TENOFOVIR MICROBICIDE GEL AS AN EFFECTIVE PROPHYLACTIC MEASURE TO HUMAN HERPES VIRUS-8

by

RAEESA ISMAIL BHORAT

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Declaration

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Dr RaeesaBhorat: Date:

Prof. ABM Kharsany [redacted] Date: 5 November 2019

Dr. D Sadhabiriss: Date:

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
ANP	Acyclic nucleoside phosphanate
APO1	Apoptosis antigen 1
ART	Antiretroviral therapy
BCBL	Body cavity-based lymphoma
B FGF	Basic Fibroblast growth factor
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CIITA	Class II transactivator
CTL	Cytotoxic T lymphocytes
DNA	DeoxyriboNucleic Acid
EMMPRIN	Extracellular matrix metalloproteinase inducer
EZH2	Enhancer of zeste 2
FDA	Food and drug administration
FGF	Fibroblast growth factor
FLICE	Fas-associated death domain like interleukin 1 beta converting enzyme
FLIP	FLICE inhibitory protein
Flk/KDR	Foetal liver kinase/Kinase insert Domain Receptor
GC	Guanine cytosine
GLD	Germinotropic lymphoproliferative disease
GSK3b	Glycogen synthase kinase 3 beta
GTEC	Genital tract epithelial cells
HEL	Human embryonic lung
HHV-8	Human Herpes Virus -8
HIV	Human immunodeficiency virus
HLA	Human Leucocyte Antigen
HSV	Herpes simplex virus
HSV-1	Herpes simplex Virus-1
HSV-2	Herpes Simplex virus 2
ICAM-3	Intercellular adhesion molecule-3
IL-13	Interleukin -13
IL-4	Interleukin –4
IRIS	Immune reactivation inflammatory syndrome
KICS	Kaposi sarcoma-associated herpes virus Inflammatory Cytokine Syndrome
KS	Kaposi sarcoma
LANA	Latency associated nuclear antigen
LTR	Long terminal repeat
MCD	Multicentric Castleman’s disease
MCP-1	Monocyte chemoattractant protein-1
MHC-II	Major histocompatability complex
MIR	Modulators of immune response
MM	Multiple myeloma
MSM	Men who have sex with men
NF	Nuclear factor
NK	Natural killer
NTP	Nucleotide triphosphate
ORF	Open Reading Frame

PEL	Primary effusion lymphoma
PMEA	Phosphonomethoxyethyladenine
PMPA	9-[2-(Phosphonomethoxy)propyl]adenine
PTEN	Phosphatase and tensin homologue
RB	Retinoblastoma
STI	Sexually transmitted infections
Tat	Transactivating regulatory protein
TDF	Tenofovir disoproxil fumarate
Th	T helper
V Cyclin	viral Cyclin
VEGF	Vascular endothelial growth factor
VGPCR	Viral G protein-coupled receptor
VZV	Varicella zoster virus

Abstract

Background: High prevalence and incidence rates of human herpes virus-8 (HHV-8) contributes significantly in the aetiology of cancers, including Kaposi sarcoma (KS). These cancers remain among leading causes of morbidity and mortality especially in people who are immunocompromised.

Aim: To determine the effectiveness of 1% topical tenofovir disoproxil fumarate (TDF) as a vaginal gel formulation on HHV-8 acquisition amongst participants enrolled in the CAPRISA 004 trial.

Methods: The CAPRISA 004 trial was a randomised controlled trial which investigated the effect of TDF gel on Human immunodeficiency virus (HIV) acquisition. A total 889 women from urban and rural settings in KwaZulu-Natal, aged 18 to 40 years were enrolled in the study and the samples from these women were tested for HHV-8 infections. Peripheral blood samples collected at enrolment and at the study exit visit were tested with Biotrin's indirect immunofluorescent system to measure antibody to HHV-8 lytic antigens. HHV-8 prevalence was evaluated by sociodemographic factors and incidence by study arm and location. Descriptive statistics included means and standard deviation for quantitative data and frequencies for categorical data with Fisher's exact test. Poisson regression was used to evaluate the incidence rate. A two-tailed p-value of less than 0.05 was considered significant in hypothesis testing and 95% confidence intervals (95% CI) are reported.

Results: At baseline testing, 54 of 889 participants were HHV-8 positive with a prevalence of 6.12%. A higher parity (p=0.014) and higher number of sexual partners (p<0.001) as well as a rural setting (p=0.014) were associated with higher prevalence. With 40 infections among the 660 participants, the overall HHV-8 incidence rate was 3.98 per 100 person-years (PY) (95% Confidence Interval [CI] 2.84-5.42). Twenty infections occurred among participants assigned to the TDF arm with an HHV-8 incidence rate of 4.05 per 100 PY (95% CI 2.48-6.26) compared to the 20 infections among participants assigned to the placebo arm with an HHV-8 incidence rate of 3.91 per 100 PY (95% CI 2.39-6.04) (Incidence rate ratio (IRR) of 1.04, 95% CI 0.56-1.93; p=0.909). There were no differences in the HHV-8 incidence rates among participants assigned to the TDF and placebo arms in rural (IRR 1.40, 95% CI 0.66-2.95), p=0.383 or urban settings (IRR.51, 95% CI 0.15-1.68), p=0.266. HIV incidence rate among participants with prevalent HHV-8 infections was 6.48 per 100 PY (95% CI 2.11-15.13) and was similar to the HIV incidence rate of 7.35 per 100 PY (95% CI 5.92-9.01) among participants who remained HHV-8 negative, IRR of 0.88 (95% CI 0.36-2.17), p=0.785.

Conclusion: The study showed that among women in rural and urban settings in KwaZulu-Natal, South Africa, the prevalence of HHV-8 infection was high and that TDF was not effective in the prevention of HHV-8 infection.

Ethical approval

Full ethical approval was obtained from the Biomedical Research Ethics Committee (BREC), University of KwaZulu-Natal (UKZN) (Reference no. BREC 030/18).

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Human Herpes Virus-8

Human herpes virus-8 (HHV-8) is one of the most recently discovered human oncogenic viruses and is aetiologically associated with Kaposi sarcoma (KS)- an acquired immunodeficiency syndrome (AIDS) defining malignancy. HHV-8 is an enveloped virus containing a double stranded deoxyribonucleic acid (DNA) genome and belongs to the rhabdinovirus genus of the herpesviridae family. It is a gamma2 – lymphotropic-oncogenic virus. HHV-8 has been linked to the development of three neoplastic disorders, primarily KS, primary effusion lymphoma (PEL) or body cavity-based lymphoma (BCBL) and a plasmablastic variant of multicentric Castleman's disease (MCD). It has also been associated with several other lymphomas including germinotropic lymphoproliferative disease (GLD), multiple myeloma (MM), angiosarcomas, malignant skin tumours and squamous cell carcinomas. Recently, a new clinical HHV-8 associated syndrome has been identified namely KS-associated herpes virus Inflammatory Cytokine Syndrome (KICS) which has similar manifestations to MCD.[1] Approximately 100 herpes viruses have been identified, with at least eight affecting humans.

All human herpes viruses are well adapted to their natural host. Disease caused by human herpes viruses tends to be relatively mild and self-limiting in immunocompetent people, although severe and quite unusual disease had been reported in people with immunosuppressed states.[2]

There are key characteristics that differentiate the herpes viruses from other viruses. These include the presence of a DNA genome ranging in size from 125 000 to 250 000 base pairs; an icosahedral capsid of approximately 125nm in diameter and consisting of 162 capsomeres; an amorphous layer of viral proteins called the tegument and a lipid bi-layer envelope containing viral glycoproteins.[1] The herpesviridae families are cytotoxic during productive infection and can establish latent infection in an infected host.[2]

The genomes of the various herpesviruses are clearly evolutionarily related however, differ in size as well as in gene content and order.[1] The herpesviridae family is biologically subdivided into three subfamilies. The alpha herpesviruses are neurotropic viruses that replicate relatively rapidly and infect a wide range of cells in the cell culture. Examples of these include herpes simplex virus-1 and 2 (HSV-1 and HSV-2) and Varicella Zoster Virus (VZV). The beta herpesviruses replicate slowly and are

restricted in the types of cells that are productively infected in cell culture. The gamma herpesviruses are lymphotropic viruses that also replicate relatively slowly.[1]

1.1.1. Mode of transmission

The molecular mechanism of HHV-8 entry is still poorly understood and remains controversial. Horizontal transmission by saliva appears to be the most common route among families in endemic regions as well as among high risk groups in western countries. Vertical transmission, sexual, blood, and transplant related transmission remains a significant concern. Studies of risk factors for HHV-8 infection in men who have sex with men (MSM) demonstrate an association with markers of sexual activity including the number of partners, unprotected sexual practices and markers of sexually transmitted infections (STI).[3] Evidence for heterosexual transmission in endemic countries has been conflicting. In a South African study, HHV-8 was marginally associated with higher number of sexual partners, but not with HIV co-infection.[4] Although it has been reported in certain African countries, associations between HHV-8, STIs and HIV have been inconsistent .[5, 6]

The transmission of HHV-8 and the influence of HIV co-infection on the infectivity of HHV-8 are still to be investigated. In sub-Saharan Africa, HHV-8 is transmitted during childhood; perhaps via saliva and rarely via breast milk.[7] The sexual transmission of HHV-8 was postulated as early as 1990 on the basis of KS among MSM compared with other HIV exposure groups; and an association of KS with more sexual partners and STI followed.[8-10]

1.1.2. Pathogenesis

HHV-8 has been found to be an aetiological agent in aggressive malignancies, namely KS, PEL and a plasmablastic variant of MCD. PEL is a high grade B cell malignancy characterised by a lymphomatous effusion tumour present in body cavities. PEL cells were found to be latently infected with HHV-8 and 70-80% were found to be EBV positive as well. Molecular mechanisms of HHV-8 induced tumorigenesis in PEL remain a subject of research. MCD is a rare relapsing, remitting B-cell lymphoproliferative disease. These lesions express both lytic and latent proteins of the virus and does not co-occur with EBV (in contrast to PEL). Oncogenesis is stimulated by expression of cellular and viral cytokines, IL-6, IL-10. Expression of v FLIP and v GPCR increase expression of VEGF and angiogenesis. The 165 kb HHV-8 genome is notable for molecular control of several homologous cellular regulatory genes that contribute to pathogenesis of KS through the intricate interplay of immunological and

endothelial systems.[11] Lesion development is associated with neoangiogenesis and the appearance of spindle-shaped endothelial cells, which are the KS tumour cells. With progression of disease, spindle cells proliferate, and neovascularisation continues. This occurs together with extravasations of red blood cells and haemosiderin which contributes to the violaceous colour of the skin lesions.[12] Nodular KS lesions have predominant spindle cells, however, immune cells are also present. A reactive polyclonal inflammatory process from previous inflammation is the hallmark in early stage KS.[13] Late stage lesions are typically oligo/monoclonal, which represents disease progression through naturally selected genetic mutations.[14]

Cellular tropism

Lesions of KS comprise immature blood vessels, inflammatory infiltrate and proliferating spindle cells and can progress to involve lymph nodes and visceral organs. The origin of spindle cell line remains controversial; immunohistochemistry studies showed that KS spindle cells are poorly differentiated cells of endothelial origin as shown by the presence of panendothelial cell markers (CD 31, CD34 and factor VIII), however some spindle cells express smooth muscle cell, fibroblast, monocyte, macrophage and dendritic cell markers. Regardless, it is accepted that spindle cells are derived from endothelial cells specifically lymphatic endothelial cell lineage with expression of markers such as D2-40, podoplanin and vascular endothelial growth factor receptor.[15, 16]

Virus entry and replication

There are at least two separate binding events required for viral entry into susceptible cells. In the setting of HHV-8 infection, glycoproteins K 8.1 and glycoprotein B bind to cell surface heparin sulphate which concentrates the virus on the cell surface which will enhance binding to a second receptor. The receptor for HHV-8 infection in vascular endothelial cells and in the fibroblasts of human foreskin is the integrin alpha 3 beta 1. HHV-8 enters myeloid dendritic cells and macrophages through the dendritic cell specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN). This receptor is a type II C-type lectin that is expressed on myeloid dendritic cells in the dermis, mucosa, lymph nodes, lung and thymus and interleukin–4 (IL-4) treated monocyte-derived dendritic cells.[17]

Latent phase

HHV-8 can exist either as a latent infection or as a productive lytic infection. These entities have distinct viral gene expression profiles. During latent infection the viral genome replicates within the host cell during the cell cycle as a circular, extra-chromosomal episome. In this conformation, few viral genes are expressed. These include latency associated nuclear antigen (LANA), open reading

frame 73 (ORF73), viral cyclin (vCyclin), ORF72, ORF 71 and viral FLIP (v FLIP) which is the homologue of FLICE inhibitory protein).[1]

Latent proteins

LANA is the main protein involved in the latent phase and is essential for episomal maintenance in latently infected cells. LANA has been shown to significantly inhibit p53, the cell cycle checkpoint protein and tumour suppressor. It interacts with G1-S checkpoint proteins, protein retinoblastoma (p RB) and glycogen synthase kinase 3 beta (GSK3b) and modulates G-S transition resulting in primary endothelial cells being less susceptible to apoptosis. Furthermore, LANA has been shown to stimulate angiogenesis.[18]

Lytic phase

During the lytic phase, the genome is linear which leads to the production infectious virions. Upon reactivation, a full repertoire of lytic viral genes including ORF50, ORF57, ORF59, K8, ORF40, ORF6, viral interleukin-6 (v IL6), ORFK2, viral G protein-coupled receptor (v GPCR), ORF74 and viral chemokines are expressed. [1] HHV-8 encoded lytic genes have been shows to play a significant role in the secretion of cytokines and growth factors and matrix metalloproteinases.[1] These molecules induce angiogenesis and inflammatory lesions in uninfected and latently infected cells: ORF74 specifically, upregulates vascular endothelial growth factor (VEGF).[1]

In vitro and *in vivo* models of latent infection, HHV-8 is not able to transform primary human cells. This suggests that host cell changes and pathogenesis depend not only on direct infection of the host cell, but time dependent changes governed by viral host interactions at a molecular level. These changes can be explained by the concept of epigenetics. This is compelling as HHV-8 encodes genes that modulate host cell chromatin.

