The barrier functions of the cervical mucus plugs and purified mucins against a panel of HIV-1 strains in the contexts of cell-free and cell-to-cell infections

Baxolele Mhlekude

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Supervisor: Professor Anwar Suleman Mall (University of Cape Town)
Co-supervisor: Jun. Prof. Dr. rer. nat. Christine Goffinet (TWINCORE)

Abstract

HIV must traverse the mucosal barriers in order to establish a clinical infection during sexual transmission in the female reproductive tract. The cervical tract of a pregnant woman produces a mucin-rich solid material called the cervical mucus plug, which prevents the entry of bacteria and other foreign substances from the vaginal tract into the uterus. Our laboratory found that the cervical mucins—but not the crude cervical mucus plugs—isolated and purified from the mucus plugs inhibit HIV-1 infection in an *in vitro* cell-free inhibition assay. We aimed to characterize and compare the inhibition of cell-free and cell-associated HIV-1 strains by the crude cervical mucus plugs and purified cervical mucins.

Fifty-two cervical mucus plugs were collected from women in labour in 6 M guanidine hydrochloride with protease inhibitors. The cervical mucus plugs were solubilised in 6 M guanidine hydrochloride with protease inhibitors, centrifuged, dialysed against distilled water and lyophilized. The mucins were purified by caesium chloride density gradient ultracentrifugation and size exclusion chromatography, dialysed against distilled water and lyophilized. The lyophilized samples were reconstituted in cell culture medium and used in cell viability assays, after which they were probed against a panel of replication-competent strains of HIV-1 using functional assays.

The crude cervical mucus plugs inhibited cell-free HIV-1 strains more potently than the purified mucins. Depending on the infecting HIV-1 strain, this potency varied between and within samples from different donors. The data from a FACS-based virus fusion assay showed that some crude cervical mucus plugs inhibit fusion between the virus and target cell. Interestingly, cell-to-cell transmission of HIV-1 partially overcame the anti-viral activity of the crude cervical mucus plugs. Furthermore, despite the donor's HIV status, the biochemical analysis of the purified cervical mucins showed comparable characteristics in terms of buoyancy in caesium chloride and guanidine hydrochloride, glycoprotein and protein contents, which were recapitulated in the HIV inhibition assays.

In conclusion, the crude cervical mucus plugs can potently inhibit different strains of HIV-1 compared to the purified cervical mucins, and this potency is more pronounced in the HIV positive cohort compared to the HIV negative cohort.

Declaration

I, Baxolele Mhlekude, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in the University of Cape Town or any other university.

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Date: 14 November 2018

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ARV	Antiretroviral
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
BlaM-Vpr	β-lactamase-Viral protein
CC	Cell control
CCR5	C-C chemokine receptor type 5
cm	Centimetre
CMP	Cervical mucus plug
CO_2	Carbon dioxide
CsCl	Caesium chloride
CXCR2	C-X-C chemokine receptor type 2
CXCR4	C-X-C chemokine receptor type 4
CYS	Cysteine
Da	Dalton
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EC	Eastern cape

EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescent activated cell sorting
Fig.	Figure
FSC-A	Forward scatter-area
FSC-H	Forward scatter-height
SSC-H	Side scatter-height
FRT	Female reproductive tract
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
GuHCl	Guanidine hydrochloride
HAART	Highly active antiretroviral therapy
HCl	Hydrochloric acid
HEK293T cells	Human embryonic kidney 293 cells with large SV40 T antigen
H.F. Verwoed Hospital	Hendricks Frensch Verwoed hospital
HIV	Human immunodeficiency virus
HMGN2	Highly-mobility group nucleosomal-binding domain 2
HTLV-lll	Human T-lymphotropic virus lll
HREC/REF	Human research ethics committee/Reference
IgA	Immunoglobulin A
IgG	Immunoglobulin G
kDa	Kilodalton
L	Litre

LAV	Lymphadenopathy-associated virus
M	Molar
mA	Milliamps
mBa	Millibars
MDa	Megadaltons
mg/ml	Milligram per millilitre
MGH	Mthatha General Hospital
ml	Millilitre
mM	Millimolar
mm^3	Cubic millimetre
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaN_3	Sodium azide
$Na_2S_2O_5$	Sodium metabisulphite
NBT/BCIP	Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate
NEM	N-ethylmaleimide
NIH	National Health Institute
N	Normal
p	Plasmid
PAS	Periodic acid Schiff
PBS	Phosphate buffer saline

PBST	Phosphate buffered saline Tween 20
PFA	Paraformaldehyde
PMSF	Phenylmethanesulfonyl fluoride
RLU	Relative light units
RNA	Ribonucleic acid
rpm	Revolution per minute
RPMI 1640	Roswell park memorial institute 1640
RSV	Respiratory syncytium virus
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIV	Simian immunodeficiency virus
SLPI	Secretory leukocyte protease inhibitor
TIF	Tagged image file
TIMP	Tissue inhibitor of metalloproteinase
UNAIDS	Joint United Nations Programme on HIV and AIDS
USA	United States of America
UTI	Urinary tract infection
v	Version
V	Volt
VC	Virus control
V_0	Exclusion volume
V_i	Inclusion volume

vWF	von Willebrand factor
μg/ml	Microgram per millilitre
μg	Microgram
μl	Microliter
μM	Micromolar

Symbols

α	Alpha
&	And
~	Approximately
β	Beta
°C	Degrees Celsius
μ	Micro
–	Minus
%	Percentage
+	Plus

Chapter – 1: Literature Review

1.1. Background

The first documented clinical accounts of Acquired Immunodeficiency Syndrome (AIDS) were reported in 1981 in Los Angeles, the United States of America (USA), when the clinicians recognized a group of five homosexual males who presented with rare pneumonias that were associated with immunodeficiency [1]. In 1982, similar reports emerged from several European countries [2-4]. Ugandan doctors in the Kasensero village recognized a new disease that predominantly affected heterosexuals. This disease was accompanied by weight loss and diarrhoea, acquiring the name of "slim disease", given to it by the locals [5]. In 1983, the South African doctors at H.F. Verwoerd hospital reported two cases of flight steward homosexual males, who had travelled to the USA and upon their return, presented with opportunistic infections that were associated with immunodeficiency [6].

The group of Luc Montagnier isolated a retrovirus called Lymphadenopathy Associated Virus (LAV) [7] whilst that of Robert Gallo isolated another retrovirus, which they called human T-lymphotropic retrovirus (HTLV-lll) [8]. Molecular evidence emerged that LAV and HTLV-lll were identical and both caused AIDS [9-11]. This prompted the International Committee on the Taxonomy of Viruses to group these viruses under one name: Human Immunodeficiency Virus (HIV). By late 1985, the AIDS wave had swept across the world, and ten years later, HIV had infected about 14 million people globally [12, 13]. In 1996, the United Nations established a Joint United Nations Programme on AIDS (UNAIDS) to spearhead a global action against the HIV/AIDS pandemic [14].

After several efforts to contain the HIV/AIDS pandemic since its outbreak, the UNAIDS recently reported a sharp decline in HIV/AIDS-related deaths between 2000 and 2016, which are more pronounced among women compared to men [15]. The less likelihood of men seeking medical attention compared to women corroborates this trend, at least in the context of sub-Saharan Africa [16]. The upscaling of the anti-retroviral therapy (ARVs) has significantly suppressed paediatric HIV infections globally [15].

Eastern and Southern Africa have seen the steepest decline in new HIV infections between 2010 and 2016, which the UNAIDS attributes to the upscaling of the ARV therapy in these two regions, where approximately 76% of people are HIV positive and 60% of whom are on ARV treatment [15]. Eastern Europe and central Asia have seen a 60% rise in new HIV infections between 1990 and 2016 [15]. In Europe, this trend correlates with decreasing condom use by the men who have sex with men, a population that is 24 times more likely to acquire HIV infection than the general male population [16].

There are about 5000 new HIV infections per day in the world and 64% of them happen in the sub-Saharan Africa. Of these daily 5000 new HIV infections, 4500 occur in young adults, 43% of whom are women [15]. In the sub-Saharan Africa, young women of child-bearing age still remain at high risk of HIV infection, while in the low-prevalence regions HIV infections are more pronounced among the key groups: drug users, transgender individuals, prisoners and sex workers. South Africa still remains the worst affected country in the sub-Saharan Africa, despite her decreasing new HIV infections [15]. These declines are attributed to the South African ARV therapy programme, which was launched in 2004 through the public health system under the initial guidance of a Comprehensive Care Management and Treatment Programme, which was succeeded in 2007 by the National Strategic Plan [17].

The new HIV infections are declining faster among women compared to men, but women still account for 59% of the HIV positive people in the sub-Saharan Africa [18]. HIV infections are predominantly established through exposure of the mucosal surfaces, which are the internal tracts of the body that are in direct contact with the outside world and portals of entry for several pathogens that cause human diseases. Of these mucosal tracts, HIV transmission is more pronounced in the rectum and genital tracts among sexually active individuals [19]. The paediatric infections occur *in utero*, intrapartum and in the postpartum period [20, 21].

The mode of HIV transmission was unknown until its isolation from the seminal fluid and saliva, which pointed towards the possibility of its mucosal transmission [22-24]. In line with these findings, Zeigler et al. [25] reported the first case of mother-to-child

transmission through breastfeeding. But several lines of evidence opposed the possibility of salivary-mediated HIV transmission [26-29]. Supporting these lines of evidence, Fultz et al [30], using two chimpanzees, showed that HIV only established systemic infection after its application on the vaginal mucosa but not on the oral mucosa, even after two rounds of oral application. The authors argued that HIV infection of the vaginal mucosa was independent of the vaginal trauma, given the absence of blood stains on the swabs following viral application. This suggested differences between these mucosae and the well-recognized capabilities of HIV to invade the intact mucosae of the ecto-cervix and endo-cervix respectively [31].

Sexual transmission of HIV is the main driving force behind the HIV/AIDS pandemic [32, 33]. To realize the UNAIDS-proposed 2030 Target [18], part of our strategy should be to invest more efforts to find alternative ways of blocking HIV transmission during sexual intercourse, without ignoring other potential routes of transmission. The low probability of HIV acquisition per coital act is suggestive of the active presence of anti-viral factors in the female reproductive tract [34]. Habte et al [35] reported the inhibition of HIV-1 infection by mucus glycoproteins (mucins), which they isolated from the crude mucus plugs of women in labour, just prior to delivery. Interestingly, the crude cervical mucus plugs did not inhibit HIV-1 infection *in vitro* [35].

Gosh et al [36] reported the inhibition of HIV infection by the cervico-vaginal lavage samples collected from HIV negative and HIV positive women. Cervico-vaginal secretions collected from non-pregnant and pregnant women also inhibited HIV infection, irrespective of the viral tropism [37]. Cervico-vaginal mucus hindered the diffusion of HIV strains [38]. The protonated lactic acid in the human cervico-vaginal fluid, which is secreted by the *lactobacilli* species residing in the vaginal canal, inhibited HIV infection in *ex vivo* experiments [39]. Together, these data suggest the presence of active endogenous anti-HIV factors throughout the female reproductive tract.

1.2. General overview of the mucus and mucosal tracts

Mucus is an adherent and viscous fluid that is secreted by epithelial cells and mucosal glands at the mucosal surfaces of the body, where it protects against pathogens, toxic insults and desiccation [40]. Depending on the mucosal surface and species, mucus predominantly contains water (~95%), mucins, proteoglycans, non-mucin proteins, inorganic salts, DNA, lipids and cell debris [41, 42]. The mucus is conserved across different species, ranging from amphibians to high order mammals [43]. Mucins are large and heavily *O*-glycosylated proteins with a molecular weight range of 0.5 – 20 MDa [40]. They exhibit a specific expression pattern across different mucosae of the human body [43], and are the major components of the mucus. The evolutionary origin of mucins dates back to as far as metazoan, with five to six gel-forming *MUC* genes conserved across vertebrates, except *Xenopus tropicalis*, which has a panel of 26 gel-forming mucins [44].

Mucus is involved in several functions across different mucosae and species. Gastropods crawl across surfaces of different textures by coating themselves with mucus to adhere to these surfaces without contacting them, after which they propel themselves over this mucus layer in a process called adhesive locomotion [45]. While allowing gaseous exchange and that of other vital molecules, mucus on the fish skin must protect against pathogens and toxins in the marine environment [46]. A group of marine creatures called mucus-mesh grazers use their mucus meshes as a fishing net to capture microscopic particles on which they feed [47]. In humans, the mucus coats the internal body tracts, where it acts on the front line of the innate immune system. These internal body tracts are broadly classified as oculo-rhino-otolaryngeal, gastrointestinal, respiratory, and genitourinary tracts respectively. The concept behind the mucus barrier functions stems from several studies reporting on the changes in mucus production in response to infections [48-51].

1.2.1. Oculo-rhino-otolaryngeal tract

The oculo-rhino-otolaryngeal tract encompasses the eyes, nose, ears and larynx. The typical mucosal disorders of this tract include dry eye syndrome, rhino-sinusitis, otitis media, and laryngitis; with differential expression of the mucins as a hallmark. The mucus layer that covers the ocular epithelium contains MUC2, MUC5AC, MUC5B and MUC19; with MUC1, MUC4, MUC16 and MUC20 forming the glycocalyx [52]. These mucins are produced by the lacrimal gland, cornea and conjunctiva of the eye to maintain homeostasis in the ocular epithelium [53]. Abnormalities in the tear film of the eye, for which mucins are the crucial components, mediate dry eye syndrome: a feeling of dryness and foreign body sensation in the eyes [52].

The mucus overproduction and goblet cell hyperplasia in the sino-nasal surface are the hallmarks of chronic rhino-sinusitis. The normal nasal mucus predominantly contains MUC2 and MUC5B, while the sinus mucus predominantly contains MUC5AC and MUC5B [54]. Ali et al [54] found the downregulation of MUC2 and upregulation of MUC5AC in the sinus mucus of patients with chronic rhino-sinusitis. The maxillary mucosa of the patients with chronic rhino-sinusitis showed elevated mRNA and protein levels of MUC5AC and MUC5B respectively [55]. The mRNA levels of *MUC4*, *MUC5AC*, *MUC5B*, *MUC7* and *MUC8* were upregulated in the sinus mucosa of patients with chronic rhino-sinusitis [56]. Together, these data highlight the modification of mucus components in chronic rhino-sinusitis.

Chronic otitis media is a leading cause of hearing loss among children and accounts for a high proportion of annual paediatric clinical visits in the USA [57, 58]. Its symptoms include accumulation of thick mucus in the middle ear cavity, which is dominated by MUC5B [59]. Preciado et al [59] noticed occasional upregulation of MUC5AC in patients with chronic otitis media. After challenging the mouse middle ear epithelial cells with *Haemophilus Influenza*, Val et al [60] found that *Muc5ac* acts as an acute responder during ear infection while *Muc5b* acts as a chronic responder. Of the membrane-bound mucins, MUC1 and MUC4 are also upregulated during ear infection [61]. These changes in *MUC* gene expression levels could be used to monitor the patient response to treatment in the clinical settings [62].

Laryngopharyngeal reflux, a backward flow of the corrosive gastric content up the oesophagus into the laryngopharynx, is a common cause of laryngitis that accounts for up to 50% of the voice disorders [63]. The mucosal barrier in the larynx is organized around a panel of mucins as discussed elsewhere [64]. The expression levels of *MUC2*, *MUC3*, *MUC5AC* and *MUC5B* were reduced in patients with laryngopharyngeal reflux-mediated laryngeal injury [64]. Given that MUC3 is a membrane-bound mucin, this shows that not only does the gastric refluxate disrupts the mucus layer covering the laryngeal epithelium, but also the underlying glycocalyx, thereby compromising MUC3-mediated restoration of the laryngeal epithelium after injury [65].

1.2.2. Respiratory tract

The respiratory tract mucus, which is in constant motion by muco-ciliary transport, is organized around MUC5AC and MUC5B as the structural components [66]. The muco-ciliary transport and alveolar macrophages in the lungs facilitate the pathogen clearance and maintain homeostasis [67]. The lung mucus layer is packed on top of the periciliary liquid layer to facilitate ciliary beating that clears the entrapped pathogens [68]. The membrane-bound mucins (MUC1, MUC4, MUC16) attached to the underlying epithelial cells are embedded into the periciliary liquid layer along with cilia to block the escaping foreign objects from reaching the underlying epithelial cells [69].

Of these two gel-forming mucins in the lung mucus, evidence from the knockout studies in mice attribute muco-ciliary clearance to Muc5b [70, 71]. Overexpression of Muc5ac protected the mice against influenza infection, while the *Muc5ac*$^{-/-}$ mice showed effective muco-ciliary clearance [70]. The *Muc5b*$^{-/-}$ mice had reduced muco-ciliary clearance compared to the wild type mice following the bacterial infection to which they later succumbed [71]. Together, these data highlight the different roles played by Muc5ac/MUC5AC and Muc5b/MUC5B in maintaining homeostasis in the respiratory tract.

1.2.3. Gastrointestinal tract

The gastrointestinal tract (GIT) is a tubular structure that stretches from the mouth to the rectum, with varying specializations in the form of organs, which are constantly exposed to foreign and commensal pathogens [72]. The mucosa of the GIT is covered by the mucus, which is produced by the glands and epithelium lining its tract. The salivary glands in the mouth produce ~1.2-1.5 L of saliva per day, which contains MUC5B and MUC7: the secreted mucins that lubricate the oral cavity and the ingested food [73, 74]. Being home to over 700 commensal bacteria, saliva ensures that these commensal bacteria live in harmony with the oral cavity, while protecting the oral mucosa against colonization by the foreign pathogens [74, 75].

In the stomach, mucus protects the epithelium against the corrosive effect of pepsin and hydrochloric acid (pH 1-2) during digestion, which are secreted by the mucosal glands [76]. The underlying epithelial cells secrete bicarbonate to create a pH gradient that maintains a neutral pH at the cell surfaces [76]. The crude mucus gel lining the stomach epithelium is organized into two layers that predominantly contain extended sheets of MUC5AC, which are interspersed with MUC6 [77]. The loosely-packed outer layer is an ecological niche for microbiota and lubricates transiting stomach content, while the regenerative inner layer is tightly-packed to protect the epithelium against toxic and pathogen insults [78, 79].

Unlike in the stomach, a single layer of loosely-packed mucus that is organized around MUC2 protects the intestinal epithelium [80]. In addition to protection, mucus in the intestines facilitates a smooth passage of the nutrients during absorption while excluding microbe populations [80]. Mucus undergoes a constant turnover to limit exposure to the virulent pathogens [72]. The Paneth cells secrete antibacterial peptides to facilitate microbial clearance in the intestinal mucus [81]. The unabsorbed waste material from the intestines transits into the colon in preparation for excretion. The colonic mucosa is covered by a double layer of mucus that is organized around MUC2 [82]. The loose outer mucus layer is an ecological niche for the microbiota, while the regenerative and tightly-packed inner mucus layer protects the epithelium [72]. How the same MUC2 behaves differently between the intestines and colon remains a mystery, which highlights the different characteristics exhibited

by the cellular machineries of the goblet cells lining these two regions. It could also be that the commensal bacterial products in the colon, e.g. lactic acid [83], which are almost absent in the small intestines, mediate the differences in MUC2 gelation.

1.2.4. Genitourinary tract

The genitourinary tract encompasses the urinary tract and reproductive tract respectively. The mucosa of the normal kidneys in the urinary tract is predominately made up of MUC1, while MUC3 dominates in neoplasia [84]. Kirby et al [85] reported a frameshift mutation in *MUC1* as a cause of autosomal dominant tubulo-interstitial kidney disease, shortly known as Mucin 1 Kidney Disease, which is the hereditary chronic nephropathy and risk factor of kidney failure. Nie et al [86] found that MUC1 facilitates renal calcium re-absorption in the distal convoluted tubules of the kidney, thereby protecting against nephrolithiasis, which is caused by impaired calcium re-absorption [87]. These data highlight an active role of MUC1 in the renal physiology.

The bladder stores urine, which carries toxins that could not be re-absorbed by the kidneys. This requires the bladder to protect its mucosa from potential toxin insults and pathogen invasion. The normal bladder epithelium consists of MUC1 and MUC4 [88-90]. Parsons et al [91], reported the antibacterial activity of a surface mucin lining the epithelium of the rabbit and human bladders. They found that this muco-polysaccharide layer would be disrupted by acid treatment, re-synthesized in less than 24 hours and replenished in about 48 hours, thereby supporting a well-recognized mucus turnover [92, 93]. Rigorous studies are lacking that investigate the direct role of the mucins in biofilm formation during urinary tract infection [94].

In the reproductive tract, a repertoire of mucins protects the epithelium against pathogens, and mechanical and chemical insults, while ensuring a smooth process of reproduction in both males and females [95, 96]. The mucins exhibit a unique expression pattern across different organs of the male reproductive tract, reflecting different specialities within this tract [95]. The female reproductive tract is covered by the mucus layer that is organized around a repertoire of mucins [96], and provide a barrier against pathogens. The secretions of the female reproductive tract protect against a repertoire of pathogens that cause human diseases [36-39, 97, 98].

1.3. Cervical mucus and the mucus plug

Cervical mucus is a heterogeneous and viscous secretion that is secreted by the mucosal cells and glands lining the epithelium of the endo-cervix [99]. It coats the epithelia of the female reproductive tract (FRT), where it predominantly protects against desiccation and invading pathogens. The mucus in the FRT operates under control of the steroid hormones (oestrogen and progesterone), which change the mucus rheology to meet the demands of the FRT [100]. The FRT must protect its epithelia against invading pathogens while tolerating the foreign spermatozoa and the foetal allograft on its endometrium, whose cells may persist in the maternal blood for decades due to foeto-maternal micro-chimerism [101].

The cervical mucus contains MUC5AC, MUC5B and MUC6 as its gel-forming mucins, while MUC1, MUC4 and MUC16 form the glycocalyx in the FRT [96, 102]. There are contrasting data on the expression of MUC2 in the human endo-cervix, with more evidence opposing its expression [35, 96, 100, 102, 103]. There is inconsistency and poor signals of MUC2 expression even in the data that support its expression, thereby making it difficult to recognize MUC2 as an endo-cervical mucin [35, 103]. Of the recognized cervical mucins, MUC5B is the major gel-forming mucin in the endo-cervix that peaks before mid-cycle, while MUC4 dominates the glycocalyx among the membrane-bound mucins [104].

