Separation and purification of mucins and tenascin-C in breast milk of patients and the investigation of the role of mucins and tenascin-C in the inhibition of HIV-1

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DECLARATION

I, *Kathleen Kehoe*, hereby declare that the work on which this dissertation/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 this or any other university.

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This study was performed under the supervision of Professor Anwar Suleman Mall in the Department of Surgery, Faculty of Health Sciences, University of Cape Town during 2016 and 2017.

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Abstract

An estimated 36.7 million people were living with HIV in 2015, with 2.1 million newly infected people with HIV in 2015 worldwide. The highest prevalence of HIV in 2015 was in the Eastern and Southern African regions. This highlights the importance for research in this field to further prevent the number of new HIV cases. Mother-to-child transmission of HIV is due to either cell-associated or cell-free virus present in the breast milk of an HIV positive mother. Most often, HIV positive mothers choose to breastfeed their infants due to the nutritional and immunological benefits outweighing that of the risk of HIV transmission. Importantly, approximately 85% of infants do not acquire HIV through daily exposure to breast milk from their HIV positive mothers who are not on ART suggesting that human breast milk has antiviral properties. Previously in our laboratory, MUC1 and MUC4 has been implicated in the inhibition of HIV-1 in an *in vitro* assay. Furthermore, crude breast milk was tested in this assay showing strong HIV-1 neutralisation. Pasteurisation (80°C for 10 minutes) of both HIV positive and negative breast milk indicated good neutralisation of HIV-1 in our laboratory.

Another breast milk protein, tenascin-C (TNC), was recently shown to strongly neutralise HIV-1 in a study performed by another group. Therefore, with this knowledge, this study was employed to firstly compare the antiviral properties of MUC1, MUC4 and TNC. Furthermore, the HIV-1 neutralisation ability of crude breast milk was sought to be investigated along with the investigation of two different pasteurisation methods including 80°C for 10 minutes and 62.5°C for 30 minutes (Holder pasteurisation).

Human breast milk was separated into milk fat and skim milk using caesium chloride density gradient ultracentrifugation. MUC1 was purified from the milk fat using gel extraction from a 4-20% sodium dodecyl sulphate polyacrylamide gel. The skim milk was chromatographed on a Sepharose 2B-CL column from which the void volume was collected to purify TNC using gel extraction from a 4-20% sodium dodecyl sulphate polyacrylamide gel. During this purification, a band consisting of MUC1 which adhered to TNC was used to co-purify the MUC1/TNC glycoprotein using gel extraction. MUC1 and TNC were individually purified using gel extraction. MUC4 was not successfully purified and from ELISA data it was concluded that the concentration of MUC4 was below the detectable limit of the ELISA kit. The average concentration of MUC1 was determined to be 307.85 ng/ml, while the

concentration of TNC could not be determined due to the majority of absorbance values (450 nm) lying above the upper limit of the curve.

The HIV neutralisation of each of the samples was tested in an *in vitro* HIV-1 assay. This assay utilises a luciferase reporter gene in modified TZM-bl/JC cells using Du422.1 virus derived from clad C of HIV-1. These assays are being performed to assess the antiviral properties of crude and heat treated breast milk and purified MUC1 and TNC separately as well as co-eluted and co-purified MUC1/TNC. The two pasteurisation methods increased the HIV-1 neutralisation when compared to crude breast milk. The HIV-1 neutralisation of these groups were compared with a Kruskal Wallis test and a statistically significant difference was detected among the crude and 62.5°C heat treated breast milk cohorts (Mann-Whitney U, p-value = 0.0021). Furthermore, a statistically significant difference in the HIV-1 neutralization was detected among the 80°C and 62.5°C heat treated breast milk cohorts (Mann-Whitney, p-value = 0.0033). From the data, and the range of IC_{50} values (50% inhibitory concentration), the HIV-1 potency was deemed the strongest in the 62.5°C heat treated breast milk. This pasteurisation method could potentially be promoted in lower resource settings to decrease mother-to-child transmission of HIV-1.

Purified MUC1 and TNC, as well as co-eluted and co-purified MUC1/TNC, was tested in the same neutralization assay in order to compare the HIV-1 potency of these glycoproteins. The difference in HIV-1 neutralisation was not statistically significant among all three groups (Kruskal Wallis, p-value = 0.13). From the range of IC_{50} values, it was suggested that TNC has a stronger HIV-1 potency when compared to MUC1. Overall, the co-eluted and co-purified MUC1/TNC showed a lower HIV-1 potency when compared to the single, purified glycoprotein. MUC1 and TNC could be purified and cloned to aid in the protection against contracting HIV-1, especially in mother-to-child transmission of HIV-1.

Histochemistry and immunohistochemistry was performed on breast tissue sections to investigate the morphology of the cells and the presence of MUC1, MUC4 and TNC respectively. The lactating breast tissue was confirmed to have wide, dilated lumina and vacuolated cytoplasm with neutral and sialomucins. The presence of MUC1, MUC4 (β subunit) and TNC were confirmed in the lactating breast tissue using immunohistochemistry.

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Abbreviations

A.P.E.S	Aminopropyl-triethoxy-silane
AIDS	Acquired immune deficiency syndrome
AMPS	Ammonium persulphate
ART	Antiretroviral therapy
ASGP-1	Ascites sialoglycoprotein-1
ASGP-2	Ascites sialoglycoprotein-2
AZT	Aziodothymidine
AZT/3TC	Combivir
CD4	Cluster of Differentiation 4
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CsCl	Caesium chloride
DAB	Diaminobenzidine
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEAE	Diethylaminoethyl
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF-like	Epidermal growth factor-like
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal bovine serum
FFPE	Formalin fixed paraffin embedded
FNIII	Fibronectin-type III
FReD	Fibrinogen related domain
GDPH	Gly-Asp-Pro-His
gp120	Glycoprotein 120
gp41	Glycoprotein 41

GuHCl	Guanidinium hydrochloride
H&E	Mayer's haematoxylin and eosin
HCl	Hydrochloric acid
HID	High Iron Diamine
HID/AB	High Iron Diamine/Alcian Blue
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
IC_{50}	50% inhibitory concentration
ICGEB	International Centre for Genetic Engineering and Biotechnology
kDa	Kilodalton
LC_{50}	50% lethal concentration
M	Major
mBar	Millibar
MFGM	Milk fat globule membrane
mRNA	Messenger ribonucleic acid
MTT	Thiazolyl blue trazolium bromide
N	Non-major and non-outlier
NaCl	Sodium chloride
NaN_3	Sodium azide
NEAA	Non-essential amino acids
NEM	N-Ethylmaleimide
NGS	Normal goat serum
NVP	Nevirapine
O	Outlier
PAS	Periodic acid Schiff's
PAS/AB	Periodic acid Schiff/Alcian Blue
PBS	Phosphate buffered saline
PBST	PBS with Tween-20
PenStrep	Penicillin Streptomycin
PMSF	Phenylmethylsulfonylfluoride

RLU	Relative light units
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sIgA	Secretory immunoglobulin A
SMC	Sialomucin complex
TAE	Tris-acetate-EDTA
TBST	Tris saline buffer with Tween-20
TEMED	N,N,N,N,'-tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TNC	Tenascin-C
TNR	Tenascin-R
TNW	Tenascin-W
TNX	Tenascin-X
Tris	Tris(hydroxymethyl)-aminomethane
V_0	Void volume
V_i	Included volume
w/v	Weight per volume

Chapter 1: Introduction

1.1 HIV: the epidemic

An estimated 36.7 million people were living with Human immunodeficiency virus (HIV) in 2015, with 2.1 million newly infected people with HIV in 2015 worldwide (UNAIDS 2016). New HIV infections among adults have not declined since 2010 with 1.9 million adults becoming infected yearly, whereas new infections among children have decreased by approximately 50% from 290 000 new HIV infections in 2010 to 150 000 new HIV infections in 2015 (UNAIDS 2016). The burden of HIV is the largest in the Eastern and Southern African region with approximately 52% of total HIV infections resulting from this region (Figure 1.1). The lowest HIV prevalence can be noted in the Eastern Europe and central Asian region (Figure 1.1).

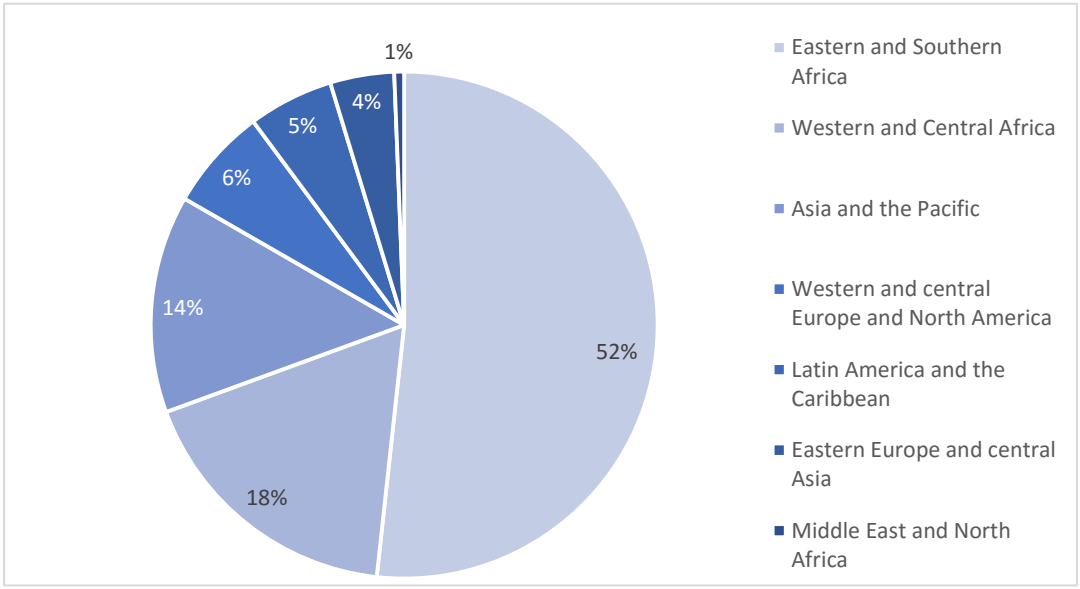

Figure 1.1. People living with HIV in different regions of the world in 2015 (figure adapted from regional data from UNAIDS, 2016).

In East and Southern Africa, 19 million people were living with HIV in 2015 of which more than half were women (UNAIDS 2016). Factors that increase the risk of HIV infection for women in Sub-Saharan Africa include biological and behavioural risk factors as well as socioeconomic and cultural risk factors (Ramjee & Daniels 2013). In this region, the new HIV infection rate decreased by 15% between 2010 and 2015 with an estimated 960 000 new HIV infections in 2015 (UNAIDS 2016). There has been a decline in new HIV infections among children by 66% between 2010 and 2015 in this region and in 2015, there was an estimated 56 000 children reported with new HIV infections (UNAIDS 2016). In South Africa itself,

there were approximately 6.19 million people living with HIV and the prevalence was approximately 11.2% (Statistics South Africa 2015). In Gauteng, an estimated 24% of the provinces population were living with HIV whereas the Northern Cape had the lowest prevalence of HIV (2.2%) in 2015 (Figure 1.2) (Statistics South Africa 2015). The burden of HIV is the greatest in Gauteng, Kwa-Zulu Natal and the Eastern Cape. South Africa currently struggles with this epidemic and effective treatment and prevention strategies are required to reduce the prevalence and incidence of HIV.

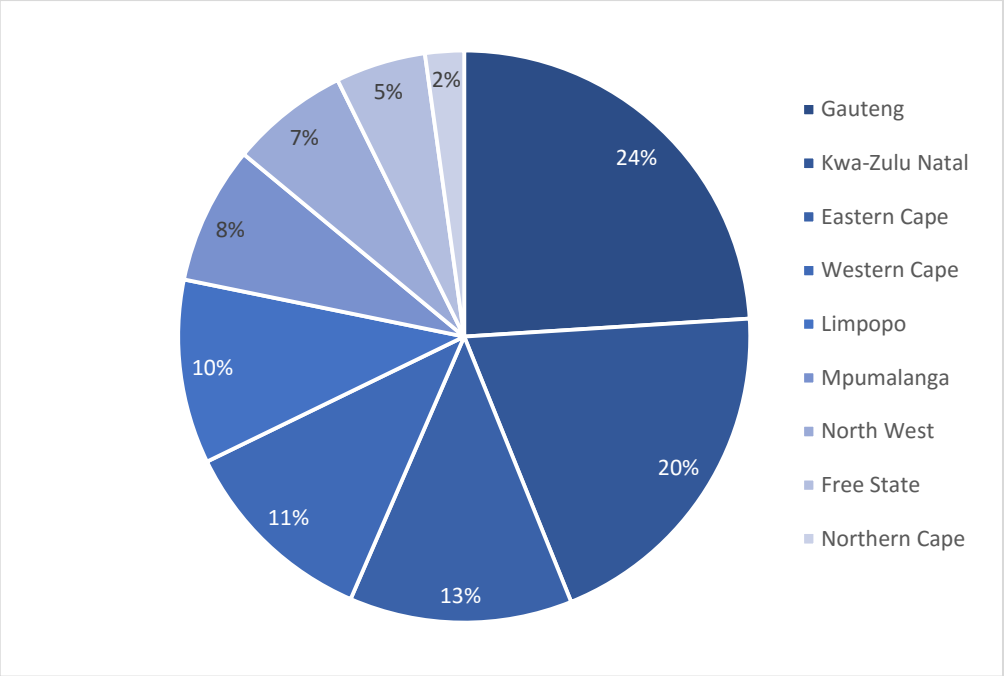

Figure 1.2. The distribution of HIV positive individuals in the nine provinces in South Africa in 2015 (Statistics South Africa 2015).