Epigenetics

Epigenetics is the term that describes the phenotypic changes that occur with preservation of genomic structure. Gene expression is altered by changes in surrounding chromatin. On the molecular level, the term is used in reference to post translational modification of histones or methylation of DNA (which in mammals, occurs almost exclusively at C G nucleotides) and therefore is also referred to as CpG methylation. While DNA methylation inactivates chromatin, histone post translational modifications can either be activating or repressive.[19]

Proteins likely to alter the epigenome

LANA has been reported to interact with myriad chromatin components associated with transcriptional activation and repression. LANA suppresses promoters of genes encoding H-cadherin as well as transformation growth factor (TGF) beta promoter via DNA methylation through recruitment of de novo methyltransferase. V-FLIP upregulates enhancer of zeste 2 (EZH2) through the nuclear factor kappa beta (NF-kappa B) pathway. EZH2 was found to be required for HHV-8 induced tube formation of endothelial cells. HHV-8 encodes 12 pre-microribonucleic acid (RNA) hairpins which are processed to produce at least 25 mature mi RNAs. This RNA targets host and viral genes that may result in maintenance of latent infection, evasion of the immune system as well as prevention of apoptosis.[19]

1.1.3. Immune response

During the primary infection, both cellular and humoral responses are activated. Neutralising antibodies are produced and during latency the virus yields different proteins that escape the cytotoxic response resulting in relative immunological inertia. In both responses, the virus expresses latent and lytic proteins that modulate adaptive responses based on their similarity to cellular regulatory proteins.[20]The immune infiltrate of HHV-8 lesions comprises CD 8+ T cells, monocytes and macrophages. The inflammatory milieu is interspersed with dendritic cells, CD 4+ T cells and B Cells. KS Lesions are characterised by elevated proinflammatory cytokines namely interferon gamma, tumour necrosis factor (TNF)alpha, IL-1, IL-6 and granulocyte- macrophage colony stimulating-factor (GMCSF).[21]

1.2. Kaposi sarcoma

Kaposi's sarcoma (KS) is a mesenchymal tumour involving blood and lymphatic vessels. KS is an AIDS-defining cancer that is associated with the advanced immunosuppressed state as a result of HIV co-infection. In individuals who were not infected by HIV, KS was reported in the Mediterranean and in Central African regions and patients on immunosuppressive therapy following organ transplantation.[22] Patients who are co-infected with HIV and HHV-8 are 10 000 times more likely to develop KS compared with persons who are not infected with HIV. About 50% of patients co-infected with HIV and HHV-8 are likely to develop a KS tumour within 10 years.[23]

1.2.1. Kaposi sarcoma and HIV co-infection

AIDS-KS in African patients often present late with extensive, systemic disease associated with increased morbidity and mortality. Without initiation of anti-retroviral therapy (ART), these patients often have a poorer response to therapy. Availability of ART in sub-Saharan Africa would have a great impact in significantly reducing the incidence of AIDS-KS and contributing to improvement in quality of life and decrease in mortality. A study by Mosam *et al,* documented the positive impact of ART initiation on the outcomes of patients diagnosed with AIDS-KS.[24]

1.3. Tenofovir disoproxil fumarate and microbicide gels

Microbicides are products that can be applied to the vagina or rectum in order to reduce transmission of STI's including HIV. An effective product as a gel formulation for prevention of HIV acquisition has the potential to alter the inexorable course of the global HIV pandemic.[25] Tenofovir is an adenosine nucleotide analogue which has is effective in preventing viral replication and potent activity against retroviruses. This, together with its long half- life as well as safety profile has made it the ideal drug to be formulated as a topical gel for the prevention of HIV.[25]

Mechanism of action of acyclic nucleotide phosphanates

TDF is an acyclic nucleoside phosphanate (ANP) which is a key class of antiviral nucleoside derivatives. ANPs behave as analogues of 2', 3'-dideoxynucleotides. Unlike the "classical" acyclic nucleoside analogues such as acyclovir, ganciclovir, penciclovir as well as dideoxynucleoside analogues such as zidovudine or lamivudine, they circumvent the initial phosphorylation step necessary for the activation of modified nucleosides which is catalysed by nucleoside kinase. In those cells in which kinase is less active or missing, nucleoside analogues are inactive, whereas ANPs are converted by nucleotide guanine monophosphate or adenosine monophosphate (AMP) kinase to the monophosphate and further by nucleoside diphosphate (NDP) kinase to the triphosphate analogue. These analogues are inhibitors of the DNA polymerase enzymes. The inhibition differs with the character of the base, the most potent being with guanine derivatives. The antiviral activity of ANPs is the result of the higher affinity of the diphosphorylated ANP metabolite for viral DNA polymerases than for the cellular DNA polymerases alpha, beta, gamma and epsilon. ANPs are active against a broad range of DNA viruses because they are not dependant on virus induced thymidine kinase for conversion from the monophosphate stage.[26]

ANPs possess a phosphonate group attached to the acyclic nucleotide moiety through a stable P-C (phosphate- carbon) bond, in contrast to the phosphate group which is attached through a P-O-C (phosphate-oxygen-carbon) bond. This structure results in evasion of cleavage by cellular hydrolases (phosphoesterases). Foremost amongst the ANPs are cidofovir (HPMPC: (S)-1(3-hydroxy-2-phosphonylmethoxypropyl)cytosine), adefovir (PMEA: 9-(2-phosphonylmethoxyethyl)adenine) and tenofovir (PMPA- (R)-9-(2 phosphonylmethoxypropyl)adenine). Due to their limited oral bioavailability, the latter two compounds have been converted to oral prodrug forms. PMPApp which is a tenofovir metabolite was found to be a poor substrate (1000 fold less efficient than adenosinetriphosphate(ATP))and a weak inhibitor of cellular DNA polymerases alpha, beta and epsilon. Therefore, it minimally interferes with nuclear DNA synthesis which could account for the low cytotoxicity and favourable safety profile.[26]

The synthesis of ANPs first started with the development of the acyclic nucleoside analogue dihydroxypropyladenine (DHPA),with a different mode of action to acyclovir.[26] DHPA serves as an adenosine analogue and occupies the adenosine binding site of S-adenosyl-L-homocysteine (SAH) hydrolase, a key regulatory enzyme in S-adenosyl-L-methionine (SAM) mediated methylations. This inhibition prevents the cleavage of SAH into homocysteine and adenosine, which are substances further metabolised into three components: AMP, adenine and inosine. Therefore, SAH accumulates thereby repressing the reaction converting S-adenosylmethionine to S-adenosylhomocysteine.[27]

1.3.1. Tenofovir disoproxil fumarate and HIV-1 prevention

Myriad biological factors and social circumstances could explain the disproportionate impact of the HIV pandemic on women.[25, 28]. Current HIV prevention behavioural messages on abstinence, faithfulness and condom promotion have had limited impact on HIV incidence rates in women, especially in sub-Saharan Africa. [25, 28]. Therefore, the Centre for the AIDS Programme of Research in South Africa (CAPRISA) 004 trial assessed the effectiveness and safety of a 1% vaginal gel formulation of tenofovir for the prevention of HIV acquisition in women.[25]. A double-blind, randomised control trial was conducted comparing tenofovir gel and a placebo gel in sexually active, HIV uninfected women aged from 18 to 40 years old in urban and rural KwaZulu-Natal, South Africa. Participants were followed up for 30 months and HIV serostatus, safety, sexual behaviour and gel condom use were assessed monthly. The HIV incidence rate in the tenofovir gel arm was 5.6 per 100 women-years compared with 9.1 per 100 women-years in the placebo arm. Tenofovir gel reduced HIV acquisition by an estimated 39% overall and by 54% in women with high gel adherence. Therefore, tenofovir gel

could have a major role to play in HIV prevention, especially amidst the reality of social injustices and lack of female autonomy.[25, 28] However, the results in subsequent studies were equivocal regarding efficacy and therefore further studies may be required.[29].

1.3.2. Tenofovir disoproxil fumarate and HSV-2 prevention

In the CAPRISA 004 trial, a significant 51% reduction of the risk of acquiring HSV-2 was observed when a TDF microbicide gel was applied. This effect was unexpected because TDF has been shown to be a highly potent antiretroviral and anti-hepadnaviral drug and had previously shown minimal, if any, anti-herpesvirus activity. It was initially thought that a complex, indirect mechanism explained this phenomenon, however, it has been proven that the mechanism is direct as well.

Tenofovir activity was demonstrated in laboratory and clinical HSV-1 and HSV-2 isolates in human embryonic lung (HEL) cell fibroblasts, primary macrophages and keratinocytes, organo-typic epithelial cell raft structures, human lymphoid, cervicovaginal tissues as well as HSV-1 and HSV-2 infected mice. The predominant anti-herpetic activity noted in macrophages is likely attributed to low pools of endogenous d NTP and/or to a low rate of HSV-2 replication within macrophages. The low d NTP pools provided a competitive advantage, allowing tenofovir to interact with herpetic DNA polymerase. The molecular mechanism of the effectiveness of TDF involves the conversion of tenofovir to its active metabolite tenofovir diphosphate, which inhibits HSV DNA polymerase more efficiently. Efficacy of tenofovir in prevention of HSV-2 acquisition was related to higher vaginal concentrations of the drug following topical application in contrast to levels reached through systemic administration. Tenofovir was effective at a vaginal concentration of approximately 10 000 to 20 000ng per millilitre. The study found that in some women with this concentration, the rate of protection against HSV-2 was 63% higher than women with no detectable levels.[30, 31]

1.3.3 Tenofovir disoproxil fumarate and HHV-8 prevention

Even though anti-herpetic activity was demonstrated with HSV-2 (in the CAPRISA 004 trial), an alphavirus similar efficacy in the context of gamma herpes virus infection remains unanswered. Despite the intrinsic structural differences of alpha and gamma herpes viruses, efficacy can theoretically be extrapolated to gamma herpes viruses namely HHV-8, given that hydroxy-PMPA, the precursor of adefovir, has been shown to have antiviral activity against HHV-8. In addition, adefovir, even though not listed as a drug candidate for the treatment of the respective infections, has a

mechanism of action similar to tenofovir and has been shown to have antiviral activity against the herpes viruses including HSV-2 and HHV-8. Furthermore, HPMPC, the cytosine counterpart of hydroxy-phosphonomethoxypropyladenine (HPMPA), has shown antiviral activity spectrum similar to that of HPMPA, which involves herpes viridae, including HSV-2 and HHV-8. The overlap of the antiviral activity of the molecule could be translated to the results of the study that is being undertaken.[26]

The microanatomy of the female genital tract has an important role in the transmission of HHV-8. The lower reproductive tract in women is comprised of the ectocervix and the vaginal tract. The mucosal lining in these compartments consists of stratified squamous epithelium and can be more than 25 layers thick.[32] In contrast, the upper reproductive tract, made up of the endocervix and endometrium, is comprised of a single layer of columnar epithelium that rests on a thin, continuous basement membrane. The columnar epithelium is differentiated by the presence of tight junctions between the cells that make it impermeable to large molecules and particulate matter, including pathogens. In contrast, the upper layers of the stratified squamous epithelium, which form the lining of the lower genital tract, have been shown to lack tight junctions resulting in increased permeability to large weight soluble mediators.[33] While the multiple layers in the lower genital epithelium may provide a better mechanical protection against viral entry, the greater surface area of the vaginal wall and ectocervix could enhance access for viral entry, particularly when a breach occurs in the epithelium, such as during sexual intercourse.[34]

The genital tract epithelial cells (GTEC) display a plethora of immunological cellular activity involving both the innate and adaptive arms of the immune system. These cells express an array of toll like receptors and can stimulate dendritic cells to activate the adaptive immune response. GTEC secrete cytokines, chemokines and numerous peptides that prevent entry of pathogens. Lymphocytes and antigen presenting cells are present throughout the female genital tract interspersed with B cells. Hormonal influence impacts greatly on immune function as oestrogen receptors on cell types of the GTEC stimulate secretion of immune modulators.[34, 35] The dendritic cells express c-type lectin receptors including DC-SIGN, providing additional receptors for the binding, uptake and dissemination of infectious virions to the T cell rich environment without the requirement for direct DC infection.[31] There is a paucity of data regarding the interaction of HHV-8 with cells of the female genital epithelium. There is no evidence of tropism of epithelial cells as is the case with HSV-2; however,HHV-8 can infect dendritic cells and macrophages, which would be a significant portal of entry for establishment of infection.[17]

1.4. Molecular link between HIV and HHV-8

Kaposi sarcoma (KS) is more aggressive in patients with advancing HIV disease. This can be explained by the depth of immunodeficiency as well as a direct effect of HIV infection. HHV-8 persists as a latent infection within its host cell, with expression of only latent genes (ORF 73). In the setting of HIV co-infection, replication occurs, and lytic genes are expressed. Immune dysregulation associated with HIV infection stimulates HHV-8 replication and reactivation of latently infected cells through the production of inflammatory mediators and growth factors.[36] Th-1-type cytokines induce activation of endothelial cells leading to spindle cell formation, angiogenesis as well as adhesion and tissue extravasation of lymphocytes and monocytes. HIV-1 transactivating gene produces transactivating regulatory protein (Tat) which leads to the proliferation of spindle cells and inhibits apoptosis via interferon gamma or transactivation of interleukin 6 genes. The Tat protein induces overexpression and synergism with b Fibroblast growth factor (b FGF), thereby stimulating the secretion of growth factors. Tat has been shown to act as an angiogenic factor that can interact with the vascular endothelial growth factor (VEGF)- receptor, foetal liver kinase, kinase insert domain receptor (Flk/KDR). Tat expression enhances interaction between HHV-8 and its receptors thereby augmenting infectivity and promoting tumorigenesis through v-GPCR.[36]

Some epidemiological studies have shown that HHV-8 infection can lead to AIDS progression. LANA can activate the long terminal repeat of HIV genome, thereby stimulating p24 production.[37] HHV-8 can increase cell vulnerability to HIV as the interaction of ORF 50 interacts with HIV Tat, induces long terminal repeat (LTR) transactivation. In addition, ORF50 increases production of IL-6 leading to increased expression of monocyte chemoattractant protein-1 (MCP-1) in macrophages, thereby increasing susceptibility to HIV infection. Furthermore, the HHV-8 viral G protein-coupled receptor synergises with HIV Tat in NF-AT (nuclear factor activation) and NF kappa B activation.[36]

The HHV-8 protein K1 has been reported to act synergistically with HIV-1 regulatory protein, NEF (negative regulatory factor) to stimulate cell proliferation and angiogenesis in a chicken chorioallantoic membrane (CAM) model. The regulation of angiogenic properties is achieved by multiple intracellular signalling transduction pathways, mainly PI3K/ATK/mammalian target of rapamycin (TOR) signalling and downregulating phosphatase and tensin homologue (PTEN).[1]

1.5. Epidemiological link between HIV and HHV-8

Since 1993, there has been a disproportionate increase in the incidence of KS in South African women. In South Africa, the incidence of KS has doubled in men and increased seven fold in women, with a decrease in ratio from 7:1 in 1988 to 2:1 in 1996, showing a stark female preponderance. The study by Mosam et al showed that the predominance of AIDS-KS cases was highest amongst women in their mid-20's, a finding that was intriguingly similar to the pattern of HIV incidence noted in past studies [(23)]. This suggests an epidemiological relationship between KS and HIV infection. In the study, it was also found that at diagnosis, women presented with more fulminant disease in keeping with studies from Zimbabwe and Italy [(23)]. This disparity was thought unlikely to be related to immunological differences as men and women had similar CD4 cell counts and viral loads. Further investigation is required to understand other relevant factors such as female hormones as well as parity in the pathogenesis of disease. This disproportionate predilection for and severity of infection in young women underscores the need for prophylaxis to prevent acquisition of HHV-8 in this group, and thereby reduce the burden of disease as well as to reduce the overall morbidity and mortality associated with KS.[23]

1.6. Sociodemographic variances in urban versus rural participants

The CAPRISA 004 trial was conducted between May 2007 and March 2010 in Kwa Zulu Natal, South Africa at the CAPRISA Vulindlela Clinical research site in Vulindlela (a rural community) and at the CAPRISA eThekwini Clinical research site in the Durban city centre. Rural participants recruited from the rural area were younger, had lower mean parity and were more likely to be living apart from their regular partner. In contrast, urban participants were older with higher levels of education and income. With regards to sexual behaviour characteristics, rural participants had a lower mean age of sexual debut, reported lower mean number of sex acts in the past seven and 30 days, were more likely to be living separately from their regular partner due to partner or self-employment options and had lower condom use compared to the urban participants. A higher proportion of urban participants reported having a new partner in the past 30 days, having higher numbers of lifetime partners and having received money in exchange for sex compared to rural participants (all p values <0.001).[38]

1.7 Research Rationale

Structural similarities between HHV-8 and HSV-2 have been demonstrated. [26, 27, 30] In addition, they have similar molecular machinery and enzymatic processes. It has been demonstrated that the application of topical tenofovir vaginal gel has been effective in preventing HSV-2 infection. [30] It is therefore reasonable to evaluate if a similar favourable outcome can be achieved with tenofovir microbicide gel as a preventative measure against HHV-8.[26, 27, 30]

CHAPTER 2

METHODS

2.1. Literature review and search strategy

An extensive literature review and search was conducted using PubMed and Medline search engines. All cited literature and referencing were performed using EndNote^{R}software. Keywords in the search for literature included Kaposi sarcoma, human herpes virus, HIV, AIDS and tenofovir.