During the oestrogen-dominated follicular phase of the menstrual cycle, cervical mucus becomes less viscous to allow sperm penetration. It then gets thicker and viscous during the progesterone-dominated luteal phase to limit further sperm penetration [105]. Pommerenke and Viergiver [106] showed that administered progesterone counteracts the effect of oestrogen on the cervical mucus of castrated women, who cannot secrete these steroid hormones due to the removal of their ovaries. Gipson et al [107] reported that MUC5B levels peak at mid-cycle. This is coupled with a 99% increase in water content of the cervical mucus at mid-cycle, thereby increasing the mucus volume, which drops to about 91% during the progesterone dominated-luteal phase to produce thick cervical mucus [108]. Mucus hydration most likely dilutes the biochemical factors that mediate mucin

entanglement and increase the mucin pore size to form a more receptive mucus gel [42, 109, 110]. MUC5B peaks at mid-cycle presumably to retain more water, thereby forming a watery mucus with high sperm receptivity in the endo-cervix [107].

It is of no doubt that this watery texture of the cervical mucus at mid-cycle is critical for sperm-mucus interactions and successful pregnancy. "*In the investigation of sterility—if we expect to proceed understandingly—we must determine whether the secretions of the cervical canal are favourable or not to the vitality of the spermatozoa*." said J. Marion Sims in 1868, and about a century and a half later, his words still reverberate throughout the field of reproductive biology (adapted from Stevenson, 1958 [111, 112]). Stevenson [112] proposed that cervical mucus must meet certain requirements for a successful pregnancy: amount, clarity, *spinnbarkeit*, and pH. Existing evidence suggest that cervical mucus protects the spermatozoa from phagocytosis in the FRT and facilitate their movement to the uterus to ensure successful fertilization [113, 114]. Together, these data highlight the role played by the cervical mucus in pregnancy regulation.

As early as mid-1940s, it became clear that not only does cervical mucus facilitates pregnancy and protects against desiccation, but also protects against genital tract infections. Koch [115] reported that cervical cultures were more likely to be negative for *Neisseria gonorrhoea* during the luteal phase and positive during the oestrogen-dominated follicular phase when mucus becomes watery and more alkaline. Moriyama et al [116] reported the presence of secretory leukocyte protease inhibitor (SLPI) in the cervical mucus of women at mid-cycle. Eggert-Kruse et al [98] reported the antimicrobial activity of the cervical mucus collected from sexually active women at mid-cycle. Ming et al [97] purified and characterized antimicrobial activity of the two antimicrobial factors (HMGN2 and SLPI) from the human cervical mucus samples, thereby highlighting the antimicrobial activity of the cervical mucus.

During pregnancy, the body of a mother-to-be undergoes physiological changes predominantly to maintain pregnancy till term [117-121]. Among these changes is a cervical remodelling that includes transformation of cervical mucus into a thick solid material called cervical mucus plug (CMP) as the progesterone levels begin to rise [122], and while bacterial load is increasing in the neighbouring vaginal canal [123].

These changes in cervical mucus during pregnancy were initially recognized in 1948, when Atkinson et al [124] reported an increased production of the cervical mucins by pregnant women. The CMP (~10 g) must tightly seal the cervical os during the course of pregnancy to prevent bacterial ascent from the vaginal canal into the cervix, failure of which may cause pre-term deliveries that are responsible for approximately 35% of the global annual neonatal mortalities [125].

At term, when a purpose of CMP is served, cervix sheds off the CMP to allow parturition. The cervix releases the CMP (in part) through a controlled action of matrix metalloproteinases (MMPs), a group of proteolytic enzymes that degrade extracellular matrix in the rigid cervix, thereby triggering cervical dilatation [126] and foetal membrane rupture [127]. The cervix maintains a balance between the MMPs and tissue inhibitors of metalloproteinases (TIMPs). This balance shifts in favour of the TIMPs during pregnancy to suppress MMPs and prevent pre-term membrane rupture, and then shift in favour of the MMPs at term to allow the process of parturition [128]. These MMPs and TIMPs are components of the CMP itself, highlighting its capabilities to remodel the same tissue in which it resides.

1.4. Mucins

Mucins are a group of heavily *O*-glycosylated proteins with a molecular weight range of 0.5 – 20 MDa [40], which are synthesized by the mucosal cells and glands lining the mucosal surfaces of the body. They exhibit extensive *O*-glycosylation and to a lesser extent, *N*-glycosylation and the still controversial *C*-mannosylation, which together contribute about 80% to an overall molecular weight of the mucins [43]. Mucins have evolved to become a family of 20 members [129], and are grouped on the basis of heavily *O*-glycosylated tandem repeat domains (http://www.hugo-international.org/hugo/). Mucins are encoded by a group of genes called *MUC* genes, which have been mapped on several chromosomes across the human genome [129]. The mucins are designated MUC, followed by a number according to the order of discovery—MUC1 in humans and Muc1 in animals. They exhibit a specific expression pattern across the human mucosae to meet the specific needs of a particular mucosa (Fig. 1).

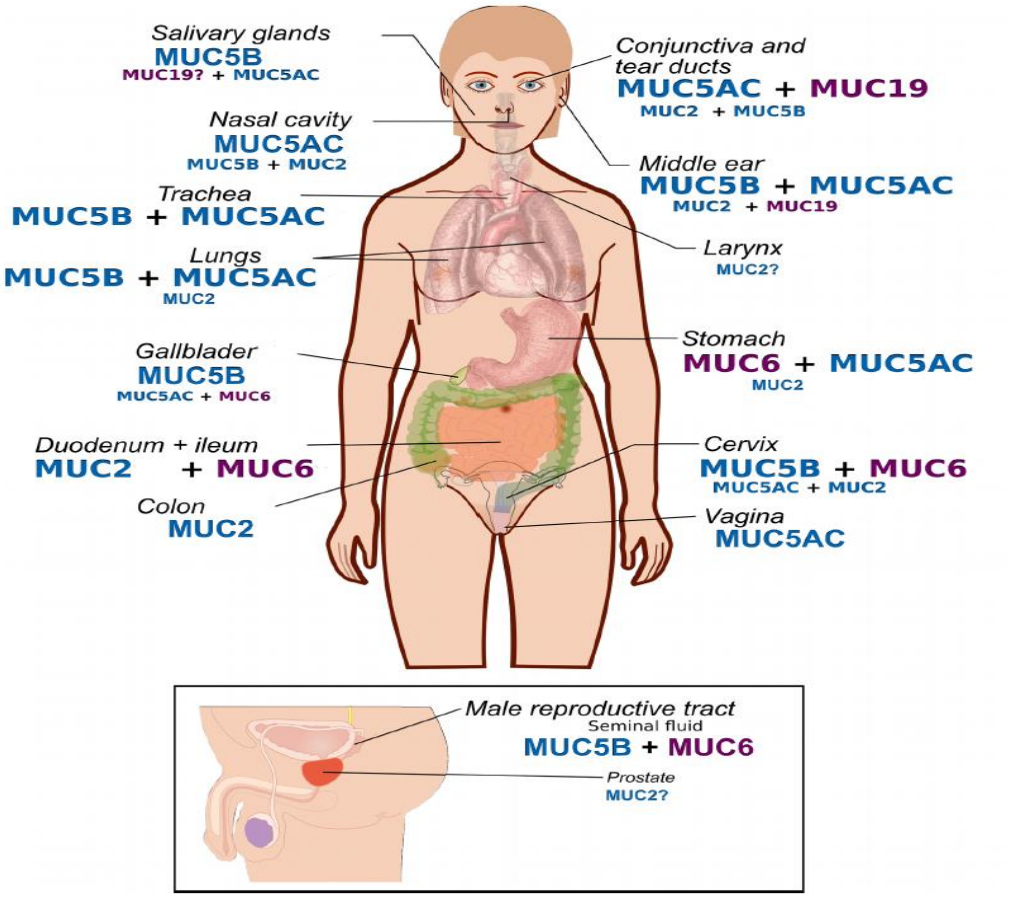


Figure 1: The expression pattern of the gel-forming mucins under normal conditions. Illustration of a constitutive expression profile of the gel-forming mucins across different mucosae of a healthy person. The blue-coloured mucins have CYS domains interspersing the mucin domains along their protein backbones while the purple-coloured mucins do not. The font size represents a degree of expression for each mucin in a particular mucosa (with permission of Elsevier, adapted from Demouveaux et al [43]).

Mucins are broadly divided into two groups: secreted and membrane-bound mucins. Secreted mucins are further subdivided into gel-forming and non-gel-forming mucins. Of these 20 mucins, five mucins—MUC2, MUC5AC, MUC5B, MUC6 and MUC19—can polymerize through their terminal ends and retain water molecules through their hydrophilic glycans or oligosaccharide side chains, to form mucus gels that coat the mucosal surfaces of the body, thus referred to as the gel-forming mucins [129]. MUC7, MUC8 and MUC9 are secreted non-gel-forming mucins, because they do not polymerize and participate in mucus gelation [129]. The remaining ten mucins are attached to the membranes of the epithelial cells where they form the glycocalyx just underneath the mucus layer [129], hence referred to as membrane-bound mucins. The membrane-bound mucins—MUC1, MUC3A/B, MUC4, MUC12, MUC13,

MUC15, MUC16, MUC17, MUC19, MUC20 and MUC21—predominantly serve to prevent toxin penetration and pathogen adherence to the underlying epithelial cells [130], and engage in molecular signalling to trigger several biological processes, a phenomenon that is well-characterized in cancer biology [131].

Of this repertoire of mucins, the endo-cervix expresses MUC5AC, MUC5B and MUC6 as its gel-forming mucins while MUC2 expression remains controversial [96, 100, 103]. MUC1, MUC4 and MUC16 are the membrane-bound mucins expressed by the endo-cervix [100, 102]. *MUC2*, *MUC5AC*, *MUC5B* and *MUC6* genes are clustered within a ~400 kb region found on the short arm of chromosome 11p15.5, with highly conserved exon-intron boundaries and amino acid terminal sequences flanking their large central mucin domains [132, 133]. Unlike other gel-forming mucins, *MUC19* is found on chromosome 12q12 [134]. Genes encoding the membrane-bound mucins in the endo-cervix are located in different chromosomes across the human genome: *MUC1* on chromosome 1q21, *MUC4* on chromosome 3q29 and *MUC16* on chromosome 19p13.2 respectively [129].

1.4.1. Secreted gel-forming mucins

The gel-forming mucins share several structural features, which are characterized by a large and heavily *O*-glycosylated central region that is flanked by naked N and C-terminal regions, thereby forming a bottle-brush like structure [40]. This large central region has a high content of tandemly repeated proline, threonine and serine amino acid residues to constitute PTS pattern, with threonine and serine acting as glycan acceptors to form extensively *O*-glycosylated mucin domains [135, 136]. These mucin domains are interspersed by short invariant cysteine domains (CYS)—except for MUC6 and MUC19—that are believed to non-covalently cross-link the mucin polymers through intramolecular disulphide bonds and determine mucus pore size [137]. The tandemly repeated sequences that encode the mucin domains differ in length and sequence for each mucin, with varying number of tandem repeats between and within individuals to constitute polymorphism, except for *MUC5B* [138]. Terminal regions of the gel-forming mucins resemble von Willebrand factor (vWF), a blood-clotting glycoprotein. Their N-terminal region consists of cysteine-rich domains called D1, D2, D' and D3 housed within a vWF-like domain. The C-terminal region

consists of cysteine-rich domains called D4, B, C domains and cysteine knot within another flanking vWF-like domain [129]. In the C-terminal region, MUC6 has a cysteine knot only and lacks vWF-like domain, while MUC19 has only C domain and cysteine knot [43, 134]. Evidence from the studies on recombinant mucins shows that the gel-forming mucin polymerize through their flanking vWF-like domains found on the terminal ends and cross link to form mucus gels [109].

1.4.2. Secreted non-gel-forming mucins

The secreted non-gel-forming mucins include MUC7, MUC8 and MUC9 [129]. These mucins do not polymerise and form gels, because neither of them has the vWF-like domains at their terminal ends. MUC7 is located on chromosome 4q13.3, carries 23 tandem repeat amino acids in its central mucin domain. It is expressed by the sublingual and submandibular glands in the mouth, and by the submucosal glands in the respiratory tract respectively [129, 139]. MUC8 is located on chromosome 12q24.3, carries about 13/14 tandem repeat amino acids in its central mucin domain and expressed by the submucosal glands in the respiratory tract [129], with less characterized expression across the FRT [140]. MUC9 is located on chromosome 1p13, with a central mucin domain of 15 tandemly repeated amino acids and expressed in the oviduct [141]. The salivary MUC7 is the most characterized mucin among the secreted non-gel-forming mucins, through its active role in microbial clearance in the oral mucosa [142-144]. There is little information about the role of MUC8 in the respiratory tract mucus, and MUC9 is believed to protect the early embryo in the oviduct and the oviduct itself against pathogen invasion [141].

1.4.3. Membrane-bound mucins

The membrane-bound mucins also do not polymerize and form gels, since they lack the vWF-like domains in their terminal ends. Instead, they have a transmembrane domain, hence found attached at the surfaces of the epithelia cells where they form a glycocalyx together with other glyco-conjugates [130]. However, to a lesser extent, the membrane-bound mucins contribute to the mucin content of the mucus through their heavily *O*-glycosylated extracellular domain, which can be cleaved from the cell membranes by the sheddases, a group of proteolytic enzymes that mediate

shedding of the membrane-bound proteins, or directly secreted into the mucus through alternative splicing [145-147]. They are characterized by a large heavily *O*-glycosylated N-terminus (α-subunit) and short naked C-terminus (β-subunit). Of the endo-cervical membrane-bound mucins, in addition to its terminal ends, MUC1 has a sea urchin sperm protein enterokinase agrin (SEA) domain located between the end of the *O*-glycosylated α-subunit and its transmembrane domain, while MUC16 has several SEA domains [131]. MUC4 lacks SEA domains, but has the nidogen (NIDO), adhesion-associated domain in MUC4 and other proteins (AMOP) and von Willebrand factor type D (vWD) (NIDO-AMOP-vWD) domains with three epidermal growth factor (EGF)-like domains [131]. Generally, the SEA domains serve as the unifying feature for the membrane-bound mucins, except for MUC4 that evolved from a different ancestor, at least when compared to MUC1 and MUC16 [148]. These SEA domains serve as the cleavage sites on which the sheddases must act in order to release the α-subunits of these mucins, while MUC4 is cleaved behind its vWD domain [131]. A mutation in the SEA sequence inhibits the shedding of MUC1 [149].

1.4.4. Structural models of gel-forming mucins

Two models of mucin structure and conformation have been proposed based on the use of different extraction procedures [150, 151]. Allen and Snary proposed the windmill model (Fig. 2A) after isolating the mucins from the pig gastric mucus, which they collected in 0.2 M NaCl and 0.02% NaN_3 without protease inhibitors. According to this model, the four glycosylated mucin subunits of $2x10^6$ Da are inter-linked through a naked 'linker' protein of $7x10^4$ Da at the C-termini, through disulphide bonds, producing a windmill structure [150, 152]. Carlstedt and Sheehan isolated the mucins from the cervical and gastric mucus, which they collected in 6 M GuHCl with protease inhibitors and proposed a random coil model [151]. In this model (Fig. 2B), the glycosylated mucin subunits are joined end-to-end through the disulphide bonds to assume a linear random coil structure [151]. The accepted genetic model of the mucin structure supports the model proposed by Carlstedt and Sheehan [151]. The *MUC* genes are characterized by the central tandemly repeated sequences encoding the mucin domains that are interspersed by the short invariant cysteine-rich domains [40]. This central region is flanked by the 5' and 3' regions respectively. The 5'

sequence encodes a vWF-like domain that houses four D-domains while the 3' sequence encodes another vWF-like domain that houses D, B and C domains with a cysteine knot [40, 109, 129]. The mucin monomers join end-to-end through their cysteine-rich vWF-like domains to form long polymers [109].

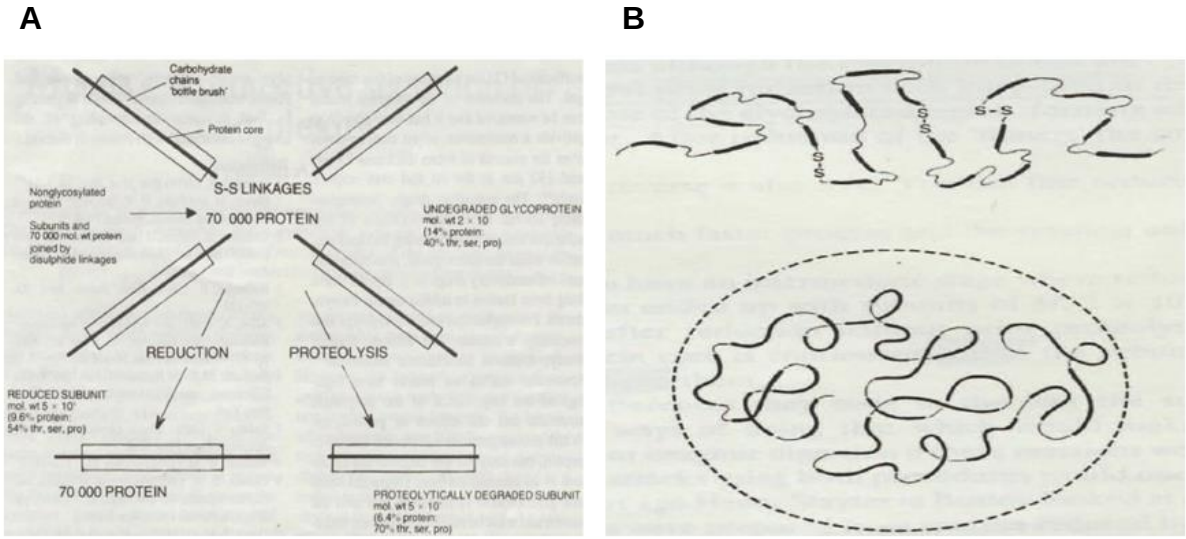


Figure 2: The illustration of the proposed biochemical models of the mucin structure. The windmill model (**A**) shows that the four mucin subunits are inter-linked by a naked 'linker' protein through disulphide bonds. On the other hand, the random coil model (**B**) argues that the mucin subunits are joined end-to-end through the disulphide bonds in a random coil fashion (Adapted from Mall, 2008 [153]).

1.5. Aspects of the cervical mucus/plug

The cervical mucus, a heterogeneous and viscous secretion that occludes the cervical os must selectively control entry of several organisms into the cervix. It acts by blocking access to pathogens while cyclically allowing entry of the spermatozoa to facilitate reproduction, failure of which may cause gynaecological and obstetrical problems [100, 123]. During pregnancy, it transforms into a solid cervical mucus plug that blocks bacterial ascent from the vaginal canal into the cervix until parturition to ensure successful pregnancy [154, 155]. The rhythmical effect of the steroid hormones changes the mucus rheology and its content, which is largely biochemical and immunological in nature [97, 155, 156]. Certain factors may be released from the foeto-maternal unit into the cervical mucus/plug during diseases or pregnancy, thereby making it a perfect breeding ground for biomarkers to assist in clinical diagnosis [157].

1.5.1. Biochemical aspects of the cervical mucus/plug

Cervical mucus must provide the spermatozoa with an environment in which they can survive for a period long enough to increase the chances of fertilization. In 1873, J. Marion Sims noticed that spermatozoa could survive in the vagina for 12 hours and in the cervix for 36 hours [158]. Lamar et al [159] noted that the spermatozoa could survive in the cervical mucus longer than they could survive in the seminal fluid, suggesting the presence of molecules needed for elongated sperm survival in the cervical mucus. It later emerged that the cervical mucus is loaded with glucose, glycogen and amylase to catabolise glycogen back into usable glucose, which like the fructose capacitates sperm motility [160]. The glucose in the cervical mucus is also a basic energy source for the implanted trophoblast in the endometrium [161].

In 1946, Papanicolaou noticed that the cervical mucus samples crystalized when viewed under microscope upon drying on a microscope slide [162]. These crystal patterns would be well-defined at mid-cycle when oestrogen levels are high and diminish post-ovulation and in pregnancy when progesterone has dominated. Campos da Paz [163] reported that progesterone inhibits crystallization of the cervical mucus. Using X-ray crystallography, Macdonald [164] confirmed these to be true crystals that are rich in sodium chloride and potassium chloride, and built around tiny amounts of organic material (proteins and carbohydrates) in the cervical mucus.

It is recognized in the GIT that the endogenous lipids are responsible for hydrophobicity of the gastric mucus [165]. Breckenridge and Pommerenke [166] reported the presence of cholesterol and lipid phosphorus in the cervical mucus samples. Singh and Swartwout [167] reported the presence of the hydrocarbons, glycerides, cholesterol, fatty acids and phospholipids in the cervical mucus samples collected from the two ovulating women. They noticed that the total lipid content of the cervical mucus decreased at mid-cycle when the oestrogen was at the peak, suggesting the effect of lipids in the receptivity of the cervical mucus.

1.5.2. Immunological aspects of the cervical mucus/plug

The FRT must maintain immunity against foreign pathogens while tolerating the foreign spermatozoa and developing foetus. Safaeian et al [168] reported the low levels of IgA and IgG in the cervical mucus collected from women at mid-cycle. A comparison between these two immunoglobulins showed more decline of the IgG levels compared to the IgA levels in the cervico-vaginal secretions collected from ovulating women at mid-cycle [169]. Another study in rhesus macaques at mid-cycle showed the decreased immunoglobulin levels in the cervical mucus [170]. Antibody screening in the cervico-vaginal secretions of 45 infertile women attending Kammal El-Sammrari Hospital in Iraq showed a 62% increase of anti-sperm antibodies among infertile women compared to 3.3% from fertile women [171]. Together, these data suggest that the cervical mucus must lower its immune defences to tolerate the spermatozoa during the receptive phase of the menstrual cycle.

During pregnancy, the FRT remains under the control of progesterone and this means a slow production of the dehydrated mucus plug with impaired turnover, thereby maximising exposure to the virulent pathogens [93]. We have learned from the mucosal biology of the respiratory tract in cystic fibrosis patients that the mucus plug impairs mucus turnover and predisposes to infections by the entrapped pathogens [69], a circumstance that would yield severe obstetrical problems in the cervix. Unlike in the respiratory tract, the mucus plug in the cervix retains more phagocytes to ensure continuous clearance of entrapped pathogens in the face of the impaired mucus turnover [155].