HIV morbidity and mortality has been effectively reduced by antiretroviral therapy (ART) which is able to reduce the viral load to below a detectable level (Dieffenbach 2009). ART improves life expectancy of those living with HIV and controls the HIV/AIDS epidemic as well as reducing the progression of HIV to Acquired immune deficiency syndrome (AIDS) (Dieffenbach 2009). As a result, AIDS-related deaths decreased from 2 million worldwide in 2005 to 1.1 million AIDS-related deaths worldwide in 2015 (UNAIDS 2016). In June 2016, there were 18.2 million HIV positive people who had access to ART which is approximately a two and a half fold increase to that observed in 2010 (UNAIDS 2016). Regional data collected in 2015 indicates the percentages of people accessing ART in three categories: adults (aged 15+), children (aged 0-14) and pregnant women to prevent mother-to-child transmission. The highest coverage across all three groups in 2015 can be noted in Western and central Europe

and North America; eastern and southern Africa and Latin America and the Caribbean (Figure 1.3). In the eastern and southern African region, 90% of pregnant women are accessing ART and 53% and 63% of adults and children are accessing ART respectively in 2015 (UNAIDS 2016). Successful ART programmes are imperative in countries with a high burden of disease, for example the eastern and southern Africa region.

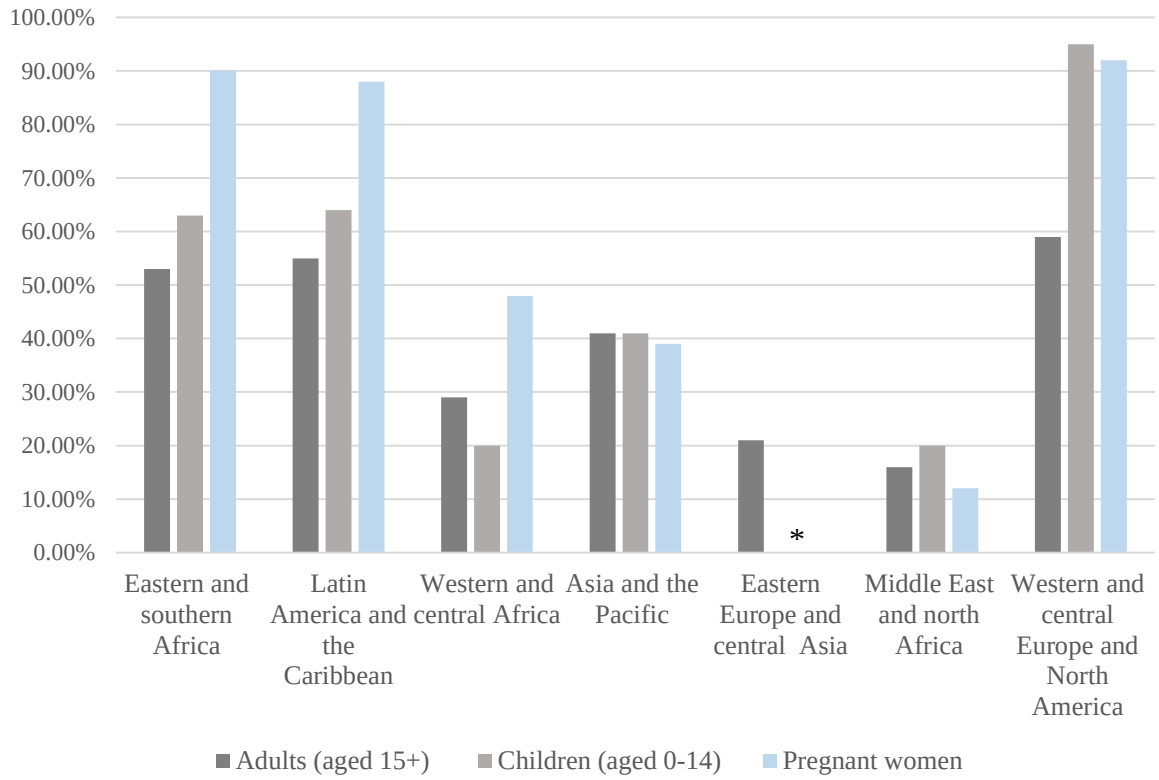

Figure 1.3. The regional distribution of antiretroviral therapy in 2015 in three categories: adults (15+), children (0-14) and pregnant women. The data is adapted from regional antiretroviral therapy – 2015 in UNAIDS 2016. The data for children and pregnant women in the Eastern Europe and central Asia region was not available at the time of publication (*).

1.1.1 HIV characteristics

HIV can be categorised into two main groups namely HIV-1 and HIV-2. The suspected origin of HIV-1 could be due to the transmission of a chimpanzee virus to humans, and the origin of HIV-2 is also due to cross-species transmission however HIV-2 is thought to have arisen from a Sooty Mangabey virus (Hahn et al. 2000; Sharp et al. 2005; Lemey et al. 2003). The variation between the two groups can be attributed to the differences in their envelope genes resulting in approximately 30% variation between these groups (Levy 2009). HIV-1 infection is more

prevalent worldwide and progresses fast to immune deficiency, whereas HIV-2 is less prevalent with a slower progression and inefficient transmission of the virus, even *in utero* (Nyamweya et al. 2013; Jaffar et al. 2004).

HIV-1 has been further categorised into three main groups based on the transmission events and include: Major (M), Outlier (O), and non-major and non-outlier (N) (Korber et al. 2000; Keele et al. 2006). The majority of HIV-1 infections are due to M group viruses which consist of nine clades namely A-D, F-H and J-K (Cohen et al. 2008). These clades contain distinct DNA sequences which differ 15-20% (McCutchan 2000). HIV-2 consists of different groups from A-E with group A and B the most common occurring (Chen et al. 1997; Cohen et al. 2008).

In North and South America, Europe and Australia, clade B of HIV-1 is the most common whereas Southern Africa is largely affected by clade C of HIV-1 (Cohen et al. 2008). Clade C accounts for 48% of worldwide HIV-1 infections and is the most common subtype in South Africa. Kwa-Zulu Natal is severely effect by HIV-1 with 95% of cases attributed to clade C (Gordon et al. 2003). Recombinant HIV-1 strains of the different clades have been noted globally with the recombinant clade A and G strain noted in West Africa and the recombinant B and C strain mostly noted in China (McCutchan 2000). HIV-2, although less prevalent, mainly affects West Africa and India and a limited prevalence can be noted in Portugal and former Portuguese colonies (Cohen et al. 2008).

1.1.2 The transmission of HIV-1

HIV-1 has spread unequally across the globe with some regions more affected than others. The multiple ways in which HIV-1 is transmitted includes blood and blood products, infected mothers transmitting the virus to their infants either during pregnancy, birth or breastfeeding and also through sexual intercourse (vagina or anal intercourse) (Cohen et al. 2008). The transmission of HIV-1 will depend on the infectiousness of the person transmitting HIV-1 and the vulnerability of the host (Galvin & Cohen 2004). The concentration of HIV-1 will depend on the stage of the disease and change throughout the stages in blood and genital secretions (Pilcher et al. 2007). The first days after infection due to exposure of HIV-1 is the stage of infection and viremia however the HIV-1 specific antibody-mediated immune response cannot be detected as yet in a reliable manner and this is known as acute HIV-1 infection (Pilcher et al. 2004). Acute HIV-1 infection accounts for a large amount of HIV-1 transmission via sexual exposure due to the strong viral replication (Wawer et al. 2005; Cohen et al. 2008). The acute

HIV-1 infection will settle as the viral load in the infected individual reaches a "set point" which is affected by other microbial pathogens such as malaria and sexually transmitted diseases (Fellay et al. 2007; Galvin & Cohen 2004).

The HIV-1 will bind to cells which have a Cluster of Differentiation 4 (CD4) glycoprotein receptor and either the CCR5 or CXCR4 co-receptor (Levy 2009). HIV-1 infects cells using two co-receptors namely CCR5 and CXCR4, with the CCR5 co-receptor utilised most of the time (Levy 1996; Berger et al. 1999). Viruses that are macrophage-tropic utilise the CCR5 co-receptor and are known as R5 viruses, whereas viruses utilising CXCR4 co-receptors are known as X4 viruses and infect T-cell lines (Berger et al. 1999; Tersmette et al. 1988). The viral envelope glycoprotein 120 (gp120) binds to the host CD4 receptor of the target cells including CD4+ thymus cells (T cells), macrophages and dendritic cells resulting in a conformational change which will expose the CCR5 and CXCR4 co-receptors allowing an interaction with high-affinity between either the co-receptors or gp120 (Haase 2011; Klasse 2012; Pancera et al. 2010). Subsequently, there are conformational changes in glycoprotein 41 (gp41) due to the receptor binding allowing the fusion of the viral envelope and the target cell membrane (Pancera et al. 2010). The virion then enters the host cell releasing its' viral core into the cytoplasm in order to convert the viral RNA to viral DNA by the activity of the viruses reverse transcriptase (Klasse 2012). Viral integrase is then responsible for integrating the viral DNA into the host cell genome by moving into the cell nucleus as a nucleoprotein complex with viral DNA and viral integrase (Klasse 2012; Chen et al. 2000). It is important to note that the infection cannot be reversed at this stage and viral replication occurs due to viral DNA which is transcribed by RNA polymerase II into viral RNA (Engelman & Cherepanov 2012).

The mucous membranes, the primary route of HIV-1 transmission, line the internal tracts of the body providing protection against pathogens such as HIV-1 (Wu & KewalRamani 2006). The delicate nature of this mucous barrier could result in potential damage thus allowing HIV-1 infection however this is not always the case for HIV-1 transmission. HIV-1 is bound by dendritic cells in the rectal mucosa and the vaginal epithelium resulting in infection and viral transmission (Wu & KewalRamani 2006). HIV transmission can occur via multiple modes with sexual intercourse being the most common mode. HIV transmission is more efficient when the virus is transmitted from male to female in which HIV infected semen comes into close contact with the mucosal membranes of the vagina or rectum (Collins et al. 2000; Duriux-Smith & Goodman 1992; Pettifor et al. 2005). A viral reservoir can be formed in the mucosal surfaces due to the presence of cell-associated virus and potentially result in latent infection (Collins et

al. 2000). Women are more susceptible to HIV infection during sexual intercourse due to high concentrations of the virus in semen and the larger mucosal surface area which could potentially result in viral transmission (Ramjee & Daniels 2013). HIV transmission can also occur through anal intercourse at a higher risk than vaginal intercourse as rectal fluids contain a high viral load and the resultant damage to the colorectal epithelium will increase viral transmission (Zuckerman et al. 2004). Mother-to-child HIV transmission can occur during pregnancy, at birth or during breastfeeding.

1.2 Human breast milk

Breastfeeding is an important source of infant nutrition and the recommended period of breastfeeding is one to two years of life, with the first six months exclusive to breastfeeding only (Gartner et al. 2005; Ballard & Morrow 2013). Human breast milk has a dynamic composition when compared to the fixed composition of infant formula milk and thus, human breast milk is suited to infants to promote health, survival and development (Ballard & Morrow 2013; Oftedal 2012). The dynamic nature of human breast milk results in differences over lactation and feeding, and among mothers of different populations due to environmental and maternal factors as well as the storage of human breast milk (Ballard & Morrow 2013).

Colostrum is first produced postpartum for 2-4 days and is important for infant's immune system by transferring acquired and innate factors to the infant (Ballard & Morrow 2013; Godhia & Patel 2013). Colostrum is rich in immunological factors including lactoferrin, secretory immunoglobulin A (sIgA), and leukocytes and the main function of colostrum is to transfer these immunological factors rather than providing nutrition to the infant (Ballard & Morrow 2013). Colostrum has a lower concentration of lactose, potassium and chloride and high concentrations of sodium and magnesium when compared to milk during the later stages of lactation (secretory activation and transitional milk) (Kulski & Hartmann 1981; Pang & Hartmann 2007). Secretory activation and the production of transitional milk is characterised by the change in lactose concentration and sodium to potassium ratio due to the closure tight junctions in the mammary epithelium (Ballard & Morrow 2013). The secretory activation occurs from 72 hours onwards after delivery and biochemical markers that induce the onset of the secretory activation that include the sodium to potassium ratio and the change in citrate, sodium and lactose concentration (Cregan et al. 2002; Ballard & Morrow 2013). Transitional milk production starts from 5 to 14 days postpartum and is similar to colostrum. However an increased production of milk is needed in order to provide the nutritional and developmental

support required by the infant to grow (Ballard & Morrow 2013). Human breast milk is now considered to be fully mature and the composition will remain relatively the same with minor changes for the duration of lactation (Ballard & Morrow 2013).

1.2.1 Storage and pasteurisation of human breast milk

Human breast milk is often expressed and stored before being fed to the infant. The storage of breast milk will affect the nutritional component of breast milk based on the storage method utilised (Ballard & Morrow 2013). The bactericidal capacity of milk is reduced due to long-term storage and freeze-thaw cycles due to significant degradation of breast milk components (Ballard & Morrow 2013). There are different methods which are used to store breast milk at home in order to maintain the integrity of the breast milk. The Academy of Breastfeeding Medicine suggest that freshly expressed milk be stored at room temperature (16-29°C) for three to four hours and could be stored between six and eight hours under conditions of extreme cleanliness (Eglash et al. 2010). Fresh milk is considered to be better than frozen milk and frozen milk should be thawed in the fridge overnight using the oldest milk first (Eglash et al. 2010). Human breast milk should be stored in the freezer for 6 months optimally at < -17°C, however 12 months is acceptable (Eglash et al. 2010).

Expressed milk can be pasteurised to reduce the transfer of pathogens especially for donor milk (Ballard & Morrow 2013). Pasteurisation techniques include high temperature, short time heating which involves heating milk at 72°C for 15 seconds; flash heating involves placing a bottle of milk in water which is brought to a boil and then the milk is removed and rapidly cooled; heating milk at 80°C for 10 minutes and lastly, Holder pasteurisation which involves heating milk at 62.5°C for 30 minutes (Ballard & Morrow 2013; Buchheim et al. 1988). Holder pasteurisation has been shown to not modify the protein profile of human milk in the majority of samples suggesting that the biological activity of the human milk proteins are maintained after this process and is the method preferred by the Human Milk Banking Association of North America (Peila et al. 2016; Ballard & Morrow 2013). The aim of flash heating is to effectively pasteurise breast milk at home, especially in poorer regions in the world as pasteurisation is thought to prevent transmission of HIV from mother to child during breastfeeding (Ballard & Morrow 2013). The biological activity of human milk components seems to be affected differently by the different pasteurisation methods used. The antimicrobial activity is better preserved in human milk using the flash heating method when compared to Holder pasteurisation (Chantry et al. 2011).