2.2. Study Population

The study population included women who were enrolled in the CAPRISA 004 trial which studied women urban and rural clinics in KZN, South Africa. We evaluated 889 participants for HHV-8 serology at baseline and at follow-up (Figure 1).

2.3. Study Design

We assessed the effectiveness of peri-coital 1% tenofovir gel applied vaginally as an antiviral microbicide, in preventing HHV-8 acquisition in women enrolled in the CAPRISA 004 study, a double-blind, randomised, placebo-controlled trial.

2.3.1. CAPRISA 004 trial study design

The overall CAPRISA 004 trial conducted from2007 to 2010 was a double-blind, placebo-controlled, randomised trial that was designed to assess whether tenofovir gel prevents HIV acquisition in women. In the CAPRISA 004 trial, 889 eligible HIV negative urban and rural South African women were randomly assigned to receive either tenofovir gel or placebo gel. Tenofovir gel consisted of approximately 40 mg of 9-[(*R*)-2-(phosphonomethoxy)propyl]adenine (PMPA) monohydrate in a solution of purified water with edetate disodium, citric acid, glycerin, methylparaben, propylparaben and hydroxyethylcellulose. Gilead Sciences donated PMPA monohydrate for the manufacture of tenofovir gel and the company did not play any additional role in the study or have access to the data presented here. By arrangement with Gilead Sciences and CONRAD (a non-profit reproductive health organisation), the biotechnology agency of the South African government received a voluntary, nonexclusive, royalty-free license for tenofovir gel for local manufacture and low-cost distribution in

Africa. The placebo was the universal microbicide placebo, hydroxyethylcellulose gel, for which there is no evidence in animal models of either a protective or susceptibility-enhancing effect to HHV-8.

Randomisation was performed in permuted blocks of 6 or 12 and was stratified according to site. The randomisation procedure was conducted by an independent statistician who issued study-drug assignments by letters in sealed, opaque envelopes, which were stored securely; each envelope was opened in sequence by the study pharmacist once the study clinician had enrolled the participant. Participants' HHV-8 serologic status was not known during the process of enrolment and randomisation, as well as during follow-up in the trial and HHV-8 serologic status was determined retrospectively.

Tenofovir and placebo gels appeared identical and were dispensed in the same prefilled vaginal applicators with identical packaging. Women were instructed to insert one dose of the gel within 12 hours before sex, a second dose as soon as possible within 12 hours after sex, and no more than two doses in 24-hour period. The women were provided with the gel on a monthly basis. The total follow-up time was 1341 person-years (mean follow-up of 18 months).

All women were followed up monthly with risk-reduction counselling, provision of condoms, pregnancy testing and contraception provision, clinical assessments, and safety assessments. At each monthly study visit, women were requested to return all used and unused applicators for an assessment of adherence. The frequency of use of tenofovir gel was measured by the mean number of returned empty applicators each month. Vaginal tenofovir concentrations were measured at a single, randomly selected time point for each participant during follow-up, with the concentration serving as a biomarker of drug exposure. Concentrations were measured in undiluted aspirated cervicovaginal fluid with the use of validated, ultra-high-performance liquid chromatography–mass spectrometry[39].

2.3.2. HHV-8 sub-study design

The HHV-8 study design uses a subset of participants from the CAPRISA 004 trial and therefore shared the baseline eligibility criteria as listed below:

2.3.2.1. Inclusion criteria

- Sexually active women from 18 to 40 years old and who were not infected with HIV. Sexual activity was defined as having engaged in vaginal sex at least twice in the preceding 30 days of screening.

2.3.2.2. Exclusion criteria

- Women who were pregnant or became pregnant during the study period.
- Women who used a barrier method of contraception.
- Any person with a history of any adverse reaction to latex.
- Any person with a planned travel away from the study site for more than 30 consecutive days or a planned relocation from the study site.
- Persons who enrolled in any other behavioural or investigational product study.
- Any persons with a creatinine clearance of 50 ml/min or less.
- Any person with a deep genital epithelial disruption.
- Any person who participated in any research related process in the preceding year.

Retrospective testing of serum samples of the 889 women involved in the CAPRISA 004 trial for HHV-8 serology were performed. Serological testing involved the use of Biotrin's indirect immunofluorescent system which was a rapid and simple method for the determination of antibodies to HHV-8 lytic antigens.

2.4. Sampling and testing methods

The testing method consisted of the following equipment and was standardised. High quality distilled or deionised water; Accurate^{R} 20, 100 and 200 microlitre pipettes and disposable tips, serum collection equipment, a timer; wash bottles and a wash tray; test tubes, racks, pipettes, microlitre plates and safety pipetting devices for making sample dilutions; an incubator set at 37 degrees Celsius;

a moist chamber for incubating slides; a slider holder rack and staining dish for washing slides; coverslips and a fluorescent microscope with 10x eyepiece and 16X or 40X objectives.

2.4.1. Specimen and reagent preparation

A consistent procedure method was employed to ensure quality control in specimen handling and the standardisation of the handling and testing of samples reduced inter-observer variability. The procedure involved the buffer being washed and the contents of the packet were added to a single litre of freshly prepared distilled or deionised water. It was stored in a clean, closed container at 2 to 8 degrees Celsius for up to 4 weeks.

To further ensure quality control, qualitative testing of the samples was standardised, and the sample was diluted in a 1:64 wash buffer. All dilutions were prepared in a minimum volume of 100 microlitres of wash buffer.

During quantitated testing the sample was diluted in at least 100 microlitres of a wash buffer. Thereafter the slide was prepared with one drop of a sample which was then incubated but not prior to the slide being washed and the addition of a conjugate having taken place. A single drop of mounting media was applied to the centre of each well before the coverslip was applied to each slide. Thereafter, each slide was examined using a fluorescence microscope of 200 to 500 times magnification.

2.5. Interpretation of test results

The results were interpreted according to the fluorescence intensity grading of the specimens. Samples were considered HHV-8 Immunoglobulin G (IgG) positive if green fluorescent staining of the infected cells was present in both the cell cytoplasm and nucleus at a dilution of greater than 1:64 and if the staining was similar to that of the positive control. Positivity reactivity ranged from brilliant to weak according to the following grading scale; +4 (brilliant); +3 (bright); +2 (moderate); +1 (weak). A sample was considered negative if fluorescent staining in the infected cells were absent. The sample titre was determined by preparing a two-fold serial dilution of the sample in the wash buffer.

2.6. Study Oversight

The trial was approved by the BREC at the University of KwaZulu-Natal, the Protection of Human Subjects Committee of FHI 360 (a non-profit human development organisation), and the Medicines Control Council of the South African government.

2.7. Statistical Analysis

Baseline characteristics of women included in the sample were analysed. Participants were stratified according to location (i.e. rural or urban) and, in subsequent analysis, by HHV-8status. Descriptive statistics included mean and standard deviation for quantitative data and frequencies and percentages for categorical data. Associations between groups were tested using the Fisher's exact test (categorical data) and the Wilcoxon rank sum test (quantitative data).

For the calculation of the HHV-8 incidence rate, the infection date was assumed to have occurred at the midpoint between the last HHV-8negative result (i.e. randomization date) and the first positive one for those women who became infected. Incidence rates, incidence rate ratios (IRR) and associated 95% confidence intervals (CI) were estimated using Poisson regression.

HIV incidence rates were calculated for those who were HHV-8-negative and those that were HHV-8positive at baseline. HIV infection date for those that seroconverted was estimated as the midpoint between the last HIV-negative result and the first HIV-positive result. IRRs were estimated using Poisson regression. Finally, the association between HIV status and HHV-8 status at study exit was assessed using Fisher's exact test.

The statistical analyses were performed using Statistical Package for the Social Sciences (SPSS); version 23, IBM Corp, Armonk, N.Y. USA.

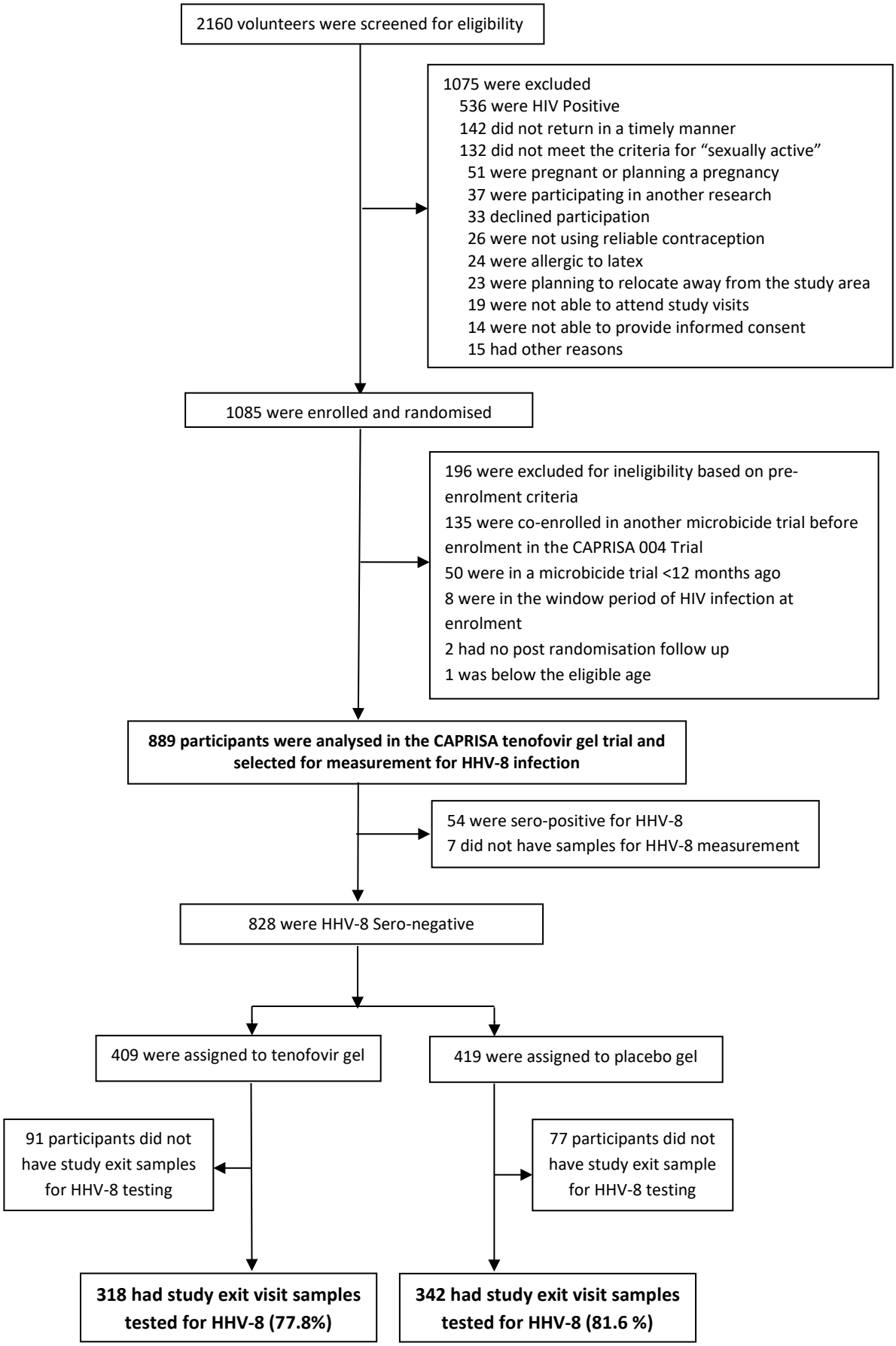

Figure 1. Sample population and distribution of participants in the CAPRISA 004 tenofovir gel trial and the HHV-8 sub-study

CHAPTER 3

DESCRIPTION OF THE STUDY SAMPLE

We evaluated 889 HIV seronegative women at baseline. Participants attended clinics in the eThekweni region, which is an urbanised setting in KZN as well the Vulindlela clinic which is set in rural KZN.

Two hundred and seventy-eight participants lived in urban regions and 611 lived in rural areas. We sought to evaluate the sociodemographic differences between participants in the urban and rural areas. The mean age (SD) in completed years of the study population was 23.87 (±5.11) 25.06 (±5.37) in the urban setting and 23.34 (±4.90) in participants comprising the rural group. (Table 1).

3.1. Social and Education Factors

The difference in the number of participants who earned a household monthly income of less than R1000 per month was statistically different according to location (86.1% of rural participants versus 69.1% of urban participants; p<0.001). Overall, 718 (80.8%) of the study participants had an income below this threshold.

The overall number of participants who had completed high school education was 365 (41.1%). There was a significant difference between urban and rural participants with 160 (57.5%) participants in urban settings had completed high school whereas 205 (33.6%) in rural areas had done so (p<0.001).

Parity in the overall population was 1.15 (SD ±0.96) children per participant. Urban participants had significantly higher parities (Mean =1.37; SD ±1.10) as compared to participants in rural areas (Mean= 1.05; SD ±0.87); p<0.001.

3.2. Relationship Status

Majority of participants were either married or in a stable relationship (833; 93.7%). Two hundred and twenty-four participants (80.6%) in urban centres were in stable relationships, however this was significantly fewer than the 609 (99.6%) of rural participants who reported being married or in a stable relationship (p<0.001). Interestingly, despite these figures, only 109 (12.3%) of participants were living

with their partner overall. The reported 58 (9.5%) participants in rural areas was significantly lower than the 51 (18.3%) participants in urban settings who lived with their partner (p<0.001).

Very few participants were able to report knowing that their partner is HIV infected (2.1%). Participants in rural areas had significantly more knowledge on their partners' HIV status (2.6%) than participants in urban regions (1.1%) p=0.042.