Hein et al [155] showed elevated levels of intact immunoglobulins and phagocytes in the cervical mucus plugs collected from women in labour when compared to the cervical mucus collected from ovulating non-pregnant women. Hordes et al [172] reported a median IgG:IgA ratio of 6:1 in the cervical mucus plugs collected from pregnant women in their second trimester of gestation. Proteomic studies showed an abundance of immunological factors in the cervical mucus plugs collected from women in labour when compared to the cervical secretions collected from non-pregnant women [121, 173]. Vornhagen et al [174] reported that when overwhelmed by the pathogens, the cervical mucus plug components can stimulate leukocytes in

the systemic blood to activate a complement system and destroy the invading pathogens. Together, these studies show a synergy between the innate and adaptive immune responses in the cervical mucus plugs to deliver good health outcomes for the mothers and their babies, while saving them from the economic burden of pregnancy complications that require frequent clinical visits [175, 176].

1.5.3. Clinical aspects of the cervical mucus/plug

The cervical secretions can retain a repertoire of biologically active factors from the systemic circulation and their surroundings [157, 174]. In addition to their biochemical and immunological nature, these secretions can be a fertile field from which we can harvest the biomarkers of clinical relevance and learn about the changes in the FRT to facilitate diagnosis. The fern pattern assumed by the cervical mucus upon drying on a microscope slide due to mucus crystallization in response to the fluctuating steroid hormones in the FRT, could be used to monitor functionality of the corpus luteum [177]. Maxwell Rowland [178] used a fern test to determine the oestrogen activity, ovulation and early pregnancy from the cervical mucus samples. A well-defined fern pattern from the cervical mucus of a woman in menopause indicates oestrogen activity and can alert the attending clinician about a possible use of oestrogen therapy or the presence of an oestrogen-secreting tumour [164].

A proteomic study reported nine downregulated proteins in the cervical mucus of women with endometriosis when compared to the normal women [179]. The authors noticed that other 15 proteins were exclusively expressed in the normal cervical mucus and absent in the endometriosis cervical mucus. This points towards the cervical mucus as a potential source of biomarkers for the diagnosis of endometriosis. Until day 7 of the menstrual cycle, MUC1 showed low but detectable signals in the uterine flushing of fertile women, after which the signals drastically increased and peaked at day 13, suggesting the loss of MUC1 by the uterine epithelium [180]. Women who had spontaneous miscarriages showed low signals of MUC1 throughout the cycle, a possible retention of MUC1 by the uterine epithelium and impairment of trophoblast's implantation [181]. These data suggest that levels of MUC1 in the uterine flushing could be used to predict the window of implantation among women of the reproductive age to improve fertility.

The foetal cells can migrate from the placenta into the maternal organs through a process called foeto-maternal cellular trafficking, which was first documented in 1893 by a German pathologist Christian Georg Schmorl after he found clumps of foetal cells in the lungs of eclamptic women on whom he performed autopsies [182]. Shettles hypothesized that the same trophoblasts could be shed from the regressing chorionic villi and leak into the cervical mucus plug during pregnancy. In 1971, he collected trophoblasts from the cervical mucus plugs of 30 pregnant women in their early pregnancy from which he accurately diagnosed the sexes of 10 foetuses using Y-chromosome fluorescein dye that is positive for a male foetus [157]. Mantzaris et al [183] extended these observations using a multiplex fluorescent polymerase chain reaction in prenatal diagnosis on the trophoblasts isolated from the cervical mucus plugs of 22 pregnant women in their early pregnancy, and accurately diagnosed the sexes of 22 foetuses. The cervical mucus plug could be used in prenatal diagnosis as an alternative method to the invasive amniocentesis and chorionic villous sampling, which carry a miscarriage risk of 0.5-1% and 5% rate of false-positive results respectively [184, 185].

1.6. The antimicrobial activities of the cervical mucins

The purified salivary MUC5B acts as an anti-biofouling agent to prevent *Streptococcus mutans* from forming biofilms in the oral cavity by keeping them in planktonic state [74]. In line with these data, Caldara et al [186] showed that purified porcine gastric mucins, dominated by Muc5ac, prevent *Pseudomonas aeruginosa* from aggregating, thereby keeping them in a planktonic state where they are more susceptible to antibiotics. *Campylobacteria jejuni* was detected in the systemic organs of *Muc1*$^{-/-}$ mice following an oral challenge, but not in the systemic organs of *Muc1*$^{+/+}$ mice [187]. Purified salivary MUC5B inhibited Influenza A virus in an *in vitro* assay [188].

Following infection with respiratory syncytial virus (RSV), chemokine receptor CXCR2 triggered an increased mucus production dominated by Muc5ac in the airways of wild type mice compared to the *CXCR2*$^{-/-}$ mice, presumably to enhance the barrier function against infection [189]. Meanwhile, in the gastric mucosa

Helicobacter pylori infection impaired the mucin production and turnover in the mice stomach, thereby paralysing mucus clearance and promoting further infection [190]. The N-terminus of MUC7 showed anti-fungal and antibacterial activities in *in vitro* assays [142-144]. The purified MUC1 isolated from the breast milk of lactating mothers inhibited poxvirus in an *in vitro* assay [191]. Collectively, these studies highlight the antimicrobial properties of the mucins in general.

Bergey et al [26] fractionated the human salivary samples using size exclusion chromatography and found that the mucin-rich fractions had anti-HV-1 activity. Accordingly, size exclusion chromatography-purified salivary mucins (MUC5B and MUC7) inhibited HIV-1 infection in *in vitro* assays [29, 192, 193], and so did the purified breast milk mucins from the breastfeeding mothers [194, 195]. Carias et al [31] reported a 2.5-fold increase of HIV penetration into the endo-cervical columnar epithelium, following a neuraminidase-mediated degradation of the cervical mucus layer from the human endo-cervical tissue explants. Habte et al [35] found that the purified cervical mucins inhibit HIV-1 in *in vitro* assays, while the crude cervical mucus plugs did not, despite a repertoire of antimicrobial factors present in these secretions [121]. Taken together, these studies show that the mucins in general and cervical mucins in particular exhibit anti-HIV-1 activity.

Literature is accumulating on the anti-viral activities of the cervico-vaginal secretions coating the vaginal canal, while largely excluding the endo-cervical mucus, which seems to provide better protection in the *ex vivo* experiments [31]. Several studies in the field rarely address the pressing issue of HIV infection in the context of cell-associated HIV-1 viruses, the most efficient physiological mode of HIV transmission [196, 197]. We aimed to characterize and define the anti-HIV-1 activity of the crude cervical mucus plugs and purified cervical mucins collected from HIV negative and HIV positive women in the contexts of cell-free and cell-associated HIV viruses.

Our approach involved probing the individual samples against a panel of replication-competent strains of HIV-1 in the context of cell-free viruses, allowing an unbiased analysis of individual variations between the individual donors. Assessing the anti-HIV-1 activity of the crude cervical mucus plug in the context of cell-free viruses using two independent readouts allowed us to show the consistency of their anti-

HIV-1 activity. We conducted infection assays in the context of cell-associated virus to investigate whether HIV-1 could overcome the barrier functions imposed by the cervical mucus plug in the context of cell-free HIV-1 viruses.

Chapter – 2: Aims and Objectives

2.1. Aims

We aimed to characterize and define the barrier functions of the crude cervical mucus plugs and their cluster of purified mucins (samples) against a panel of replication-competent HIV-1 strains in the contexts of cell-free and cell-associated viruses.

2.2. Objectives

- Investigate the anti-HIV-1 activity of the crude cervical mucus plugs and purified mucins against a panel of replication-competent strains of HIV-1 in the context cell-free virus using luciferase and flow cytometry-based GFP readouts.
- Pin-point the mechanism of action behind the anti-HIV-1 activities of the study samples using time-of-addition and β-lactamase-based assays.
- Investigate the anti-HIV-1 activity of the crude cervical mucus plugs and purified mucins against HIV-1 in the context of cell-associated virus using a flow-cytometry-based GFP readout.

Chapter – 3: Materials and Methods

3.1. Materials

Guanidine hydrochloride, ethylenediaminetetraacetic acid, N-ethylmaleimide, phenylmethanesulfonyl fluoride, caesium chloride, Sepharose CL-2B beads, periodic acid, pararosaniline hydrochloride, ammonium persulfate, glycerol, bromophenol blue, tetramethylethylenediamnie, dialysis tubing, acetic acid, sodium metabisulphite, hydrochloric acid, β-mercaptoethnaol, Tris, sodium chloride, sodium azide, *N*,*N'*-methylenebisacrylamide, alkaline phosphatase-conjugated goat anti-rabbit polyclonal antibody and NBT/BCIP Detection Kit were purchased from Sigma-Aldrich, South Africa. Bradford assay reagent was purchased from Bio-Rad, South Africa and Vacutec AquaStain from Vacutec, South Africa. Nitrocellulose membrane was purchased from Amersham Biosciences, South Africa. Rabbit polyclonal anti-MUC5AC and anti-MUC5B antibodies were a gift from Thornton's Lab, University of Manchester, United Kingdom. Sucrose, RPMI 1640, Dulbbeco's Modified Eagle's Medium, Phosphate Buffered Saline were purchased from Sigma-Aldrich, Germany. Luciferase Assays System and CellTiter-Glo Luminescent Cell Viability Assay Kit were purchased from Promega, Germany. CalPhos Mammalian Transfection Kit was purchased from Takara Bio Europe, Germany. CCF4 staining kit was purchased from Invitrogen, Germany. Penicillin, streptomycin, L-glutamine, trypsin and CO_2 independent medium were purchased from Gibco, Germany. TZMbl cells, parental Jurkat cells, T20, efavirenz and maraviroc were obtained from NIH AIDS Reagent Programme, USA. PFA was purchased from Carl Roth, Germany. The cell culture flasks were purchased from Sardstedt and the cell culture plates from Geriner Bio One, Germany. HEK293T and PM1 cells were obtained from ATCC, USA.

3.2. Ethics statement

The study was approved by the Human Research Ethics Committee of the University of Cape Town (HREC REF: 102/2013) and the provincial health department of the Eastern Cape (EC_2016RP7_393). The samples were collected according to the principles expressed in the Declaration of Helsinki.

3.3. Study population and sample collection

A group of 52 pregnant women of African black ethnicity, with a mean age of 25.5 years, were recruited from the maternity unit of Mthatha General Hospital (MGH) in the Eastern Cape province between October 2016 and April 2017. Of these 52 women, 28 (53.8%) were HIV negative while 24 (46.2%) were HIV positive. There were no inclusion or exclusion criteria set out during the recruitment process and the donation of samples was voluntary. The cervical mucus plugs of women in labour were either shed spontaneously or retrieved by a midwife during the routine cervical examinations and collected in 6 M guanidine hydrochloride (GuHCl) with protease inhibitors (10 mM EDTA, 5 mM NEM and 1 mM PMSF) [198]. The samples were kept at 4°C until the end of the sample collection process, after which they were shipped to us at -80°C.

3.4. Preparation of the crude cervical mucus plugs

Of the 52 samples, 16 (half from each cohort) were selected for preparation of the crude cervical mucus plugs based on the cervical mucus plug quantity (≥ 5 g), and throughout this study, the samples were analysed individually. The crude cervical mucus plugs were stirred slowly overnight at 4°C. The solubilised material was centrifuged at 6000 rpm for 10 minutes at 4°C to remove cell debris. The soluble supernatant was dialyzed against three changes of distilled water and freeze-dried at -50°C/0.021 mBa using a Freeze Dryer (Labconco, USA) [35].

3.5. Preparation of the purified cervical mucins

Purification of the mucins from another batch of 16 samples (half from each cohort) was performed by caesium chloride density gradient ultracentrifugation and Sepharose CL-2B gel-filtration [198]. The samples were adjusted to a density of 1.4 g/ml using caesium chloride (CsCl) and 4 M GuHCl. Ultracentrifugation was carried out in a 70Ti rotor using a Beckman ultracentrifuge (Beckman Coulter, South Africa) at 40000 rpm for 48 hours at 4°C. After centrifugation, each sample was divided into nine 1 cm fractions. These fractions were weighed for the determination of density,

after which they were assayed for glycoproteins and proteins using periodic acid Schiff (PAS) and Bradford assays respectively. The absorbance values readings were measured at 585 nm [199] using an Anthos HT3 plate reader (Anthos Labtec Instruments, USA) and graphs generated by Microsoft Excel (Microsoft, 2016).

The glycoprotein positive fractions, which fractionated at a density of 1.39 to 1.42 g/ml, were pooled and subjected to Sepharose CL-2B gel-filtration on a 100 ml column (100 x 2 cm) and eluted with 0.2 M NaCl and 0.02% sodium azide at a flow rate of 1 ml/minute using a Miniplus 3 peristaltic pump (Gilson, South Africa). About 110 fractions of 2 ml each were collected from each sample with FC 204 fraction collector (Gilson, South Africa), assayed for glycoprotein and protein contents using PAS and Bradford assays respectively, as mentioned above. The graphs were generated by Microsoft Excel (Microsoft, 2016). The glycoprotein-rich fractions that eluted in the exclusion volume (V_0) were pooled and dialysed against three changes of distilled water at 1:30 ratio, frozen at -80°C for 2 hours and freeze-dried at -50°C/0.021 mBa using a Freeze Dryer (Labconco, USA) [198].

3.6. Sodium dodecyl sulphate polyacrylamide-gel electrophoresis

The crude cervical mucus plugs and purified mucins were characterized by 4-20% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The freeze-dried samples were reconstituted in 1% of SDS loading buffer (62 mM Tris-HCl, pH 6.8; 2.5% SDS; 10% glycerol; 0.01% bromophenol blue and 1% β-mercaptoethanol) to a final concentration of 100 μg/ml and resolved on 4-20% of SDS-PAGE at 120 V, 300 mA for 1 hour using the Laemmli method [200]. To visualize proteins, the gels were washed with distilled water for 10 minutes and incubated with Vacutec Aqua stain for 1 hour. To visualize sugars, the gels were washed with distilled water for 1 hour and fixed with 50% ethanol for 30 minutes. After 10-minute wash with distilled water, sugars in the gel were oxidized with 50% periodic acid for 30 minutes and reduced with 0.1% sodium metabisulphite in 10 mM HCl for 20 minutes. The pink colour was developed by incubating the gels with a decolourized Schiff reagent for 1 hour [201]. The gels were covered with 3M flip-frame transparency protector (216 x 279 mm) and digitized using canon scanner (Canon, South Africa) to generate the TIF images.

3.7. Western blot analysis of the crude cervical mucus plug and purified mucins

To identify the major gel-forming mucins in the cervix by the Western blots, the samples were prepared and resolved on 4-20% SDS-PAGE gels as mentioned above [200]. Following resolution, the samples were blotted onto nitrocellulose membranes at 4 V, 64 mA for 2 hours using electro-blot SV20-SDB (Sigma-Aldrich, UK). The membranes were rinsed with distilled water and blocked with 5% skimmed milk in PBS at 4°C overnight. The membranes were then washed three times with PBST and probed with polyclonal antibodies (1:5 000) to MUC5AC (MAN-5ACI) and MUC5B (MAN-5BI) in 5% skimmed milk for 1 hour at room temperature [35]. The unbound primary antibodies were washed three times with PBST, after which the membranes were probed with alkaline phosphatase-conjugated secondary antibody (1:10 000) in 5% skimmed milk. The unbound secondary antibody was washed off three times with PBST, after which the blots were developed using nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indonyl phosphate (NBT/BCIP) as a substrate. The membranes were covered with 3M flip-frame transparency protector (216 x 279 mm) and digitized using canon scanner (Canon, South Africa) to generate the TIF images.

3.8. CellTiter-Glo luminescent viability assays

The CellTiter-Glo Luminescent Cell Viability Assay Kit, which quantifies the ATP molecules released by the metabolically active cells as a sign of viability, was used to assess the effect of the crude cervical mucus plugs and purified cervical mucins (samples) on the viability of TZMbl cells, clones of HeLa cells that have been engineered to overexpress CD4, CCR5 and luciferase [202]. The individual samples from the HIV negative and HIV positive groups were reconstituted in 10% Dulbbeco's Modified Eagle's Medium (DMEM), supplemented with penicillin-streptomycin (100 units/ml-100 μg/ml) and 2 mM L-glutamine, to a final concentration of 1 mg/ml. TZMbl cells were seeded overnight at $1x10^5$ cells/well in 96-well plates and incubated in triplicates with 1 mg/ml of the individual samples. Ten percent of SDS in 1 M HCl was used as a positive control for induction of cytotoxicity. The cells were incubated at 37°C/5% CO_2 for 48 hours. Post-incubation, the CellTiter-Glo substrate

was added directly into the cultures at 1:2 ratio and mixed for 2 minutes, followed by a 10-minute incubation in the dark to stabilize the luminescent signals. The samples (100 μl) were then transferred into 96-well luminometer plates in which the relative light units (RLUs) were measured using luminometer (Sirius, Berthold Detection Systems). The RLUs were background (media only control) subtracted and normalized to the negative control wells (untreated cells).

3.9. Production of the virus stocks and titration

HEK293T cells, which express SV40 T-antigen, were seeded in 10% DMEM at $2x10^6$ cells/10 cm cell culture dish overnight at 37°C/5% CO_2 and transfected with 60 μg of pro-viral plasmid DNA encoding full length SIV and HIV-1 strains. To produce HIV-1 strains carrying β-lactamase-Vpr, HEK293T cells were transfected with three DNA plasmids per 10 cm cell culture dish: pYU-2/pNL4.3 (60 μg), pBlaM-Vpr (20 μg) and pAdvantage (8 μg) [203, 204]. All the transfections were carried out using the CalPhos Mammalian Transfection Kit. Following a change of medium after 4-hour incubation, the cultures were incubated at 37°C/5% CO_2 for 48-72 hours. Post transfection, the cell-free virus particles were harvested from the supernatants, filtered through 0.45 μm filters (Millipore, Germany) and concentrated using 20% sucrose cushion method (30000 rpm, 4°C, 1.5 hours) [202]. The concentrated viruses were titrated on TZMbl cells using β-galactosidase-based blue cell assay [202]. The primary isolate of $HIV\text{-}1_{BaL}$ was obtained from the NIH AIDS Reagent Programme (USA) and passaged in PM1 cells cultured in 10% Roswell Park Memorial Institute (RPMI) 1640 medium [205].

3.10. TZMbl cell-based luciferase assays

TZMbl cells in 10% DMEM were seeded at $1x10^5$ cells/well in 96-well plates and incubated at 37°C/5% CO_2 overnight. The viruses were incubated with two-fold serially diluted samples for 1 hour at 37°C/5% CO_2, before infecting TZMbl cells for 48 hours at 37°C/5% CO_2. Post-infection, the supernatant was aspirated, after which the cells were lysed with 1% Cell Culture Lysis Buffer for 10 minutes at room temperature. Ten microliters of the cell lysate were transferred into 96-well

luminometer plates and mixed with 30 μl of Luciferase Assay Substrate. The RLUs were measured using luminometer (Sirius, Berthold Detection Systems). The background was subtracted using the untreated wells, divided by the RLUs from the virus control wells and multiplied by 100 to generate percentage infection: % infection = [average RLU (treated)—average RLU (CC)/average (VC)—average RLU (CC)] [206]. The data were analysed using GraphPad Prism v5 (La Jolla, CA, USA).

3.11. Heat inactivation assays

To investigate the pro-SIV_{mac239} activity of sample 47, which was absent in other samples, an aliquot (1 mg/ml) was heated at 96°C for 20 minutes at 600 rpm using Eppendorf Thermomixer (Eppendorf, Germany) and cooled down for 10 minutes at 4°C. SIV_{mac239} was incubated with the heated and unheated aliquots of sample 47 for 1 hour at 37°C/5% CO_2. In 96-well plates, TZMbl cells ($1x10^5$ cells/well) were infected in triplicates with the mucus-virus mixtures for 48 hours at 37°C/5% CO_2. Post-infection, the luciferase assays were conducted to quantify luciferase expression in TZMbl cells as mentioned above. The data were analysed using GraphPad Prism v5 (La Jolla, CA, USA).

3.12. Time-of-addition assays

In 10% DMEM, TZMbl cells were seeded in 96-well plates overnight at $1x10^5$ cells/well prior to infection with $HIV\text{-}1_{NL4.3}$. These assays were conducted under three independent conditions to characterize the mode of action by which the crude cervical mucus plugs potently inhibit HIV-1. Firstly, TZMbl cells were incubated with cervical mucus plugs for 1 hour at 37°C/5% CO_2, prior to infection with $HIV\text{-}1_{NL4.3}$ for 48 hours. Secondly, TZMbl cells were infected with $HIV\text{-}1_{NL4.3}$ for 1 hour at 37°C/5% CO_2 and incubated with cervical mucus plugs for 48 hours. Thirdly, $HIV\text{-}1_{NL4.3}$ was incubated with cervical mucus plugs for 1 hour at 37°C/5% CO_2 prior to infection of TZMbl cells for 48 hours. Following 48-hour incubations at 37°C/5% CO_2, the luciferase assays were conducted to quantify the luciferase expression in TZMbl cells as mentioned above. The data were analysed using GraphPad Prism v5 (La Jolla, CA, USA).

3.13. The β-lactamase-based HIV-1 fusion assay

TZMbl cells in 10% DMEM were seeded at $2x10^5$/well in 12-well plates overnight at 37°C/5% CO_2. On the next day, the BlaM-Vpr HIV-1 strains were incubated with samples (1 mg/ml) for 1 hour at 37°C/5% CO_2. As the positive controls for inhibition of infection, the cells were either incubated with 25 μM maraviroc (HIV-1_{YU-2} BlaM-Vpr) or 50 μM T20 (HIV-$1_{NL4.3}$ BlaM-Vpr) for 1 hour prior to infection. The cells were infected with the virus-sample mixture for 4 hours at 37°C/5% CO_2, after which the infections were stopped. Post-infection, the cells were washed with 10% CO_2 independent medium and then phosphate buffered saline (PBS) before trypsinization for 4 minutes at 37°C/5% CO_2. The cells were then re-suspended in 10% CO_2 independent medium and centrifuged (5000 rpm, 5 minutes, 4°C). The cell pellets were washed, re-suspended in 10% CO_2 independent medium, centrifuged (5000 rpm, 5 minutes, 4°C) and stained with CCF4 overnight at room temperature. The stained cells were washed with PBS, centrifuged (5000 rpm, 5 minutes, 4°C) and fixed with 4% PFA for 90 minutes at room temperature. The cells were given a final PBS wash, centrifuged (5000 rpm, 5 minutes, 4°C) and re-suspended in PBS. The CCF4 spectral shift was analysed by acquiring 20 000 events in FITC-A and Horizon V450-A channels using a multi-parameter LSR II flow cytometry (BD Biosciences). The data were analysed by FlowJo v10 (Tree Star, Ashland, USA) and GraphPad Prism v5 (La Jolla, CA, USA).