1.2.2 Components of human breast milk

Components of human breast milk include lactose, lipids, oligosaccharides and proteins with lactose being the most abundant component and proteins the fourth most abundant component (Froehlich et al. 2010). The concentration of protein decreases in breast milk over the duration of lactation and typically ranges between 10 and 20 mg/ml (Froehlich et al. 2010). The proteins are divided into whey, casein and mucins (Liao et al. 2011; Lönnerdal 2003). The mucins are found in the fat portion of breast milk and the concentration is most likely to remain consistent throughout lactation however the concentration of whey proteins is high during the early stages and the concentration of casein proteins is undetectable during the first few days of lactation (Lönnerdal 2003; Kunz & Lönnerdal 1990; Kunz & Lönnerdal 1992). Lactoferrin, α-lactalbumin, secretory immunoglobulin IgA, lysozyme and serum albumin are the most abundant proteins present in human breast milk (Lönnerdal 2004; Ballard & Morrow 2013). Human milk nitrogen is comprised of non-protein nitrogen containing proteins such as urea, uric acid, creatine, creatinine, amino acids and nucleotides (Ballard & Morrow 2013).

1.2.3 Milk fat globule membrane

The proteins and glycoproteins along with phosphor-, glycero-, glyco- and sphingolipids are present in the milk fat globule membrane (MFGM) of breast milk (Figure 1.4) (Dewettinck et al. 2008). Glycoproteins consists of 20-60% of the MFGM, glycerophospholipids consist of 15-33% of the MFGM and sphingo- and glycolipids, triacylglycerols and sterols consists of 0.3% of the MFGM with the remaining MFGM comprising of enzymes and other minor components (Lopez et al. 2010). As the stages of lactation progress, the expression or glycosylation levels of glycoproteins differ and this dynamic glycoprotein component of breast milk suggests glycoproteins perform a more-structure specific role influencing cell-cell interactions, protein functions, stability and susceptibility to proteolysis (Froehlich et al. 2010). The MFGM is derived from the apical plasma membrane and contains a lipid core consisting of triglycerides which is surrounded by a single layer of polar lipids and proteins and an outer, double layer of polar lipids and proteins (Lopez et al. 2010; Dewettinck et al. 2008). Mucins such as MUC1 and MUC4, present in both human and bovine milk are located in the MFGM, with MUC15 expressed in the MFGM of bovine milk (Wilson et al. 2008; Lopez et al. 2010).

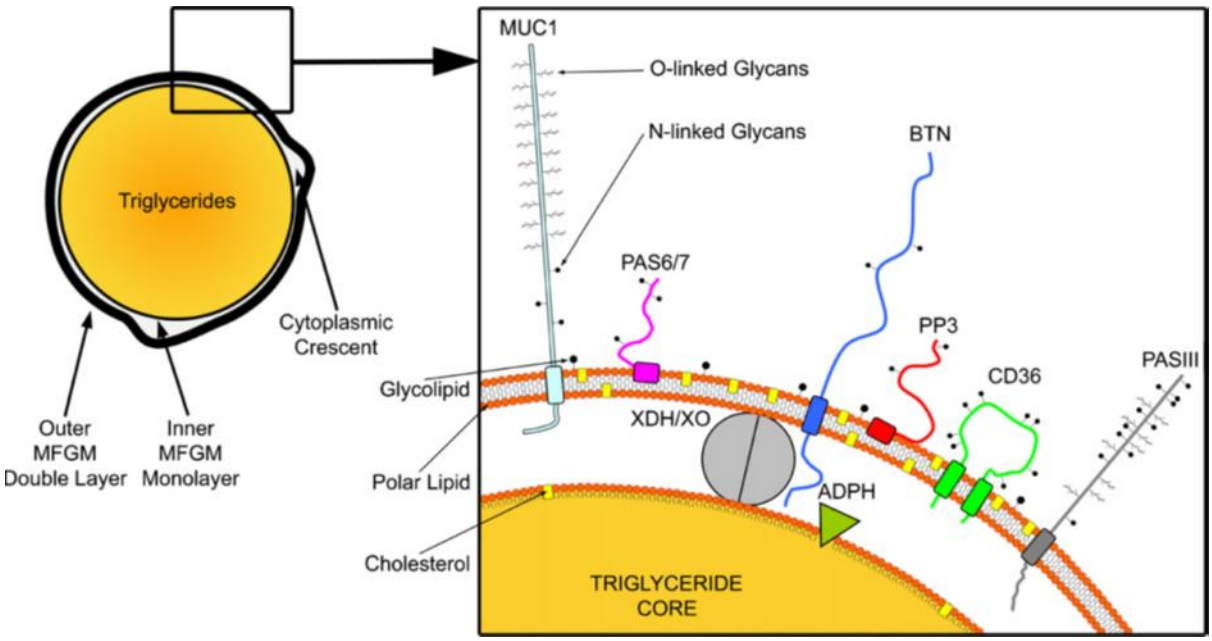

Figure 1.4. A schematic representation, not to scale, of the organisation of the milk fat globule membrane with an inner monolayer of polar lipids surrounded by a double layer of polar lipids (Dewettinck et al. 2008).

1.2.4 Antimicrobial activity of human breast milk

Human breast milk has the ability to inhibit the activity of pathogenic bacteria, viruses, and fungi with most of the breast milk proteins inhibiting the same pathogen suggesting that the immune system of a breastfed infant acts on multiple layers to ensure infection does not occur (Lönnerdal 2003; Dewey et al. 1995). Secretory immunoglobulin A (sIgA) is the predominant immunoglobulin expressed in high concentrations in the colostrum and a substantial concentration in mature human breast milk (Liu & Newburg 2013). This immunoglobulin is sensitive to pasteurisation, with approximately 70% activity remaining after Holder pasteurisation, still allowing sIgA to specifically bind to a pathogen to inactivate it (Czank et al. 2009; Lönnerdal 2003). These antibodies target bacterial pathogens including *Escherichia coli, Haemophilus influenzae, Streptococcus pneumoniae* and *Salmonella* as well as a host of viruses such as rotavirus, HIV, influenza virus and cytomegalovirus (Goldman 1993). Lactoferrin helps the infant absorb free iron by chelation which also inhibits bacterial growth and other microorganisms including viruses, parasites and fungi (Liu & Newburg 2013). Lactoferrin has been implicated in the inhibition of HIV and *C. albicans* (Lönnerdal 2004). After Holder pasteurisation, the activity of lactoferrin is affected and only 39% of it remains

(Czank et al. 2009). Lactoferrin responds to the population of bacteria in the infants gut due to the changes in glycosylation of the protein which is genetically regulated to provide protection (Barboza et al. 2012). Lysozyme, together with lactoferrin, are able to target and kill gram-negative bacteria *in vitro* (Ellison & Giehl 1991). Lysozyme, in an *in vitro* assay, can inhibit the growth of HIV however in human breast milk lysozyme may act on the free virus rather than the cell-associated virus (Lee-Huang et al. 1999). κ-Casein inhibits the adhesion of *Helicobacter pylori* in gastric mucosa specifically at younger ages, and κ-Casein in breast milk aids this protection (Lönnerdal 2004). The inhibition of *Helicobacter pylori* can be attributed to lactoferrin as well as sIgA and κ-Casein, suggesting that these proteins work together to inhibit the growth and adhesion of *Helicobacter pylori* (Lönnerdal 2004). Other breast milk proteins that have been implicated in antimicrobial activity include lactoperoxidase, haptocorrin and α-Lactalbumin (Lönnerdal 2004).

1.2.5 Breast milk and HIV

Importantly, breast milk has nutritional benefits for infants to promote health, however mother-to-child transmission of HIV results from breast milk carrying the infectious agents (Jones 2001; Wahl et al. 2015). HIV-positive mothers in developed countries tend to use formula milk as a substitute for breast milk however in developing countries and regions with limited resources, HIV positive mothers continue to breastfeed their infants as nutritional and immunological benefits of breastfeeding surpass the potential HIV transmission (Semba et al. 1999; Wahl et al. 2015). The World Health Organisation suggests that HIV-positive mothers exclusively breastfeed for the first six months of the infants life in combination with antiretroviral therapy (ART), whether it be infant or maternal ART (WHO & UNICEF 2011). Approximately 260 000 are affected by paediatric HIV-1 infection annually, with half of infections attributed to breastfeeding (UNAIDS 2013). It is important to note that most infants, approximately 85%, however do not acquire HIV through daily exposure to breast milk from their HIV positive mothers who are not on ART (WHO et al. 2008). The risk of HIV transmission through breastfeeding is thus low and is thought to be due to the activity of the antiviral factors present in breast milk (Wahl et al. 2015). Human breast milk contains a high concentration of antibodies that are alleged to provide protection to infants in order to prevent them from contracting HIV through breast milk (Hanson et al. 2003).

Human breast milk of HIV positive mothers contains both cell-associated and cell-free virus which increases the risk of HIV transmission for the duration of breastfeeding (Koulinska et

al. 2006; John et al. 2001; Van de Perre et al. 1993). HIV-1 can either be transmitted through HIV-1 moving from the vascular system into the breast milk or HIV-1 itself replicating in breast milk and mammary glands (Van de Perre et al. 2012). The frequency of either cell-associated or cell-free virus in breast milk is not known for the various stages of lactation and it is therefore important to ensure that the viral load and number of infected cells are reduced in breast milk to prevent and reduce the transmission of HIV through breastfeeding. Furthermore, HIV transmission could be targeted at the oral mucosa of the infant to prevent mother-to-child transmission (Wahl et al. 2012). Cell-free HIV-1 particles in breast milk can mediate HIV-1 transmission, particularly in late lactation, from HIV positive mothers to infants (Van de Perre et al. 2012). HIV transmission due to breast milk is predominantly due to cell-associated HIV-1 virus producing T cells (latently infected or activated) (Van de Perre et al. 2012). There is a reservoir of cell-associated virus present in breast milk which plays a part in mother-to-child transmission and this reservoir is not completely eliminated by ART (Van de Perre et al. 2012). The reservoirs of cell-associated virus in breast milk are responsible for the low-level of mother-to-child transmission of HIV despite effective preventative ART programmes (Danaviah et al. 2015). Therefore, it is important to monitor the viral load of mothers during pregnancy and breastfeeding as well as monitoring the viral load in infants to ensure the prevention of HIV transmission and early diagnosis (Danaviah et al. 2015).

HIV positive mothers that are eligible for ART need to be compliant to their ART regime for the duration of their pregnancy and breastfeeding to ensure that HIV transmission is successfully prevented (Van de Perre et al. 2012). WHO has three recommendations of ART treatment to prevent HIV transmission from HIV positive mothers to their breastfed infant. Option A includes HIV positive mothers receiving aziodothymidine (AZT) daily as early as 14 weeks of gestation until the onset of labour at which time the mother receives nevirapine (NVP) and their first dose of combivir (AZT/3TC), which they continue to receive for seven days postpartum (UNICEF 2012; Van de Perre et al. 2012). The infant receives NVP daily for four to six weeks if the mother is on treatment with one week of treatment after breastfeeding is terminated (UNICEF 2012; Van de Perre et al. 2012). Option B includes triple antiretroviral treatment starting at 14 weeks of gestation which is continued throughout pregnancy and labour and if the mother is breastfeeding, the treatment continues for the duration of breastfeeding and furthermore, for one week after breastfeeding is terminated (UNICEF 2012; Van de Perre et al. 2012). From birth, infants will receive NVP or AZT for four to six weeks regardless of whether the infant is breastfed or formula fed (UNICEF 2012). Option B+ was later introduced

and includes mothers receiving triple antiretroviral treatment as soon as they are diagnosed and is continued for life (UNICEF 2012). The infant receives the same treatment as described in option B. Antiretroviral drugs can diffuse into breast milk and therefore could result in infants overdosing if the treatment is not correctly adhered to as described by one of the three options recommended by WHO (Van de Perre et al. 2012). Due to the reservoir of cell-associated virus present in breast milk, mother-to-child transmission cannot completely be eliminated by the mothers ART regime only (Van de Perre et al. 2012). Therefore, it is necessary for infants to adhere to a prophylaxis regime during breastfeeding to ensure that the possible routes of HIV-1 transmission are protected (Van de Perre et al. 2012). It is important to understand mother-to-child transmission in order to effectively implement treatment options for mothers and prophylaxis programmes for infants.

1.3 Mucus

Mucus lines epithelial surfaces with a viscoelastic gel lining secreted by the mucosa, protecting epithelial surfaces from injury and infection as well as hydrating the internal tracts of the body (Allen 1981). This crude mucus functions as a protective layer on the mucosal surfaces to protect the mucosa from the hostile luminal environment in the respiratory and gastrointestinal tract, auditory and urogenital systems, cervix and the conjunctiva of the eye (Allen 1978; Perez-Vilar & Mabolo 2007). The location and pathophysiological conditions of mucus will determine the composition of the mucus, but it is normally composed of water, inorganic salts, immunoglobulins, mucins and secreted proteins (Rachagani et al. 2009). There are multiple locations of the crude mucus highlighting the importance and versatility of this secretion. Foreign particles are trapped in this mucus in the respiratory tract to remove harmful substances with the help of ciliary action (Allen 1981). The crude mucus layer in the stomach is important for protecting the epithelial surfaces from the mechanical force and hydrochloric acid involved in digestion (Allen 1981). Vaginal epithelium is protected by mucus during sexual intercourse and a cervical plug of mucus aids protection against infection during the menstrual cycle, which will change consistency during the mid-cycle to facilitate fertilisation (Allen 1981).

1.3.1 Mucins

Mucus is comprised of mucous glycoproteins known as mucins which are responsible for the gel forming and viscous properties of the crude mucus (Allen 1981; Sellers et al. 1988). These high molecular weight glycoproteins along with the protein core (apomucins) is encoded by

mucin genes which undergo a number of post-translational modifications resulting in a heavily glycosylated glycoprotein (Zalewska et al. 2000). There are three categories of mucins namely secreted mucins, which can either be gel forming or non-gel forming, and transmembrane mucins. MUC2, MUC5AC, MUC5B, MUC6 and MUC19 are gel forming, secreted mucins forming oligomeric structures which are more cysteine rich when compared to non-gel forming mucins (Rose & Voynow 2006; Rousseau et al. 2008; Rachagani et al. 2009; Zalewska et al. 2000). Non-gel forming secreted mucins include MUC7, MUC8 and MUC9 which are soluble (Rachagani et al. 2009). The transmembrane mucins include MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20 (Mall 2008; Rachagani et al. 2009). These mucins are tethered to cells with a cytoplasmic tail and are important in signal transduction due to their association to cytosolic proteins and cytoskeleton proteins (Rachagani et al. 2009).