3.3. Sexual History

Overall, the mean age of sexual debut was 17.42 (±2.03) years. There was no significant difference in the mean age of sexual debut between participants in the urban setting (Mean=17.67; SD±2.03) and participants in the rural settings (Mean= 17.31; SD ±2.03) p=0.140.

The mean number of lifetime sexual partners for the sample population was 3.30 per patient (SD±2.03). The mean number of lifetime sexual partners in participants from urban areas (Mean= 5.98; SD±18.40) was significantly higher than in those from rural areas (Mean=2.08; SD±1.24) p<0.001. Participants in urban regions also demonstrated significantly more frequent sexual activities when evaluated over the preceding one month (Mean =12.09; SD±13.56) when compared to participants from rural areas (Mean =6.67; SD±5.22) p<0.001.

Table 1. Baseline characteristics of participants analysed for HHV-8 infection stratified by study location

	Total (N=889)	Urban (n=278)	Rural (n=611)	*p-value**
Mean Age - years *(SD)* **	23.87 (±5.11)	25.06 (±5.37)	23.34 (±4.90)	**<0.001**
High school education or more *n, (%)*	365 (41.1)	160 (57.5)	205 (33.6)	**<0.001**
Income per month <1000 rands *n, (%)*	718 (80.8)	192 (69.1)	526 (86.1)	**<0.001**
Parity – *mean (SD)* **	1.15 (±0.96)	1.37 (±1.10)	1.05 (±0.87)	**<0.001**
Married or Stable partner –*n, (%)*	833 (93.7)	224 (80.6)	609 (99.6)	**<0.001**
Living with a regular partner –*n, (%)*	109 (12.3)	51 (18.3)	58 (9.5)	**<0.001**
Knows Partner is HIV positive –*n, (%)*	19 (2.1)	3 (1.1)	16 (2.6)	**0.042**
Age at first sexual experience- Mean (SD)**	17.42 (±2.03)	17.67 (±2.03)	17.31 (±2.03)	**0.140**
No. of lifetime sexual partners – Mean *n, (SD)***	3.30 (±2.03)	5.98 (±18.40)	2.08 (±1.24)	**<0.001**
No. of sex activities in last 30 days – *Mean (SD)***	8.37 (±9.08)	12.09 (±13.56)	6.67 (±5.22)	**<0.001**
Always condom use –*n, (%)*	259 (29.1)	119 (42.8)	140 (22.9)	**<0.001**
Hormonal contraception –*n, (%)*	719 (80.9)	188 (67.6)	531 (86.9)	**<0.001**
TDF gel arm *no. – n, (%)*	445	137 (49.3)	308 (50.4)	
Placebo gel arm *no. – no. (%)*	444	141 (50.7)	303 (49.6)	

** *Using Wilcox Rank Sum test*

CHAPTER 4

PREVALENCE OF HHV-8 INFECTION

4.1. Introduction

KS is the most common cancer in men and second most common in women in multiple African countries. It was found that HHV-8 seroprevalence early in adulthood was 10.8% in South Africa, but increased with age.[40]

4.2. Results

In our sample population of 889 women, 882 had serological testing for HHV-8 at baseline. Of the 882 participants tested for HHV- 8 at baseline, 54 women were positive with a prevalence of 6.12%. We sought to analyse women who were baseline positive against those who were baseline negative.

We found no significant difference in the groups by age, level of education, or monthly household income (p=0.311, 0.396 and 0.457 respectively). Similarly, we demonstrated no significant difference in prevalent subgroups for the following characteristics, namely: marital status, living with a regular partner, knowledge of partners' HIV status, age of sexual debut and contraceptive use (barrier and hormonal).

Participants with baseline HHV-8 seropositivity had significantly higher parity, p=0.014. A rural setting was also a significant factor associated with baseline HHV-8 seropositivity (p=0.022). The most remarkable significant association with HHV-8 seroprevalence was a higher mean number of sexual partners (p<0.001) (Table 2).

4.3. Discussion

A prevalence of 6.12% was found in our study which was lower than the national prevalence of 10.8%. This could be due to the sample population which only included women and were HIV negative at initiation of the study.

Socio-economic factors had no direct causal association but could have indirectly influenced HHV-8 acquisition. In rural areas, socio-economic barriers and major structural issues could potentially lead to psychological stress, poor living conditions and disruption of family support network within

communities. Poor socio-economic conditions promote transmission of HHV-8 because of lack of facilities leading to poorer hygiene practices.

Water supply has been shown to be a risk factor for HHV-8 transmission. Mbulaiteye et al in 2005, studied associations between HHV-8 seropositivity in Ugandan children and their mothers as well as socio-economic and environmental characteristics (39). The study showed an association between HHV-8 seropositivity and source of drinking water. Limited access to water translated to poor hygiene practices such as infrequent hand washing, bathing, use of saliva to clean children's faces; factors which could contribute to increased saliva-related transmission of HHV-8.[41]

The significant increase associated with a higher parity may be related to genital tract microanatomy changes which may predispose to a higher infectious transmission rate. Furthermore, increased parity could influence hormonal balance thereby impacting on the immunological milieu resulting in cells being more susceptible to infection by HIV or HHV-8 and augmenting the molecular synergism between the two viruses. Interestingly it was found that urban participants had a higher mean number of partners as well as a higher mean parity consistent with the findings of Abdool Karim *et al*.[42] This seems contradictory as rural location was a significant factor in increased baseline prevalence of HHV-8. This finding could indicate that there are other factors associated with HHV-8 prevalence in the rural population that remain largely unknown. Possible reasons could include the effect of poor socioeconomic circumstances as well as increased use of hormonal contraception (which could perturb immunological cellular interactions within the genital tract epithelium).

The reported evidence for sexual transmission of HHV-8 is mixed with some studies suggesting evidence for sexual transmission but much more finding conflicting evidence. However, HIV incidence is directly correlated to increased exposure and because of epidemiological as well as molecular associations between HIV and HHV-8 co-infection. Therefore, an increased number of sexual partners could lead to an increased rate of HHV-8 acquisition.

Table 2: Association of HHV-8 infection with socio-demographic and behavioural characteristics

	HHV-8 sero-negative (N=828)	HHV 8 sero-positive (N=54)	p-value
Age in years - mean (SD)	23.8 (±5.1)	24.4 (±5.1)	0.311
High-school education - n (%)			
High school not complete	486 (58.7%)	35 (64.8%)	0.396
High school complete	342 (41.3%)	19 (35.2%)	
Income per month - n (%)*			
<R1000 Per Month	670 (90.8%)	44 (88.0%)	0.457
>R1000 Per Month	68 (8.9%)	6 (10.0%)	
Parity – mean (SD)	1.1 (±1.0)	1.5 (±1.0)	**0.014**
Relationship status– n (%)			
Married/in stable relationship	814 (98.3%)	52 (96.3%)	0.256
Other	14 (1.7%)	2 (3.7%)	
Living with a regular partner – n (%)			
Yes	106 (12.8%)	3 (5.6%)	0.137
No	722 (87.2%)	51 (94.4%)	
Age at first sexual experience – mean (SD)	17.4 (±2.0)	17.0 (±2.0)	0.169
No. of lifetime sexual partners – mean (SD)	3.2 (±10.7)	4.4 (±7.3)	**<0.001**
No. of sex activities in last 30 days – mean (SD)	8.3 (±9.1)	9.4 (±8.6)	0.202
Condom use – n (%)			
Always	240 (29.0%)	18 (33.3%)	0.537
Not always	588 (71.0%)	36 (66.7%)	
Using hormonal contraception – n (%)			
Yes	809 (97.7%)	52 (96.3)	0.372
No	19 (2.3%)	2 (3.7%)	
Location of study site – n (%)			
Rural	577 (69.7)	29 (53.7)	**0.022**
Urban	251 (30.3)	25 (46.3)	
Study arm n (%)			
Tenofovir	409 (49.4%)	34 (63.0%)	0.067
Placebo	419 (50.6%)	20 (37.0%)	

**94 participants missing income data*
p –values are testing for differences

CHAPTER 5

INCIDENCE OF HHV-8 INFECTION

5.1. Introduction

A South African study examined trends in the incidence of KS in black participants in KZN spanning a 23 year period [23]. It noted the striking rise in the age standardised incidence rate of KS from the pre-HIV/AIDS era to the HIV/AIDS era: there was a documented increase from less than 1 per 100 000 in the year 1990 to 15 per 100 000 in 2006 respectively. The age standardised incidence rates increased in males and females: 20 fold in men and 50 fold in women with a 30 fold combined increase. This marked and rapid increase directly corresponded to the concurrent rise in the HIV seroprevalence rate: 1.6% in 1989 which increased to 39% in 2006.[24]

5.2. Results

Of the 828 women whose baseline HHV-8 serology was negative, 168 did not have exit testing and were excluded from incidence testing. There were 40 incident infections in the remaining 660 women resulting in an incidence rate of 3.96 per 100 women-years (Figure 2). The incident rate ratio was 1.02 for women using TDF gel compared to women using the placebo gel. Women using TDF gel therefore did not demonstrate a protective outcome compared to those in the placebo group. Similarly, when stratified by location, there were no significant differences stratified for Vulindlela and eThekweni (Table 3).

5.3. Discussion

TDF microbicide gel has been shown to be ineffective against HHV-8 acquisition when compared to placebo. These findings are interesting given the structural similarities of HHV-8 and HSV-1, and the proven efficacy in prevention of HSV-1 acquisition. There are several reasons that could explain the negative result which include: differing cell types and cellular tropism; discrepant drug concentrations within the cells; laboratory methodology and lastly the microenvironment of the female genital tract.

The greatest anti herpetic tenofovir activity has been shown to occur in macrophages; therefore, intuitively, inhibition of viral replication within these cells would prove to be most effective. However, viral entry into macrophages is prohibited by various factors: Firstly, there are barriers to entry of the virus into the lamina propria (the site where macrophages and dendritic cells are found).[31, 32] Secondly, certain conditions need to exist for entry into and replication within macrophages. Thirdly, even if levels are detectable within macrophages, the effectiveness of tenofovir on HHV-8 replication cannot be entirely extrapolated because of intrinsic structural differences between HHV-8 and HSV 2. In addition, differing cellular tropisms may preclude the effectiveness of tenofovir. For example, keratinocytes and B cells may escape the effect of tenofovir.[22]

The structures of the respective herpesviruses are different. HSV-2 is an alpha herpesvirus, whereas HHV-8 is a gamma herpesvirus. The genomes differ in size, gene content and order: these variations determine the structure of DNA polymerases, the affinity for nucleotides and hence effectiveness of tenofovir.

Even though the mechanism of action is similar to that of adefovir, there are differences that make the effects of tenofovir unpredictable. Tenofovir has been found to be a weak inhibitor of the cellular DNA polymerases alpha, beta, and epsilon. Tenofovir has been subject to crystallographic studies which revealed the structure of HIV-1-RT complexes before incorporation of tenofovir or after incorporation. The PMPA residue in the binary complex showed multiple structural conformations and unlike the more static anti-HIV agents such as 3TC and AZT, the molecule seemed to evade resistance. Even though this is favourable, the protean conformations could translate into unpredictable effectiveness if applied to different substrates, and this pliability could account for the discrepancy seen in efficacy between HSV 2 and HHV-8.[27, 43, 44]

The phase of the host cell cycle is an important determinant of anti-herpetic drug activity. In a study that investigated the activity of cidofovir against KS, it was shown that cidofovir had no effect.[45] Cidofovir inhibits late lytic genes, not latent or early lytic genes. Therefore, cell proliferation stimulated by early lytic gene transcripts would not have been affected by the drug. Furthermore, because the episomal conformation of the viral genome in latently infected cells relies on cellular DNA polymerase, antiviral drugs have no effect during latent infection. This concept could be extrapolated to the HHV-8 study where the phase of host cell cycle is unknown. Tenofovir would not influence

latently infected cells and replication of cells expressing early lytic genes would not be suppressed leading to inefficacy of the drug.

In vitro studies have shown that the concentrations of drug vary depending on the cell type. HHV-8 is lymphotropic and has been detected in KS spindle cells, which are of mixed vascular and lymphatic endothelium as well as macrophage origin. The HSV-2 virus is neurotrophic and can infect epithelial cells, including the cells lining the female genital tract. This is explained by the presence of specific receptors on host cells for viral entry. HHV-8 uses heparin sulphate for initial cell adhesion whereas entry into cells via endocytosis requires interaction of HHV-8 glycoprotein B with integrin alpha three beta 1. For HSV, initial attachment to the cell required interactions of viral glycoproteins B and C with heparin sulphate like receptors.[33, 46]

While all sexually transmitted diseases need to cross the female genital epithelium to cause infection in the host; their specific interactions with the epithelial lining of the genital tract are quite different. HSV- 2 directly infects the genital epithelium and undergoes replication within the cells. It then infects adjacent epithelial cells and other cell types located under the epithelium subsequently infecting peripheral nerves where it can become latent. The latent virus reactivates from time to time to replicate; the lining is shed in genital secretions thereby leading to further transmission. There is a paucity of data regarding the entry of HHV-8 into cells of the female genital tract, however HHV-8 has been shown to infect dendritic cells and macrophages through the DC-SIGN receptor: cells that form part of the mucosal immunity of the female genital tract. However, in order to infect dendritic cells that reside within the lamina propria, it is imperative for the virus to cross an intact epithelial cell layer. The mechanism of entry of HIV has been studied extensively and describes an alternative mechanism which could possibly be applied to HHV-8. Even though similar receptors are used such as heparin sulphate and proteoglycans, the main target of HIV-1 replication appears to be primarily the Langerhans cells and T cells underlying the epithelium. The virus does not infect the epithelium per se, but is able to cross the epithelium to infect immune cells, including CD4 and DCs and T cells in the lamina propria of the mucosa. However, HHV-8 is not known to infect Langerhans cells (as they do not express the DC-SIGN receptor) and therefore is unable to cross the epithelial barrier to enter the dendritic cells.[17, 33, 34, 47]

Myriad factors influence infectivity of macrophages. Unstimulated macrophages are susceptible to a low level of infection. Activation of macrophages with IL-13 greatly enhanced expression of DC-SIGN

and rendered them highly permissive to HHV-8 infection. IL-13 is produced by T helper 2 cells, as well as eosinophils, mast cells and basophils. HHV-8 encodes a viral macrophage inhibitory protein II that is associated with infiltration of Th2 and eosinophils in KS lesions. Production of IL-13 by these infiltrating cells could enhance infection of HHV-8 by monocytes and macrophages by upregulating expression of DC-SIGN. The complex interplay of cytokines within the molecular milieu can lead to altered gene expression and receptor upregulation, thereby influencing susceptibility of macrophages to HHV-8 infection.[17, 20, 34, 48, 49]

Furthermore, limitations of the experimental method could have influenced the result. The assay detects antibody only to lytic antigens. This means that latently infected cells would not be detected by the fluorescent antibody test. Microarray analysis has shown that HHV-8 infection of fibroblasts and endothelial cells does not result in a productive, replicative cycle. Instead, there is expression of a subset of lytic cycle gene transcripts which quickly subsides, followed by persistent expression of latency gene transcripts, which would not be detected by the antibody test[17]

The negative result could be due to several factors: the complex biological microenvironment, immunological interactions as well as limitations of the experimental method. However, this does not negate the effectiveness of tenofovir drug. Perhaps a different formulation, change in dosage and different route of administration would have impacted on the results and therefore more studies are needed to investigate these possibilities in the future.