3.14. Cell-to-cell infection assays

As the donor cells, HEK293T cells in 10% DMEM were seeded overnight at $2x10^5$ cells/well in 24-well plates and transfected with 2 μg of pro-viral DNA plasmid encoding full length HIV-$1_{NL4.3-GFP}$ using the CalPhos Mammalian Transfection Kit. After 4 hours of incubation, the medium was changed followed by incubation of cultures for 24 hours at 37°C/5% CO_2. The virus-producing HEK293T cells were incubated (1:2 ratio) with 1 mg/ml of crude cervical mucus plug for 1 hour at 37°C/5% CO_2 and co-cultured with parental Jurkat T-cells ($1x10^6$ cells/well), which are the immortalized $CD4^+$ human T-lymphocytes that are sensitive to HIV-1 infection, for 24 hours at 37°C/5% CO_2 in the presence or absence of efavirenz.

Parental Jurkat T-cells were maintained in 10% RPMI 1640 medium supplemented with Pen-Strep and L-glutamine prior to co-culture experiments. As the negative and positive controls, parental Jurkat T-cells were co-cultured with HEK293T cells in the absence or presence of efavirenz respectively. Post-infection, the cells were harvested, PBS-washed and fixed with 4% PFA for 90 minutes at room temperature. The GPF signal from parental Jurkat T-cells was measured by acquiring 50 000 events in FL1-H and FL4-H channels using FACS-Calibur flow cytometry (BD Bioscience), The data were analysed using FlowJo v10 (Tree Star, Ashland, USA) by gating on the live population of parental Jurkat T-cells to exclude HEK293T cells and other cell debris. Statistical analysis was performed using GraphPad Prism v5 (La Jolla, CA, USA).

3.15. Jurkat T-cell-based infection assays in the context of cell-free virus

Cell-free HIV-$1_{NL4.3\text{-}GFP}$ was incubated with 1 mg/ml of cervical mucus plugs at 1:2 ratio for 1 hour at 37°C/5% CO_2, prior to 48-hour infection of parental Jurkat T-cells at $1x10^6$ cells/well in 12-well plates. As the positive control for inhibition of infection, parental Jurkat T-cells were incubated with 25 μM efavirenz for 1 hour at 37°C/5% CO_2 prior to infection with HIV-$1_{NL4.3\text{-}GFP}$ for 48 hours. Post-infection, the cells were harvested, washed with PBS, centrifuged (5000 rpm, 5 minutes, 4°C) and fixed with 4% PFA for 90 minutes at room temperature. The GFP signal was measured by acquiring 50 000 events in FL1-H and FL4-H channels using FACS-Calibur flow cytometry (BD Bioscience). The data were analysed using FlowJo v10 (Tree Star, Ashland, USA) and GraphPad Prism v5 (La Jolla, CA, USA).

3.16. Statistical analysis

One-way ANOVA (nonparametric) coupled to Dunnett's multiple comparison test was used to compare the means. The descriptive statistics is represented by the mean and standard error of the mean (SEM), where $P \leq 0.05$ was considered significant. The statistical tests were conducted using GraphPad Prism v5 (La Jolla, CA, USA).

Chapter – 4: Results

4.1. Clinical profile of the study cohort

This study comprised 52 women of African black ethnicity with a mean age of 25.5 years, 28 (53.8%) of whom were HIV negative and 24 (46.2%) were HIV positive. There are no clinical data for 16 of the purified cervical mucin samples, eight from each cohort of HIV negative and HIV positive women. For the crude cervical mucus samples, one of the HIV negative women presented with an *E. coli* positive urinary tract infection, which was consecutively treated with a cocktail of antibiotics: 500 mg amoxicillin, 375 mg augmentin, 960 mg co-trimoxazole and 2 g metronidazole. The rest of the HIV negative women presented with no symptoms of genitourinary tract infection during hospitalization. One HIV positive woman presented with minor genital warts for which she was not treated prior to sample collection. Another one presented with an offensive vaginal discharge for which she was treated with 500 mg amoxicillin, 1 g ampicillin, 400 mg metronidazole and one nystatin vaginal pessary. The CD4 count ranged between 270-727 cells/mm^3 (mean 413 cells/mm^3) for the HIV positive cohort, whose members were all on HAART treatment, with a duration range of 1 to 36 months. The viral load ranged from being undetectable to 4980 RNA copies/ml.

4.2. Biochemical purification of the cervical mucins

Mucin purification and isolation from individual samples was performed by a single density gradient ultracentrifugation step in CsCl, followed by gel-filtration on a Sepharose CL-2B column to enrich the population of large polymeric mucins eluting in the excluded volume (V_0) of the column, and separate from the degraded subunits and any low molecular weight protein contaminants eluting in the included volume (V_i) [198]. In agreement with Carlstedt et al [198], the cervical mucins fractionated at an average density of ~1.4 g/ml, high in PAS positive material and low in protein content (Fig. 1A-1B). The glycoprotein-rich fractions (fractions 3-6) were pooled from individual samples for subsequent assays. The purification graphs show that there were no biochemical differences between the HIV negative and HIV positive cohorts.

The gel-filtration graphs showed a clear separation of V_0 and V_i for PAS positive material and the protein content for both cohorts (Fig. 1C & 1D). The high molecular weight mucin polymers eluted in the V_0 fractions while the low molecular weight proteins and glycoproteins eluted in the V_i fractions (Fig. 1C & Fig. 1D) [201]. The glycoprotein-rich V_0 fractions were pooled, dialyzed against three changes of distilled water and freeze-dried. The samples from both cohorts showed comparable glycoprotein and protein content, which eluted at about the same fractions, thereby suggesting similar biochemical characteristics of mucins from the HIV negative and HIV positive cohorts.

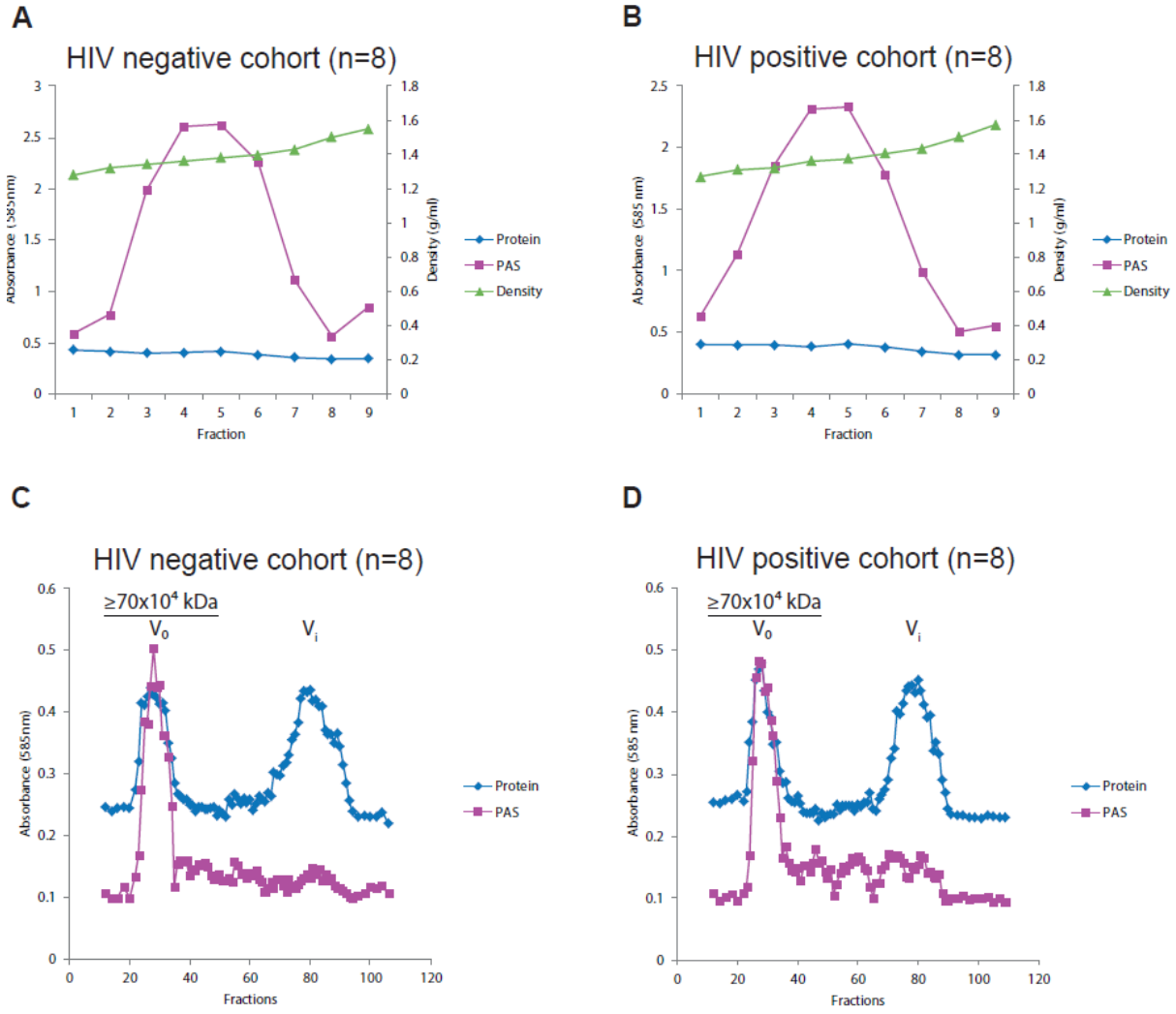


Figure 1: Biochemical preparation of the cervical mucins. Mucins were purified from individual cervical mucus plugs that were collected from the HIV negative and HIV positive cohorts by caesium chloride density gradient ultracentrifugation (**A** & **B**). Purified cervical mucins from the HIV negative (**A**) and HIV positive (**B**) cohorts were further subjected to Sepharose CL-2B gel-filtration (cut-off $70x10^4$ kDa) (**C** & **D**) and assayed by PAS and Bradford assays for glycoproteins and proteins respectively.

4.3. Sodium dodecyl polyacrylamide-gel electrophoresis

The crude cervical mucus plugs and purified cervical mucins were resolved on 4-20% gradient SDS-PAGE gels in parallel and the gels were stained for glycoproteins and proteins with PAS and Vacutec AquaStain respectively, to assess the degree of purity of mucins following gel-filtrations. Following protein staining (Fig. 2A & 2C), the crude cervical mucus plugs showed a spectrum of proteins with different electrophoretic mobilities spanning the entire length of the gels. The protein staining intensified with decreasing molecular weight (Fig. 2A & 2C), suggesting a high presence of fast-migrating species. Some low intense material was seen stuck in the spacer gel, suggesting the presence of high molecular weight species, likely glycoproteins with inaccessible protein backbones, hence the poor protein staining (Fig. 2A & 2C). The individual samples exhibited different intensities, with ~130 kDa band among certain samples (Fig. 2A; samples 1, 2, 4, 18 & 32; Fig. 2C; samples 12, 28 & 48) regardless of the donor's HIV status. There was also a ~250 kDa band exclusively present among certain samples from the HIV positive cohort (Fig. 2C; samples 7, 12, 14 & 28). The PAS stain (Fig. 2B & 2D) was clearly of large size, with material in the spacer gel, at the beginning of the running gel and even entering the running gel, suggesting presence of slowly migrating and heavily glycosylated species, a typical electrophoretic pattern of the mucins in SDS-PAGE [198, 201, 207]. The intensities of the ~250 and ~130 kDa bands decreased following PAS stain (Fig. 2B; samples 1, 2, 4, 18 & 32; Fig. 2D; samples 12, 28 & 48), suggesting a lesser degree of glycosylation. These two bands correspond with a molecular weight range of intact IgA and IgG [208], the dominating immunoglobulins in the cervical mucus plugs [155]. Several protein bands of lower molecular weight were lost in several samples following PAS staining, while certain samples retained some PAS positive bands of ~55 kDa (Fig. 2B; samples 1, 2, 4, 18 & 39; Fig. 2D; samples 12, 14 & 28), corresponding to the molecular weights of heavy chains of IgA and IgG [155, 208], and α-1 acid glycoprotein [209].

Compared to the crude cervical mucus plugs, protein staining of the purified cervical mucins in the V_0 fractions showed an effective removal of the low molecular weight species and retention of the high molecular weight species (Fig. 2E; samples 8, 10,

11 & 24; Fig. 2G; samples 30, 40, 43 & 44). There were low molecular species in the V_i fractions as expected, dominated by a ~55 kDa band (Fig. 2E; samples 8, 10 & 11; Fig. 2G; samples 30, 40, 43 & 44), suggesting protein separation based on the molecular weights as reflected in the gel-filtration profiles (Fig. 1C & 1D). This ~55 kDa band in the V_i fractions was independent of the donor's HIV status and could be a heavy chain of an immunoglobulin [155, 208] or α-1 acid glycoprotein [209]. PAS staining (Fig. 2F & 2H) confirmed that the slowly migrating species on top of the gels are heavily glycosylated species of higher molecular weight. Also, the intensity of ~55 kDa band in the V_0 fractions decreased following PAS (Fig. 2F; samples 8, 10 & 11; Fig. 2H; samples 30, 40, 43 & 44), suggesting a lesser degree of glycosylation.

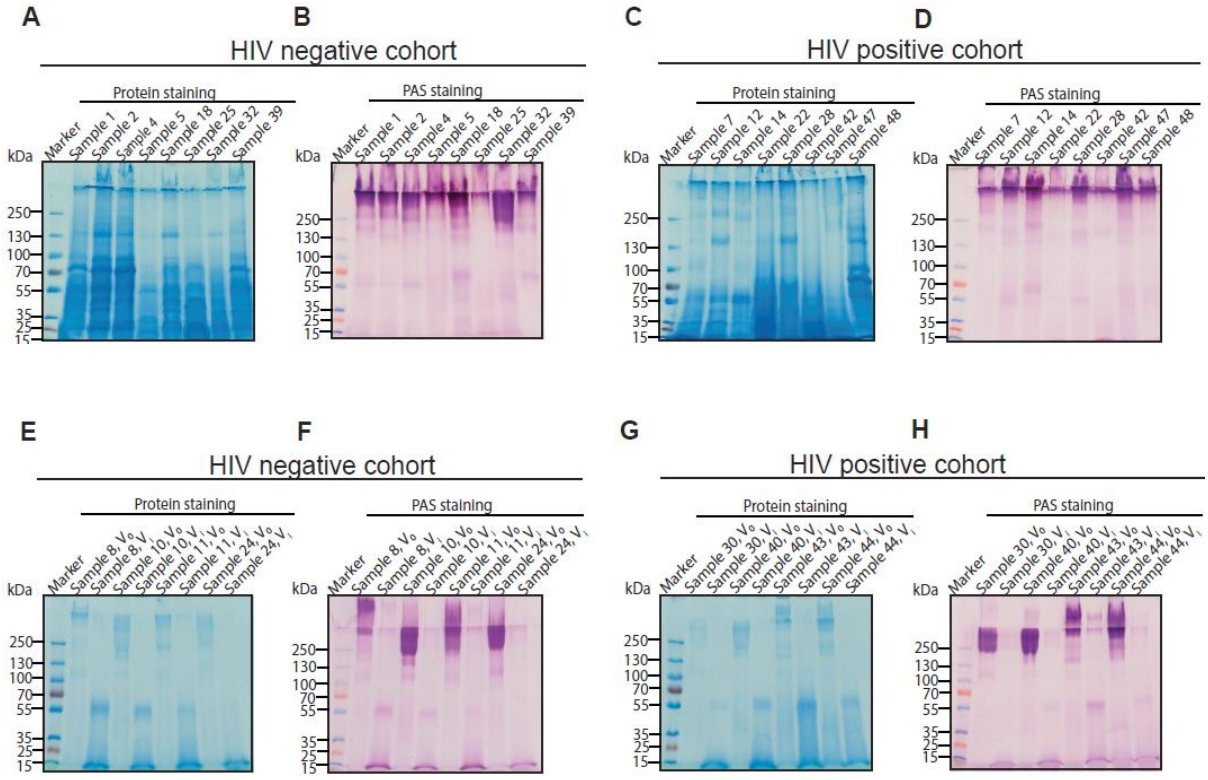


Figure 2: Characterization of the crude cervical mucus plugs and purified cervical mucins by SDS-PAGE. The individual crude cervical mucus plugs from the study cohorts were prepared by slow stirring overnight at 4°C and centrifuged at 600 rpm for 10 minutes at 4ºC to remove cell debris. The supernatants were dialyzed against distilled water (ratio1:30) and freeze-dried. The freeze-dried crude cervical mucus samples (**A-D**) from the HIV negative (**A-B**) and HIV positive (**C-D**) cohorts were reconstituted in 1% SDS loading buffer and resolved on 4-20% gradient gels. The gels were stained with Vacutec AquaStain and PAS for proteins and glycoproteins respectively. The purified cervical mucins (**E-H**) from HIV negative (**E-F**) and HIV positive (**G-H**) cohorts were characterized on 4-20% gradient SDS-PAGE as mentioned above. For each sample, the V_0 and V_i were analysed in parallel.

4.4. Western blot identification of the gel-forming cervical mucins

MUC5AC and MUC5B, the predominant gel-forming mucins in the endo-cervix [96] were identified using Western blots. The crude cervical mucus plugs from the HIV negative cohort showed a positive signal for both MUC5AC (Fig. 3A; samples 1, 2, 4, 5, 18, 25, 32 & 39) and MUC5B (Fig. 3A; samples 2, 4, 5, 18, 25, 32 & 39), with varying intensities between individual samples. However, sample 1 was negative for MUC5B (Fig. 3B). The samples from the HIV positive cohort were also positive for MUC5AC (Fig. 3C; samples 7, 12, 14, 22, 28, 42 & 48) and MUC5B (Fig. 3D; samples 7, 12, 14, 22, 28, & 48), with samples 42 and 47 showing trace amounts of MUC5B. Generally, the gels show a high intensity of bands from the HIV positive cohort (Fig. 3C & 3D). Purified cervical mucins generally showed cleaner and intense bands for the respective mucins (Fig. 3E-3H) compared to the crude cervical mucus plugs (Fig. 3A-3D). This suggests an improved access of the antibodies to the mucin antigens due to effective removal of other hundreds of proteins found in the cervical mucus plugs as reported by the mass spectrometry studies [121, 174]. The samples from HIV negative cohort showed low signals for MUC5AC (Fig. 3E) compared to MUC5B (Fig. 3F). The intensity of MUC5AC (Fig. 3G) signal was high in the HIV positive cohort, while that of MUC5B also increased among certain samples (Fig. 3H; samples 9, 15, 30 & 43). In the context of MUC5AC, these data confirm a recognized effect of HIV infection on *MUC* gene regulation [210].

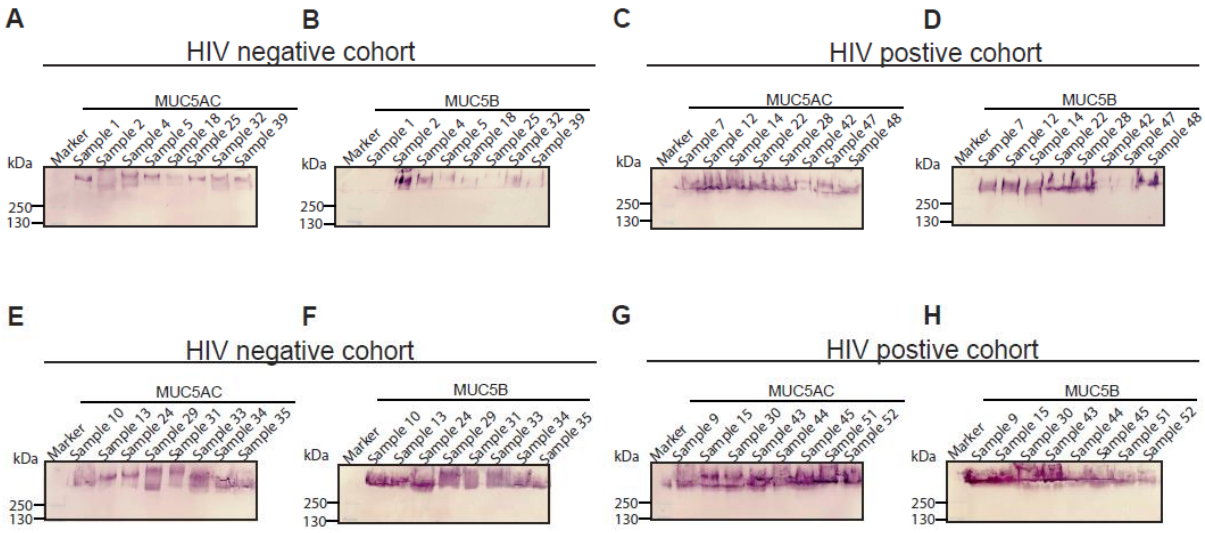
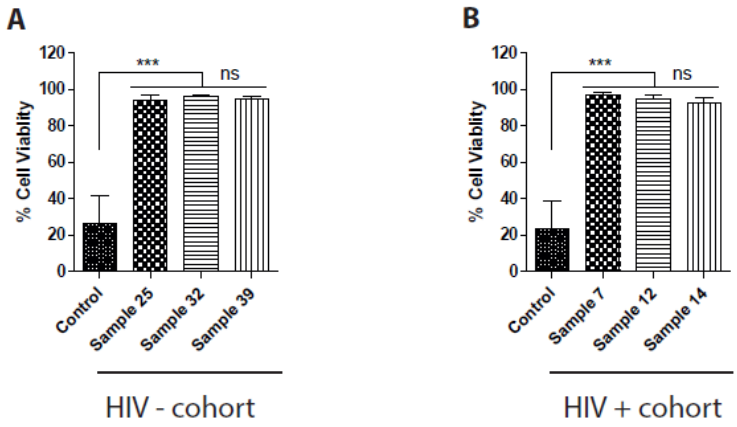


Figure 3: Identification of the major gel-forming cervical mucins. The freeze-dried crude cervical mucus plug (**A-D**) and purified cervical mucins (**E-H**) samples

from the HIV negative and HIV positive cohorts were reconstituted in 1% SDS loading buffer and resolved on 4-20% SDS-PAGE. The proteins were blotted onto nitrocellulose membranes, which were probed with anti-MUC5AC and anti-MUC5B rabbit polyclonal antibodies, washed and re-probed with goat anti-rabbit monoclonal secondary antibody. The protein bands were developed using NBT/BCIP substrate.