1.3.2 Structure of mucins

A protein core of the mucin is linked to carbohydrate side chains via O-glycosylated bonds to serine and threonine residues resulting in a highly glycosylated mucin (Allen 1981; Zalewska et al. 2000). The polymers will determine the viscosity of gel forming properties of mucins and approximately 70% of the mucin's molecular weight can be attributed to these carbohydrate side chains (Allen 1981). The regions of the mucins can either be glycosylated or not, with the glycosylated regions providing protection to the protein core from proteolytic enzymes (Carlstedt & Sheehan 1984). A number of tandem repeats rich in serine, threonine and proline residues are variable in the protein core and can be altered with many O-linked oligosaccharides and few N-glycan chains (Rachagani et al. 2009). N-acetylgalactosamine, N-acetylglucosamine, sialic acid, fucose and galactose are present in the glycosylated regions and importantly uronic acid, mannose and glucose are not present in mucins (Allen 1981). N-acetylgalactosamine is primarily responsible for linking carbohydrate side chains to the protein core via serine and threonine residues, whereas proline residues are important in maintaining a tight packed conformation of the carbohydrate side chains (Allen 1981). Disulphide bonds are formed between cysteine residues in the naked protein regions to link glycoprotein subunits with carbohydrate side chains repelling one another and these mucin polymers form gels which increase in viscosity as the concentration of mucins increase (Carlstedt & Sheehan 1984; Sellers et al. 1988).

1.3.3 Mucin conformation

The conformation and structure of mucins are surrounded by some controversy. The 'windmill' model of the mucin polymer was proposed by Allen and Snary (Figure 1.5) using pig gastric mucus (Allen & Snary 1972). From their experimental work, it was deduced that pure mucins weighed 2×10^6 daltons and when the disulphide bonds were reduced, four subunits resulted with each weighing 5×10^5 daltons (Allen & Snary 1972). Therefore, this model proposed that the C-terminal region of each of the four subunits are linked at the naked regions to a low molecular weight protein via disulphide bonds which appear as a windmill-like structure (Allen & Snary 1972).

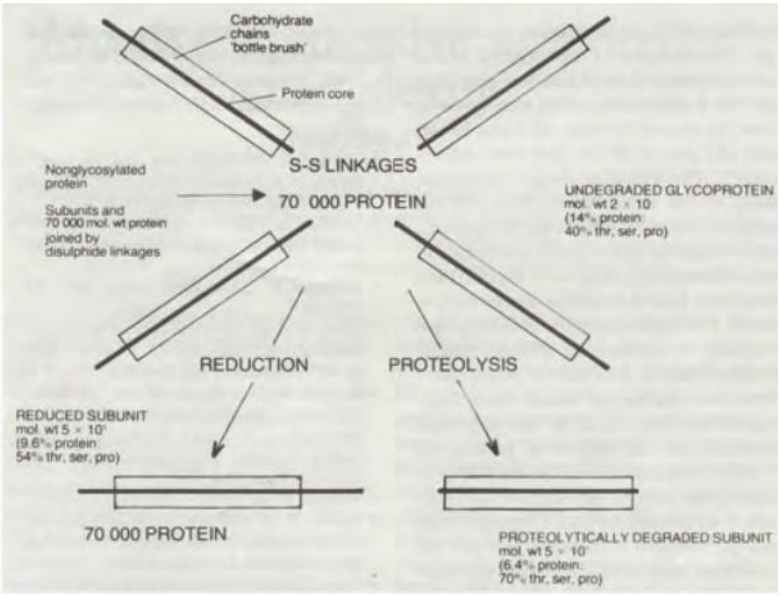

Figure 1.5. The 'windmill' model of the mucin polymer proposed by Allen and Snary (1972).

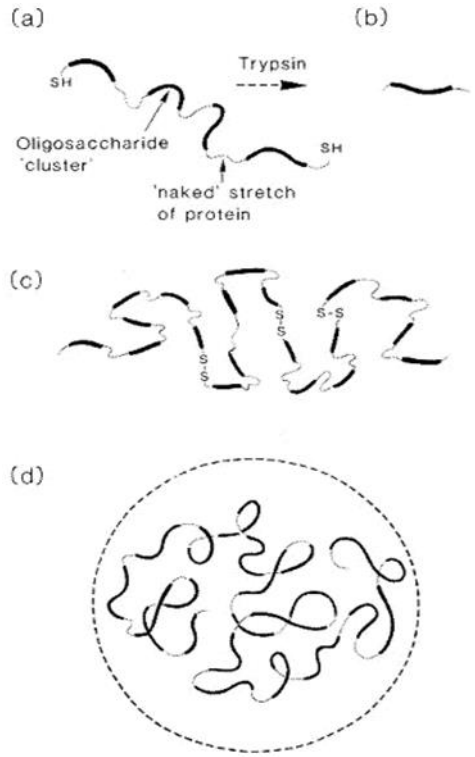

Figure 1.6. The linear model of the mucin polymer proposed by Carlstedt and Sheehan (1984). (a) A mucin subunit with interspersed naked regions of protein between a oligosaccharide cluster; (b) Separate oligosaccharide clusters due to trypsin treatment; (c) The oligosaccharide clusters join end-to-end, via disulphide linkages, to form mucins; (d) A random coil formation creating an exclusion zone.

An alternative model proposed that the conformation of mucins were joined in a linear arrangement with sporadic naked regions in between these glycosylated regions resulting in a random coil (Figure 1.6) (Carlstedt & Sheehan 1984). From experimental observations, Carlstedt and Sheehan noticed that the molecular weight of mucins was substantially larger than described by Allen and Snary with the mucins weighing between 10×10^6 and 45×10^6 daltons (Carlstedt & Sheehan 1984). Different sized fragments resulted when whole mucins were reduced or digested, supporting the idea that the naked protein regions are spread sporadically between the glycosylated regions (Carlstedt & Sheehan 1984).

The difference in the two models of mucin structures was noted by Mall *et al.* (Mall et al. 1988). It was noted that two different extraction methods were employed and could be a source of this variation. Allen and Snary used 0.2 M sodium chloride and 0.02% sodium azide and thereby not accounting for endogenous proteolysis (Allen & Snary 1972). A smaller mucin size was thus noted by the authors due to the active proteolytic enzymes. However, Carlstedt and

Sheehan accounted for proteolytic enzymes by using guanidine hydrochloride which would denature these enzymes retaining the linear conformation of the mucin structure (Carlstedt & Sheehan 1984). Furthermore, this extraction medium caused the aggregation of mucins and thus the authors noted a bigger structure (Carlstedt & Sheehan 1984). The linear model proposed by Carlstedt and Sheehan is widely accepted as the model that more accurately depicts the mucin conformation.

1.4 Breast milk mucins

The best characterised breast milk mucin is MUC1, but there is another breast milk mucin originally identified as MUCX and was thought to be heavily glycosylated with a higher molecular weight (Rossi et al. 1996). Further investigation into MUCX resulted in this mucin being identified as MUC4 (Liu & Newburg 2013). These mucins are contained within the MFGM in breast milk.

1.4.1 MUC1

MUC1 is located on chromosome 1q21 and the protein core's estimated molecular weight is 120-225 kDa with the molecular weight increasing to 250-500 kDa once glycosylation has occurred (Patton et al. 1995; Brayman et al. 2004). The *MUC1* gene, spanning 4-7 kb, contains seven exons which can be alternatively spliced resulting in transcripts that range from 3.7 kb to 6.4 kb (Brayman et al. 2004; Patton et al. 1995). There are a variable number of tandem repeats of 20 amino acids which are rich in serine, threonine and proline residues which allows for extensive O-glycosylation of the glycoprotein (Brayman et al. 2004; Peat et al. 1992). Human MUC1, found in human breast milk, contains approximately 50% carbohydrates with 30% being sialic acid (Patton et al. 1995).

A smaller fragment of MUC1, known as MUC1-CT which consists of three regions namely an extracellular stem region of 58 residues with O- and N-glycosylated sites, a short transmembrane domain and a 72 amino acid tyrosine phosphorylated cytoplasmic tail (Hattrup & Gendler 2008; Lacunza et al. 2009). The size of MUC1-CT can range between 25 kDa and 30 kDa once glycosylated and phosphorylated, while the protein core with the three regions explained above has a molecular weight of 14 kDa (Hattrup & Gendler 2008).

1.4.1.1 MUC1's normal and pathological functions

Epithelial tissues that express MUC1 under normal conditions include the mammary gland, the female reproductive tract excluding the ovary, the prostate and testis, the gastrointestinal tract, the salivary gland, the digestive tract excluding the large intestine and colon, the pancreas, the liver bile ducts, the lung, the kidney (both the distal tubules and collecting ducts), the bladder and the eye (Brayman et al. 2004; Gendler 2001). Furthermore, MUC1 is also expressed by hematopoietic cells and activated T cells thus modulating immune responses. (Brayman et al. 2004). MUC1 is responsible for blocking cell-cell interactions and cell-extracellular interactions, and furthermore the cytoplasmic tail interacts with signal transduction molecules (Gendler 2001). These cytoplasmic tails of MUC1 inhibit the E-adherin/β-catenin complex from forming by competing with β-catenin (Rakha et al. 2005). General functions of MUC1 include those that are generally attributed to mucins, such as hydration, protection and lubrication.

MUC1 is present in the milk fat globule membrane in milk and is known to bind bacteria (Patton et al. 1995; Rakha et al. 2005). The concentration of mucins (both MUC1 and MUC4) in human breast milk is 729±75 mg/ml and this will be concentrated in the MFGM (Liu & Newburg 2013). Maximal MUC1 expression in humans is present in the implantation phase (Hey et al. 1994). MUC1 is present in human breast milk and plays a role in the prevention of mother-to-child-transmission of HIV-1 (Brayman et al. 2004). It has been shown that MUC1 and MUC1 co-eluted with MUC4 can inhibit HIV-1 in an *in vitro* assay from both HIV positive and negative human breast milk (Habte et al. 2008; Mthembu et al. 2014). MUC1, which is abundantly present in breast milk, efficiently binds the Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor on the dendritic cells to effectively prevent DC-SIGN mediated transmission of HIV-1 (Saeland et al. 2009). This prevention of mother-to-child transmission is thought to be due to the repetitive Lewis-X structures within the MUC1 domain which are recognised by the DC-SIGN receptor and are responsible for specifically blocking the DC-SIGN mediated HIV-1 transmission from dendritic cells to CD4+ cells (Saeland et al. 2009). Therefore, MUC1 is a naturally occurring protective mechanism to reduce the transmission of HIV-1 to infants during breastfeeding.

An overexpression of MUC1 is noted in various cancers once malignant transformation has occurred often resulting in a loss of apical restriction and in an alteration of glycosylation and mRNA spliced variants (Brayman et al. 2004). Aberrant MUC1 expression can be noted in

breast, lung, pancreatic, ovarian, colon and prostate cancer (Taylor-Papadimitriou et al. 1999; Goode et al. 2017). MUC1 expression in breast milk is an important marker for the progression and metastasis of breast cancer (Sekine et al. 1985). Patients with breast cancer have a better prognosis overall when there is an increased MUC1 expression (Ellis et al. 1987). MUC1 is overexpressed in approximately 90% of early triple negative breast cancer cases and regulates the glutamine metabolism of triple negative breast cancer cells aiding the tumour growth and metastasis of the cancer (Goode et al. 2017). A core 1 structure is created when galactose is added to the first N-acetylgalactosamine in the MUC1 oligosaccharide. However this is modified to a core 2 structure when N-acetylglucosamine is added by the enzyme core 2 β1,6-N-acetylglucosaminyltransferase to the MUC1 in epithelial cells in breast tissue (Burchell et al. 1999). This conversion of core 1 to core 2 is altered in breast carcinomas such that the MUC1 is shorter and less complex (Burchell et al. 1999).

1.4.2 MUC4

The *MUC4* gene, first cloned from a human pancreatic cell line and the human tracheobronchial cDNA library, is located on chromosome 3q29 and the molecular weight varies from 550 kDa to 930 kDa (Gross et al. 1992; Singh et al. 2004). The molecular weight of MUC4 is dependent on the size of the polymorphic central tandem-repeat domains (Chaturvedi et al. 2008). The *MUC4* gene, spanning 26.5 kb, contains 26 exons and the amino acid sequence contains a signal sequence with 27 residue peptides which is followed by a repeated motif, which is imperfect, ranging between 126-130 residues and ends in 554 residues of a unique sequence (Moniaux et al. 2007; Chaturvedi et al. 2008). There are two, functional subunits of MUC4 namely MUC4α and MUC4β due to the proteolytic cleavage at the Gly-Asp-Pro-His (GDPH) site (Moniaux et al. 2007). The MUC4α mucin-type subunit is highly glycosylated and the MUC4β transmembrane subunit is the growth factor-like subunit (Zhang et al. 2005; Moniaux et al. 2007; Chaturvedi et al. 2008). Human MUC4 is a homologous to sialomucin complex (SMC), which was originally isolated from rat mammary adenocarcinoma ascites cells which are highly metastatic (Pflugfelder et al. 2000). The SMC is a heterodimeric glycoprotein in which ascites sialoglycoprotein-1 (ASGP-1) is equivalent to MUC4α which is non-covalently bound ascites sialoglycoprotein-2 (ASGP-2). ASGP-2 is equivalent to MUC4β (Zhang et al. 2005). SMC does not contain a 16 amino acid repeat sequence in ASGP-1, which is however present in human MUC4 (Pflugfelder et al. 2000). The transcript of SMC spans 9 kb and the polypeptide is cleaved to yield the ASGP-1 and ASGP-2 subunits (Pflugfelder et al. 2000).

