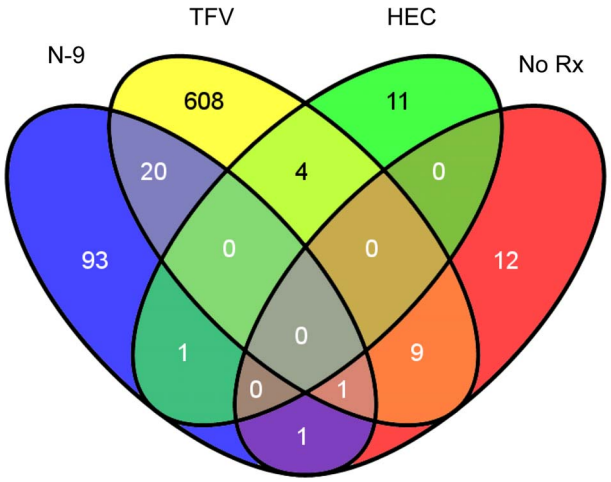

Figure 2. Number of genes in rectal biopsies (9 cm) with significant expression changes between baseline and after 7 days of treatment. The four ellipses represent each of the four treatment arms, Nonoxynol 9 (N-9) gel, tenofovir (TFV) gel, hydroxyethylcellulose (HEC) gel or no gel (No Rx). The numbers of genes changing expression exclusively in one study arm are indicated in fields of the diagram that do not overlap with another field (N-9, 93 genes; TFV, 608 genes; HEC, 11 genes; No Rx 12 genes). When identical genes changed in more than one treatment arm, their number is placed into the respective overlapping fields of the diagram. Choice of colors is arbitrary to help visually distinguish each separate field of the diagram. cRNA preparation, gene chip hybridization, and data significance analysis was carried out as described in the Materials and Methods section of the paper.
doi:10.1371/journal.pone.0060147.g002

Gender Specific and Regional Variations in Mucosal Biomarkers

MTN-007 enrolled 20 women (31% of the study population) all of whom reported RAI. Suggestive differences in T cell phenotype (CD8+/CD69+) and mucosal cytokine/chemokine gene expression (TNF-α and IL-23) were also present when the data were stratified by gender (manuscript in preparation). Regional differences were seen in T cell phenotype (CD3+/CD8+, CD8+/CD69+, CD4+/CD69+, CD4+/CXCR4+) and mucosal cytokine/chemokine gene expression (IL-1β, IL-6, IL-8, MIP-1α, MIP-1β, RANTES, IL-12p40, IL-17, and IL-23) between samples collected at 9 cm and 15 cm (manuscript in preparation).

Discussion

This study demonstrates that the RG formulation of TFV gel is safe and acceptable when administered for up to seven days in sexually abstinent participants with a history of RAI. Gastrointestinal AEs such as abdominal pain, rectal urgency, diarrhea, and flatulence were the most common AEs seen but generally mild or moderate. The preparatory enema and subsequent flexible sigmoidoscopy may have contributed to these symptoms. However, the overall rates of these symptoms were less than those seen in the RMP-02/MTN-006 study which evaluated a more hyperosmolar formulation of TFV gel [8]. Use of TFV gel did not appear to be associated with mucosal damage as assessed by a broad range of histological, immunological, and microbiological parameters. Significant changes in gene expression were identified in the TFV gel arm using microarray technology. The biological significance of these changes, and more importantly whether they represent a mucosal safety signal, is not clear and will require further evaluation. A recent preclinical study identified intestinal injury associated with *in vitro* exposure of colorectal explants to the hyperosmolar vaginal formulation of TFV [9]. However, similar histopathological changes were not seen in the TFV gel arm of the MTN-007 study.

The inclusion of N-9 as a positive control was associated with evidence of mild mucosal injury but was not associated with significant changes in epithelial sloughing. Indeed epithelial sloughing was seen at baseline in several participants. Non-human primate studies of rectal exposure to N-9 also failed to demonstrate epithelial sloughing [24]. Fecal calprotectin was also not elevated

throughout the study. This assay is useful in discriminating between irritable bowel syndrome and IBD [19] but appears to have limited utility in the evaluation of RM. Based on these data, the MTN will not use these two assays in future Phase 1/2 studies of candidate RM.

It is unclear whether the changes in mucosal gene expression assessed by microarray are related to the TFV or its associated formulation. Future RM studies may answer this question. The CHARM-01 study (NCT01575405) will explore the impact of three different TFV formulations on rectal safety and acceptability. Each formulation has a different osmolality ranging from 479 to 3111 mOsmol/kg) and the study should be able to evaluate the impact of osmolality on mucosal safety. The MTN-017 study (NCT01687218) will be a Phase-2 RM study in which participants will receive 8 weeks of TFV gel (either daily or with sex) followed by 8 weeks of oral Truvada®. This study will provide information on the consequences of extended exposure to rectal TFV as well as a crossover comparison of oral and topical exposure to TFV. Both studies are expected to start in 2013.

Importantly, 31% of the participants in MTN-007 were women; emphasizing the need for RM for both men and women. We observed some modest gender specific differences in mucosal safety biomarkers but a larger study would be required to provide more definitive data on the impact of gender on the gastrointestinal mucosa. Differences in mucosal safety parameters between the 9 cm and 15 cm samples were more marked and in keeping with previous studies demonstrating regional heterogeneity in colonic mucosal biology [18,25].

This study demonstrated that it is possible to collect adequate mucosal samples for Phase 1 RM studies via anoscopy. This observation will potentially simplify the design and execution of future Phase 1 RM studies although it is difficult to collect more than 7 rectal biopsies via anoscopy and so studies requiring collection of rectal samples for mucosal safety, pharmacokinetic, and pharmacodynamic assessment may still require flexible sigmoidoscopy.

It is encouraging that the RG formulation of TFV is well tolerated as the vaginal formulation used in previous studies was associated with a high frequency of gastrointestinal side effects when given rectally [8]. This safety profile together with evidence from the RMP-02/MTN-006 study showing that rectal use of TFV is associated with *ex vivo/in vitro* inhibition of HIV-1 viral replication [8] provides a compelling rationale for progression of this product into Phase 2 development. Collectively, these data will determine the longer term safety profile of this product and help decide whether it is a suitable agent to take into Phase 3 effectiveness studies.

Supporting Information

Table S1 qRT-PCR primer and probe sequences.
(DOC)

Table S2 Study retention and adherence.
(DOC)

Checklist S1 MTN-007 CONSORT Checklist.
(DOC)

Protocol S1 MTN-007 Trial Protocol.
(PDF)

Acknowledgments

Preliminary findings from this study were presented at the 19th Conference on Retroviruses and Opportunistic Infections (CROI), March 2012. The authors would like to thank the MTN-007 participants for volunteering their time for this study. The MTN-007 study was registered at www.Clinical-Trials.gov (NCT01232803) and the protocol can be found at http://www.mtnstopshiv.org.

Author Contributions

Conceived and designed the experiments: IM CH RDC PA LJ JYD AC-D JP FH KM. Performed the experiments: IM CH RDC PA KM LJ RKNA FH. Analyzed the data: JYD AC-D FH. Contributed reagents/materials/analysis tools: AC-D FH. Wrote the paper: IM CH RDC LJ JYD AC-D JP FH KM.

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