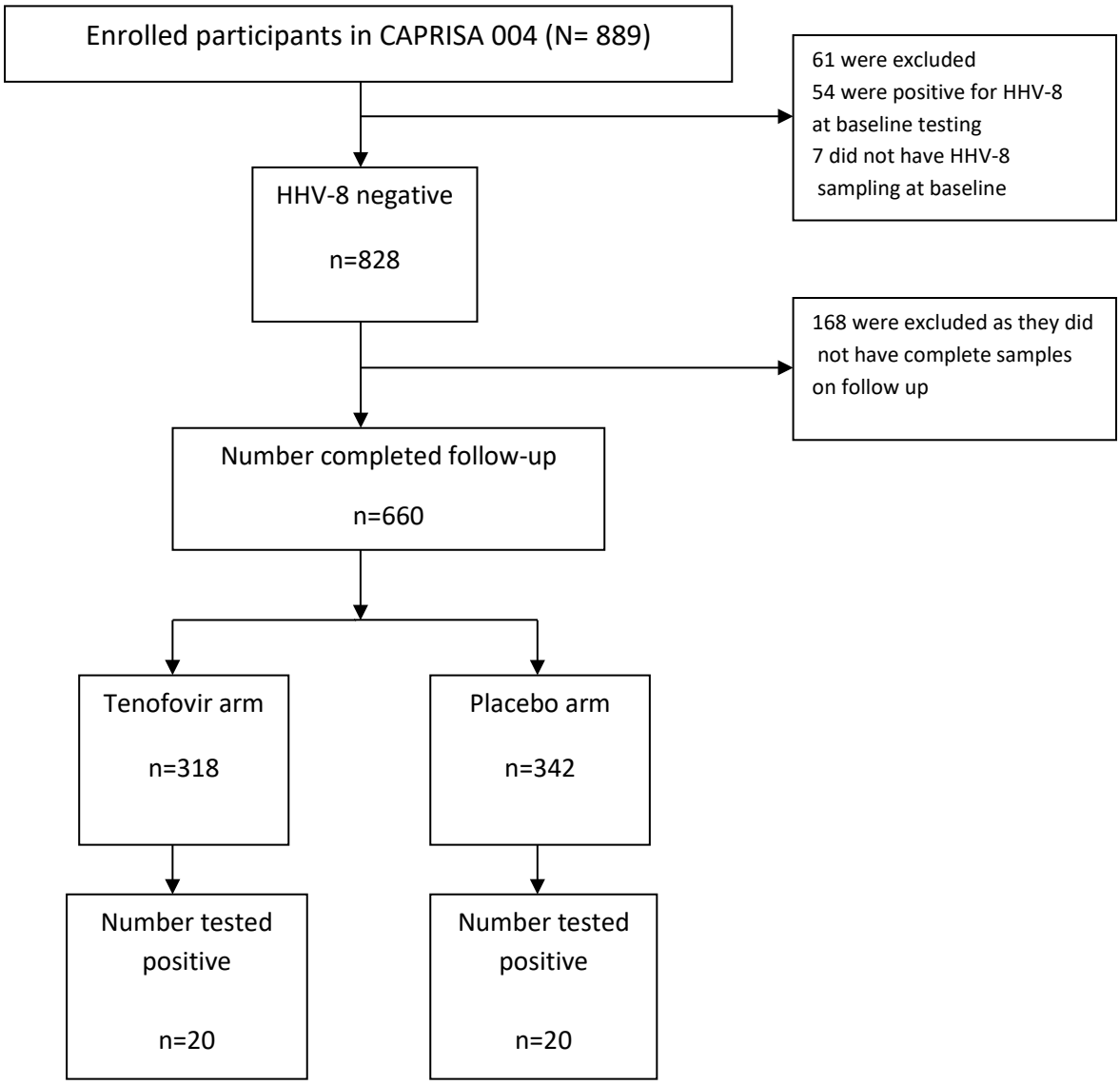

Figure 2: Number of incident HHV-8 infections in study sample stratified by treatment arm

Table 3: Incidence of HHV-8 stratified by treatment arm and location

	Total			Rural Vulindlela			Urban eThekwini		
	Tenofovir	Placebo	Total	Tenofovir	Placebo	Total	Tenofovir	Placebo	Total
Participants (n)	318	342	660	224	242	466	94	100	194
HHV-8 infections* (n)	20	20	40	16	12	28	4	8	12
Person-years (PY)	493	512	1005	357	374	731	136	138	275
Incidence rate per 100 PY 95% CI	4.05 (2.48-6.26)	3.91 (2.39-6.04)	3.98 (2 84-5.42)	4.48 (2.56-7 28)	3.21 (1.66-5.61)	3.83 (2.55-5.54)	2.93 (0.8-7.5)	5.79 (2.5-11.41)	4.37 (2.26-7.63)
Incidence rate ratio (95% CI)		**1.04 (0.56-1.93)**			**1.40 (0.66-2.95)**			**0.51 (0.15-1.68)**	
p value		**p=0.909**			**p=0.383**			**p=0.266**	

**HHV-8 seropositivity at study exit testing*

CI-Confidence Interval

CHAPTER 6

ASSOCIATION OF HIV AND HHV-8 INFECTION

6.1. Introduction

Prior to the AIDS epidemic, KS was common in South Africa (up to 5 per 1000 population at risk per year), however the incidence of KS has increased exponentially in parallel with AIDS epidemic.[50] In a case control in South Africa between 1995 and 1999, incidence rates of 20 per 1000 per year were documented, which was markedly higher than developed countries (rates of 0.005 per 1000 per year[46]. HIV promotes tumorigenesis: In participants who have dual infection with HIV and HHV-8, approximately 50% would develop KS within a decade. With the advent of ART, the incidence of KS has decreased in the US and Europe. However, the impact of ART in Africa with an already high baseline prevalence of HHV-8, has yet to be elucidated.[51, 52]

6.2. Results

In evaluating for an association of HIV incidence with HHV-8 seroprevalence, we found that five (9.3%) of the 54 participants who seroconverted for HIV were HHV-8 sero-positive at baseline. The remaining 92 HIV positive participants were HHV-8 seronegative at baseline. HIV incidence rate among participants with prevalent HHV-8 infections was 6.48 per 100 PY (95% CI 2.11-15.13) and was similar to the HIV incidence rate of 7.35 per 100 PY (95% CI 5.92-9.01) among participants who remained HHV-8 negative, IRR of 0.88 (95% CI 0.36-2.17), p=0.785. Therefore, there was no significant association with HIV seroconversion in participants with HHV-8 baseline seropositivity (Table 4a). Of the 40 participants who seroconverted for HHV-8, five participants seroconverted for HIV. Thus, we demonstrated no significant association between HIV incidence and HHV-8 incidence in our study population (p=0.58) (Table 4b).

6.3. Discussion

A molecular as well as an epidemiological link has been established between KS and HIV. Immune dysregulation associated with HIV potentiates HHV-8 infection through immunological interactions. HIV associated Tat, Nef proteins stimulate the production of proinflammatory cytokines that promote tumorigenesis. HHV-8 has been postulated to influence proliferation of HIV infection through latently

expressed antigens which bind the LTR regions of the virus and co-ordinate gene transcription.[48] However, our results did not show a significant association between HIV incidence and HHV-8 incidence. This could be because the sample population is not representative of the entire population and therefore does not underscore the epidemiological link. In addition, it is unclear as to whether the trial participants' cells were latently infected with HHV-8 or within the lytic phase of the viral cycle. Phases of the viral cycle are characterized by a specific molecular signature determined by gene transcription. During the lytic phase, gene expression results in secretion of cytokines and upregulation of growth factors that stimulate angiogenesis. Importantly, the result could indicate gaps our current understanding of the pathogenesis of HHV-8 infection, angiogenesis, and interaction with HIV. There may be as yet undetermined inhibitory and stimulatory molecules which unpredictably influence cellular proliferation, latency and specific gene expression profiles.

An additional contributing factor could be the prophylactic efficacy of tenofovir gel on HIV transmission. Prevention of HIV acquisition would decrease susceptibility to HHV-8, as the molecular synergistic effects of HIV and HHV-8 coinfection would not exist.

Table 4a: Incidence of HIV among participants with prevalent (n=54) HHV-8 infection

	Total		Placebo		Tenofovir	
	HHV-8 negative	HHV-8 positive	HHV-8 negative	HHV-8 positive	HHV-8 negative	HHV-8 positive
Participants (n)	828	54	419	20	409	34
HIV infections (n)	92	5	56	3	36	2
Person-years (PY)	1252	77	623	28	629	49
Incidence rate per 100 PY (95% CI)	7.35 5.92-9.01	6.48 2.11-15.13	8.99 6.79-11.68	10.65 2.2-31.13	5.72 4.01-7.92	4.09 0.49-14.76
Incidence rate ratio (95% CI) p value	0.88 (0.36-2.17) p=0.785		1.18 (0.37-3.78) p=0.775		0.71 (0.17-2.97) p=0.643	

Table 4b: Incidence of HIV among participants with incident (n=40) HHV-8 infections

		HHV-8 test result Positive	Negative	p-value
Incident HIV infection at study exit	Positive n (%)	5 (12.5)	59 (9.5)	0.58**
	Negative n (%)	35 (87.5)	561 (90.5)	

**p-value analysis by Poisson regression model*

***p-value by Fishers exact test*

HIV; Human Immunodeficiency Virus, CI; Confidence Interval

CHAPTER 7

STUDY LIMITATIONS

This study has several limitations. First, the trial was not originally designed to assess the effect of tenofovir gel on HHV-8 infection and therefore randomisation at enrolment was not stratified according to HHV-8 status. Further, TDF concentrations at the time of HHV-8 acquisition could not be assessed. TDF concentration for each woman was measured at one randomly selected study visit, and the result may therefore not accurately reflect overall exposure to TDF concentration at the time of HHV-8 exposure. An important limitation of this study is that it is a regional centre study and although we believe it may be representative of the community it may not necessarily be representative nationally. Similar studies in the future in various regions could be conducted to generate a more representative finding. Samples were evaluated using a single test method and the interpretation thereof was reliant on a brightness grading scale which may influence user objectivity. This was minimised by performing standardised testing of all samples. Finally, there are multiple factors that influence the pathogenesis of the disease entities tested and these include epigenetics, genetic susceptibility, host microenvironment and others, all of which may be influential in test findings and lie beyond the scope of this study.

CHAPTER 8

CONCLUSION

A high prevalence of HHV-8 was demonstrated in rural areas. Contributing factors to increased prevalence could include the effect of poor socioeconomic conditions on HHV-8 transmission, increased use of hormonal contraception and resultant perturbation of the immunological milieu of the female genital tract epithelium. Tenofovir microbicide gel was not an effective prophylactic measure to HHV-8. There are myriad explanations for the negative result, namely, differing cell types and cellular tropism; discrepant drug concentrations within the cells; laboratory methodology as well as the microenvironment of the female genital tract.

In the study, there was no link established between HIV incidence and HHV-8 incidence, despite the well described synergistic effects of co-infection. Possible explanations include limitations in methodology, sample representation and gaps in current knowledge: existence of as yet unknown inhibitory factors and cytokines which may unpredictably hinder or promote angiogenesis depending on as yet undetermined gene expression profiles.

The complexity of the biological microenvironment and limitations of simple extrapolation from *in vitro* to *in vivo* conditions needs to be further investigated and specific experimental methods employed to help achieve a more insightful understanding of TDF and its prophylactic role to HHV-8.

CHAPTER 9

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CONFLICTS OF INTEREST AND DISCLOSURES

The CAPRISA 004 trial and the herpes simplex virus type 2 (HSV-2) sub study were supported by the US Agency for International Development, FHI 360 (cooperative agreement GPO-A-00-05-00022-00, contract 132119), and the Technology Innovation Agency, the biotechnology organisation of the Department of Science and Technology of the South African government. The CAPRISA 004 trial and the HSV-2 sub study were also supported by CONRAD for product manufacturing and packaging and by grants from the US National Institutes of Health Comprehensive International Program of Research on AIDS (AI51794) and the Columbia University–Southern African Fogarty AIDS International Training and Research Programme (D43TW00231) for research infrastructure and training.

ANNEXURES

ANNEXURE A: ETHICS CERTIFICATE

TRREE

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Certificate

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November 29, 2017

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ANNEXURE B: BREC APPROVAL LETTER

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

09 March 2018

Dr RI Bhorat (209501211)
School of Clinical Medicine
College of Health Sciences
Raeesabhorat.1@gmail.com

Dear Dr Bhorat

Protocol:Tenofovir microbicide gel as an effective prophylactic measure to Human Herpes Virus-8
Degree: MMed **BREC Ref No: BE030/18**

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee **of** the Biomedical Research Ethics Committee has considered and noted your application received on 21 December 2017.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 22 February 2018 to BREC correspondence dated 06 February 2018 has been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval** and may begin as from 09 March 2018.

This approval is valid for one year from **09 March 2018.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **10 April 2018.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

[redacted]

Professor V Rambiritch
Deputy Chair: Biomedical Research Ethics Committee

Postgraduate administrator: [illegible]@ukzn.ac.za/SCMpgrad@ukzn.ac.za
Supervisor: Ayesha.khanyile@caprisa.org Co-supervisor: dhiren.sadhabirdas@gmail.com

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2486 Facsimile: +27 (0) 31 260 4609 Email: brec@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

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ANNEXURE C: STUDY PROTOCOL

Title of the study

Tenofovir microbicide gel as an effective prophylactic measure to Human Herpes Virus-8.

Aim of the study

To establish an association between the tenofovir microbicide gel and the prevention of Human Herpes Virus-8 (HHV-8) infection.

Specific Objectives

To investigate the link between HIV and HHV-8

To investigate the pathogenesis of HHV-8 infection

To determine if preventing the acquisition of HHV-8 by TDF microbicide gel is possible

To determine if there may be a decreased exacerbation of HIV due to the interaction between HIV and HHV-8

To reduce the negative impact on the quality of life at individual, community and national levels

To contribute toward an intervention strategy for the prevention of disease progression of HIV, it's associated mortality and thereby reduce the burden of disease

To provide insight into disease processes to contribute toward the development of future and novel therapeutic interventions in disease prevention

Background and literature

Introduction

Kaposi's sarcoma (KS) is a mesenchymal tumour involving blood and lymphatic vessels of multifactorial origin. Viral oncogenesis by human herpesvirus 8 (HHV-8) and cytokine-induced growth together with an immunocompromised state represent important conditions for the development of this tumour (1). KS has become relevant in South Africa through the grim backdrop of human immunodeficiency virus (HIV) co-infection. The HIV pandemic remains one of the major challenges to the country's socio-economic development. Using the Spectrum model, the 2009 HIV prevalence in the non-paediatric population of people aged from 15 years to 49

years was estimated at 17.8% or 5.63 million adults and children were infected with HIV and AIDS. The highest HIV prevalence by province was recorded in KwaZulu-Natal (KZN) which increased from 38.7% (95%CI 37.2% – 40.1%) in 2008 to 39.5% (95%CI: 38.1 – 41.0) in 2009(1).