1.4.2.1 MUC4's normal and pathological functions

MUC4 is expressed in various normal epithelial tissues including the eye, vagina, the parotid and submandibular gland, trachea, ectocervix, mammary epithelium, and uterus (Chaturvedi et al. 2008; Liu et al. 2002). At the epithelial barrier, MUC4 has anti-adhesive properties and/or anti-recognition properties and furthermore, MUC4 can alter signalling pathways by binding tyrosine receptor kinase ErbB2 (Carraway et al. 2001). MUC4 expression is both transmembrane and secretory with transmembrane expression of MUC4 localised to the cell surface to provide protection and the secretory expression of MUC4 involved in lubricating and protecting the luminal surfaces (Chaturvedi et al. 2008). MUC1 and MUC4 are expressed in the parotid and submandibular glands of the oral cavity, and is thought to play a role in signal transduction pathways and protecting the epithelia of the oral cavity (Liu et al. 2002). The tear film in the eye is maintained and stabilised by the expression of MUC4 in both the conjunctival stratified epithelium and the corneal epithelial cells (Corrales et al. 2003). MUC4 is expressed in the reproductive tract along with MUC1 (Gipson et al. 1997).

MUC4, originally described as MUCX, is expressed in breast milk and plays a role in anti-HIV-1 activity along with MUC1 (Rossi et al. 1996; Mthembu et al. 2014). MUC4 secretion during pregnancy and lactation occurs due the posttranscriptional modifications of transforming growth factor beta (TGF-β) and the basement membrane in the mammary gland (Carraway et al. 2001).

As with MUC1, MUC4 is also involved in diseases and malignancies. MUC4 is not expressed in the normal pancreas nor in chronic pancreatitis, but the overexpression of MUC4 can be noted in pancreatic cancers thus making MUC4 a pathological marker for these cancers (Andrianifahanana et al. 2001). Crohn's disease, an inflammatory bowel disease, is characterised by mucosal ulcerations with a decreased MUC4 expression in the intestinal epithelial cells (Buisine et al. 1999; Buisine et al. 2001). Salivary gland tumours, which are high-grade, have a lower MUC4 expression when compared to intermediate and low-grade salivary gland tumours (Chaturvedi et al. 2008). Inflammatory diseases such as cystic fibrosis and chronic obstructive pulmonary disease involve aberrant MUC4 expression (Chaturvedi et al. 2008). MUC4 expression is negligible in the ovary however MUC4 is expressed in epithelial ovarian carcinomas in the early stages of ovarian tumours and MUC4 may be used for detecting late-stage ovarian tumours along with MUC16 (Chauhan et al. 2006).

1.5 Tenascins

Tenascins are a large family of extracellular matrix glycoproteins found in various tissues (Brellier & Chiquet-Ehrismann 2012; Adams et al. 2015). These glycoproteins are composed of an N-terminal with heptad repeats, repeated epidermal growth factor-like (EGF-like) domains, a variable number of fibronectin-type III (FNIII) domains and a large C-terminal fibrinogen related domain (FReD) (Chiovaro et al. 2015; Adams et al. 2015). The four tenascins in vertebrates include tenascin-C (TNC), tenascin-W (TNW), tenascin-R (TNR) and tenascin-X (TNX). Tenascin subunits are formed via disulphide bonds at their N-terminus either forming homo-trimers (TNX and TNR) or homo-hexamers (TNC and TNW) ((Pas et al. 2006; Chiovaro et al. 2015).

1.5.1 Tenascin- R (TNR)

The *TNR* gene is located on chromosome 1q23-q24 with 21 exons (Carnemolla et al. 1996; Leprini et al. 1996). Molecular isoforms of TNR, TNR 160 (160 kDa) and TNR 180 (180 kDa), differ based on molecular weight due to the sixth FNIII domain which is alternatively spliced (Woodworth et al. 2004). Each monomer contains 4.5 EGF-like domain repeats and 8 FNIII domains centred between the N-terminus cysteine rich region and the C-terminus FReD region respectively (Carnemolla et al. 1996). TNR expression is limited to the nervous system found in motor neurons and axons, the hippocampus, the cerebellum, olfactory bulbs, myelinating oligodendrocytes and type 2 astrocytes (Rathjen et al. 1991; Carnemolla et al. 1996).

1.5.2 Tenascin-W (TNW)

The *TNN* gene for TNW glycoprotein is located on chromosome 1q23-q24 with 19 exons next to the *TNR* gene (Tucker et al. 2006; Degen et al. 2007; Chiovaro et al. 2015). Each monomer has an N-terminus cysteine rich region followed by 3.5 EGF-like domain repeats and 9 FNIII domains. The C-terminus comprises of a FReD region (Scherberich et al. 2004). TNW is transiently expressed during embryonic development and stem cell niches with TNC (Scherberich et al. 2004). In adult bone, it localizes to the periosteum and is involved in osteogenesis (Scherberich et al. 2004). Degen et al. (2007) showed that TNW in the activated stroma of low-grade breast cancer cells alters the migratory behaviour of these cancer cells aiding tumorigenesis. TNC and TNW are highly expressed in the stroma of colon and breast cancer, with TNW not corresponding to normal tissue. Therefore, TNW is proposed as a

biomarker for colon and breast cancers and potentially, other solid tumours (Brellier et al. 2012).

1.5.3 Tenascin-X (TNX)

The *TNX* gene, includes *TNXA*, a truncated copy, and *TNXB*, which is located on chromosome 6p21.3 with 38 exons coding for the intact TNX protein (Speek et al. 1996). Subunits have 18.5 EGF-like domain repeats followed by 32 FNIII domains flanked by a cysteine rich N-terminal and a C-terminal FReD region respectively (Brösicke & Faissner 2015). TNX interacts with extracellular matrix molecules including fibrillary and fibril-associated collagen and decorin (Egging et al. 2007; Elefteriou et al. 2001). The deficiency of TNX results in Ehler-Danlos Syndrome, a recessive heritable connective tissue disorder affecting the connective tissues, which results in hyperextensible skin and hypermobile joints (Burch et al. 1997; Heights & Francisco 2001; Bristow et al. 2005).

1.5.4 Tenascin-C (TNC)

TNC is the first glycoprotein discovered in this family (Chiquet-ehrismann & Tucker 2011). The *TNC* gene contains 29 exons, 9 which can be alternatively spliced, and is located on chromosome 9q33 (Sriramarao & Bourdon 1993; Gherzi et al. 1995; Mighell et al. 1997). TNC is a hexameric glycoprotein which consists of six monomers, with a molecular weight of 180-250 kDa, linked by their N-termini with disulphide bonds (Gulcher et al. 1991). The subunits consist of a globular cysteine rich N-terminal, 14.5 EGF-like domain repeats, 8 consecutive FNIII domains and a FReD which ends the monomer at the C-terminal (Gulcher et al. 1991; Adams et al. 2002; Tsunoda et al. 2003). The C-terminal sequence shows homology to the β- and γ-chains in fibrinogen (Gulcher et al. 1991). Nine alternatively spliced FNII domains (A1-A4, B, AD1, AD2, C, D) can be integrated into the glycoprotein between the fifth and sixth FNIII domain resulting in various isoforms (Adams et al. 2002; Tsunoda et al. 2003; Brösicke & Faissner 2015). TNC's function is dependent on the cell type in which it is expressed in rendering it either pathological or physiological (Pas et al. 2006). TNC plays an important role in embryonic development with its marked expression during neural, skeletal and vascular morphogenesis (Hsia & Schwarzbauer 2005). During pathological processes, such as mechanical or chemical injury, the expression of TNC increases in tissues during inflammation and tissue repair (Chiquet-Ehrismann & Chiquet 2003). Adversely, TNC expression changes during pathological events such as various cancers, asthma, interstitial pneumonia and

pathological bone marrow. Malignant tumours with TNC expression include carcinomas of the breast, colon and lung, gliomas and prostatic adenocarcinoma tumours (Pas et al. 2006; Brösicke & Faissner 2015; Brellier et al. 2012). Brellier et al (2012) showed that TNC and TNW are both biomarkers for solid colon and breast cancer tumours and gliomas, with TNW being the preferential biomarker as it is not normally expressed in human tissues. Higher molecular weight TNC isoforms are expressed in breast cancers increasing migration and invasion (Adams et al. 2002).

During lactation, mammary glands have an increased expression of TNC with these levels decreasing in breast milk during involution (Qin et al. 2016). Sustained TNC levels during involution could mediate in tumour progression (O'Brien et al. 2011). It is hypothesized that there is a possible interaction between decreased TGFβ2 levels, thought to induce apoptosis, and increased levels of TNC during involution in the progression to pregnancy associated breast cancer (Qin et al. 2016). In breast milk, TNC has innate mucosal properties which neutralise HIV-1 by binding to a chemokine co-receptor site on the HIV-1 envelope protein reducing mucosal transmission of HIV-1 from infected mothers to uninfected infants (Fouda et al. 2013). However, the concentration of TNC in semen and cervical fluids is insufficient to neutralise HIV-1 in uninfected individuals (Mansour et al. 2016).

1.6 Aims and Objectives

Human breast milk offers both protection and nutrition to infants during the first few months of life. Although the transmission of HIV-1 through breast milk is low, there is still a risk of HIV-1 transmission due to reservoirs of HIV-1 that can form in breast milk (Collins et al. 2000). Purified mucins, such as MUC1 and MUC4, as well as purified TNC have previously been implicated in HIV inhibition using *in vitro* assays (Habte et al. 2008; Mthembu et al. 2014; Fouda et al. 2013). Moreover, pasteurisation is an option for mothers in more rural areas to potentially reduce the risk of HIV-1 transmission due to the membrane-bound mucins which potentially could be exposed due to the heat exposure. It is important to develop preventative strategies to allow safe breastfeeding by HIV positive women, due to the nutritional and immunological benefits which outweigh those of formula milk.

The aims and objectives of this study are:

- To separate, purify and characterize breast milk mucins (transmembrane mucins including MUC1 and MUC4) from HIV negative milk samples and to determine the

differences in anti-HIV-1 activity patterns using an HIV neutralisation assay against the strain of HIV-1 subtype C derived from patients in Durban, KwaZulu Natal.

- To isolate and assess TNC in human, HIV negative breast milk and test it against the HIV-1 strain subtype C from Durban, KwaZulu Natal using an HIV neutralisation assay.
- To expose breast milk samples from HIV to two pasteurisation methods (80°C for 10 minutes and 62.5°C for 30 minutes) and test the pasteurised milk against the HIV-1 .
- To identify the presence of MUC1, MUC4 and TNC and the morphology of cells in lactating breast tissue using histochemistry and immunohistochemistry respectively.

Chapter 2: Materials and Methods

2.1 Ethics

This study was approved by the Human Research Ethics Committee, Faculty of Health Sciences University of Cape Town: HREC 117/2016 and written, informed consent was received from all participants.

2.2 Materials

Guanidinium hydrochloride (GuHCl), caesium chloride (CsCl), disodium phosphate (Na_2PO_4), sodium azide (NaN_3), monosodium dihydrogen phosphate (NaH_2PO_4), Sepharose CL-4B, pararosaniline chloride, pararosaniline hydrochloride, glycerol, sodium chloride, methyl orange, N,N'-methylenebisacrylamide (Bis/Acrylamide), acrylamide, Thiazolyl blue trazolium bromide (MTT), N-Ethylmaleimide (NEM), sodium bicarbonate, potassium metabisulphite, anti-mouse IgG and anti-rabbit IgG alkaline phosphatase antibodies, Penicillin Streptomycin, acetic acid, diethylaminoethyl (DEAE) dextran, dialysis tubing, trypsin and ethylenediaminetetraacetic acid (EDTA) were from Sigma-Aldrich (St. Louis, Missouri, USA). Dulbecco's Modified Eagle's Medium (DMEM) with L-Glutamine and phosphate buffered saline (PBS) without calcium and magnesium was purchased from Lonza (Cape Town, South Africa). NBT/BCIP Tablets were obtained from Roche (Basel, Switzerland). Phenylmethylsulfonylfluoride (PMSF), glycine, trypan blue, ammonium persulphate (AMPS), N,N,N,N,'-tetramethylethylenediamine (TEMED), hydrochloric acid (HCl), butanol, periodic acid, tri-sodium citrate and Tween 20 Detergent were obtained from Merck (Darmstadt, Germany). Tris(hydroxymethyl)-aminomethane (Tris), sodium dodecyl sulfate (SDS), sodium metabisulphate, bromophenol blue and nitrocellulose membrane were supplied by Kimix Chemical and Lab Suppliers (Cape Town, South Africa). Activated charcoal was from BDH Chemicals (London, UK). Quick StartTM Bradford dye was from Bio-Rad Laboratories (Hercules, California, USA). Protein Acqua Stain was obtained from Vacutec (Johannesburg, South Africa). PageRuler Prestained Protein Ladder was from Thermo Fisher Scientific (Cape Town, South Africa). Human MUC1 (E-EL-H0616), human MUC4 (E-EL-H0715) and human TNC (E-EL-H1423) ELISA kits were obtained from Elabscience Biotechnology Co., Ltd (Wuhan, China). Non-essential amino acids (NEAA) and Fetal bovine serum (FBS) were from Gibco (Massachusetts, USA). Bright-Glo Luciferase Assay Substrate and Buffer was purchased from Promega (Madison, Wisconsin, USA). The International Centre for Genetic

Engineering and Biotechnology (ICGEB) supplied the TZM-bl/JC cells and Du422.1 pseudovirus (Cape Town, South Africa). The polyclonal rabbit Tenascin-C (H-300) and the monoclonal mouse MUC4 (1G8) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, United States). The monoclonal mouse MUC1 (HMFG1) primary antibody was purchased from Abcam (Cambridge, United Kingdom). Envision secondary anti-mouse and anti-rabbit antibodies and Chromogen DAB were from Dakocytomation (Agilent Technologies, Santa Clara, United States of America).

2.3 Sample Collection

Human breast milk samples were collected from HIV negative lactating mothers from the post-natal and neonatal wards at Groote Schuur Hospital and the Panaroma Breastfeeding Clinic (Cape Town, South Africa) and their HIV status was recorded to ensure only HIV negative breast milk was collected and utilised for subsequent experiments. Human breast milk samples were immediately frozen at -20°C. Preceding this, 1-5 ml of human breast milk was aliquoted for heat treatment and ELISA analysis and frozen at -20°C.