4.5. Cell viability assays

The CellTiter-Glo Luminescent Cell Viability Assay was used to investigate the extent to which TZMbl cells tolerate treatment with the crude cervical mucus plugs and purified cervical mucins (samples). This assay quantifies the ATP molecules generated by the metabolically active cells as a measure of cell viability. The samples were reconstituted in 10% DMEM to a final concentration of 1 mg/ml. TZMbl cells were incubated with the samples for 48 hours at 37°C/5% CO_2, followed by quantification of the generated ATP molecules in the form of relative light units (RLUs) using luminometer (Sirius, Berthold Detection System), which were normalized to the negative control. Following incubation with the crude cervical mucus plug and purified cervical mucins (Fig. 4A-D), TZMbl cells showed an average 99% of cell viability compared to those incubated with 10% SDS in 1 M HCl (positive control), which showed significantly decreased cell viability. There were no well-defined differences on the cell viabilities between the crude cervical mucus plugs and purified cervical mucins nor were there any HIV status-based differences. These data show that TZMbl cells can equally tolerate the crude cervical mucus plugs and purified cervical mucins regardless of the donor's HIV status, while reflecting the efficiency of dialysis in removing high salt content used during the biochemical preparation of the study samples.



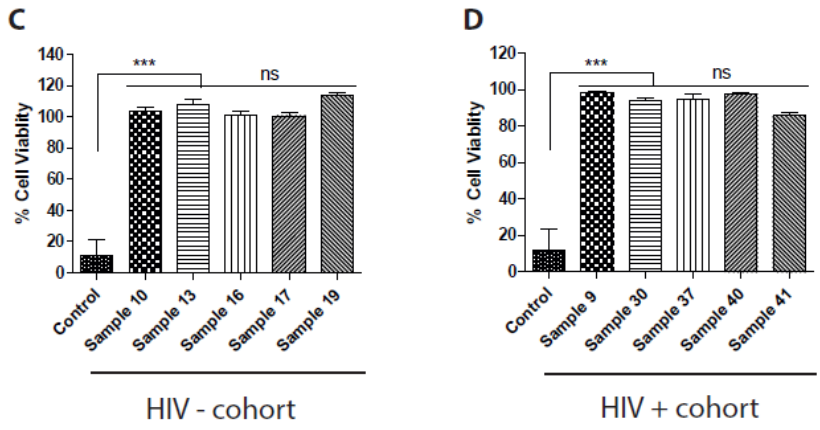


Figure 4: Effect of the crude cervical mucus and purified cervical mucins on the viability of TZMbl cells. The freeze-dried crude cervical mucus plugs (**A-B**) and purified cervical mucins (**C-D**) from the HIV negative and HIV positive cohorts were reconstituted in 10% DMEM to a final concentration of 1 mg/ml. The individual samples were used to inoculate TZMbl cells in triplicates for 48 hours, after which the ATP molecules were quantified from the cell lysates using a CellTiter-Glo Luminescent Cell Viability Kit. The data were analysed by GraphPad Prism v5 using One-way ANOVA (nonparametric) to compare the means. Error bars represent the mean ± SEM of the triplicates from one experiment.

4.6. Anti-HIV-1 activity of the crude cervical mucus plugs in the context of cell-free virus: Firefly luciferase-based reporter assay

The aim was to extend the findings of Habte et al [35] in our laboratory, who reported that the crude cervical mucus plugs from the HIV negative women do not inhibit HIV-1 in the *in vitro* assays, despite comprising a spectrum of immunological factors [121], some of which are absent in the cervical mucus of non-pregnant women [173]. This study further had crude cervical mucus plugs from the HIV positive cohort, while Habte et al [35] only used samples from the HIV negative cohort. We hoped to detect the difference, if any, between samples from the HIV negative and HIV positive cohorts. Unlike Habte et al [35], the samples were probed individually against a panel of HIV-1 (plus SIV_{mac239}) replication-competent strains from different subtypes and tropisms to learn more about their anti-viral coverage from different aspects. Infection efficiency was analysed in TZMbl cells using luciferase assays that quantify infection on the basis of increasing or decreasing signals in HIV's Tat-driven fire fly luciferase reporter gene expression following infection.

TZMbl cells were incubated with 2-fold serially diluted samples for 1 hour at 37°C/5% CO_2, followed by 48-hour infection under similar conditions, after which the infections were analysed using luciferase assays. Unlike Habte et al [35] who showed that the crude cervical mucus plugs do not inhibit HIV-1 in an *in vitro* assay, the cervical mucus plugs generally inhibited several strains of HIV-1, irrespective of the donor's HIV status. The samples from different donors exerted a selective inhibition against HIV-1 infection, depending on the infecting strain. Most samples collected from the HIV positive cohort were more potent compared to most samples collected from the HIV negative cohort, probably due to the ability of cervical mucus plugs to retain HIV-specific antibodies from the circulatory system [174] and HAART regimens [211]. However, sample 25 (Fig. 5F) from the HIV negative cohort was just as potent against several strains of HIV-1. This suggest that this potency is not exclusively mediated by HIV infection, but presumably by certain factors that may vary between individuals in terms of antimicrobial content of the cervical mucus plugs.

From a panel of study viruses, HIV-1_{BaL} proved to be the most refractory strain, and its refractoriness was more pronounced in the HIV negative cohort (Fig. 5A-5H), where several samples showed a slight enhancement of HIV-1_{BaL} infection. Sample 7 (Fig. 5I) from the HIV positive cohort exhibited a similar characteristic, suggesting that this refractoriness of HIV-1_{BaL} is not exclusively mediated by HIV infection, but probably by the inter-individual variations on which HIV infection may impact. While certain samples showed slight enhancement of HIV-1_{BaL} infection, sample 47 (Fig. 5O) showed a potent inhibition compared to all the study samples. Surprisingly, the same sample 47 (Fig. 5O) drastically enhanced SIV_{mac239} infection, highlighting the heterogeneity between and within cervical secretions [36, 212].

Depending on the infecting HIV-1 strain, certain samples potently suppressed HIV-1 infection, but that was hardly seen for SIV_{mac239}, even from the most potent samples despite the donor's HIV status. Except for sample 47, the inhibition profile of SIV_{mac239} consistently began with high sensitivity to the crude cervical mucus plugs and quickly gained resistance before the HIV-1 strains, after which further increase in cervical mucus plug concentration had little or no effect on it. SIV_{mac239}, a rhesus macaque immunodeficiency-causing virus, was included to extend our investigation

to other non-HIV mucosal transmitted viruses. From a tropism point of view, the X4 strains (HIV-$1_{NL4.3}$ & HIV-1_{LAI}) were more susceptible to inhibition by the crude cervical mucus plugs compared to the R5 strains of HIV-1 and SIV (HIV-1_{BaL}, HIV-1_{YU-2}, HIV-1_{CH198} & SIV_{ma239}), coherent with a recognized predominant exclusion of the X4 viruses and selective transmission of the R5 viruses at the female genital mucosal surfaces during the initial stages of HIV infection [213, 214].

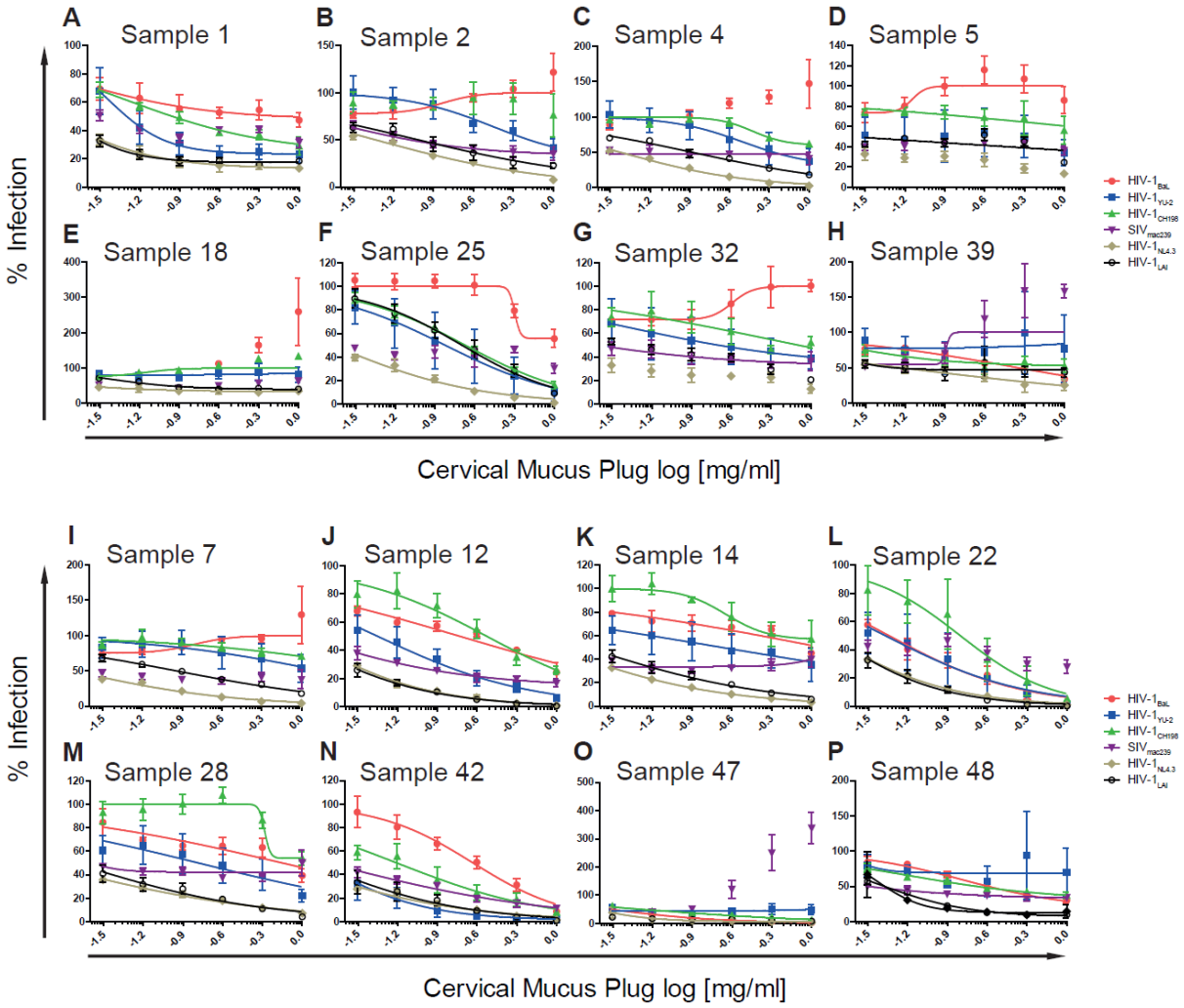


Figure 5: Luciferase reporter assay-based analysis of the anti-viral activity of the crude cervical mucus plugs in the context of cell-free virus. Crude cervical mucus plugs from the HIV negative (**A-H**) and HIV positive (**I-P**) cohorts were probed against a panel of replication-competent strains of HIV-1 and SIV_{mac239} in the context of cell-free virus. The viruses were incubated with two-fold serially diluted individual crude cervical mucus plugs for 1 hour at 37°C/5% CO_2 in a humidified incubator prior to infection of TZMbl cells for 48 hours, after which luciferase expression was measured using Luciferase Assay System. The dose-response curves were generated by GraphPad Prism v5 using One-way ANOVA (nonparametric) analysis to compare the means. The error bars represent the mean ± SEM of duplicates from three experiments.

4.7. Anti-HIV-1 activity of the purified cervical mucins in the context of cell-free virus: Firefly luciferase-based reporter assay

The anti-HIV-1 activity of the purified cervical mucins from the HIV negative cohort has been reported [35], but not in comparison to that of the purified cervical mucins from the HIV positive cohort. Due to limited quantity of the purified cervical mucins, the samples were probed three times in duplicates against two HIV-1 strains that were less (HIV-1_{BaL}) and more (HIV-$1_{NL4.3}$) susceptible to inhibition by the crude cervical mucus plugs respectively (Fig. 5). The inhibition assays were conducted as mentioned above, after which the purified cervical mucins showed comparable anti-HIV-1 activity regardless of the donor's HIV status, suggesting that HIV infection does not appreciably change the anti-HIV-1 activity of the cervical mucins (Fig. 6). However, there was a noticeable evidence of the inter-individual variations [121].

Unlike the crude cervical mucus plugs, there was no well-defined selective inhibition against the infecting HIV-1 strains in the context of purified cervical mucins and the inhibition profiles were comparable. There was no enhancement of HIV-1 infection by the purified cervical mucins, suggesting that at least this enhancement is not exclusively mediated by the mucins. The purified cervical mucins did not show selective transmission of the R5 strains over X4 strains, suggesting that the mucins alone do not mediate this gatekeeping character, and if they do at all, they likely do so synergistically with other components of the cervical mucus plug. Interestingly, unlike the crude cervical mucus plugs from the HIV negative cohort that could barely inhibit HIV-1_{BaL} (Fig. 5A-5H), the purified cervical mucins from the HIV negative cohort inhibited HIV-1_{BaL} (Fig. 6A-6H). On the other hand, the crude cervical mucus plugs showed high potency against HIV-$1_{NL4.3}$ (Fig. 5A-5H), while the purified mucins showed less potency (Fig. 6A-6H), suggesting that the mucins alone are not responsible for the varying potencies seen in the context of crude cervical mucus plugs. In overall, the purified cervical mucins were less potent compared to the crude cervical mucus plugs, most likely due to the elimination of non-mucin factors found in the cervical mucus plugs [121], which could probably synergize with mucins against HIV-1.

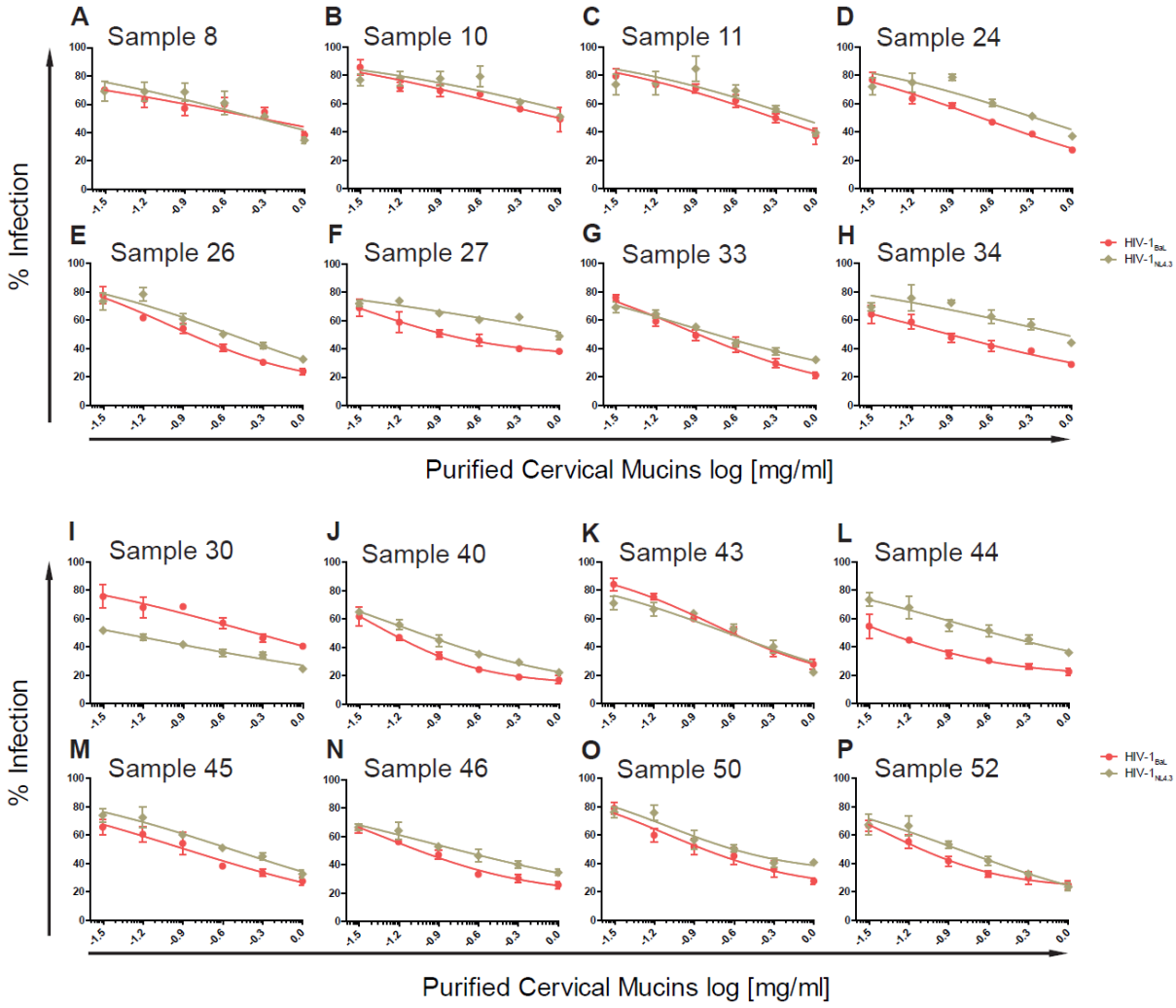


Figure 6: Luciferase reporter assay-based analysis of the anti-viral activity of the purified cervical mucins in the context of cell-free virus. Purified cervical mucins from the HIV negative (**A-H**) and HIV positive (**I-P**) cohorts were probed against a panel of replication-competent strains of HIV-1 in the context of cell-free virus. The viruses were incubated with two-fold serially diluted purified cervical mucins for 1 hour at 37°C/5% CO_2 in a humidified incubator prior to infection of TZMbl cells for 48 hours, after which luciferase expression was measured using a Luciferase Assay System. The dose-response curves were generated by GraphPad Prism v5 using One-way ANOVA (nonparametric) analysis to compare the means. The error bars represent the mean ± SEM of duplicates from three experiments.

4.8. Enhancement of SIV_{mac239} infection by crude cervical mucus plug

The pro-SIV_{mac239} activity of sample 47 was investigated under heated (96°C, 20 minutes, 600 rpm) and unheated conditions. The aim was to heat-inactivate the potential infection-enhancing factors and reduce their activity if they are proteins/glycoproteins, since they are generally susceptible to heat inactivation.

SIV_{mac239} was incubated with heated (but cooled down) and unheated aliquots of sample 47 for 1 hour at 37°C/5% CO_2, before infecting TZMbl cells for 48 hours under similar conditions. Post-infection, the firefly luciferase assays were conducted to assess the level of infection. Interestingly, the enhancement of SIV_{mac239} infection was significantly reduced after heat inactivation of sample 47 compared to its unheated aliquot (Fig. 7B), suggesting that these pro-SIV_{mac239} factors are more likely to be proteins or glycoproteins.

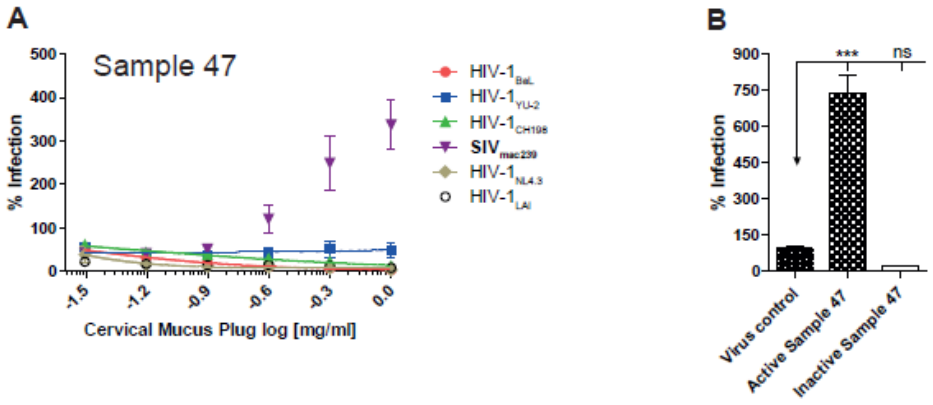


Figure 7: Pro-SIV_{mac239} activity of sample 47 from an HIV positive patient. After detecting an enhanced infectivity of SIV_{mac239} (**A**), sample 47 was probed against SIV_{mac239} under unheated (active) and heated (inactive) conditions before infecting TZMbl cells in triplicates for 48 hours. Post-infection, luciferase expression was measured from the cell lysates of TZMbl cells using Luciferase Assay System. The data from triplicates of one experiment were analysed by GraphPad Prism v5 (**B**) using One-way ANOVA (nonparametric). The error bars represent the mean ± SEM.

4.9. Characterizing the mode of action of the crude cervical mucus plug against HIV-1

The mode of action that the cervical mucus plugs must employ in order to provide an efficient barrier against the invading pathogens was investigated under three conditions: incubation of TZMbl cells with cervical mucus plugs for 1 hour prior to HIV-1 infection, HIV-1 infection of TZMbl cells for 1 hour prior to incubation with cervical mucus plugs and incubation of HIV-1 with the cervical mucus plugs for 1 hour prior to infection of TZMbl cells respectively (Fig. 8A). The cultures were incubated at 37°C/5% CO_2 for 48 hours, followed by the luciferase assays to measure the level of infection.

Compared to the virus only control, there was significant inhibition of HIV infection across all the conditions despite the donor's HIV status (Fig. 8B-8E). The incubation of TZMbl cells with cervical mucus plugs prior to infection showed the least inhibition of HIV-1 infection across all samples. One-hour infection of TZMbl cells prior to incubation with cervical mucus plugs showed a second-best inhibition of HIV-1 infection, which partly varied between samples since there was no such evidence in sample 4 (Fig. 8C). The incubation of HIV-1 with cervical mucus plugs prior to infection of TZMbl cells showed the most potent inhibition of HIV-1 when compared to other conditions. Together, these data point to the cervical mucus plug as more of a virus-directed inhibitor than a cell-directed inhibitor, thereby supporting its role in the selective transmission of HIV-1 strains seen in the luciferase assays (Fig. 5).