These statistics highlight the burden of disease in South Africa and particularly in KZN and therefore render prevention therapy as an imperative intervention to decrease the morbidity and mortality of HIV- associated Acquired Immunodeficiency Syndrome Kaposi sarcoma (AIDS/KS). As such, this may contribute to an already over-burdened and overwhelmed national healthcare system as a result of the HIV/AIDS pandemic (2). A preventative strategy may have a foreseeable positive impact across all levels of healthcare.

There is evidence that HHV-8 may increase HIV progression, therefore, prevention strategies related to Kaposi sarcoma could potentially reduce mortality and morbidity of HIV co-infection.

The growing availability of combined anti-retroviral therapy (ART) in Sub-Saharan Africa offers the potential for dramatically reducing the incidence of AIDS-KS, as well as improving the overall survival and quality of life of patients living with AIDS-KS. Since 2006, the combined ART coverage has continued to expand, with an estimated 40% of those who require combined ART, receiving therapy. However, despite promising trends, only 44% of patients with KS were prescribed the indicated combined ART in the period from 2003 to 2006 (1). Therefore expansion of combined ART to patients remains an important goal. This is a further indication for the significance of an intervention to prevent the spread and exacerbation of KS.

Human Herpes Virus

HHV-8 is one of the most recently discovered human oncogenic viruses and is aetiologically associated with Kaposi sarcoma- an AIDS defining malignancy (3). HHV8 is an enveloped virus containing a double stranded DNA genome and belongs to the rhadinovirus genus of the Herpesviridaefamil (3)y. It is a gamma2 – lymphotropic-oncogenic virus. HHV8 has been linked to the development of three neoplastic disorders, primarily KS, Primary Effusion Lymphoma (PEL) or body cavity-based lymphoma (BCBL) and a plasmablastic variant of multicentricCastleman's disease (MCD). It has also been associated with several other lymphomas including germinotropic lymphoproliferative disease (GLD), multiple myeloma, angiosarcomas, malignant skin tumours and squamous cell carcinomas. Recently, a new clinical HHV8 associated syndrome has been identified namely Kaposi sarcoma –associated herpes virus Inflammatory Cytokine Syndrome (KICS) which has similar manifestations to MCD) (3). KS is a reactive, angioproliferative chronic inflammatory lesion. KS is characterised by latently infected spindle cells of endothelial origin, fibroblasts and infiltrating inflammatory cells. The microenvironment of KS is associated with growth factors, chemokines and inflammatory cytokines which are integral to the pathogenesis of KS.

Approximately 100 herpesviruses have been identified, with at least eight affecting humans. All human herpes viruses are well adapted to their natural host, being

endemic in all human populations studied and carried by a significant proportion of persons in each sub-population. Disease caused by human herpesviruses tends to be relatively mild and self-limiting in immunocompetent persons, although severe and quite unusual disease had been reported in immunosuppressed persons (3,4).

There are key characteristics that differentiate the herpes viruses from other viruses. These include the presence of a double stranded linear DeoxyriboNucleic Acid genome ranging in size from 125 000 to 250 000 base pairs, an icosahedral capsid of approximately 125nm in diameter and consisting of 162 capsomeres as well as an amorphous layer of viral proteins which are called the tegument and a lipid bi-layer envelope containing viral glycoproteins (3,4). The herpesviridae families expresses a large number of viral enzymes, assemble the nucleocapsid in the cell nucleus and are able to destroy the cell during productive infection as well as establish latent infection in an infected host (4)

Latency means that the genome of the invading virus is maintained in a stable state by the host cell and with the limited expression of viral genes there is no production of progeny virus and no evident virus-induced cytotoxicity. Latent infections are converted to productive infections by factors and stimuli that have not as yet been clearly identified (3).

The family of herpesviridae is further divided into three subfamilies based on biological differences. The alpha herpesviruses are neurotropic viruses that replicate relatively rapidly and infect a wide range of cells in the cell culture. Examples of these include Herpes simplex Virus-1 and 2 (HSV-1 and HSV-2) and Varicella Zoster Virus (VZV). The beta herpesviruses replicate slowly and are restricted in the types of cells that are productively infected in cell culture. Cells infected by beta herpes virus often become enlarged. The gamma herpesvirusses are lymphotropic viruses that also replicate relatively slowly and are restricted in the types of cells that are productively infected (3).

The genomes of the various herpesviruses are clearly evolutionarily related but differ in size and in the organisation of unique and repeated sequences as well as in gene content and order (3). Although each virus encodes unique genes, a large fraction of these genes are conserved among members of the family. These homologous genes are arranged in several collinear blocks which are themselves arranged in the same order and orientation for members of any one subfamily of herpesviruses and in different orders and orientations for viruses from different families. Genes specific for a virus family tend to be at the genomic termini or in clusters between the blocks of homologous genes. Certain key regulatory proteins and genes expressed in latency tend to be different for members of the different subfamilies (5).

Epidemiology

Kaposi Sarcoma is an AIDS-defining cancer that is associated with the advanced immunosuppressed state as a result of HIV co-infection. In individuals who were not infected by HIV, KS was reported in the Mediterranean and in Central African regions and in organ transplant recipients on immunosuppressant therapy (1). Patients who are co-infected with HIV and HHV-8 are 10 000 times more likely to develop KS

compared with persons who are not infected with HIV and 50% of patients co-infected with HIV and HHV-8 are likely to develop a KS tumour within 10 years (2). Although HIV infection is neither necessary nor sufficient for KS development, a strong association has been reported and a suggested increased incidence and more aggressive course when compared to patients who are not co-infected.

The incidence of KS in South Africa has increased with the exponential spread of HIV and this combined with the poor ART coverage in the general population in South Africa exacerbates this. It is the most common cancer in men and second most common in women in a number of countries in Africa. Kwa Zulu Natal is the province with the highest HIV seroprevalence and incidence in South Africa and has an estimated 1.8 million cases and an antenatal HIV-1 seroprevalence rate of 39.1% (2). In 2006, A conservative estimate of the incidence of KS was 19.7 per 100 000 and 11.5 per 100 000 in men and women respectively (2). Adult seroprevalence rates of HHV-8, the causative agent of KS, have ranged from 38% to 75%, with the majority of seropositive persons being co-infected with HIV-1 (2).

Mode of transmission

The molecular mechanism of HHV-8 entry is still poorly understood and remains a subject of controversy among experts. Horizontal transmission by saliva appears to be the most common route among families in endemic regions as well as among high risk groups in western countries. Vertical transmission, sexual and blood and transplant related transmission, however, still remain a significant concern. Studies of risk factors for HHV-8 infection in men who have sex with men (MSM) demonstrate an association with markers of sexual activity including the number of partners, unprotected sexual practices and markers of sexually transmitted infections (STI) (6). Evidence for heterosexual transmission in endemic countries has been conflicting. In a South African study, HHV-8 was marginally associated with higher number of sexual partners, but not with HIV co-infection (6,7). Also, several reports have shown associations between HHV-8, STIs and HIV in Ugandan and Zambian populations (6). Other studies conducted in the same African countries however, have not shown these associations (6).

The transmission of HHV-8 and the influence of HIV co-infection on the infectivity or vulnerability of HHV-8 are not well known. In Sub-Saharan Africa, HHV-8 is transmitted during childhood; perhaps via saliva and rarely via breastmilk. HIV co-infection was unrelated to HHV-8 serocoincidence among 485 infants in Zambia, whereas it was associated with a two-fold higher seroprevalence of HHV-8 among 1165 children in South Africa. These limited data suggest a limited synergistic effect on the risk of HHV-8 infection with HIV co-infection. The sexual transmission of HHV-8 was postulated in 1990 on the basis of KS among MSM compared with other HIV exposure groups and an association of KS with more sexual partners and sexually transmitted infections followed. Molecular studies however, pointed to saliva as perhaps the major vehicle of transmission, as HHV-8 DNA is detected much more frequently and at much higher levels in saliva than in any other body fluids (6,7).

Pathogenesis

HHV-8 infection involves a complex cascade of events from binding of target cells to viral gene expression. These events could be sequentially categorised into six indiscrete phases. Phase one involves binding of the virus to target cells. Herpesvirus infection is initiated by attachment of the virus to the susceptible cell (3). Viral glycoproteins in the virion envelope bind to components of the cell surface in a cascade of interactions that culminates in the penetration of the nucleocapsid into the cell cytoplasm. Thereafter, the nucleocapsid is transported to the cell cytoplasm. In productively infected cells, herpesvirus gene expression proceeds in a well co-ordinated cascade. Expression of the immediate-early regulatory genes is necessary for subsequent transcriptional activation and expression of the delayed-early and late genes of the lytic cascade is required for viral replication. In latently infected cells, the immediate-early regulatory genes will not ordinarily be expressed. The factors, viral DNA replication and nucleocapsid assembly are in the cell nucleus. Subsequent envelopment occurs by budding of the nucleocapsid through the inner nuclear membrane prior to the release from the cell (3).

HHV-8 is the first human gamma herpesvirus and has a broad cellular tropism. This may be, in part, due to its ubiquitous interaction with the proteoglycan heparin sulphate, which is similar to several other herpesviruses (8). HHV-8 has been detected in KS spindle cells, which are of mixed vascular and lymphatic endothelium and macrophage origin and monocytes that are found in proximity to KS lesions (9). The virus persists in a latent form in these cell types as well as in B lymphocytes. Herpesvirus entry into susceptible cells requires at least two separate binding events. In HHV-8 infection, the viral glycoproteins K 8.1 and g B bind to cell surface heparin sulphate and infection of endothelial cells can be blocked by soluble heparin. This binding likely enhances the efficiency of viral infection by concentrating the virus on the cell surface and may serve to position the virus for binding to a second receptor involved in the entry of the virus into the cell. The integrin alpha 3 beta 1 has been shown to serve as a receptor for HHV-8 infection of vascular endothelial cells and human foreskin fibroblasts (9). HHV-8 enters myeloid dendritic cells and macrophages through the dendritic cell specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN). This receptor is a type II C-type lectin that is expressed on myeloid dendritic cells in the dermis, mucosa, lymph nodes, lung and thymus and interleukin –4 (IL-4) treated monocyte-derived dendritic cells (9). It is also expressed on macrophages of lung alveolae, the placenta and inflammatory lesions as well as IL-13 activated, monocyte-derived macrophages. DC-SIGN and other C-type lectins act as pathogen recognition receptors and alert macrophages and dendritic cells to take up and process pathogens for antigen presentation to T cells. Certain viruses, parasites, yeast and bacteria can subvert this immune function by using DC-SIGN as a receptor for infection of myeloid lineage dendritic cells.

The protein capsid structure of HHV-8 is surrounded by an amorphous tegument and a lipid bilayer. The genome is 165 kb long and contains a central region of low GC DNA flanked by multiple repetitive high GC DNA. The genome is circular during latent infection and linear during the lytic phase. Not unlike other herpesviruses, replication of HHV-8 has been phylogenetically classified into strains A, B and C.

Strain A was more often seen in classic Mediterranean KS and B and C in African KS (8,9).

Similar to other herpesviruses, HHV8 has a linear, double stranded DNA genome, which is enclosed within a large icosahedral capsid, enveloped by an amorphous tegument layer consisting of several host and viral proteins and an outer glycoprotein -rich lipid bilayer. HHV8 can infect various cell types and exhibit either a lifelong, immunologically silent, latent infection or productive, lytic infection; all with distinct viral gene expression profiles (3).

During latent infection , the viral genome is maintained as a circular, extra – chromosomal episome which replicates along with the host cell in a cell cycle dependent manner with expression of a few viral genes, including latency associated nuclear antigen (LANA, ORF73), viral cyclin (vCyclin, ORF72) viral FLIP (v FLIP, ORF71: FLIP is the homologue of FLICE inhibitory protein), and microRNA's, whose cooperative effects drive cell survival and cell proliferation (3).

Latent infection is the predominant state infection state of HHV8 and in it the viral genome is maintained at 100-150 copies which are tethered to the host chromosome. In contrast, during the lytic phase the virus reactivates from latency leading to the production infectious virions. Upon reactivation, a full repertoire of lytic vital genes including ORF50, ORF57, ORF59, K8, ORF40, ORF6, viral interleukin-6 (v IL6, ORFK2), viral G protein-coupled receptor (v GPCR, ORF74) and viral chemokines (3, 8,9). HHV8 encoded lytic genes are documented to play a significant role in the secretion of multiple paracrine factors, including cytokines and growth factors, vascular endothelial growth factors and interleukin-6, interleukin-8, platelet derived growth factor , fibroblast growth factor and matrix metalloproteinases, which induce angiogenesis, lymphatic programming, and inflammatory lesions in uninfected and latently infected cells (3,8,9).

Infection of endothelial cells with HHV8 plays an important role in viral dissemination and paracrine induction of angiogenesis and Kaposi Sarcoma (KS) lesions. Furthermore, HHV8 infection can upregulate various cellular signalling pathways to increase endothelial cell proliferation and vascular permeability during angiogenesis and vasculogenesis (3).

Immune response

The host immune response against HHV-8 is critical for three distinct steps of the infection. During the primary infection, the host develops innate responses based on natural antibodies, the complement system and innate cytotoxic cells (10). Second, neutralising antibodies are produced and during latency, the virus yields different proteins that escape the cytotoxic response and enable its persistence in a relatively silent mode. In a third step, the virus is present in infected cells and in tumours (10). In both conditions, the virus expresses latent and lytic proteins that allow various degrees of adaptive responses. The virus genome codes for immunomodulators that interfere with innate and adaptive immune responses because they are homologous to cellular proteins. LANA, which is critical for episomal maintenance in latently infected cells yields target peptides for cytotoxic T lymphocytes (CTL). It also regulates proteosomal degradation, thus reducing the number of immunogenic peptides (11).

LANA has been shown to significantly inhibit p53, the cell cycle checkpoint protein and tumour suppressor (3,12). LANA also interacts with G1-S checkpoint proteins , p RB (retinoblastoma) and GSK3b (glycogen synthase kinase 3 beta) and modulates G-S transition (3,12). In addition, LANA increases the longevity of primary endothelial cells and makes them less susceptible to apoptosis. LANA has been shown to modulate c-Myc oncogene thereby affecting Myc phosphorylation. LANA has been shown to upregulate the expression of EMMPRIN (extracellular matrix metalloproteinase inducer), a modulator of metastasis and angiogenesis. LANA suppresses MHCII gene expression by interacting with RFX proteins and barring the recruitment of class II transactivator (CIITA) to the site of the MHCII promoter (12).

HHV-8 encodes zinc finger membrane proteins, K3 and K5, expressed during the early lytic cycle of viral replication (3). These proteins downregulate surface expression of major histocompatibility complex-1 (MHC-1) molecules through their rapid endocytosis. Although this mechanism may protect infected cells from cytotoxic T cells, it leaves the cells susceptible to natural killer (NK) cells. To prevent this, K5 downregulates ICAM and b7-2 which are ligands for NK cell-mediated cytotoxicity receptors (3).