2.4 Caesium chloride density gradient ultracentrifugation

Human breast milk samples were thawed at room temperature and prepared for a caesium chloride (CsCl) density gradient ultracentrifugation spin. A CsCl gradient is created down the tube when CsCl dissociates due to a high centrifugal force causing a density gradient because of the resulting diffusion force opposing the centrifugal force. After 48 hours, the densest material will be situated at the bottom of the tube and the lowest density material will be situated at the top of the tube. During the period of the spin, the density gradient causes protein and mucins to migrate to the fraction correlating to its' density (Creeth & Denborough 1970).

The density of the milk samples was adjusted to 1.39 g/ml using CsCl and 4M guanidine hydrochloride (GuHCl) with proteolytic inhibitors (5 mM N-Ethylmaleimide (NEM), 1 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol and 10 mM ethylenediaminetetra-acetic acid (EDTA)), pH 6.5. Milk samples were heat sealed in centrifuge tubes, placed in a Ti70 rotor and subjected to a 48 hour ultracentrifugation spin at 40 000 revolutions per minute (rpm) at a temperature of 4°C in a Beckman L45 ultracentrifuge (Beckman Coulter, Brea, California, United States).

Thereafter, the yellow milk fat layer situated at the top of each tube (Figure 2.1) was collected and frozen at -20°C. The remaining nine 1 centimetre (cm) fractions were collected from each

tube with a syringe, which were referred to as skim milk. The density of each fraction was recorded by weighing a volume of 1 millilitre (ml). The fractions were further tested for the glycoprotein and protein content using a Periodic acid Schiff's (PAS) and Bradford assay respectively.

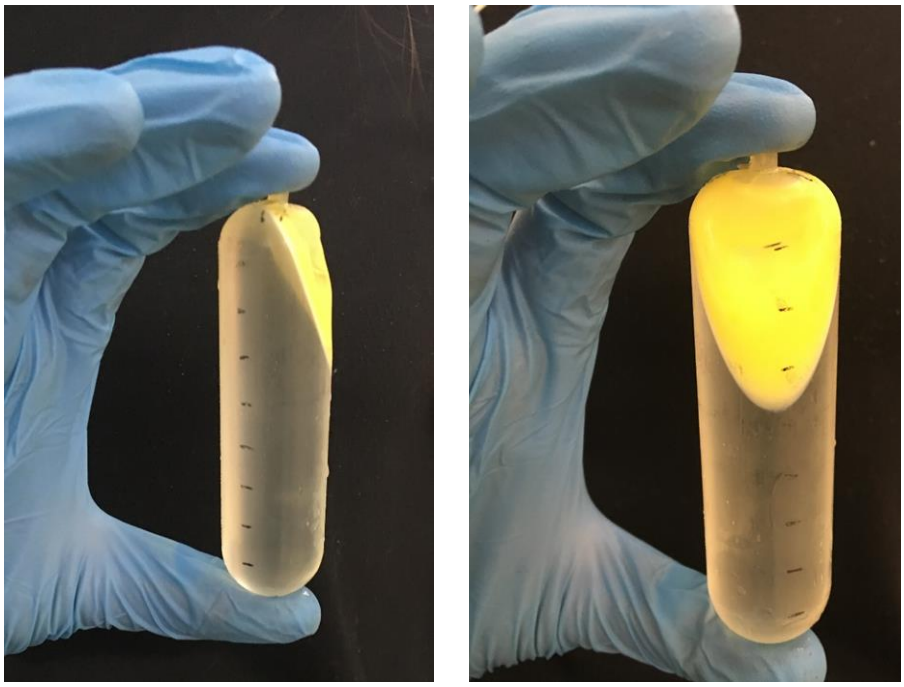

Figure 2.1. The milk fat layer at the top of the ultracentrifuge tube after a 48 hour spin (40 000 rpm at 4°C) is collected and stored at -20°C for the milk fat extraction and the remaining nine fractions were collected and referred to as skim milk.

2.4.1 PAS assay for glycoprotein determination

Glycoproteins are detected in the presence of periodic acid causing the oxidation of a hydroxyl group (-OH) to an aldehyde (-CHO). A colour change from clear to purple/pink is observed in the presence of glycoproteins due to the -CHO group reacting with the Schiff's reagent (Thornton et al. 1996). In a 96-well microtiter plate, 20 µl of sample was pipetted into each well with the addition of 100 µl of periodic solution (7% acetic acid with 50% periodic acid) and incubated at 37°C for 1 hour. Thereafter, a 30 minute incubation period at room temperature with 100 µl of decolourised Schiff's reagent in each well was performed. Finally, the absorbance was read at 585 nm on an Anthos HTII Plate Reader (GoIndustry DoveBid, Lancashire, United Kingdom).

2.4.2 Bradford assay for protein detection

A modified Bradford assay utilizes a Bradford dye which exists in three forms namely cationic (red), neutral (green) and anionic (blue) (Bradford 1976; Compton & Jones 1985). Under acidic conditions, the red dye is converted to its bluer form when bound to a protein via Van der Waals forces and hydrophobic interactions. Using a 96-well microtiter plate, 200 µl of Bradford reagent was added to 10 µl of sample in each well and incubated at room temperature for 5 minutes. The absorbance was read at 585 nm on an Anthos HTII Plate Reader.

2.5 Dialysis of samples

It is important to remove substances from the samples which could potentially interfere with subsequent experiments through the process of dialysis. Dialysing samples removes remaining salts and soluble, small molecular weight molecules to ensure the purity of the sample. Dialysis tubing, a 25 millimetre (mm) wide nitrocellulose membrane, was boiled in water in the presence of 1 mM EDTA and 2% (w/v) sodium bicarbonate until the dialysis tubing floated to the top. The samples of interest were dialysed against three changes of distilled water (dH_2O) at 4°C with continuous stirring. A minimum of 5 hours was required before changing the dH_2O.

2.6 Freeze drying

Freeze drying removes the water from samples in order to concentrate samples and determine the weight of the samples. After dialysis, samples were poured into plastic tubes suitable for the volume and sealed with a lid containing holes and subsequently frozen at -20°C overnight. Freeze drying of all samples was done in a Freeze Zone 6 Freeze Dry System (Labconco, Kansas City, United Sates). Samples were placed in the vacuum chamber, under full vacuum of 0.021 millibars (mBar), at -50°C until the sample was completely dehydrated. Freeze dried samples were stored at 4°C until required.

2.7 Gradient (4-20%) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A 4-20% SDS-PAGE method was used to confirm the presence of mucins and tenascin-C and estimate their molecular weights for further purification. The SDS-PAGE gels were prepared as 4-20% gradient gels according to a modified method of Laemmli (Laemmli 1970). The running gel was prepared using a 4% light and 20% heavy solution. Briefly, the 4% light

solution was prepared as follows: 0.8 ml 30% Bis/Acrylamide, 1.5 ml 1.5 M Tris-buffer with 0.1% SDS, pH 8.8, 3.7 ml dH_2O, 30 µl 10% ammonium persulphate (AMPS) and 5 µl N, N, N, N'-tetramethylethylenediamine (TEMED). The 20% heavy solution was prepared as follows: 4 ml 30% Bis/Acrylamide, 1.5 ml 1.5 M Tris-buffer with 0.1% SDS, pH 8.8, 0.5 ml dH_2O, 30 µl 10% AMPS and 5 µl TEMED. Using a 10 ml serological pipette, 2.3 ml 4% light solution was pipetted following 2.3 ml 20% heavy solution. The gradient in the solution was created by allowing a single bubble to move through the solution by letting some air enter the serological pipette. The running gel solution was poured into the glass plates (BioRad) and topped with a layer of butanol to create a neat interface ensuring polymerization. Briefly, the 3% stacking gel was prepared as follow: 0.5 ml Bis/Acrylamide, 2.8 ml spacer buffer, 73 µl AMPS and 5 µl TEMED. Once the 4-20% running gel had fully polymerised, the stacking gel solution was added after removing the butanol and the comb was inserted.

Freeze-dried samples (1 mg/100 µl) were prepared in sample application buffer (50% glycerol in 1× TAE with bromophenol blue to colour with no mercaptoethanol) and 15 µl was aliquoted into each well. Pre-stained protein ladder (molecular weights in descending order: 250, 130, 95, 72, 55, 36, 28, 17 and 10 kDa), 5 µl, was loaded into the respective well. Before running the gels, the appropriate amount of tank buffer (0.025 M Tris, 0.19 M glycine with 0.1% SDS, pH 8.8) was added and electrophoresis of the gels was run at 200 V for approximately 1.5 hours or until the bromophenol blue was roughly 1 cm above the bottom of the gel.

2.7.1 PAS staining of 4-20% SDS-PAGE gel

A glycoprotein staining method by Dubray and Bezard was used to visualise glycoproteins in the gel using Periodic acid Schiff (PAS) solution (Durbay & Bezard 1982). The gel was incubated at room temperature for 30 minutes with 50% ethanol and thereafter, the gel was washed with dH_2O for 10 minutes before being placed in a 3% acetic acid with 1% Periodic acid solution for 30 minutes. It was then left overnight in dH_2O at 4°C. The gel was washed with 0.1% sodium metabisulphite in 10 mM hydrochloric acid (HCl) for 2 × 10 minutes. Schiff's reagent was poured over the gel before being placed in the dark for 1 hour. For the following hour, the gel was covered with 0.1% sodium metabisulphite in 10 mM hydrochloric acid and placed in the dark. Glycoprotein protein bands were visualized on the gel as purple/pink bands and this was subsequently photographed.

2.7.2 Protein staining of 4-20% SDS-PAGE gel

Acqua stain (Vacutec) was used to visualise the protein bands on the gel by submerging the gel in Acqua stain for 10-15 minutes. Finally, the gel was photographed showing the protein positive bands.

2.8 Western blotting

The presence of specific mucins in the skim milk and milk fat of each sample was verified using Western blotting, specifically MUC1, MUC4 and TNC. Proteins and mucins were transferred from the gradient SDS-PAGE to a nitrocellulose blotting membrane (BioTrace^{TM} NT membrane, Pall Corporation, New York, United States). Six layers of filter paper were soaked in Towbin's buffer as well as the nitrocellulose blotting membrane. Using an electro-blotter, the transfer was set up as follows: three layers of filter paper, a nitrocellose membrane, the 4-20% gradient SDS-PAGE gel and another three layers of filter paper. The mucins and proteins were transferred to the nitrocellulose blotting paper at 40 V for 1 hour.

To block any non-specific binding, a 5% (w/v) low-fat milk powder in 1× TBST (Tris saline buffer with 0.05% Tween-20) solution was used to cover the nitrocellulose membrane for 30 minutes at room temperature. The appropriate primary antibody (Table 2.1), diluted in 5% (w/v) low-fat milk powder in 1× TBST, was poured over the nitrocellulose membrane and incubated overnight at 4°C. The solution was removed and membranes were washed with 1× TBST (4 × 3 minutes). The alkaline phosphatase conjugated secondary antibody was diluted in 5% (w/v) low-fat milk powder in 1× TBST and incubated for 30 min at room temperature. An anti-mouse IgG alkaline specific secondary antibody was used for both MUC1 and MUC4 (1:30 000) and an anti-rabbit IgG alkaline specific secondary antibody was used for TNC (1:50 000) (Table 2.1). Lastly, the nitrocellulose membranes were washed with 1× TBST (3 × 10 minutes) and rinsed with dH_2O. Half a NBT/BCIP tablet was dissolved in 5 ml sterilized phosphate-buffered saline (PBS) and placed over the membrane in the dark for 10 minutes or until developed.

Table 2.1. Primary and secondary antibodies utilised for Western blotting and slot blots.

Primary antibody	**Manufacturer**	**Clone**	**Type of antibody**	**Dilution**
MUC1	Abcam	HMFG (aka 1.10.F3)	Mouse monoclonal	1:100
MUC4	Santa Cruz Biotechnology	1G8	Mouse monoclonal	1:500
TNC	Santa Cruz Biotechnology	H-300	Rabbit polyclonal	1:400
Secondary antibody	**Manufacturer**	**Clone**	**Type of antibody**	**Dilution**
Anti-mouse IgG alkaline specific antibody	Sigma-Aldrich	Anti-mouse IgG (polyclonal)	Goat polyclonal	1:30 000
Anti-rabbit IgG alkaline specific antibody	Sigma-Aldrich	Anti-rabbit IgG (polyclonal)	Goat polyclonal	1:50 000

2.9 Sepharose CL-2B gel filtration chromatography

Sepharose CL-2B beads were degassed and packed into a glass column. The column was washed with three column volumes of 0.2 M sodium chloride (NaCl) and 0.02% sodium azide (NaN_3) buffer. The first two fractions of the skim milk from caesium chloride density gradient ultracentrifugation, solubilized in 0.2M NaCl and 0.02% NaN_3, were chromatographed and eluted with 0.2M NaCl and 0.02% NaN_3 with 0.5 ml fractions being collected. Samples were solubilised in 15 ml of 0.2M NaCl and 0.02% NaN_3 and 6 ml was applied to the column each time with each sample chromatographed twice. The void (V_0) volume and included (V_i) volume fractions were identified using PAS and Bradford assays and respectively pooled, dialysed and freeze dried. Western blotting confirmed the presence of TNC in the V_0 however this fraction was contaminated with MUC1 and other smaller proteins. Due to a low sample yield and sample variation, it was decided to extract the TNC from the V_0 using gel extraction.

2.10 Milk fat preparation

A modified method from Kenny et al was used to remove lipids and triglycerides from the milk fat obtained from the caesium chloride density gradient ultracentrifugation (Kenny et al. 2011). Milk fat was thawed at room temperature and aliquoted into Eppendorf tubes. The milk fat was vortexed, at full speed, for 2×10 minutes with 3 minutes rest in between. The milk fat was

subsequently incubated at 55°C for 5 minutes and then centrifuged for 10 minutes at 2 000 g at room temperature. The oil layer was removed from the tube and replaced with an equal amount of dH_2O. Samples were vortexed for 10 minutes at full speed followed by an incubation step at 55°C for 5 minutes. The milk fat samples were then centrifuged for 10 minutes at 2 000 g at room temperature. The water was removed from the tubes and samples were frozen at -20°C for a minimum of three hours before freeze drying. MUC1 and MUC4 was identified by Western blotting in the milk fat and was subsequently extracted using gel extraction.