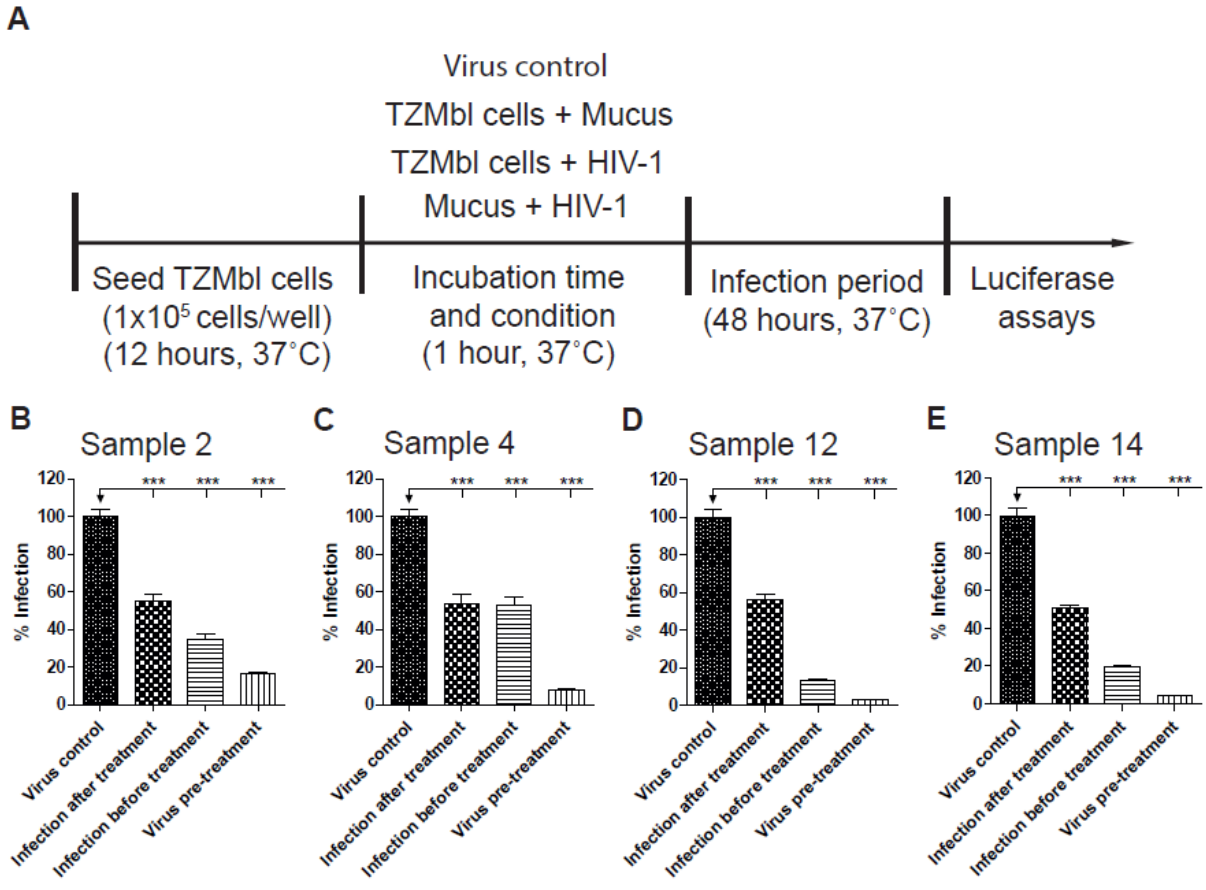


Figure 8: Mode of action through which the crude cervical mucus plugs inhibit HIV-1 infection. Schematic illustration showing a sequence of events conducted during the time-of-addition assays (**A**). Mode of action by the crude cervical mucus plugs from the HIV negative (**B-C**) and HIV positive (**D-E**) cohorts was studied using replication-competent HIV-$1_{NL4.3}$ under three conditions: one-hour TZMbl cells incubation with mucus prior to infection (infection after treatment), one-hour TZMbl cells infection with virus prior to incubation with mucus (infection before treatment)

and one-hour incubation of HIV-$1_{NL4.3}$ with mucus prior to infection of TZMbl cells (virus pre-treatment) at 37°C/5% CO_2. Cell lysates from TZMbl cells were used to measure luciferase expression after 48 hours by Luciferase Assay System. The data were analysed by GraphPad Prism v5 using One-way ANOVA (nonparametric) and the error bars represent the mean ± SEM of triplicates from one experiment.

4.10. β-lactamase-Vpr fusion assay in the context of HIV-1_{YU-2}: Crude cervical mucus plugs

The β-lactamase-based fusion assay was performed to investigate the stage of HIV-1 replication at which the cervical mucus plugs inhibit HIV-1 infection, with the aim of recapitulating the data from the luciferase assays (Fig. 5). This flow cytometry-based fusion assay measures a spectral shift in CCF4 due to the β-lactamase (BlaM)-mediated cleavage following HIV fusion with a target cell [203, 204]. HIV-1_{YU2} BlaM-Vpr was incubated with cervical mucus plugs (1 mg/ml) for 1 hour at 37°C/5% CO_2 before infecting TZMbl cells in triplicates for 4 hours under similar conditions. The cells were CCF4-stained overnight at room temperature, after which the CCF4 spectral shift was analysed by acquiring 20 000 events using a multi-parameter LSR II flow cytometry (BD Biosciences). The untreated cells, HIV-1_{YU-2} BlaM-Vpr and maraviroc-treated cells were used as controls, and the data were analysed using FlowJo v10 (Tree Star, Ashland, USA) and GraphPad Prism v5 (La Jolla, CA, USA).

Using unstained mock TZMbl cells, a scatter plot (FSC-A/FSC-H) was generated, from which the live population of singlets was gated and a CCF4-stained mock used to set up a gate. The gate was applied to the test samples to determine the spectral shift of CCF4 as the results of β-lactamase-mediated cleavage, which indicates successful membrane fusion between HIV and a target cell. The data (Fig. 9A) show that, irrespective of the donor's HIV status, certain crude cervical mucus plugs inhibit membrane fusion between HIV-1 and the target cells.

However, this may not be the only stage of HIV replication at which these cervical mucus samples inhibit HIV infection, since we have only looked at the fusion stage that typically comes after attachment—two essential events that HIV performs outside a target cell. Given the degree of heterogeneity between and within the samples, it could also be that some samples block viral attachment to the target cell

before fusion or even act at the post-entry stages. Accordingly, samples 4 (partially) and 22 (potently) inhibited HIV-1$_{YU-2}$ infection in the luciferase assays (Fig. 5C & 5L), but quantification of the fusion assays (Fig. 9B) shows intact membrane fusion of HIV-1$_{YU-2}$ with TZMbl cells in the presence of the very same samples. This suggests a possibility of acting at the post entry stages of HIV-1 replication, a mystery that awaits further investigation.

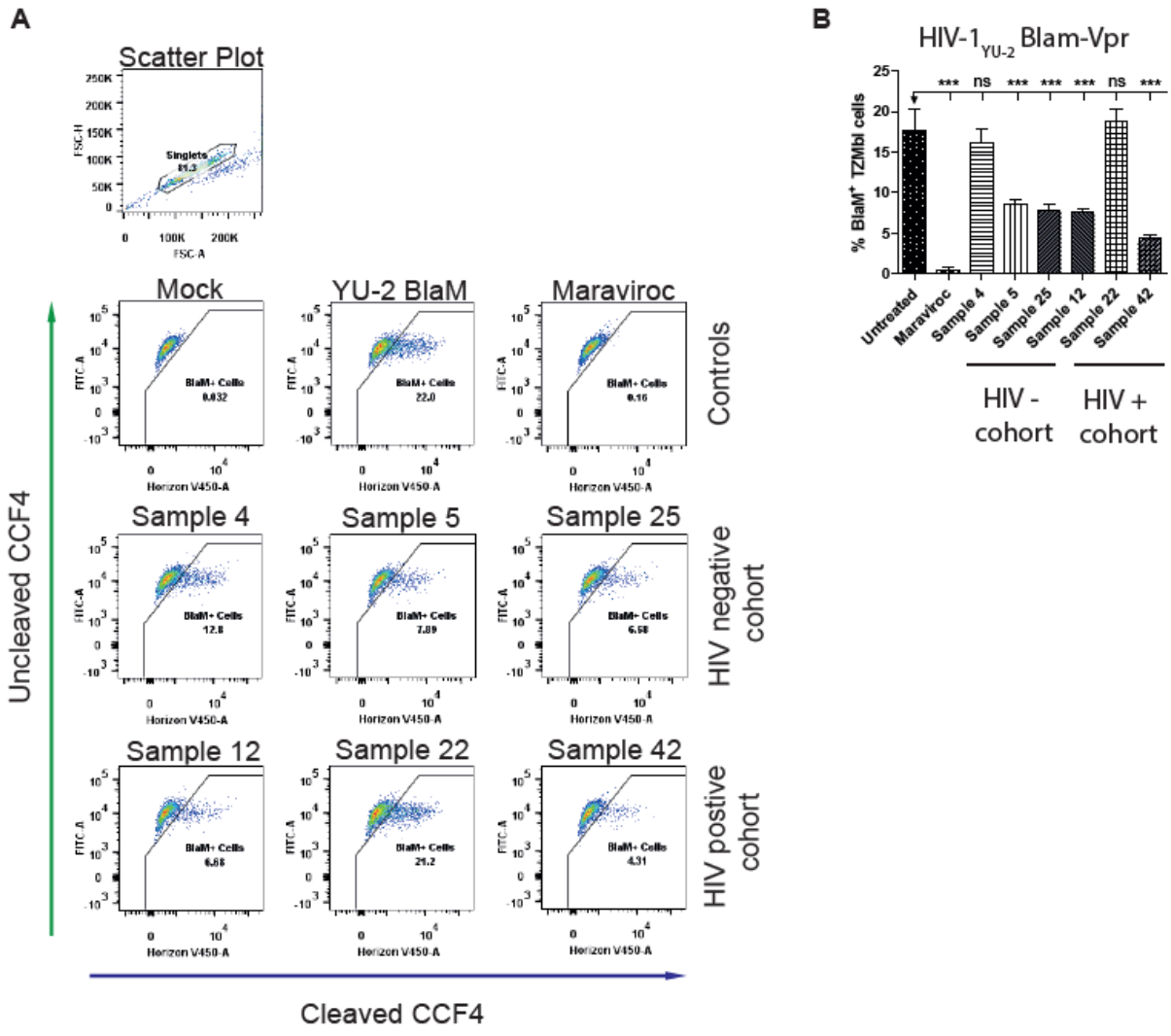


Figure 9: Mechanism behind the anti-HIV-1 (HIV-1$_{YU-2}$) activity of the crude cervical mucus plugs. Crude cervical mucus plugs from the study cohorts were probed in triplicates against HIV-1$_{YU-2}$ BlaM-Vpr for 1 hour before infecting TZMbl cells for 4 hours. Post-infection, the cells were washed with 10% CO_2 independent medium and PBS, after which they were trypsinized and re-suspended in 10% CO_2 independent medium. Re-suspended cells were pelleted, PBS-washed and stained with CCF4 overnight at room temperature. The CCF4 stained cells were PBS-washed and fixed with 4% PFA prior to analysis of a β-lactamase-mediated CCF4 cleavage using a multi-parameter LSR II flow cytometry (BD Biosciences). FACS dot plots show the degree of CCF4 cleavage in untreated and treated cells as a

reflection of HIV-1 fusion with the target cells (**A**). CCF4 cleavage was quantified by GraphPad Prism v5 using One-way ANOVA analysis (nonparametric) (**B**). The error bars represent the mean ± SEM of triplicates from one experiment.

4.11. β-lactamase-Vpr fusion assay in the context of HIV-$1_{NL4.3}$: Crude cervical mucus plugs

To investigate whether these data (Fig. 9) were influenced by tropism of the infecting strains, the fusion assays were repeated using a CXCR4 strain (HIV-$1_{NL4.3}$ BlaM-Vpr) of HIV-1, including T20 treatment as a positive control. The fusion assays showed that most cervical mucus plugs potently inhibited HIV-$1_{NL4.3}$ fusion with the target cells despite the donor's HIV status (Fig. 10A). It should be noted that it was impossible to use the same set of samples for all experiments due to limited amount of the study samples, some of which got finished before the completion of this study. Generally, the percentage fusion was low for HIV-$1_{NL4.3}$ compared to HIV-1_{YU-2} (Fig. 9), thereby recapitulating evidence of selective transmission of R5 viruses over X4 viruses as reflected by the data from the luciferase assays (Fig. 5). Again, sample 22 and now with sample 32 but not sample 4, did not block HIV-$1_{NL4.3}$ fusion with TZMbl cells, despite the inhibitory activity they showed in the luciferase assays (Fig. 5). Collectively, these data suggest that viral tropism does not influence the inhibition of HIV fusion by the cervical mucus, and this rather reflects the variations between and within samples.

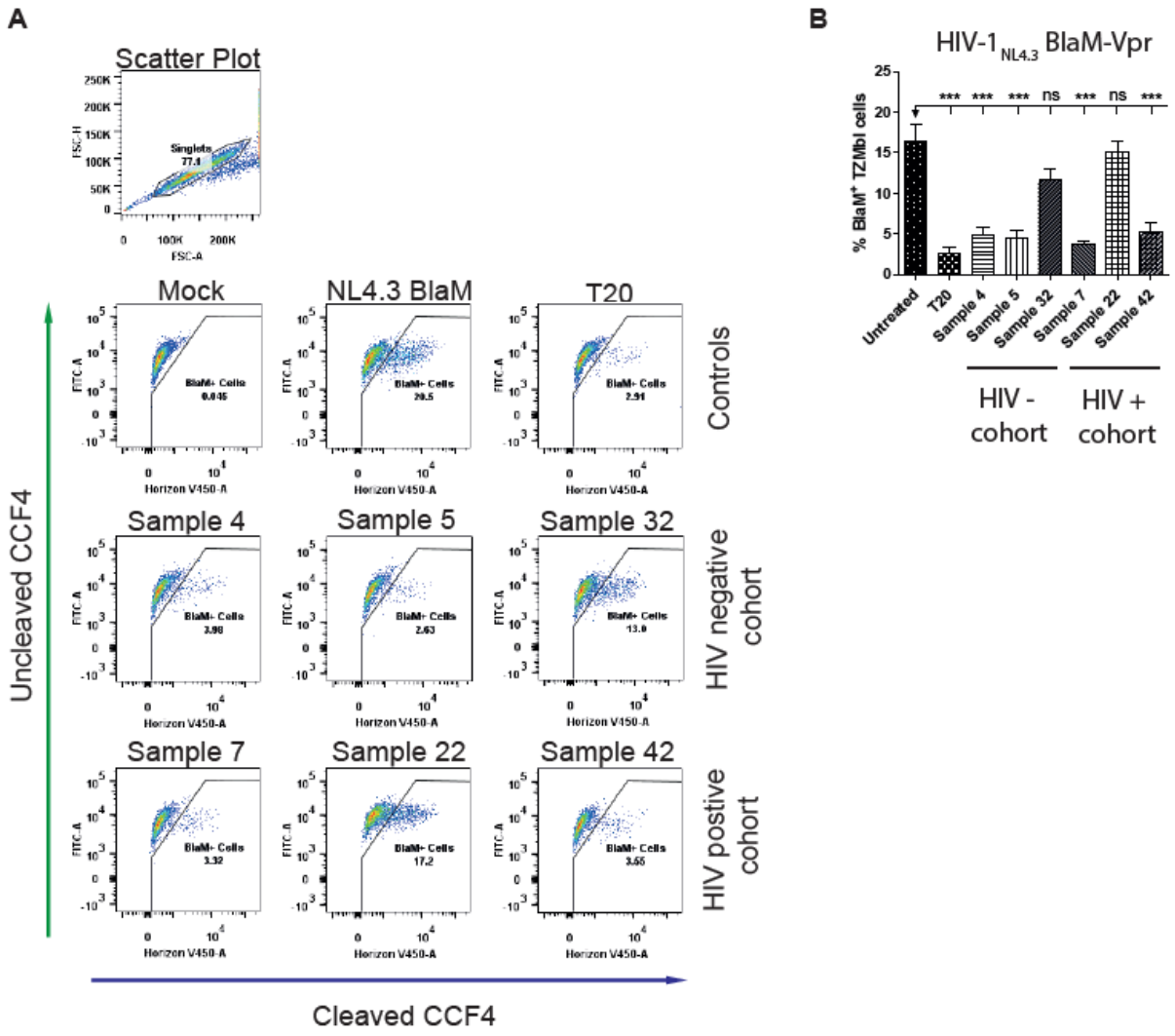


Figure 10: Mechanism behind the anti-HIV-1 (HIV-1$_{NL4.3}$) activity of the crude cervical mucus plugs. Crude cervical mucus plugs from the study cohorts were probed in triplicates against HIV-1$_{NL4.3}$ BlaM-Vpr for 1 hour before infecting TZMbl cells for 4 hours. After infection, the cells were washed with 10% CO_2 independent medium and PBS, after which they were trypsinized and re-suspended in 10% CO_2 independent medium. Re-suspended cells were pelleted, PBS-washed and stained with CCF4 overnight at room temperature. The CCF4 stained cells were PBS-washed and fixed with 4% PFA prior to analysis of a β-lactamase-mediated CCF4 cleavage using a multi-parameter LSR II flow cytometry (BD Biosciences). FACS dot plots show the extent of CCF4 cleavage in untreated and treated cells as a reflection of HIV-1 fusion with the target cells (**A**). CCF4 cleavage was quantified by GraphPad Prism v5 using One-way ANOVA analysis (nonparametric) (**B**). The error bars represent the mean ± SEM of triplicates from one experiment.

4.12. β-lactamase-Vpr fusion assay in the contexts of HIV-1_{YU-2} and HIV-$1_{NL4.3}$: Purified cervical mucins

The purified cervical mucins showed less potency in HIV-1 inhibition compared to the crude cervical mucus plugs (Fig. 5 & Fig. 6). For this reason, the purified cervical mucins were subjected into BlaM-Vpr fusion assays at a concentration of 1 mg/ml as mentioned above to investigate if this reduced potency relates to a massive influx of the HIV-1 strains into the target cells due to mild hindrance of virus-cell fusion by the purified cervical mucins. The above-mentioned gating strategy (Fig. 10) was used during flow cytometry analysis and shown here (Fig. 11) are the representatives of the quantified FACS data using GraphPad Prism v5 (La Jolla, CA, USA).

There was little or no evidence of fusion inhibition in the context of purified cervical mucins when compared to the crude cervical mucus plugs (Fig. 11A & 11B). Certain samples showed less inhibition of fusion, which could not reach statistical significance. Even in the cases of statistical significance, it is very low. For the most part and not entirely, the data from the fusion assays recapitulate the data from the luciferase assays that crude cervical mucus plugs are more potent in HIV inhibition compared to the purified cervical mucins. Together, these data show that the purified cervical mucins are less potent because they do not efficiently interfere with the influx of HIV-1 strains into the target cells to the same extent as the crude cervical mucus plugs.

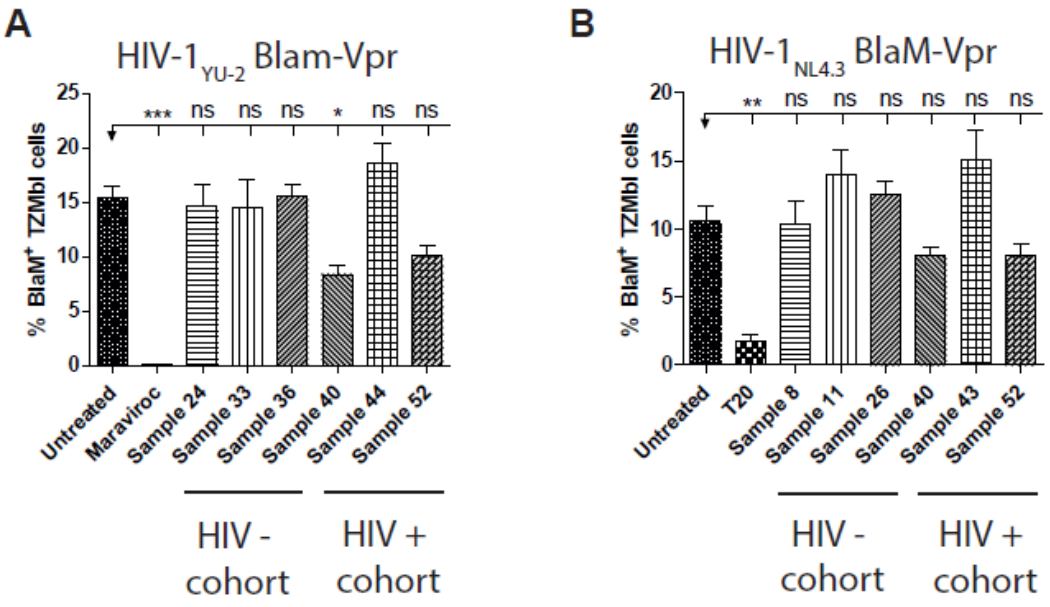


Figure 11: Mechanism behind the anti-HIV-1 (HIV-1_{YU-2} and HIV-$1_{NL4.3}$) activity of the crude cervical mucins. Purified cervical mucins from the study cohorts were probed in triplicates against HIV-1_{YU-2}/HIV-$1_{NL4.3}$ BlaM-Vpr for 1 hour before infecting TZMbl cells for 4 hours. After infection, the cells were washed with 10% CO_2 independent medium and PBS, after which they were trypsinized and re-suspended in 10% CO_2 independent medium. Re-suspended cells were pelleted, PBS-washed and stained with CCF4 overnight at room temperature. The CCF4 stained cells were PBS-washed and fixed with 4% PFA prior to analysis of a β-lactamase-mediated CCF4 cleavage using a multi-parameter LSR II flow cytometry (BD Biosciences). The same gating strategy used in Fig. 9 and 10 was used during flow cytometry analysis. The column graphs (**A-B**) show the extent of CCF4 cleavage in untreated and treated cells as a reflection of HIV-1 fusion with the target cells. CCF4 cleavage was quantified by GraphPad Prism v5 using nonparametric One-way ANOVA analysis. The error bars represent the mean ± SEM of triplicates from one experiment.