HHV-8 was found to encode two novel proteins that are modulators of immune response (MIR), namely MIR-1 and MIR-2. These block the display of MHC class 1 chains on the cell surface by increasing their endocytosis which enables immune evasion during viral infection. HHV-8 can modulate Human Leucocyte Antigen (HLA) class 1 restricted antigen presentation to CTL's, which may allow latently infected cells to escape CTL recognition and persist in the infected host. The virus has developed elaborate mechanisms to facilitate persistent infection or prolong the life of infected cells to counter the cell protective mechanism and therefore subvert apoptosis. This contributes to maximal production of viral progeny (3).

HHV-8 encodes two anti-apoptotic genes namely v-FLIP and v-BCL2. v-FLIP is a homologue of the cellular fas-associated death domain like interleukin 1 beta converting enzyme) inhibitory protein (FLICE).It has been shown to protect cells from Fas/APO1 mediated apoptosis by inhibiting activation of caspase 3,8,9 (13).

Molecular link between HIV and HHV-8

KS is more aggressive in patients with AIDS. This can be explained by the depth of immunodeficiency as well as a direct effect. HIV stimulates HHV-8 replication and reactivation of latently infected cells through the production of several inflammatory and growth promoting cytokines because of immune dysregulation (14). Th-1-type cytokines induce a generalised activation of endothelial cells leading to adhesion and tissue extravasation of lymphocytes and monocytes, spindle cell formation and angiogenesis. Co-infection of monocytes with HIV and interaction of these cells with endothelial cells as well as the secretion of cytokines and growth factors serve to disseminate the leading of the proliferation of KS cells. HIV-1 transactivating gene produces Tat (transactivating regulatory protein) protein that stimulates the proliferation of spindle cells and inhibits apoptosis via interferon gamma or transactivation of interleukin 6 gene. The Tat protein induces overexpression of and synergises with b FGF, thereby stimulating the expression of growth promoting cytokines. Tat has been shown to act as an angiogenic factor that can interact with the vascular endothelial growth factor (VEGF)- receptor, Flk/KDR (foetal liver kinase, kinase insert domain receptor). Tat expression also enhances HHV-8 infectivity through a better interaction of the virus with its receptors and by accelerating tumourigenesis via v-G protein coupled receptors (GPCR) expression (14).

Some epidemiological studies have shown that HHV-8 infection can lead to AIDS progression. Latency associated nuclear antigen has been shown to activate the long terminal repeat of HIV genome, stimulating p24 production (13). Furthermore, ORF 50 interacts synergistically with HIV Tat, inducing long terminal repeat (LTR) transactivation, leading to increased susceptibility of cells to HIV infection. ORF50 activates several heterologous promoters, including IL-6 which has been shown to induce monocyte chemoattractant protein-1 (MCP-1) expression in macrophages, increasing susceptibility to HIV infection. The HHV-8 viral G protein coupled receptor co-operates with HIV Tat in NF-AT and NF kappa B activation (14). HHV-8 encodes viral products are partially homologous to chemokines and can therefore induce cellular inflammatory cytokine production in monocytes, macrophages, dendritic and endothelial cells which may in turn enhance HIV replication.

The HHV8 protein K1 has been reported to act synergistically with HIV 1 regulatory protein, NEF (negative regulatory factor)to induce cell proliferation, vascular tube formation and excessive angiogenesis in a chicken CAM model. The regulation of ofangiogenic properties is accomplished by activating PI3K/ATK/m TOR signaling and downregulating phosphatase and tensin homolog (PTEN) (3).

Co-infected endothelial cells may serve as a reservoir for HIV spread and the presence of HHV-8 may activate HIV during passage through the endothelial barrier (13).

Epidemiological link between HIV and HHV-8

Studies have shown an unequal distribution of AIDS/KS amongst different age groups. The finding that KS occurs at an earlier age in women when compared with men has been reported in smaller retrospective studies in Europe and the United States of America. The study showed that the proportion was highest amongst women in their mid-20's. These findings are consistent to include young African women with heterosexually acquired subtype C HIV -1 infection. This was associated with a shift in the female-to-male ratio from 4.5 to 1.0 in patients less than 25 years of age. This age specific distribution pattern paralleled the epidemiological studies of HIV-1 infection, indicating that the risk of developing female KS is linked to HIV-1 epidemiology. Further, it was found that women had more extensive KS, which was consistent with studies from Zimbabwe and Italy, showing that women with AIDS/KS were more symptomatic and had a more progressive disease course (2). The reason is unknown, but does not appear to be related to virological or immunological differences (2). However, this disproportionate influence of infection on young women underscores the need for prophylaxis for acquisition of HHV-8 to protect young women, reduce the burden of disease as well as to reduce the overall morbidity and mortality of KS which has become increasing prevalent in parallel with the HIV pandemic.

Tenofovir trial and the social implications of prophylaxis

Myriad biological factors and social issues could explain why women are disproportionately affected by the AIDS pandemic in Africa (15, 16). Current HIV prevention behavioural messages on abstinence, faithfulness and condom promotion have had limited impact on HIV incidence rates in women, especially in Sub-Saharan Africa. Therefore, the Centre for the AIDS Programme of Research in South Africa (CAPRISA) 004 trial assessed the effectiveness and safety of a 1% vaginal gel formulation of tenofovir which is an adenosine nucleotide analogue, for the prevention of HIV acquisition in women (15). Microbicides are products that can be applied to the vagina or rectum with the intention of reducing the acquisition of STI's, including HIV. An effective microbicide has the potential to alter the relentless course of the global HIV pandemic. Tenofovir has potent activity against retroviruses and was initially developed and tested as a prophylactic in monkeys and was subsequently formulated in oral use as tenofovirdisoproxil fumarate (TDF) which is now widely used in the treatment of HIV. The drug's efficacy in suppressing viral replication, it's favoured safety profile and long half-life made it an ideal choice as the first antiretroviral drug to be formulated as a microbicide.

A double blind, randomised control trial was conducted comparing tenofovir gel and a placebo gel in sexually active, HIV uninfected women aged from 18 to 40 years old in urban and rural KZN, South Africa. HIV serostatus, safety, sexual behaviour and gel condom use were assessed at monthly follow-up visits for 30 months. The HIV incidence rate in the tenofovir gel arm was 5.6 per 100 women years compared with 9.1 per 100 women years in the placebo arm (15). Tenofovir gel reduced HIV acquisition by an estimated 39% overall and by 54% in women with high gel adherence (15). Therefore, tenofovir gel could have a major role to play in HIV

prevention, especially for women unable to negotiate mutual monogamy or condom use.

Tenofovir and HSV-2 infection

The trial showed that the microbicide prevented transmission of HSV-2. In the CAPRISA 004 trial, a significant 51% reduction of the risk of acquiring HSV-2 was observed. This effect was unexpected because the highly potent antiretroviral and antihepadnaviral drug had previously shown minimal, if any, antiherpesvirus activity. It was initially thought that a complex, indirect mechanism explained this phenomenon, however, it has been proven that the mechanism is direct..

Tenofovir is an acyclic nucleoside phosphanate (ANP) which is a key class of antiviral nucleoside derivatives. Phosphonate derivatives are bioactive nucleosides in which the oxygen atom is placed in the nearest position adjacent to the alpha carbon atom (17). The rationale for developing ANP's was to create catabolically stable, isopolar and possibly isostearic nucleotide analogues so as to circumvent the first phosphorylation step necessary for the transformation to its active metabolite. As the acyclic nucleoside phosphanates already contain a phosphate mimetic group, they need only two instead of three phosphorylation steps to reach the active metabolite stage. In bypassing the primary nucleoside kinase step, ANP's can be expected to act against a broad range of DNA viruses. Foremost amongst the ANP's are cidofovir, adefovir and tenofovir (PMPA- phosphonomethoxypropyladenine)). Due to their limited oral bioavailability, the latter two compounds have been converted to oral prodrug forms (17, 18).

Because the polar nature of nucleotides precludes their crossing the cellular membrane, isopolar compounds were developed in which the oxygen atom was placed in the nearest position adjacent to the alpha-carbon atom to transform the phosphoric ester group grouping to its isomeric phoshphonmethyl ether, which must also withstand cleavage by cellular enzymes- in contrast to the phosphate group, a phosphonate group cannot be cleaved by cellular hydrolases (17). The antiviral activity of ANP's is the result of the higher affinity of the diphosphorylated ANP metabolite for viral DNA polymerases than for the cellular DNA polymerases alpha, beta, gamma and epsilon (17). The formation of ANP's first started with the development of the acyclic nucleoside analogue dihydroxypropyladenine (DHPA), the mode of action of which was clearly distinct from that of acyclovir (17). DHPA behaves as an adenosine analogue by occupying the adenosine binding site of S-adenosyl-L-homocysteine hydrolase (SAH), an important regulatory enzyme S-adenosyl-L-methionine (SAM) mediated methylations, and can be termed SAH hydrolase inhibitors (17, 19). These inhibitors block the cleavage of SAH into its two components- homocysteine and adenosine, which itself can be further metabolised into three components- AMP, adenine and inosine. Therefore SAH accumulates and the reaction converting S-adenosylmethionine to S-adenosylhomocysteine will be suppressed. SAM is essential for the methylation of viral messenger RNA's, which means that if the methylation reaction starting with SAM as the methyldonor is blocked, maturation will be suppressed and so will be the production of progeny virus particles (16).

DHPA is representative of an alkyl group linked to N1 (in pyrimidines) and N9 (in purines) that bear hydroxyls necessary for activation by phosphorylation (17). The phosphonomethyl ether of the unprotected racemic DHPA gave a mixture of two enantiomeric pairs of 2'- and 3'- regioisomers, of which one compound proved to have potent activity against DNA viruses and cellular parasites (17).

By enantio and region-specific synthesis, the active form of this ANP was shown to be the 2'-Sisomer termed HPMPA (9-(3-hydroxy-2-(phosphonomethoxy)propyl)adenine). A structurally simplified, non-chiral adenine derivative PMEA also proved to have antiviral activity and soon after, the cytosine counterpart of HPMPA, HPMPC was reported to show an antiviral activity spectrum similar to that of HPMPA. Tenofovir is PMPA, and has shown to have acitivity against hepadnaviruses and retroviruses (17).

Tenofovir activity was demonstrated in both laboratory and clinical HSV-1 and HSV-2 isolates in HEL cell fibroblasts, primary macrophages and keratinocytes, organontypic epithelial cell raft structures, human lymphoid and cervicovaginal tissues and HSV-1 and HSV-2 infected mice (20). The study showed that the drug has anti-herpetic activity in a variety of experimental models at drug concentrations that are lower than the median concentration achieved in cervico-vaginal fluid following the administration of 1% tenofovir gel and that were non- toxic to non-exposed cells.

Tenofovir activity was demonstrated both laboratory and clinical HSV-1 and HSV-2 isolates in HEL cell fibroblasts, primary macrophages and keratinocytes, organontypic epithelial cell raft structures, human lymphoid and cervicovaginal tissues and HSV-1 and HSV-2 infected mice (20). The most pronounced antiherpetic activity was seen in macrophages and this is likely due to the low endogenous d NTP pools and/or to low HSV-2 replication in this cell type. Low endogenous d NTP pools give tenofovir a competitive advantage to interact with herpetic DNA polymerase activity. Several publications reported varying concentrations of tenofovir in the female genital tract in vivo which may be the reason why HSV-2 transmission prevention was 51% and not absolute. Also, the extrapolation of drug concentrations ex vivo to the environment in vivo has its limitations (20).

Shortly after the CAPRISA 004 trial, oral Truvada^{R}, was reported to have a 44% reduction in the incidence of HIV in a case of post exposure prophylaxis (PEP) in MSM. In contrast to topical application, steady state tenofovir concentrations in the genital tract following oral administration (300mg/day) have been shown to be 100ng/ml. In other experiments, tenofovir suppressed HSV activity at concentrations of approximately 10-200 mg. Although tenofovir concentrations generated during oral drug administration may be sufficient for an effective systemic inhibition of HIV infection, they are substantially lower than those necessary to inhibit herpesviruses. Accordingly, prevention of HSV-2 was not recorded in this trial. Also, no epidemiological evidence has emerged of concomitantly decreased incidence of HSV-2 infection with HIV infected individuals treated with oral tenofovir.

Even though anti-herpetic activity was demonstrated with regards to HSV-2, an alphavirus, the question of one expecting the same efficacy towards a gamma herpesvirus remains unanswered. Despite the differences in alpha and gamma

herpesviruses, there may be some overlap discovered in the effectiveness of tenofovir between HSV-2 and HHV-8. HPMPA, the precursor of adefovir, has been shown to have antiviral activity against HHV-8; and adefovir, even though not listed as a drug candidate for the treatment of the respective infections, has a mechanism of action similar to, if not identical to tenofovir and has been shown to have antiviral activity against the herpesviruses including HSV-2 and HHV-8. Furthermore, HPMPC, the cytosine counterpart of HPMPA, has shown antiviral activity spectrum similar to that of HPMPA, which involves herpesviridae, including HSV-2 and HHV-8. The overlap of the antiviral activity of the molecules could perhaps be translated to the results of the project (17).

Viral entry and cells of female genital tract The lower reproductive tract in women is composed of the ectocervix and the vaginal tract. The mucosal lining in these compartments consists of stratified squamous epithelium and can be more than 25 layers thick (21). In contrast, the upper reproductive tract, made up of the endocervix and endometrium, is composed of a single layer of columnar epithelium that rests on a thin, continuous basement membrane. The columnar epithelium is characterised by the presence of tight junctions between the cells that make it impermeable to entry of any large molecules and particulate matter, including pathogens. In comparison, the upper layers of the stratified squamous epithelium, which form the lining of the lower genital tract and have been shown to lack tight junctions and are relatively permeable to large weight soluble mediators (22). While the multiple layers in the lower genital epithelium may provide a better mechanical protection against viral invasion than the single columnar epithelium that lines the upper reproductive tract, at the same time, the greater surface area of the vaginal wall and ectocervix, arguably allows greater access for viral entry, particularly when a breach occurs in the epithelium, such as during sexual intercourse (23).

The mucosal immune system in the lower female genital tract consists of resident population of dendritic cells, monocytes/macrophages, T cells and B cells, in the lamina propria of the vagina and cervix (23). These cells express c-type lectin receptors including DC-SIGN, providing additional receptors for the binding, uptake and dissemination of infectious virions to the T cell rich environment without the requirement for direct DC infection (20). There is a paucity of data regarding the interaction of HHV-8 with cells of the female genital epithelium. There is no evidence of tropism of epithelial cells as is the case with HSV-2, however HHV-8 can infect dendritic cells and macrophages (9), which would be a significant portal of entry for establishment of infection.

Summary

There are various factors that would favour a positive outcome of the experiment. HHV 8 and HSV-2, even though gamma and alpha herpesviruses respectively, have an intrinsic similar structure which could result in overlap of effectiveness of tenofovir on prevention of HHV 8 transmission. Also, as the mechanisms of action of adefovir and tenofovir are similar, there could be some overlap in antiviral activity with HSV-2 and HHV 8. Also, Another reason is that the cells of the female genital tract that form part of the mucosal immunity do exhibit tropism for the virus and would serve as an establishment for infection (21, 22). The virus has been shown to infect macrophages, a cell line in which tenofovir has been most effective (18).