2.11 Gel extraction

To obtain purified MUC1, MUC4 and TNC, these proteins were extracted from a 4-20% SDS-PAGE gel. Freeze dried samples (1 mg/100 µl) were prepared in SDS sample application buffer (50% glycerol in 1× TAE with bromophenol blue to colour with no mercaptoethanol) and 15 µl was loaded into each well. A pre-stained protein ladder (5 µl) was loaded into the first lane as a template and marker for protein extraction. Tank buffer (0.025 M Tris, 0.19M Glycine with 0.1% SDS, pH 8.8) was added and electrophoresis occurred at 200 V for approximately 1.5 hours or until the bromophenol blue was 1 cm above the bottom of the gel.

Before extraction, one of the gels was placed in Acqua stain to create a template for subsequent gel extractions. MUC1, a band at the top of the running gel, and MUC4 around 80 kDa was extracted from the prepared milk fat. TNC, a ±250 kDa band, was extracted from the V_0 from the sepharose CL-2B column. The gel pieces cut from the gel were crushed using a mortar and pestle before being placed in a gel extraction buffer (150 mM NaCl, 0.1 mM EDTA and 50 mM Tris-HCl, pH 7.5) overnight at room temperature. The samples were then centrifuged at 3 000 rpm for 20 minutes and the supernatant was removed, dialysed with three changes of dH_2O and freeze dried. The purity of the samples was confirmed using PAS and Acqua staining of 4-20% SDS-PAGE gels and slot blots.

2.12 Slot blots

The identity of purified MUC1, MUC4 and TNC from the skim milk and milk fat was confirmed using a slot blot. The samples, MUC1, MUC4, MUC1/TNC and TNC, were diluted in sample application buffer (50 µg/50 µl) and applied directly on a section of nitrocellulose membrane soaked in 4× saline sodium citrate buffer (0.6M NaCl, 0.06M sodium citrate, pH 7) and vacuum blotted at 40 mBar using a Pharmacia LKB Vacugene XL vacuum blotter (Kalamazoo, United States) for approximately 1 hour. Thereafter, the nitrocellulose

membranes were probed for anti-MUC1 and anti-TNC primary antibodies and detected using alkaline phosphatase secondary antibodies following the same method described in section 2.8 Western blotting.

2.13 Enzyme-linked Immunosorbent Assay (ELISA) kits

Due to the trouble experienced with the MUC4 purification, it was hypothesized that the concentration of MUC4 in crude breast milk might be lower than that of MUC1 and TNC. To test this hypothesis, ELISA kits for human MUC1, human MUC4 and human TNC were purchased from Elabscience Biotechnology Co., Ltd (Wuhan, China). The reagents were supplied with the kit and prepared according to the manufacturer's instructions. The methodology was the same across all three kits with only the primary antibodies differing. Firstly, the reference standards provided was reconstituted with 1 ml of diluent provided and subsequently serially diluted. The starting concentration for both MUC4 and TNC standards were 20 ng/ml, whereas the starting concentration for the MUC1 standard was 10 ng/ml. The crude breast milk samples were serially diluted using the diluent provided starting with a 1 in 4 dilution. The 100 µl of the standard, the sample or the blank was added per well and incubated at 37°C for 90 minutes. The liquid was removed from each well and 100 µl of biotilynated detection antibody was added to each well for 1 hour at 37°C. Thereafter, the liquid was removed from each well and the wells washed three times with approximately 350 µl of wash buffer. After the last washing step, the micro ELISA plate was inverted to ensure all of the liquid was removed from the wells. Horse radish peroxidase (HRP) conjugate (100 µl) was added to each well and incubated for 30 minutes at 37°C. The wash process was then repeated five times using the same method described previously. In shaded light, 90 µl of substrate solution was added to each well for 15 minutes and a blue colour change was noted. Thereafter, 50 µl of stop solution was added to each well in the same order in which the substrate solution was added resulting in a colour change to yellow. Finally, absorbance was measured at 450 nm on an Anthos HTII Plate Reader. The micro ELISA plate was covered with a new plate sealer between every step. The standard curve was drawn on Excel by subtracting the average of the blank from the average of the crude and different dilutions. The concentration of MUC1, MUC4 and TNC was determined from their respective standard curves by taking the dilution factor into account.

2.14 Crude and the heat treatment of human breast milk

The breast milk was thawed at room temperature in preparation for the heat treatment in order to pasteurise the breast milk. Approximately 3 ml of breast milk for each sample was used for the respective heat treatments. Firstly, 3 ml of breast milk sample was placed in 80°C in a water bath for 10 minutes and then immediately cooled on ice. The second heat treatment involved heating 3 ml of breast milk sample at 62.5°C in a water bath for 30 minutes and then cooled immediately on ice. All of the samples including 1 ml of crude breast milk was then frozen at -20°C overnight before being placed in the freeze dryer. Thereafter, they were stored at 4°C.

2.15 Pseudoviral neutralisation and cell toxicity assays

The antiviral activity of purified MUC1 and TNC as well as crude and heat treated breast milk were tested using a modified HIV neutralisation assay based on the expression of the luciferase reporter gene using TZM-bl/JC cells (Montefiori 2009). The cell toxicity of all the above mentioned samples was tested using an MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay using the same cell line. The difference between the MTT assay and the pseudoviral neutralisation assay is that no HIV is added throughout the MTT assay. MUC4 was not detected in the majority of the samples and was therefore not further investigated. These experiments were completed in a P2 laboratory facility in the International Centre for Genetic Engineering and Biotechnology (ICGEB) at the Health Science Faculty, UCT, South Africa.

2.15.1 Thawing and maintenance of TZM-bl/JC cells

TZM-bl/JC cells were utilised for both the pseudoviral neutralisation assay and the MTT assay to assess cell toxicity. The TZM-bl/JC cells, a HeLa cell line, express CCR5 and CXCR4 co-receptors and CD4 receptors (Platt et al. 1998). In order to measure the viral infection in the neutralisation assay, the TZM-bl/JC cells were transfected with luciferase and β-galactosidase gene sequences. The transcriptional control of the sequences is due to the initiation of long terminal repeat by HIV-1 Tat (Wei et al. 2002).

The TZM-bl/JC cells, stored at -80°C, were quickly thawed and transferred from the cryotube to a sterile 15 ml Falcon tube to which 10 ml 5% Dulbecco's Modified Eagle's Medium (DMEM) (containing DMEM with L-glutamine, 5% fetal bovine serum, 1% non-essential amino acids ((NEAA), 0.5% 200 × PenStrep) was added. The contents in the Falcon tube were

centrifuged for 5 minutes at 4 000 rpm at 21°C and the supernatant was removed. The cell pellet was resuspended in 5 ml of 5% DMEM and subsequently added to a sterile culture dish and topped up to 13 ml with 5% DMEM. The cells were cultured at 37°C with 5% carbon dioxide (CO_2).

The TZM-bl/JC cells were examined under an inverted microscope every third day to determine the confluency of the cells. Until they were 70-80% confluent, the media was replaced with fresh 5% DMEM after washing the plate with 10 ml sterile PBS. Once the cells were 70-80% confluent and there was no fungal or microbial infection, the cells were ready to be split. The old media was removed by aspiration and discarded before the culture dish was washed with 5 ml PBS in a gentle swirling motion. The PBS was removed by aspiration and 2-5 ml of trypsin-EDTA was added to cover the entire surface of the culture plate which was then incubated at 37°C for 5 minutes. The cells should have detached from the surface of the culture dish during this incubation period and an "oily" residue floating on the surface of the media represents this. The cells were then suspended in 8 ml of 5% DMEM, transferred to a sterile Falcon tube and centrifuged at 1 200 rpm for 5 minutes at 21°C. The supernatant was removed and the cells resuspended in 6-10 ml of 5% fresh DMEM. During the centrifugation step, the culture dish should be washed with 10 ml of sterile PBS to ensure that traces of the trypsin-EDTA were removed. The cells were counted on a haemocytometer by adding 15 µl of trypan blue containing stained cells (10 µl of resuspended cells were added to 30 µl of trypan blue) to obtain the $X \times 10^4$ of cells/ml. One million cells from the suspension are returned to the plate and topped up to 13 ml with 5% DMEM and placed in the 37°C incubator (with 5% CO_2). Depending on when the cells were required, 10% DMEM (containing DMEM with L-glutamine, 10% Fetal bovine serum, 1% NEAA, 0.5% 200 × PenStrep) could be used to culture and maintain cells. TZM-bl/JC cells were prepared this way in preparation for both the MTT and neutralisation assay however they were not plated back into the culture dish and were resuspended in 10% DMEM.

2.15.2 Production of the pseudovirus

The pseudovirus utilised in the neutralisation assay was a Du422.1 strain derived from a clade C HIV-1 virus from Kwa-Zulu Natal, South Africa. This moderately infective pseudoviral construct was produced and titrated at the Dorfman laboratory at ICGEB, South Africa. The pseudovirus was prepared using 293T cells, the packaging cells, which were co-transfected with an envelope plasmid and a SG3- Δenv (an inactivated envelope gene) plasmid (Figure

2.2). The pseudovirus cannot replicate more than once due to the lack of the envelope gene sequence.

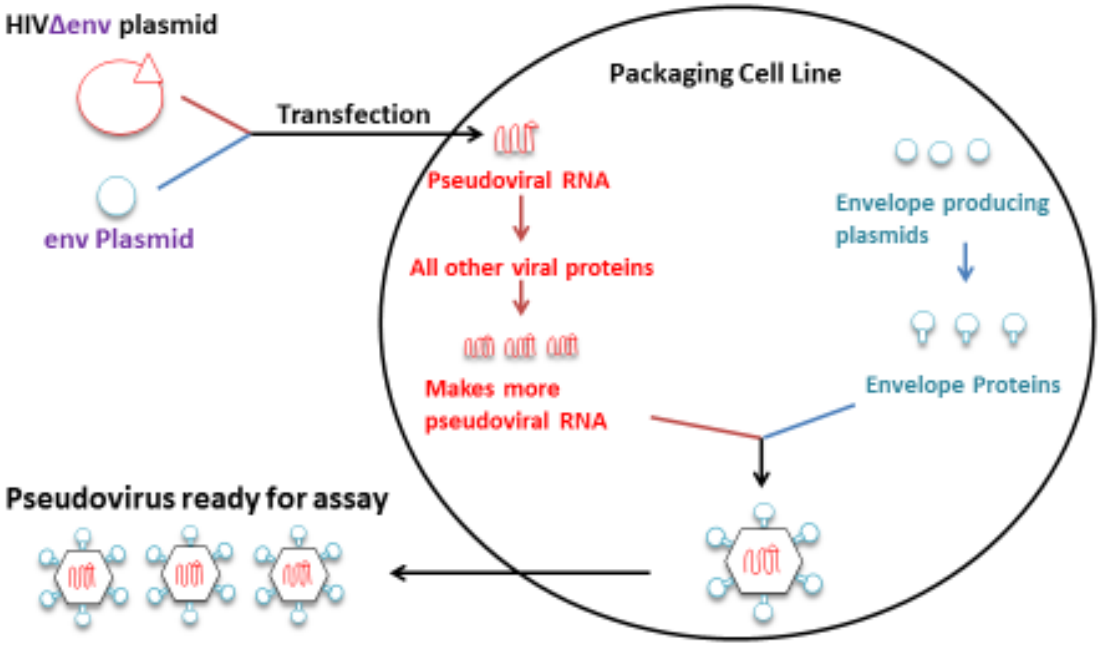

Figure 2.2. Preparation of the pseudovirus, DU422.1, using an envelope plasmid and a SG3-Δenv (an inactivated envelope gene) plasmid in the packaging cell line 293T cells.

2.15.3 Pseudoviral assay

HIV-1 infection of TZM-bl/JC cells were tested in the presence of the purified glycoproteins and crude breast milk with different heat treatments using a modified HIV neutralisation assay by Montefiori (2009).

Once TZM-bl/JC cells were confluent, they were prepared as described above and were resuspended in 10% DMEM, with Diethylaminoethyl-Dextran (DEAE-Dextran) at a concentration of 7.5 mg/ml. This is added to the media in order to remove any potential natural charge repulsion between the Du422.1 virus and TZM-bl/JC cells. The cells were plated in 96 well flat bottomed plates at a concentration of 1 million cells per plate on the first day of the assay and placed in the incubator at 37°C. The samples were diluted in 10% DMEM. The starting concentration of MUC1, MUC1/TNC and TNC was 0.5 mg/ml whereas the starting concentration of crude and heat treated milk was 1 mg/ml.

The samples were serially diluted in duplicate on the second day in a separate 96 well flat bottomed plate with a starting volume of 350 µl. The replication deficient virus, Du422.1, was

diluted in 10% DMEM according to the viral titration so that when incubated with the virus, approximately 50 000 relative light units (RLU) will be produced (Sarzotti-Kelsoe et al. 2014). The diluted virus was added to each well (82.5 µl per well) including the viral control wells and subsequently incubated at room temperature for one hour. Thereafter, 150 µl of the virus/mucin solution for the samples or virus solution for the viral control was transferred to the corresponding wells of the TZM-bl/JC cells and placed in the incubator for 48 hours. The cell control wells were topped up with 150 µl of 10% DMEM. Following the incubation period, 100 µl of the wells' contents were removed and 100 µl of Bright-Glo luciferase substrate was added before an incubation period of 2 min in the dark. Finally, 100 µl from each well was transferred to a 96 well black plate and the luminescence of each well was measured in RLU GloMax®-96 Luminometer (Promega, France).