4.13. Anti-HIV-1 activity of the crude cervical mucins in the context of cell-associated virus

The anti-HIV-1 activity of the crude cervical mucus plugs was next investigated in the context of cell-to-cell transmission, the most efficient mode of transmission through which HIV-1 can escape the therapeutic pressure to which the cell-free HIV-1 strains are more susceptible [196, 215, 216]. To model cell-to-cell transmission of HIV-1, HEK293T cells were transfected with 2 μg of pro-viral plasmid DNA encoding full length HIV-$1_{NL3.4-GFP}$ for 24 hours at 37°C/5% CO_2 [196], after which the transfectants were co-cultured with the parental Jurkat T-cells (1:2 ratio) in the presence of the crude cervical mucus plugs for the next 24 hours under similar conditions. The inhibitory effect of the crude cervical mucus plugs was investigated in the presence or absence of efavirenz, a non-nucleoside reverse transcriptase inhibitor, to remove GFP background. The anti-viral activity of the crude cervical mucus plugs was calculated by subtracting the sample + efavirenz readings from the sample – efavirenz readings.

Post-infection, the cells were harvested, washed and fixed with 4% PFA, after which the GFP signal was quantified by acquiring 50 000 events using FACS-Calibur flow cytometry (BD Biosciences). The data were analysed using FlowJo v10 (Tree Star, Ashland, USA) and GraphPad Prism v5 (La Jolla, CA, USA). During analysis, the mock treated cells were used to generate a scatter plot (SSC-H/FSC-H) from which the live parental Jurkat T-cells were gated while excluding HEK293T cells and cell debris (Fig. 12A). The mock treated cells were used to set up a gate, which was

applied to the treated cells to determine the GFP signal. Interestingly, with respect to the positive control (–Efavirenz), the representative FACS data show that the cervical mucus plugs can inhibit cell-to-cell transmission of HIV-1 (Fig. 11A).

Quantification of the FACS data show that this inhibition is independent of the donor's HIV status, since samples from the HIV negative and HIV positive cohorts significantly inhibited cell-to-cell transmission of HV-1 (Fig. 11B). However, these data do not tell the mechanism of inhibition. But it could be that the components of the crude cervical mucus plugs interfere with the virus egress from the producer cells (HEK293T) or even aggregate/destroy the released virus particles, thereby depleting virus titers available to engage the target cells for induction of potent HIV-1 infection.

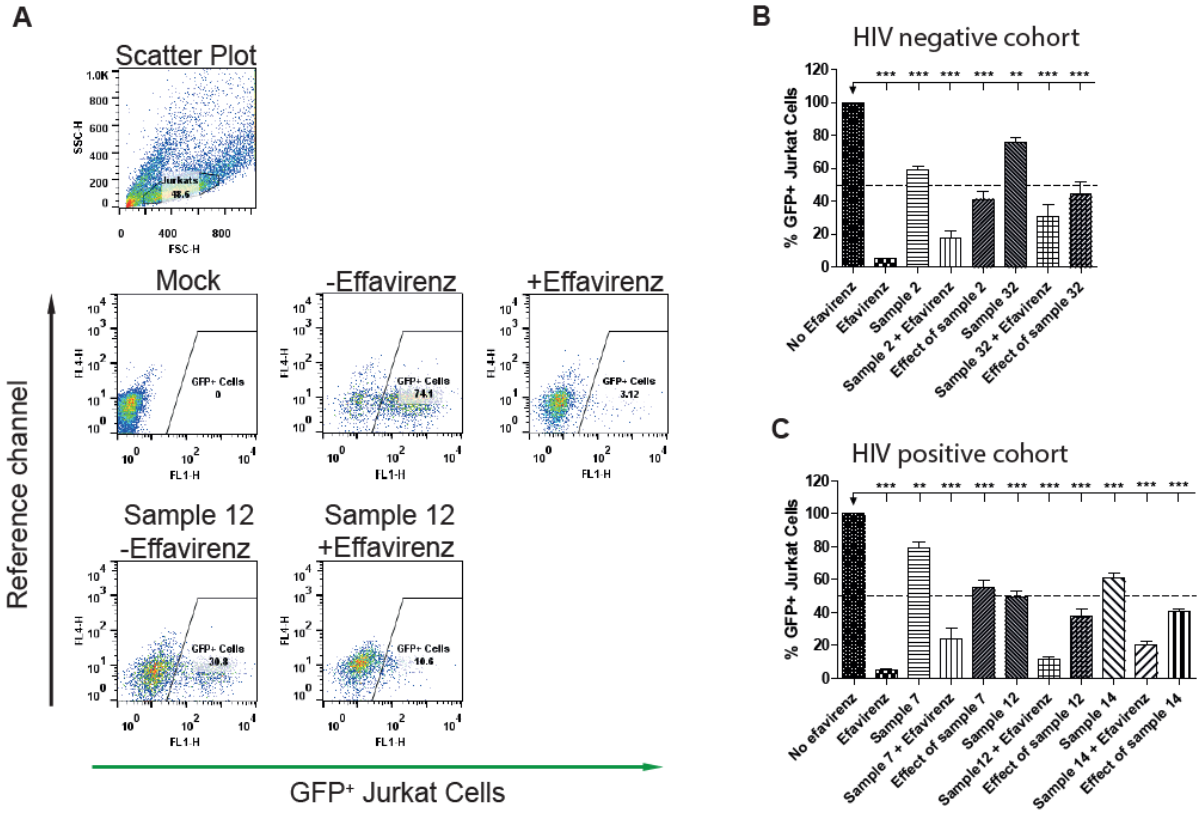


Figure 12: Anti-HIV-1 activity of the crude cervical mucus in the context of cell-associated virus. HEK293T cells were transfected for 24 hours with pro-viral DNA encoding full length HIV-$1_{NL4.3\text{-}GFP}$ and co-cultured with parental Jurkat T-cells for 24 hours in the presence or absence of cervical mucus plugs from the study cohorts. Efavirenz, a non-nucleoside reverse transcriptase inhibitor, was used as a positive control. After infection, the cells were harvested, PBS-washed, PFA-fixed and analysed by flow cytometry for a GFP signal (**A**). Samples were assayed in the presence or absence of efavirenz, the conditions from which true activity of the cervical mucus plugs was derived. GFP signal from parental Jurkat T-cells was

normalized to 100% and quantified by GraphPad Prism v5 (**B-C**). One-way ANOVA analysis (nonparametric) was used to compare the means, where the error bars represent the mean ± SEM from the triplicates of one experiment.

4.14. Anti-HIV-1 activity of the crude cervical mucus plugs in the context of cell-free virus: GFP-based reporter assays

The final step aimed to address a well-recognized phenomenon that cell-to-cell transmission can overcome the defensive barriers imposed by the crude cervical mucus plugs against cell-free HIV-1 strains [196]. Therefore, we used crude cervical mucus plugs against cell-free virus using the same readout as in the context of cell-to-cell transmission reported in Fig. 12, to allow for an unbiased side-by-side comparison of the data from the two assays. HEK293T cells were transfected in 6-well plates with 2 μg of pro-viral plasmid DNA encoding full length HIV-$1_{NL4.3\text{-}GFP}$ for 48 hours at 37°C/5% CO_2, after which the cell-free HIV-1 particles were harvested and passed through a 0.45 μm filter (Millipore, Germany) to remove cell debris. Next, HIV-$1_{NL4.3\text{-}GFP}$ was incubated with the cervical mucus plugs (1:2 ratio) for 1 hour at 37°C/5% CO_2 before infecting parental Jurkat T-cells ($1x10^6$ cells/well) in triplicates for 48 hours under similar conditions.

Following infection, the cells were washed with PBS, fixed with 4% PFA, after which the GFP signal was measured by acquiring 50 000 events using FACS-Calibur flow cytometry (BD Biosciences). The flow cytometry data analysis was conducted as mentioned above (Fig. 12). Like in the luciferase assays (Fig. 5), the crude cervical mucus plugs potently inhibited HIV-1 infection, now in a different readout using a different cell line, thereby highlighting the consistency of their anti-HIV-1 activity (Fig. 12A). Quantification of the GFP signal shows that HIV-1 inhibition by all the samples is statistically significant (Fig. 13B & 13C). The side-by-side analysis between Fig. 12 and Fig. 13 shows that, like in several cases [196, 197], cell-to-cell transmission of HIV-1 is able to partially overcome the barrier functions imposed by the crude cervical mucus plugs against the cell-free HIV-1 strains.

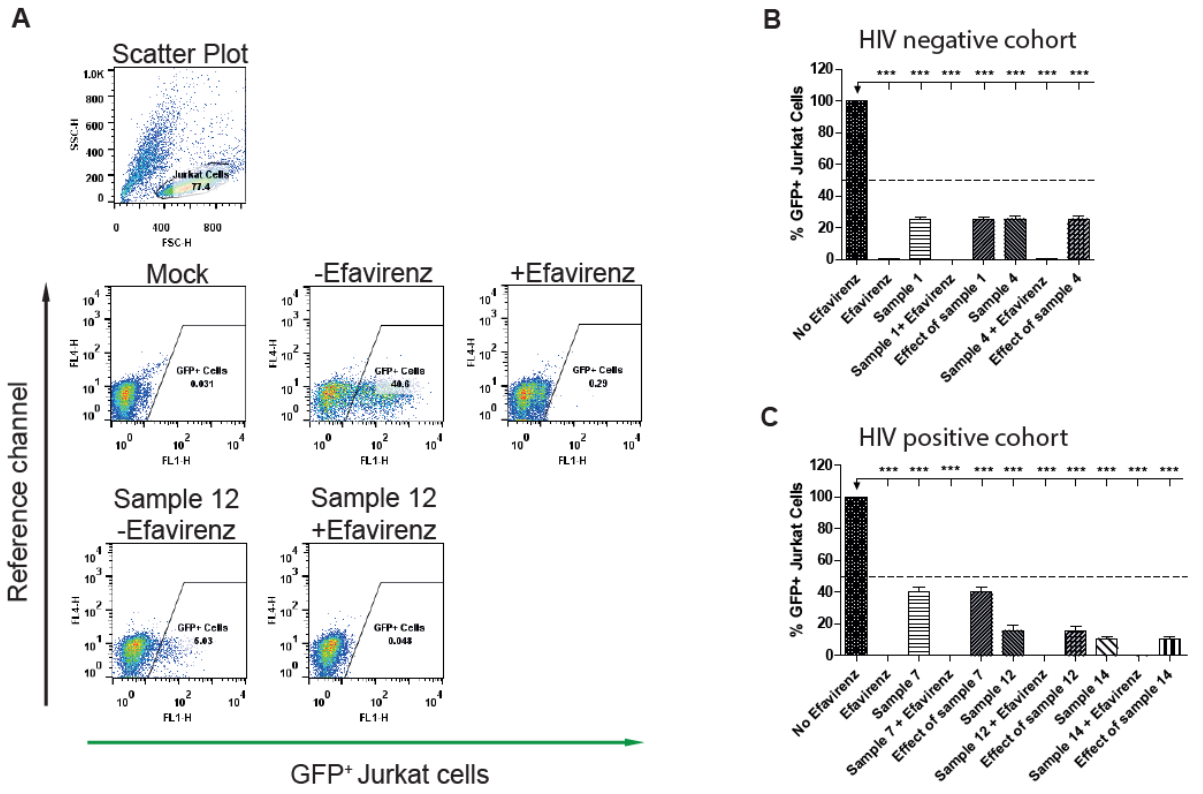


Figure 13: GFP reporter assay-based analysis of the anti-HIV-1 activity of the crude cervical mucus plugs in the context of cell-free virus. Cervical mucus plugs were probed against HIV-$1_{NL4.3\text{-}GFP}$ in the context of cell-free virus and their anti-viral activity was analysed in parental Jurkat T-cells using FACS-Calibur flow cytometry (BD Bioscience). After 48 hours, parental Jurkat T-cells were washed with PBS and fixed with 4% PFA before flow cytometry analysis. Representative FACS dot plots show the levels of GFP signal as a reflection of HIV-1 infection (**A**). Cervical mucus plugs were assayed in the presence or absence of efavirenz, the conditions from which their true anti-viral activity was derived. GFP signal was normalized to 100% and quantified by GraphPad Prism v5 (La Jolla, CA, USA) (**B**). The error bars represent the mean ± SEM from the triplicates of one experiment.

Chapter – 5: Discussion

Some years ago, our laboratory set out to investigate why HIV-1 was not transmitted through the exchange of oral fluids. At that time, evidence in the literature implicated macromolecules such as salivary mucins in the prevention of HIV-1 transmission through the exchange of oral fluids [26, 27]. Early studies from our laboratory found that crude saliva from the HIV negative subjects and its purified mucins inhibited HIV-1 in an *in vitro* assay, while purified salivary mucins from HIV positive subjects did not [29, 192, 193]. Further studies showed that crude breast milk and crude cervical mucus plugs did not inhibit HIV-1 in an *in vitro* assay, but the purified mucins from these secretions inhibited HIV-1, regardless of the donor's HIV status [35, 194, 195]. These early studies from our laboratory were limited by sporadic pooling of samples, use of samples from the subjects who self-declared their HIV status, a lack of descriptive statistics for data quantification and the absence of diverse HIV strains to reveal inter-individual variations amongst the subjects [36, 121].

This study investigated the anti-HIV-1 activities of crude cervical mucus plugs shed during labour, before and after isolation and purification of the cervical mucins from HIV negative and HIV positive pregnant women. The cervical mucus plug is exclusively produced by the cervix during pregnancy to protect the foeto-maternal unit against ascending pathogens from the vaginal canal, failure of which can cause life-threatening obstetrical problems [156, 217]. HIV must overcome the mucosal barriers before establishing clinical infection [31], and lining these barriers is the mucus, a gel-like secretion that covers the epithelia of the female reproductive tract (FRT).

Habte et al [35] in our laboratory reported that crude cervical mucus plugs collected from HIV negative women in labour do not inhibit HIV-1 infection in an *in vitro* assay. In contrast, using a panel of HIV-1 replication-competent strains, we found that crude cervical mucus plugs inhibit cell-free HIV-1 infection, irrespective of the donor's HIV status (Fig. 5). However, the anti-HIV-1 activity showed inter-individual variations between individual samples, suggesting a heterogeneity between and within the cervical secretions [36, 121, 212]. The cervical mucus plugs exerted a selective anti-

viral activity against HIV-1, depending on the infecting strain, and showed a gatekeeping characteristic that selects for transmission of the R5 strains while excluding the X4 strains [214]. There was more potent HIV-1 inhibition within the HIV positive cohort compared to the HIV negative cohort, presumably due to retention of the HAART regimens [211] and HIV-specific antibodies [174] by the cervical mucus plugs collected from the HIV positive cohort. This donor's HIV status-dependent potency of the samples was not seen in the context of cervico-vaginal secretions [36]. To a certain extent, this could be due to the exclusion of participants who were on ARV drugs, which could be retained by the cervico-vaginal secretions from HIV positive women and boost their anti-HIV-1 activity.

HIV-1_{BaL} was more resistant to the cervical mucus plugs from the HIV negative cohort (Fig. 5). This resistance was also seen in sample 7 (Fig. 5l) from the HIV positive cohort, suggesting that this phenotype is not necessarily determined by the donor's HIV status, but most likely by several factors on which the HIV positive subjects may differ: CD4 count, viral load, opportunistic infections and duration of HAART. The donor from whom sample 7 was collected had been on HAART treatment for 3 years, which is the longest period compared to other donors within the HIV positive cohort. Therefore, the suppressive effect of HAART treatment could have significantly lowered the HIV-specific antibody titers in this donor's circulatory system [218, 219] and reduced shedding into the cervical mucus plug [174]. Sample 47 (Fig. 5O) from the HIV positive cohort enhanced SIV_{mac239} infection compared to all other samples, while it consistently proved to be the most potent inhibitory sample against the resistant HIV-1_{BaL}. This enhancement was lost following heat inactivation of the sample, suggesting that these pro-SIV_{mac239} factors in sample 47 are proteins or glycoproteins. These data highlight the high degree of heterogeneity between and within the cervical secretions of the study cohorts as previously reported elsewhere [36].

SIV_{mac239} was included to extend this study to other lentiviruses that also transmit through genital mucosa. When compared to HIV-1 and HIV-2, it resembles HIV-2 in several aspects: structural components [220], response to therapy [221] and entry mechanisms [222]. Except for sample 47, SIV_{mac239} assumed a unique and rather consistent inhibition profile that began with high sensitivity to mucus, but suddenly

reached a steady state earlier than the HIV-1 strains (roughly at around 0.125 mg/ml), after which further increase in cervical mucus plug concentration had little or no effect on it (Fig. 5). Presumably, this suggests that SIV_{mac239} exhibits multiple entry mechanisms into cells, upon which it capitalizes to maintain the detectable infection levels in the face of increasing cervical mucus plug concentrations. Unlike several HIV-1 strains, most HIV-2 and SIV strains have been reported to infect the target cells independent of the CD4 receptor and utilize several co-receptors that most HIV-1 strains do not utilize [222]. These characteristics, to a certain extent, allow these slowly mutating viruses to escape the therapeutic pressure to which several HIV-1 strains are more susceptible [223, 224]. HIV-2 and SIV were also resistant against submandibular salivary samples that effectively inhibited HIV-1, thereby further highlighting the high degree of resemblance between them [225].

The genital mucosa in the FRT exhibit a strict HIV-1 sieve mechanism, which selects for transmission of specific HIV-1 strains out of other HIV-1 strains present in the ejaculate of an infected male partner [226]. This sieve mechanism selects for transmission of the R5 strains while predominantly excludes the X4 strains [213, 214, 227], thereby explaining why the less diverse R5 strains usually dominate during the early stages of HIV infection [228, 229]. In addition to the R5 versus X4 transmission, our data show that this gatekeeping phenomenon also exists within the R5 strains, thus highlighting the high degree of the selective stringency at the genital mucosa.

Saba et al [214] reported that the differentiation status of the effector memory CD4 T-cells is one of the restriction factors that select against the X4 HIV-1 strains in the FRT. Our *in vitro* data suggest that the cervical mucus plugs collected from women in labour also exhibit this gatekeeping phenomenon that selects against the CXCR4-using strains of HIV-1. Following HIV infection, this transmission gate becomes more stringent, even for the resistant R5 strains, suggesting an enhanced anti-HIV-1 specific activity in the cervical mucus plugs collected from the HIV positive cohort. Whether this gate keeping phenomenon is exclusively exhibited by the cervical mucus plug compared to usual cervical mucus remains to be investigated, but it was not observed in the context of cervico-vaginal secretions, regardless of the donor's

HIV and pregnancy statuses respectively [36, 37, 230]. Consequently, *in vivo* studies are needed to investigate the relevance of these data at the physiological level.

In agreement with Habte et al [35], the purified cervical mucins inhibited infection by the cell-free HIV-1 strains. This study further showed that the purified cervical mucins from the HIV positive cohort also inhibited cell-free HIV-1 strains, suggesting that inhibition was independent of the donor's HIV status. Limited amount of purified cervical mucin samples restricted their analysis to two strains of HIV-1 (HIV-1_{BaL} and HIV-$1_{NL4.3}$). The purified cervical mucins showed less potency against cell-free HIV-1 strains compared to the crude cervical mucus plugs (Fig. 5 & 6). In the case of purified cervical mucins, there was no evidence of selective inhibition against the infecting HIV-1 strains, nor was there any evidence of tropism-based selective transmission (Fig. 6). In summary, the findings of this study differ from those of Habte et al [35] in that crude cervical mucus plugs from HIV negative and HIV positive cohorts inhibit HIV-1 with inter-individual variations between and within samples, and more potently than the purified cervical mucins.

Together, these data suggest that the purified cervical mucins exhibit less potent anti-HIV-1 activity compared to the crude cervical mucus plugs and do not mediate the tropism-based gatekeeping phenomenon as reflected by the crude cervical mucus plugs. Furthermore, if they mediate this phenomenon at all, they probably do so synergistically with other components of the crude cervical mucus plugs. Also, there was a lesser degree of variation between and within the study subjects in terms of the anti-HIV-1 activity of the purified cervical mucins and this was independent of the donor's HIV status. Our results show a certain degree of similarity in the anti-viral properties of the cervical mucins between and within the study cohorts, independent of the donor's HIV status.

The anti-HIV molecules typically exert their inhibitory activities in different fashions, displaying a broad category of virus-directed and cell-directed inhibitors [197]. To investigate the category of inhibitors in which the crude cervical mucus plugs belong, the samples were probed against HIV-$1_{NL4.3}$ under three different conditions using the time-of-addition assays (Fig. 8). In condition one, incubation of the target cells with cervical mucus plugs for one hour, followed by aspiration of the mucus and

infection with HIV-1 for 48 hours showed the least inhibition of infection. This inhibition could have been mediated by the cervical mucus plug residues, since the cells were not washed following sample aspiration. In condition two, infecting the target cells with the virus for one hour prior to incubation with the cervical mucus plugs for 48 hours showed the second-best inhibition of infection, most likely due to mucus-mediated destabilization of the free-floating and even attached but not yet fused HIV-1 particles, thereby depleting the titers available to induce potent infection.

In condition three, the data showed that the crude cervical mucus plugs inhibited HIV-1 more potently when the virus was first incubated with the mucus samples for one hour prior to infection of the target cells for 48 hours. Together, these data suggest that *in vivo*, in order to effectively protect the foeto-maternal unit, the cervical mucus plug must block the pathogens from the vaginal canal before they ascend into the cervix and establish the clinical infections. Supporting these observations is the orientation assumed by the cervical mucus plug *in vivo*, whereby the protease, immunoglobulin and phagocyte-rich compartment faces towards the pathogen-infested vaginal canal to clear off the infections while the largely mucoid compartment of low cellular content faces towards the supposedly sterile uterus [155, 231].

The next step was to address the mechanism through which the crude cervical mucus plugs and purified cervical mucins inhibit HIV-1. This step was approached by looking at the fusion stage of HIV replication, using the flow cytometry based β-lactamase fusion assay (Fig. 9). Interestingly, most crude cervical mucus plugs inhibited the fusion between HIV-1 and the target cells, independent of the donor's HIV status. The crude cervical mucus plugs showed more potent inhibition of fusion against HIV-$1_{NL4.3}$ (Fig. 10) compared to HIV-1_{YU-2} (Fig. 9), thereby, to a certain extent, reflecting similarity to luciferase assays. However, this approach was limited by the fact that it was not conducted in parallel with HIV binding assays, thus restricting us from distinguishing between the anti-binding and anti-fusion activities of the crude cervical mucus plugs. Consequently, it could also be that the HIV-1 strains were destabilized or aggregated by the cervical mucus plug components before they could even engage the target cells.