However, there are numerous confounding factors that would impact negatively on the effectiveness of tenofovir as prophylaxis for HHV 8 transmission. These factors include the mode of transmission of the virus- molecular studies have pointed to saliva being the main mode of transmission as HHV 8 DNA is detected much more frequently and at much higher levels in saliva than other bodily fluids. Also, the differences in structure of alpha and herpes viruses could lead to differences in efficacy of replication in certain environments and influence the effectiveness of tenofovir- the interactions with the drug. Other factors to consider include the variations of the cytokine milieu of the female genital tract as well as endocrine changes that would have an effect on polarisation of mucosal adaptive immunity and hence viral entry and infectivity. HIV infection would cause a disturbance in the immunological microenvironment influencing the conclusions drawn from results. The mechanism of action of tenofovir itself- the concentration within the cells would be different as well as the rate of replication of the virus in the different cells which would impact on the experiment. Also, there are limitations in direct application of results obtained in vitro to in vivo conditions- the trial showed a 51% effectiveness and was therefore not absolute (18).

However, even if results are not positive for an intervention, the data could provide more insight into the pathogenesis of HHV8 as well as its interaction with HIV to serve as a catalyst for research for the development of novel therapeutic interventions. The results could also be used as epidemiological data. The results of the project could provide insight into the means of transmission of the virus. How HHV8 is transmitted and whether HIV influences HHV8 infectivity or vulnerability is as yet unknown.

Nationally, an intervention to decrease the morbidity and mortality associated with AIDS-KS may decrease the burden on the national health system; at a community level, the intervention may address social issues of gender inequality and power struggles through the empowerment of young women; individually, morbidity and mortality of AIDS-KS could be decreased (2). However, regardless of outcome, the data would be valuable to describe pathogenesis of disease as well as the interaction with HIV, thereby providing insight required for the development of novel therapeutic interventions. The results could be translated into epidemiological data as well as provide information regarding HHV8 transmission

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Study design

Search strategy

An extensive literature review and search was conducted using PubMed and Medline search engines. All cited literature and referencing was performed using EndNote software.

Keywords in the search for literature included Kaposi sarcoma, Human herpes virus, HIV, AIDS and tenofovir.

Study population

Women who were enrolled at CAPRISA 004 which is an urban and rural clinic in KZN, South Africa.

Sampling strategy and sample size

This study is a two-arm, double-blind and placebo randomized control trial. The sampling of the study population will include all patients attending the CAPRISA 004 rural and urban clinic in KZN. We believe that this location is representative of the population in the province and therefore has generalisable potential.

The participants will be selected consecutively as we believe this is the best design to represent generalisability. Inclusion and exclusion criteria have been decided and are outlined further in this report. The inclusion criteria set is broad and this will make it easier to recruit adequate subjects and thereby make the findings more representative of the larger population.

The random assignment of subjects into one of two groups will be concealed from the investigator and this will ensure an unbiased investigation.

Inclusion criteria

Women from 18 to 40 years old and who are not infected with HIV and are sexually active (which we defined as having engaged in vaginal sex at least twice in the preceding 30 days of screening).

Exclusion criteria

Women who are pregnant or become pregnant during the study period

Women who use a barrier method of contraception

Any person with a history of any adverse reaction to latex

Any person with a planned travel away from the study site for more than 30 consecutive days or a planned relocation from the study site

Persons who enrol in any other behavioural or investigational product study

Any persons with a creatinine clearance of 50ml/min or less

Any person with a deep genital epithelial disruption

Any person who participated in any research related process in the preceding year

Data collection methods

1. Samples from the CAPRISA 004 trial will be used. The sampling strategy for this has been described earlier in this report.

2. Serological testing involves the use of Biotrin's indirect immunoflourescent system which is a rapid and simple method for determination of antibody to HHV-8 lytic antigens

Sampling and testing apparatus

The testing apparatus consist of the following equipment and will be standardised to reduce confounding factors:

- High quality distilled or deionised water

- Accurate 20, 100 and 200 microlitre pipettes and disposable tips

-Serum collection equipment

-Timer

- Wash bottles and wash tray

-Test tubes, racks, pipettes, microlitre plates and safety pipetting devices for making sample dilutions

- Incubator set at 37 degrees Celsius

- Moist chamber for incubating slides

- Slider holder rack and staining dish for washing slides

- Coverslips

- Fluorescent microscope with 10x eyepiece and 16x or 40x objectives

Specimen and reagent preparation

A consistent procedure method with be employed to ensure quality control in specimen handling and the standardisation of the handling and testing of samples will reduce inter-observer variability. The procedure will involve the buffer being washed and the contents of the PBS packet will be added to a single litre of freshly prepared distilled or deionised water. It will be stored in a clean, closed container at 2 to 8 degrees Celsius for up to 4 weeks.

To further ensure quality control, qualitative testing of the samples will be standardised and the sample will be diluted in a 1:64 wash buffer. All dilutions will be prepared in a minimum volume of 100 microlitres of wash buffer.

During quantitave testing the sample will be diluted in at least 100 microlitres of a wash buffer. Thereafter the slide will be prepared with one drop of a sample which will then be incubated but not prior to the slide being washed and the addition of a conjugate taking place. A single drop of mounting media will be applied to the centre of each well before the coverslip is applied to each slide. Thereafter, each slide will be examined using a fluorescence microscope of 200 to 500 times magnification.

<u>Reliability</u>

Blinding at the stage of applying the intervention (tenofovir microbicide gel) and measuring the outcome will occur in order to reduce any bias. Further, the intervention and the control will be made to appear similar.

Variability

Patient demographic factors will contribute toward variability in results on the basis of the pathogenesis, epi/genetics, anatomy and microanatomy of each subject. The intervention itself, namely tenofovir, may have unpredictable and variable outcomes.

Variability of the study is sought to be minimized by ensuring a double-blind approach and true randomness in allocating subjects to the different groups by processes that are consistent. Furthermore, variability will be minimized in sampling technique and data capture using a set standard procedure that will be applied to all subjects.

Confounding factors

There will be effective concealed randomization of the subjects to the intervention or control group which will eliminate selection bias and reduce confounding variables. Both groups will be treated identically in all respects except for the intervention and this will means that both the patient and the investigator will be blind to the allocation process. This will further reduce any unforeseeable confounding factors. Attrition of subjects is difficult to predict and may be a confounding factor. We sought to reduce this by excluding any subjects who may plan travel away or relocation from the study site which would exclude them from completing the study, however other factors like intervention intolerance resulting in "drop-outs" may remain a possibility of a confounding factor.

Data analysis and statistical methods

The *sampling apparatus and technique* has been described earlier in this report.

Interpretation of samples

The results will be interpreted according to the fluorescence intensity grading of the specimens. Samples would be considered HHV-8 Immunoglobulin G (IgG) positive if green fluorescent staining of the infected cells is present in both the cell cytoplasm and nucleus at a dilution of greater than 1:64 and if the staining is similar to that of the positive control. Positivity reactivity will range from brilliant to weak according to the following grading scale; +4 (brilliant); +3 (bright); +2 (moderate); +1 (weak). A sample would be considered negative if fluorescent staining in the infected cells were absent. The sample titre will be determined by preparing a two-fold serial of the sample in the wash buffer.

Data Capture

Data will be captured and collated onto a Microsoft Excel® spreadsheet.

Statistical analyses

Descriptive statistics will be used to describe the cohort.

The prevalence of HHV8 will be measured.

The incidence of HHV8 will be measured overall.

The association of HHV8 and HIV acquisition will be examined.

All data will be analysed by the IBM Statistical Package for the Social Sciences (SPSS) for Windows®, version 23 (IBM Corp, Armonk, N.Y. USA) software program. Descriptive statistics will include mean and standard deviation for quantitative data and frequencies for categorical data. The microscopic interpretation of samples has been described earlier and the results that are expected will be categorical. As such, the chi-square (x^2) test will be applied if the data demonstrate a linear uniformity which will be determined by the visualized Q-Q plot and/or P-P plot. If no uniformity is demonstrated then non-parametric testing with the Kruskal-Wallis test will be performed.

Analyses of variance will be conducted for comparison of normally distributed variables with post hoc analysis by Tukey honestly significant difference for more than two subgroups. If the hypothesis testing is proven then further testing for intention to treat will be conducted. Using the Bonferroni correction, a two-tailed p-value of less than 0.05 will be considered significant in hypothesis testing.

Study location

The study will be conducted and analysed at CAPRISA which is situated at the Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal in South Africa.

Study period

Sampling will be conducted upon ethical approval and is anticipated to take up to six months to complete. Data analyses is expected to take a up to further six months with the write-up and finalization of the findings and document to be concluded within a further six months.

Limitations to the study

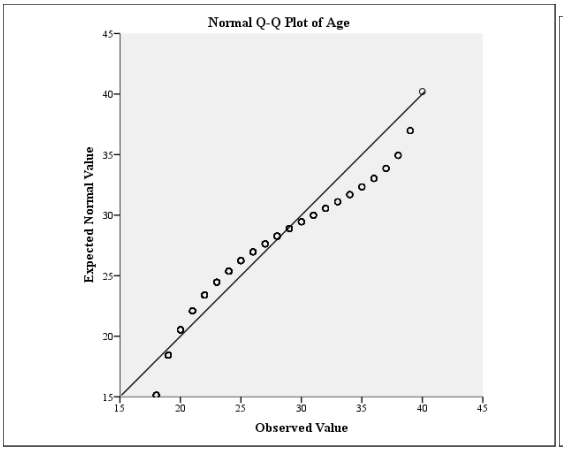
The main limitation of this study is that it is a single centre study and although we believe it may be representative of the community it may not necessarily be representative to a more general region. Similar studies in the future in various regions could be conducted to generate a more representative finding. Furthermore, we intend to evaluate the samples using a single test method and the interpretation thereof is reliant on a brightness grading scale which may influence user objectivity. We intend to minimize this by performing standardized testing of all samples. Finally, there are multiple factors that influence the pathogenesis of the disease entities being tested and these include epigenetics, genetic susceptibility, host microenvironment and others, all of which may be influential in test findings though lie beyond the scope of this study.

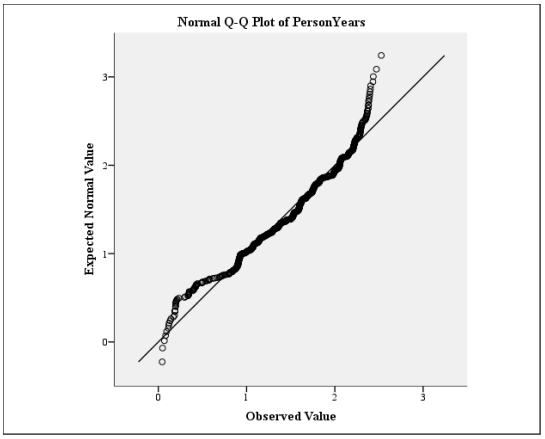
Ethical considerations

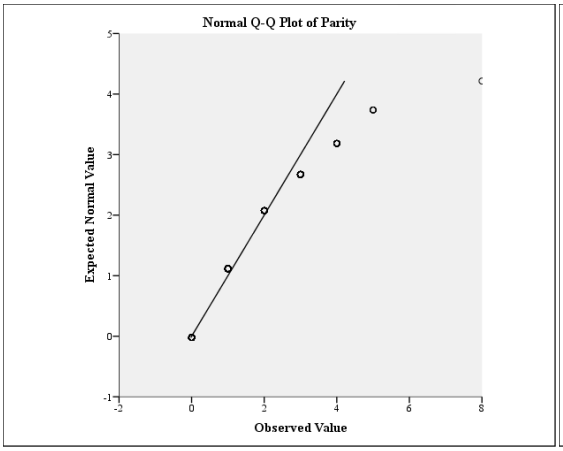
All research participants will have to voluntarily provide the relevant consent following a transparent communication regarding the study aims and objectives and methods prior to any portion of the study being conducted. The intervention will be applied to patient samples thereby ensuring that no participant is subjected to harm. The protection of the privacy of the participants is paramount in this study and the anonymity as well as a high level of confidentiality of the participants is ensured throughout.

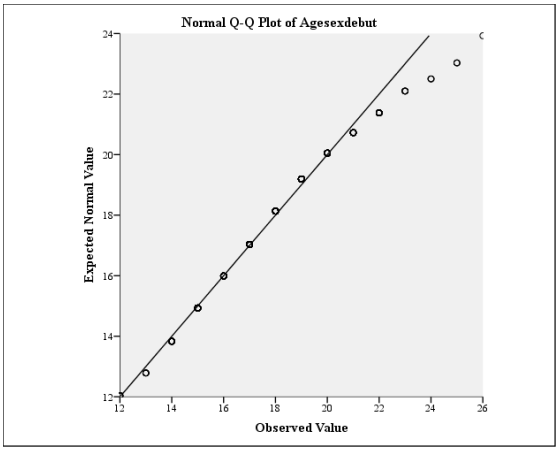
All work by other authors will be acknowledged and will be referenced in line with the Vancouver /APA/Harvard referencing system. The highest level of objectivity possible in discussions and analyses will be maintained.

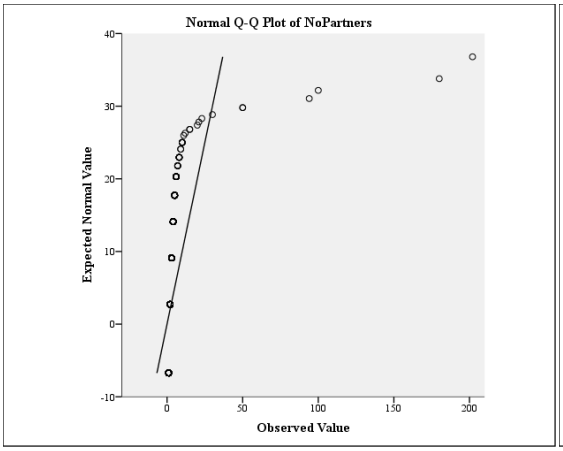
ANNEXURE D: PROBABILITY PLOTS OF QUANTITATIVE DATA

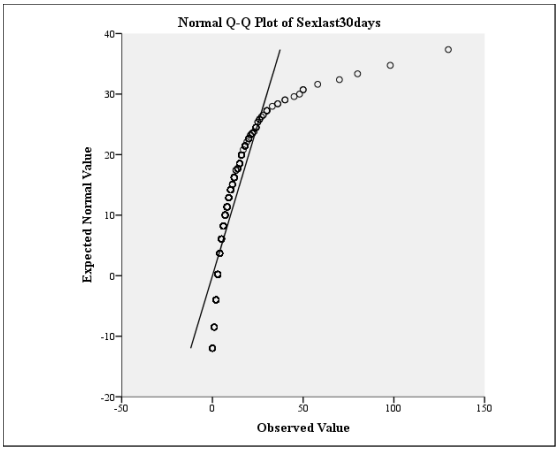












ANNEXURE E: SCRIBBER PLAGIARISM CHECKER