Some crude cervical mucus plugs showed no evidence of fusion inhibition, despite having inhibited the same HIV-1 strains in the luciferase assays. The fact that these samples inhibited HIV-1 infection in the luciferase assays but failed to show inhibition in the fusion assays suggests a possible action at the post entry stages of HIV-1 replication, the stages that remain to be investigated. The purified cervical mucins showed less to no fusion inhibition of HIV-1 strains, and this activity was comparable between the R5 and X4 HIV-1 strains (Fig. 11). Generally, this reflects the less potent inhibition of HIV-1 seen in the luciferase assays (Fig. 6).

The discrepancies shown by these specific crude cervical mucus plugs between the luciferase and fusion assays highlight another level of heterogeneity between samples, which is not only based on the potency of the anti-viral activities but also on the mechanisms through which they exert such activities. These discrepancies are well-defined between two crude cervical mucus plugs (32 and 22) from the HIV negative and HIV positive cohorts respectively (Fig. 9 & 10). The case of sample 22 can be attributed to the fact that the donor was HIV positive and with three months on HAART treatment, which predominantly consists of the regimens that act at the post fusion stages of HIV replication [232]. The cervical secretions can retain several orally administered HAART regimens [211], antibodies from the maternal blood [174] and even foetal cells from the regressing chorionic villi [183].

These data highlight the possible retention of the HAART regimens into sample 22 [211], which are lacking in fusion inhibitors [232], a circumstance that could explain the effect seen in the fusion assays. In the HIV negative cohort, the donor from whom sample 32 was collected stood out as the only one who was on antibiotic treatment (including co-trimoxazole) due to an *E. coli* urinary tract infection. The direct anti-HIV-1 activity of co-trimoxazole that could also be retained in the cervical mucus plugs [233] has not been reported yet, but this antibiotic is used to treat the HIV-specific opportunistic infections among the HIV positive individuals [234].

The co-trimoxazole prophylaxis significantly lowered the viral load and improved the CD4 counts among the HIV positive Ugandan patients, likely by stabilizing the immune system to effectively supress HIV replication [235]. These data highlight the anti-HIV role of co-trimoxazole prophylaxis, which could have influenced the anti-

HIV-1 activity of sample 32. Whether the co-trimoxazole-mediated anti-HIV activity is direct or indirect remains to be investigated. The question of why these two samples showed differences in anti-HIV-1 activity compared to other samples could be better explained by the mass spectrometry analysis, which would presumably reveal whether they were deprived of the endogenous or exogenous (in the HIV positive cohort) molecules that mediated the anti-fusion activity seen in other samples.

Interestingly, Habte et al [35] in our laboratory did not find the anti-HIV-1 activity in the crude cervical mucus plugs, despite them having a repertoire of immunological factors [121, 155] and the findings of this study that point to the cervical mucus plug as a potent virus-directed inhibitor of HIV-1. Perhaps the difference is that, compared to that of Habte et al [35], this study used more sensitive HIV inhibition assays. This study was broader, more well-defined in that there were two distinct cohorts, no pooling of samples and several independent inhibition assays were conducted. Habte et al [35] had fewer samples, which they pooled prior to the inhibition assays. The current study shows evidence of a virus-specific pro-viral activity in most samples from the HIV negative cohort (Fig. 5), the only cohort that Habte et al [35] investigated. The pooling of samples, use of one HIV strain and lack of descriptive statistics to effectively judge the differences between the samples and the controls limited Habte et al [35] from distinguishing between the pro-viral activity and lack of anti-viral activity in their study samples. The use of dosages as the dependent variable instead of time would have better illustrated the activities of the study samples. So, their data could also be reflecting a pro-viral activity, and not necessarily a lack of anti-viral activity by the crude cervical mucus plugs.

The Phase ll report of the International Network for Comparison of the HIV Neutralization Assays (NeutNet Report ll) emphasized that there is no single neutralization assay that maintains the same sensitivity across a wide spectrum of different viruses and neutralizing agents [236]. However, in agreement with Report l of NeutNet [237], report ll also showed that the luciferase readout exhibit a broader sensitivity across a large spectrum of HIV strains and neutralizing agents compared to the p24 readout, which exhibit a narrow sensitivity [236]. Nagashunmugam et al [225], whose method was used by Habte et al [35], did not find anti-SIV activity in the human submandibular salivary samples, where the highest anti-viral activity of the

saliva is found [27]. Using the *in vitro* luciferase assays, Thomas et al [238] reported that the whole human saliva can inhibit SIV in the *in vitro* assays, thereby highlighting the limitations of p24 antigen assay compared to luciferase assay.

The low infection rate of HIV per coital act suggests the active presence of endogenous antimicrobials in the crude secretions of the FRT [34]. Proteomic studies have identified a spectrum of these antimicrobials in the FRT secretions [121, 173, 174], whose ability to supress HIV infection has been reported by several independent laboratories [36, 37, 39]. The data presented by Habte et al [35] do not support these findings in the context of crude cervical secretions, and their approach along with the use of qualitative p24 assays of narrow sensitivity may have limited the detection of anti-HIV-1 activity in the crude cervical mucus plugs. The high anti-HIV-1 potency of the purified cervical mucins reported by Habte et al [35] compared to the one reported in this study could be due to the inter-individual variations between samples or the differences between the sensitivities of the inhibition assays used.

This study went on to characterize the anti-HIV-1 activity of the crude cervical mucus plugs in the context of cell-to-cell transmission, the physiological mode of transmission through which HIV can escape the therapeutic pressure to which the cell-free HIV-1 strains are more susceptible [196, 197]. Due to a limited quantity of purified cervical mucin samples, these assays were only carried out using the crude cervical mucus plugs. The data showed that the crude cervical mucus plugs can also inhibit cell-to-cell transmission of HIV-$1_{NL4.3\text{-}GFP}$ irrespective of the donor's HIV status, but with inter-individual variations between samples as seen in the previous assays (Fig. 12). For the second time, the crude cervical mucus plugs were probed against the cell-free HIV-$1_{NL4.3\text{-}GFP}$, but using a GFP readout as used in the cell-to-cell transmission assays to allow an unbiased side-by-side comparison between the data from these two modes of HIV transmission. The crude cervical mucus plugs potently inhibited HIV-1 infection in the context of cell-free virus (Fig. 13), compared to cell-to-cell transmission (Fig. 12).

The reduction in the anti-HIV-1 activity of the crude cervical mucus plugs in the context of cell-to-cell transmission compared to cell-free virus suggest that HIV-1 overcomes the barrier functions imposed by the cervical mucus plugs against cell-free strains of HIV-1. This is coherent with several lines of evidence in the context of broadly neutralizing antibodies [196, 197, 239]. The mechanism(s) behind the anti-viral activity of the crude cervical mucus plugs in the case of cell-to-cell transmission is not yet known, but it's more likely that the cervical mucus plug components impair the virus egress from the producer cells, destabilize or aggregate the released cell-free HIV-1 particles and ultimately deplete the viral titers available to engage the recipient cells for induction of potent infection.

The fact that the crude cervical mucus plugs consistently maintained a potent inhibition against cell-free HIV-1 infection across two independent inhibition assays suggests that they are indeed inhibitory against HIV-1 and that their activity is not influenced by the use of different inhibition assays. The maintenance of this inhibition across these two inhibition assays could be supported by the report that the luciferase readout is just as sensitive as the GFP readout, and more standardized compared to the error-prone p24 readout [240]. This is one of the strengths of this study over that of Habte et al [35], who reported that the crude cervical mucus plugs do not inhibit HIV-1 after using a single inhibition assay of narrow sensitivity.

The strength of this study lies in the utilization of more samples, with cohorts of well-defined HIV status, panel of replication-competent strains of HIV-1 and different inhibition assays of high sensitivity. Together, these parameters enabled the investigation of the consistency of anti-viral activity among the study samples and statistical quantification of the differences between them, thereby uncovering crucial information that Habte et al [35] did not find. However, it must be noted that this study did not have strict exclusion and inclusion criteria, since collection of samples depended on the goodwill of medical staff in busy clinics, thus bringing confounding variables into the collected data, which make it difficult to explain the underlying inter-individual variations seen between and within the study samples. Further limiting this study was a loss of material during purification of the cervical mucins, which only enabled probing the purified cervical mucins against two strain of HIV-1. This hampered a side-by-side comparison of the anti-viral activities between the

crude cervical mucus plugs and purified cervical mucins across the full spectrum of the conducted experiments. At a proteomic level, the mass spectrometry analysis of the study samples would most likely explain the underlying differences in their anti-viral activities. Therefore, its use in future studies is highly recommended for rigorous investigation of such differences and, in part, identification of the molecules that mediate the anti-viral activity in the crude cervical mucus plugs.

The crude cervical mucus plugs were collected in 6 M GuHCl with protease inhibitors to inhibit proteolysis of the mucins [198]. The cervical mucins were purified by CsCl density gradient ultracentrifugation once, and subjected to Sepharose CL-2B gel-filtration to enrich the target mucin polymers [201]. The mucin purification profiles for both HIV negative and HIV positive cohorts in CsCl were comparable in terms of density, glycoprotein and protein contents (Fig. 1A & 1B). This was also the case for the Sepharose CL-2B gel-filtration profiles (Fig. 1C & 1D), which were comparable for both cohorts, with glycoprotein and protein fractions eluting at approximately the same fractions irrespective of the donor's HIV status. Another comparable activity between the purified cervical mucins was seen in the dose-response curves against HIV-1 infection (Fig. 5), whereby the purified cervical mucins showed comparable inhibition profiles of the two HIV-1 strains, which were independent of the donor's HIV status. Together, these data suggest that the biochemical characteristics of the cervical mucins and their anti-HIV-1 activity are comparable between different individuals and not necessarily affected by the donor's HIV status.

The SDS-PAGE was performed to confirm purity of the cervical mucins compared to the crude cervical mucus plugs. Following the protein staining, the data showed more intense bands for crude cervical mucus plugs, whose intensities increased with decreasing molecular weights (Fig. 2A & 2C). However, following the glycoprotein staining (Fig. 2B & 2D), the intense bands were seen on top of the gels, after which the intensities decreased with decreasing molecular weights, suggesting the dominant presence of heavily glycosylated species of higher molecular weight. Generally, in terms of protein and glycoprotein intensities, there were no well-defined differences between the HIV negative and HIV positive cohorts, other than the inter-individual variations that could not necessarily be attributed to the donor's HIV status. There was a ~130 kDa band common between certain samples irrespective

of the donor's HIV status (Fig. 2A-2D), which could be either IgA or IgG with respect to the molecular weight [155, 208].

Another ~250 kDa band was exclusively present among certain samples (samples 7, 12, 14 & 28) from the HIV positive cohort, with varying intensities between them (Fig. 2C). There was nothing common between these donors in terms of the clinical data (see appendix 7.5) and HIV inhibition profiles, and this inconsistent band could be better explained by the inter-individual variations on which HIV infection may impact, and not exclusively by the HIV infection. Following the mucin purifications (Fig. 2E-2H), the protein staining confirmed removal of the low molecular species in the V_0 fractions, some of which were retained in the V_i fractions (Fig. 2E & 2G). Species of higher molecular weight were seen on top of the running gel among the V_0 fractions, which were confirmed by the PAS staining to be the heavily glycosylated species that assumed a typical electrophoretic pattern of the mucins (Fig. 2F & 2H) [201]. Several samples showed a ~55 kDa band following protein staining regardless of the donor's HIV status, whose intensity decreased following the PAS staining, suggesting a lesser degree of glycosylation. This band could be a heavy chain of an immunoglobulin [155, 208] or α-1 acid glycoprotein [209], at least with respect to the molecular weight.

The presence of MUC5AC and MUC5B in the crude cervical mucus plugs and purified cervical mucins was confirmed by the Western blots (Fig. 3A-3H). The crude cervical mucus plugs from the HIV negative cohort showed the positive bands for both MUC5AC and MUC5B, with varying intensities between the individuals (Fig. 3A-3B). However, sample 1 was negative for MUC5B, even though it was positive for MUC5AC, and we cannot rule out from these data whether this was due to the absence of MUC5B or its concentration was below the detection level of the antibody. Sample 2 showed a more intense band for MUC5B compared to all other samples within the HIV negative cohort. Except donor 32, other donors from the HIV negative cohort presented with no genitourinary tract infections during hospitalization, to which these discrepancies could be attributed. Using immunohistochemistry and *in situ* hybridizations, this inconsistent expression pattern of the endo-cervical mucins between individuals was also noted elsewhere [96, 103].

Therefore, it does not necessarily reflect the variations between the detection assays but rather the inter-individual variations between the donors from whom the samples were collected. More intense bands for MUC5AC and MUC5B were seen in the HIV positive cohort (Fig. 3C & 3D), even though some low intense bands were also seen among certain samples.

The purified cervical mucins (Fig. 3A-3D) generally showed more intense bands for MUC5AC and MUC5B compared to the crude cervical mucus plugs (Fig. 3E-3H). This could be due to the mucin enrichment during purification and the removal of other molecules found in the crude cervical mucus plugs [121], which could interfere with the antibody binding to the mucin antigens. The purified cervical mucins were all positive for MUC5AC and MUC5B, irrespective of the donor's HIV status (Fig. 3E-3H). There was a well-defined increase in the intensities of MUC5AC from the HIV negative to the HIV positive cohort. This observation is in line with the findings of Gundavarapu et al [210], who reported an upregulation of MUC5AC expression by the HIV's gp120 in the human bronchial epithelial cells. Such a well-defined shift was not seen for MUC5B, other than an inter-individual variation between samples.

These data highlight the selective regulation of *MUC5AC* and *MUC5B* genes and suggest that their expressions are driven by the different promoters [241]. It is recognized that these Western blots were conducted following protein resolution in SDS-PAGE gels, as opposed to the typical agarose gels that give better mucin resolution [35, 242], and therefore our data do not reflect the best mucin resolution. However, our aim was to confirm the presence of the major gel-forming cervical mucins in our samples and not necessarily the changes in their electrophoretic patterns, an objective that would necessitate the use of agarose gels. It is further noted that these data are interpreted in the absence of a loading control due to absence of the endogenous controls in the cervical secretions [121]. However, the sample loading was controlled by standardizing the sample volume and concentration across the study samples.

Chapter – 6: Conclusion

The crude cervical mucus plugs can potently inhibit HIV-1 compared to the purified cervical mucins. In the context of crude cervical mucus plugs, depending on the infecting HIV-1 strain, this anti-HIV-1 activity varies between and within the individual samples, and is more pronounced in the HIV positive cohort. However, such variations are absent in the context of the purified cervical mucins. Despite the donor's HIV status, the crude cervical mucus plugs act at different stages of HIV-1 replication. In the context of cell-to-cell transmission, HIV-1 partially overcomes the barrier functions imposed by the cervical mucus plugs in the context of cell-free virus.

Chapter – 7: Appendices

7.1. Consent form

REQUEST FOR CERVICAL MUCUS PLUG TO INVESTIGATE THE ANTI-HIV-1 and HIV-2 ACTIVTIES OF THE CERVICAL MUCINS

Research Laboratory
Division of General Surgery
OMB Groote Schuur Hospital
UCT Medical School, Observatory 7925

Tel: (021) 406 6168/6227 Fax: (021) 448 6461

Please fill in all the information requested:

Folder No. _____

Date of Birth Y: _____ M: ____ D: ___ Ethnic group: _____

For Laboratory use only:

Date Received: Y: _____ M: ____ D: ___

CONSENT FOR CERVICAL PLUG SAMPLE

Please note that your HIV status will **NOT** be recorded in this form

1. I give permission that a cervical plug be taken for research purposes in the investigation of HIV/AIDS.

2. I give permission that a portion of the sample be stored anonymously for:
   a) possible re-analysis;
   b) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

3. I authorize / do not authorize my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the researchers.

4. All of the above information has been fully explained to me in a language that I understand and my questions were answered.

SIGNATURE: _____

7.2. Patient information sheet

PATIENT INFORMATION SHEET

Dear Patient,

□ We are doing a study on the mucus produced in your cervix.

□ Mucus is a sticky substance mixed with bodily secretions and is necessary for lubrication and protection of the body tracts. Therefore, we investigate the role of mucus plug from the cervix of HIV negative and HIV positive individuals in neutralizing HIV-1 and HIV-2.

□ The long-term goal of this study is to synthesize a mucin-based vaginal microbicide, which will prevent HIV infection of the vagina.

We need to take a sample of cervical plug mucus from your vagina. **This will be done just before you deliver your baby by a trained nurse/doctor.**

Please note that:
1. An informed consent will be taken from each participant prior to sample collection.

2. We will need to take your folder number (**NOT YOUR NAME**), through which we will get your clinical data. This will be done by an authorized nurse/doctor **ONLY**.

3. All this information will be kept **absolutely confidential** and we promise not to use your sample for any other purposes, but ONLY for what we have mentioned.

4. You have a right to say that you **do not** wish to take part in this study.

5. If you agree to participate in this research now and then decide at a later time that you **do not** wish to continue, that is also fine. That will not affect the medical care you receive.

6. It is possible that we will publish the findings of this study, but your identity and any information that will identify you will not be disclosed.

7. We further emphasize that all the samples donated to this study will not carry your name, but your folder number **ONLY**.

8. The samples will be kept in the laboratory for re-analysis.

If there are any questions, or if you think of any questions at a later stage, please contact Baxolele or Professor Mall at:

Professor A Mall, Tell: 021 406 6168
Mr B Mhlekude, Tell: 021 406 6227

7.3. Ethics approval by the University of Cape Town

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS017: Annual Progress Report / Renewal

Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries

HREC office use only (FWA00001637; IRB00001938)			
This serves as notification of annual approval, including any documentation described below.			
☑ Approved	Annual progress report	Approved until/next renewal date	30/03/2019
☐ Not approved	See attached comments		
Signature Chairperson of the HREC		Date Signed	10/4/2018

Principal Investigator to complete the following:

1. Protocol Information

Date (when submitting this form)	04.04.2018		
HREC REF Number	102/2013	Current Ethics Approval was granted until	30.03.2018
Protocol title	The anti-HIV activities of human cervical mucins		
Principal Investigator	Professor Anwar Mall		
Department / Office Internal Mail Address	Groote Schuur Hospital, OMB, J-Floor, Dep. Surgery, Division of General Surgery, Observatory, 7925.		
1.1 Does this protocol receive US Federal funding?		☐ Yes	X No

2. Protocol status (tick ✓)

X	Research-related activities are ongoing
☐	Data collection is complete, data analysis only
Please indicate (in the block below) the titles and HREC reference numbers of any projects currently making use of the Database/registry/repository	

3. Protocol summary

Total number of records or specimens collected, reviewed or stored since the original approval		100
Total number of records or specimens collected, reviewed or stored since last progress report		60
Have any research-related outputs (e.g. publications, abstracts, conference presentations) resulted from this research? If yes please list and attach with this report	X Yes	☐ No

4. Signature

Signature of PI		Date	4.4.2018

7.4. Ethics approval by the provincial health department of the Eastern Cape

Eastern Cape Department of Health

Enquiries:	Zonwabele Merile	Tel No:	040 608 0830
Date:	12 February 2016	Fax No:	043 642 1409
E-mail address:	zonwabele.merile@echealth.gov.za		

Dear Mr B.Mhlekude

RE: Defining and comparing the anti-HIV-1 and anti –HIV-2 activities of the native cervical mucins and equivalent cloned mutants in the context of cell-free and cell-associated HIV strains. (EC_2016RP7_393)

The Department of Health would like to inform you that your application for conducting a research on the abovementioned topic has been approved based on the following conditions:

1. During your study, you will follow the submitted protocol with ethical approval and can only deviate from it after having a written approval from the Department of Health in writing.
2. You are advised to ensure, observe and respect the rights and culture of your research participants and maintain confidentiality of their identities and shall remove or not collect any information which can be used to link the participants.
3. The Department of Health expects you to provide a progress on your study every 3 months (from date you received this letter) in writing.
4. At the end of your study, you will be expected to send a full written report with your findings and implementable recommendations to the Epidemiological Research & Surveillance Management. You may be invited to the department to come and present your research findings with your implementable recommendations.
5. Your results on the Eastern Cape will not be presented anywhere unless you have shared them with the Department of Health as indicated above.

Your compliance in this regard will be highly appreciated.

SECRETARIAT: EASTERN CAPE HEALTH RESEARCH COMMITTEE

Ikamva eliqaqambileyo!

7.5. Clinical profiles of the study cohorts: Crude cervical mucus plugs

HIV negative cohort

Code Name	Date of Birth	Race	HIV Status	HAART Duration	CD4 Count (cells/mm^3)	Viral Load (RNA copies/ml)	Vaginal Infection
CM1	18.12.1990	Black	Negative	N/A	N/A	N/A	None
CM2	29.11.1993	Black	Negative	N/A	N/A	N/A	None
CM4	23.03.1995	Black	Negative	N/A	N/A	N/A	None
CM5	15.09.1992	Black	Negative	N/A	N/A	N/A	None
CM18	15.12.1995	Black	Negative	N/A	N/A	N/A	None
CM25	16.12.1995	Black	Negative	N/A	N/A	N/A	None
CM32	06.06.1991	Black	Negative	N/A	N/A	N/A	*E. coli* UTI
CM39	10.06.1994	Black	Negative	N/A	N/A	N/A	None

HIV positive cohort

Code Name	Date of Birth	Race	HIV Status	HAART Duration	CD4 Count (cells/mm^3)	Viral Load (RNA copies/ml)	Vaginal Infection
CM7	29.01.1984	Black	Positive	36 months	347	Undetectable	Genital warts
CM12	21.03.1996	Black	Positive	5 weeks	Missing	Missing	None
CM14	15.12.1991	Black	Positive	6 months	388	Undetectable	None
CM22	25.06.1989	Black	Positive	3 months	270	Undetectable	None
CM28	05.10.1988	Black	Positive	1 month	367	36	None
CM42	23.06.1993	Black	Positive	18 months	406	4980	Vaginal discharge
CM47	27.05.1985	Black	Positive	6 months	727	55	None
CM48	31.07.1994	Black	Positive	6 months	388	Undetectable	None

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