In order to include the sample in further data analysis, a minimum of four points at different concentrations were required for the sample. For the data points for the duplicates to be considered reliable, the RLU of the duplicates for the sample should not differ by more than 35% when compared to the higher value. The average RLU was calculated as a percentage of the viral, positive control minus the negative cell control in order to determine the viral invasion. The percentage HIV neutralisation was then calculated by subtracting the percentage HIV invasion from 100. Thereafter, the 50% inhibitory concentration (IC_{50}) was calculated on Graph pad prism (GraphPad, La Jolla, CA, USA) using the log(inhibitor) vs. normalized response -- variable slope curve fit function. The IC_{50} was used to identify the concentration (µg/ml) at which 50% of HIV was inhibited. The IC_{50} and 95% confidence interval was reported for the samples along with the log dose response curves. The X-axis of these curves was altered to the anti-logged concentration of the sample in order to better indicate the relationship between the concentration of the sample and HIV-1 neutralisation. The IC_{50} values were log transformed and a Kruskal Wallis test was performed, with a Mann-Whitney U test used in the post hoc analysis.

2.15.4 Cell toxicity assay

The toxicity of purified MUC1 and TNC, crude as well as heat treated milk was tested with an MTT assay using TZM-bl/JC cells. It is important that this assay mimics the neutralisation assay with the exclusion of the virus. The metabolic activity of the TZM-bl/JC cells is measured at 585 nm by a colour change from yellow MTT to purple formazan, an MTT derivative which is insoluble (Denzoit & Lang 1986).

Once TZM-bl/JC cells were confluent, they were prepared as described above and were resuspended in 10% DMEM for the assays. The cells were plated in 96 well flat bottomed plates at a concentration of 1 million cells per plate on the first day of the assay and placed in the incubator at 37°C. The samples were diluted in 10% DMEM. The starting concentration of MUC1, MUC1/TNC and TNC was 0.5 mg/ml whereas the starting concentration of crude and heat treated milk was 1 mg/ml.

On the second day, the samples, plated in duplicate, were serially diluted in a separate 96 well flat bottomed plate with a starting volume of 350 µl per well. The serially diluted samples (150 µl) were transferred to the corresponding wells with TZM-bl/JC cells and placed in the incubator at 37°C. The first column was the negative control containing only 10% DMEM and the second column was the positive control with TZM-bl/JC cells and MTT added on the last day. After 44 hours, 20 µl MTT at 5 mg/ml in 10% DMEM was added into each well and incubated for a further four hours. Thereafter, 100 µl was removed from each well (excluding the cell control) and replaced with 100 µl of a solubilising agent (10% SDS in 0.01 M HCl). The plates were placed in the incubator for 1 hour before being read on the plate reader at 585 nm.

Four data points, at different concentrations, are required for each sample in order to include the sample in the analysis. For data points to be considered reliable, the difference between the duplicates should not exceed 35% of the highest value. The data was entered into Microsoft Excel to determine the percentage viability using the following calculation:

$$\text{Percentage viability} = \frac{\text{absorbance value of sample} - \text{absorbance of media}}{\text{average cell control absorbance values} - \text{absorbance of media}} \times 100$$

The cell viabilities were considered to be nontoxic if all of the cell viabilities were above 70% with no downward trend for the increasing concentrations of the sample. If these conditions were not met, the cell viability and correlating concentrations were imported into GraphPad Prism (La Jolla, CA, USA) to calculate the medium lethal concentration (LC_{50}) where the LC_{50} is obtained from the log (inhibitor) vs. normalized response - variable slope graph fit function. The LC_{50} is indicative of the concentration of the sample required to result in 50% cell death. The toxicity of the samples was reported according to this criteria. The X-axis of these curves was altered to the anti-logged concentration of the sample in order to better indicate the relationship between the concentration of the sample and HIV-1 neutralisation.

2.16 Histology

Formalin fixed paraffin embedded (FFPE) tissue blocks were received from Anatomical Pathology, Health Sciences UCT (NHLS). The histochemistry and immunochemistry methods used were modified from previous work in our laboratory (Kehoe 2015).

2.16.1 Histochemistry

To identify chemical components within the cells and tissues, different histological stains were used. Mayer's haematoxylin and eosin (H&E) was used to determine the cell morphology of tissue sections. Xylol was used to dewax the tissue sections 3 × 3 min and then rehydrated with 100%, 96% and 70% graded alcohols to tap water. Tissues sections were placed in Mayer's haematoxylin for 9 minutes, blued with Scott's water (a tap water substitute) and again rinsed with tap water. The tissue sections were subsequently stained with 1% eosin for 3 minutes and again rinsed quickly with tap water. Lastly, the tissue sections were dehydrated through ascending (70%-100%) graded alcohols to xylol before being cover slipped using Entellan.

Periodic acid Schiff/Alcian Blue (PAS/AB) was used to identify acidic and neutral mucins. Alcian Blue solution stained the sections for 5 minutes after the tissue sections were moved from xylol through graded alcohols to distilled water. They were then treated with a Periodic acid solution for 2 minutes and thoroughly washed with three changes of dH_2O. Schiff's reagent was added for 8 minutes and rinsed with tap water. Tissue sections were subsequently covered with Mayer's haematoxylin for 35 second, washed with tap water and blued with Scott's water for 1 minute. The tissue sections were rinsed thoroughly with running tap water. Tissue sections were then dehydrated through the graded alcohols, cleared with xylol and cover slipped using Entellan.

A High Iron Diamine/Alcian Blue (HID/AB) stain was used on the tissue sections to identify sulphated and non-sulphated mucins. Paraffin was removed from the sections using xylol and the tissue sections were rehydrated with the graded alcohols (100% - 70%) to distilled water. Tissue sections were covered with a High Iron Diamine (HID) solution for 16-18 hours and subsequently washed with dH_2O. Lastly, the tissue sections were stained with 1% AB for 15 minutes and dehydrated, cleared and cover slipped using Entellan.

2.16.2 Immunohistochemistry

Immunohistochemistry was used to detect MUC1, MUC4 and TNC, in the cells of tissue sections, with the use of antibodies, which would bind to the corresponding antigen in the biological breast tissue if present. Firstly, the breast tissue sections were cut from FFPE blocks and fixed onto A.P.E.S (aminopropyl-triethoxy-silane) slides by heating them at 55-60°C in an incubator for 30 minutes.

The tissue sections were subsequently dewaxed in xylol for at least 15 minutes with 3-4 changes before the sections were moved through 100%, 96% and 70% graded alcohols to tap water for 1-2 minutes each in order to rehydrate them. Endogenous peroxidase activity was blocked for 15 minutes using 1% hydrogen peroxide. The tissue sections were rinsed with tap water before antigen retrieval was performed in the pressure cooker with citrate buffer (0.01 M citrate, pH 6). The citrate buffer was first boiled and the tissue sections, in metal racks, were added until full pressure was maintained for 2 minutes. The pressure was released and slides were immediately cooled in running tap water for approximately 15 minutes.

The tissue sections were then rinsed with PBST (PBS with Tween-20), pH 7.4 – 7.6. Normal goat serum (NGS) diluted 1/20 in PBS was used to block any non-specific binding. For MUC1, the NGS incubation was for 10 minutes at room temperature, whereas for MUC4 and TNC the incubation time was increased to 20 minutes with the same dilution. Subsequently, the normal goat serum was drained from all the tissue sections and the primary antibodies diluted in PBS (Table 2.2) were applied and incubated appropriately. MUC1 (1/1 000) was incubated for 30 minutes at room temperature. MUC4 (1/100) and TNC (1/100) were incubated overnight at 4°C.

Table 2.2. Summary of primary and secondary antibodies used for immunohistochemistry.

Primary antibody	**Manufacturer**	**Clone**	**Type of antibody**	**Dilution**
MUC1	Abcam	HMFG (aka 1.10.F3)	Mouse monoclonal	1/1 000
MUC4	Santa Cruz Biotechnology	1G8	Mouse monoclonal	1/100
TNC	Santa Cruz Biotechnology	H-300	Rabbit polyclonal	1/100
Secondary antibody	**Manufacturer**	**Clone**	**Type of antibody**	**Application**
Envision anti-mouse secondary antibody	Dako Products	EnVision+ System- HRP Labelled Polymer Anti-Mouse	Anti-Mouse	2-3 drops to cover tissue section
Envision anti-rabbit secondary antibody	Dako Products	EnVision+ System- HRP Labelled Polymer Anti-Rabbit	Anti-Rabbt	2-3 drops to cover tissue section

MUC4 and TNC were brought up to room temperature then rinsed with PBST twice along with MUC1 before the secondary antibody was added to the sections. Envision anti-mouse secondary antibody was used for MUC1 and MUC4 (Table 2.2). Envision anti-rabbit secondary antibody was used for TNC (Table 2.2). The incubation period was 30 minutes at room temperature for all three antibodies and then rinsed thoroughly with PBST.

Diaminobenzidine (DAB), a chromogen, was applied 7-10 minutes to develop the colour on the sections before being rinsed with PBST and then distilled water. Mayer's haematoxylin was used as a counter stain (2-4 minutes). Scott's water, a tap water substitute, was used to achieve blueing of the sections for 3 minutes and quickly rinsed with tap water. Lastly, all sections were quickly moved through the graded alcohols to dehydrate and cleared in xylol before cover slipping using Entellan.

All staining procedures included an appropriate control tissue, for the positive and negative, to test the functioning of the antibody. Breast cancer tissue was used for the MUC1 sections, normal colon tissue was used for the MUC4 sections and breast cancer and normal endocervix tissue was used for TNC.

2.16.3 Scoring of immunostaining of the sections

The slides were assessed with the assistance of Professor D. Govender, a pathologist at UCT Medical School. The proportion of cells stained was scored as follows: 0 for 0-<5% positively stained cells, 1+ for 5-25% positively stained cells, 2+ for 26-50% positively stained cells, 3+ for 51-75% positively stained cells and 4+ for 76-100% positively stained cells (Kehoe 2015). The score assigned to the tissue sections was used as a guide to photograph the stained areas.

Chapter 3: The comparison of the anti-HIV-1 potency of breast milk glycoproteins

3.1 Purification of breast milk mucins (MUC1 and MUC4) and tenascin-C

3.1.1 Caesium chloride (CsCl) density gradient ultracentrifugation spin

The milk fat was separated from the human breast milk using a CsCl density gradient ultracentrifugation spin (Figure 2.1). The remaining skim milk was divided into nine fractions to separate the glycoproteins and proteins according to their density. The glycoprotein and protein content of each of the nine fractions was determined using a PAS and Bradford assay and the corresponding density was measured (g/ml) (Figure 3.1). The glycoprotein content decreased throughout the nine fractions, whereas the protein profile remained consistently low across the nine fractions (Figure 3.1). The density of the fractions increased with the densest fraction located at the bottom of the centrifuge tube.

The mucins, located in the milk fat membrane globule (MFGM) (Figure 2.1), isolated from the milk fat obtained from this spin was frozen at -20°C in preparation for the gel extraction. The fractions of skim milk from the CsCl density gradient ultracentrifugation spin were utilised to isolate tenascin-C (TNC).

Each of the nine fractions were dialysed and freeze dried for SDS-PAGE (PAS and Acqua stain) and Western blot analysis of the fractions (Figure 3.2). The strong glycoprotein staining at the top of the running gel indicated a low electrophoretic mobility (Figure 3.2a grey arrow), which correlates to MUC1 detected by Western blotting in the majority of the fractions (Figure 3.2c grey arrow). There was a large amount of protein present in all of the fractions (Figure 3.2b grey arrow). TNC was strongly detected by Western blotting in the first two fractions (Figure 3.2d lane 2 and 3 grey arrow). These first two fractions, richest in TNC, were pooled and prepared for gel filtration in order to separate the MUC1 from the TNC. There was no MUC4 present in any of the nine fractions.

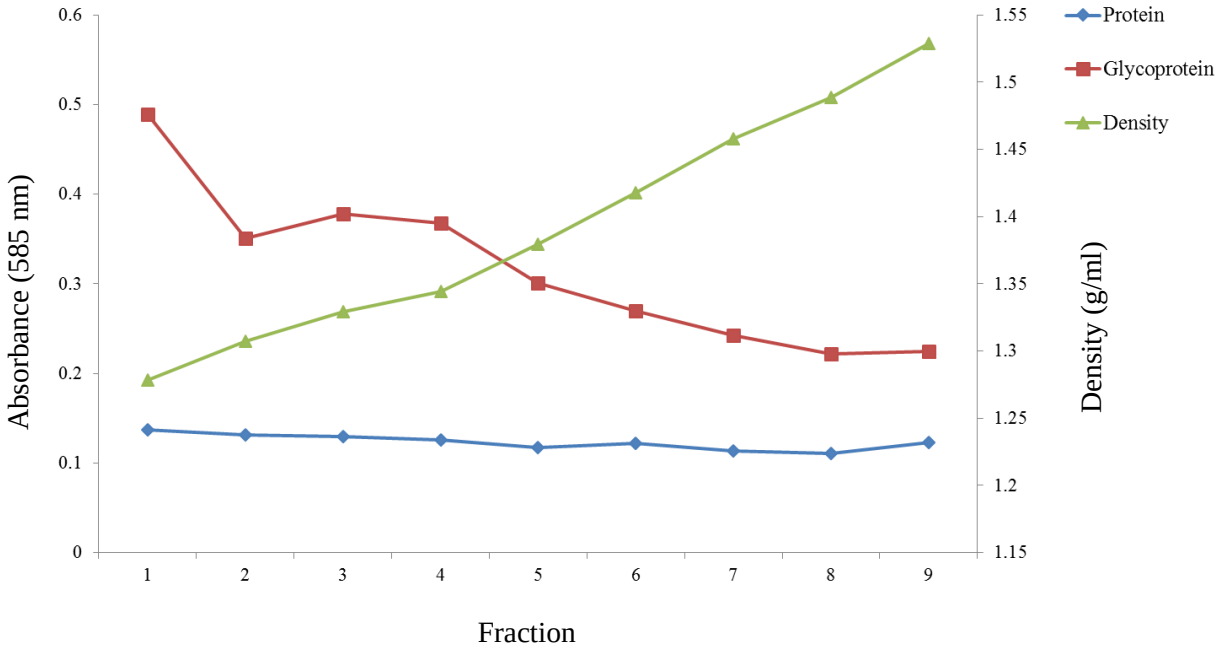

Figure 3.1. The representative protein, glycoprotein and density profiles from the CsCl density gradient ultracentrifugation spin. A PAS and Bradford assay were utilised to determine the glycoprotein and protein content of each of the skim milk fractions.

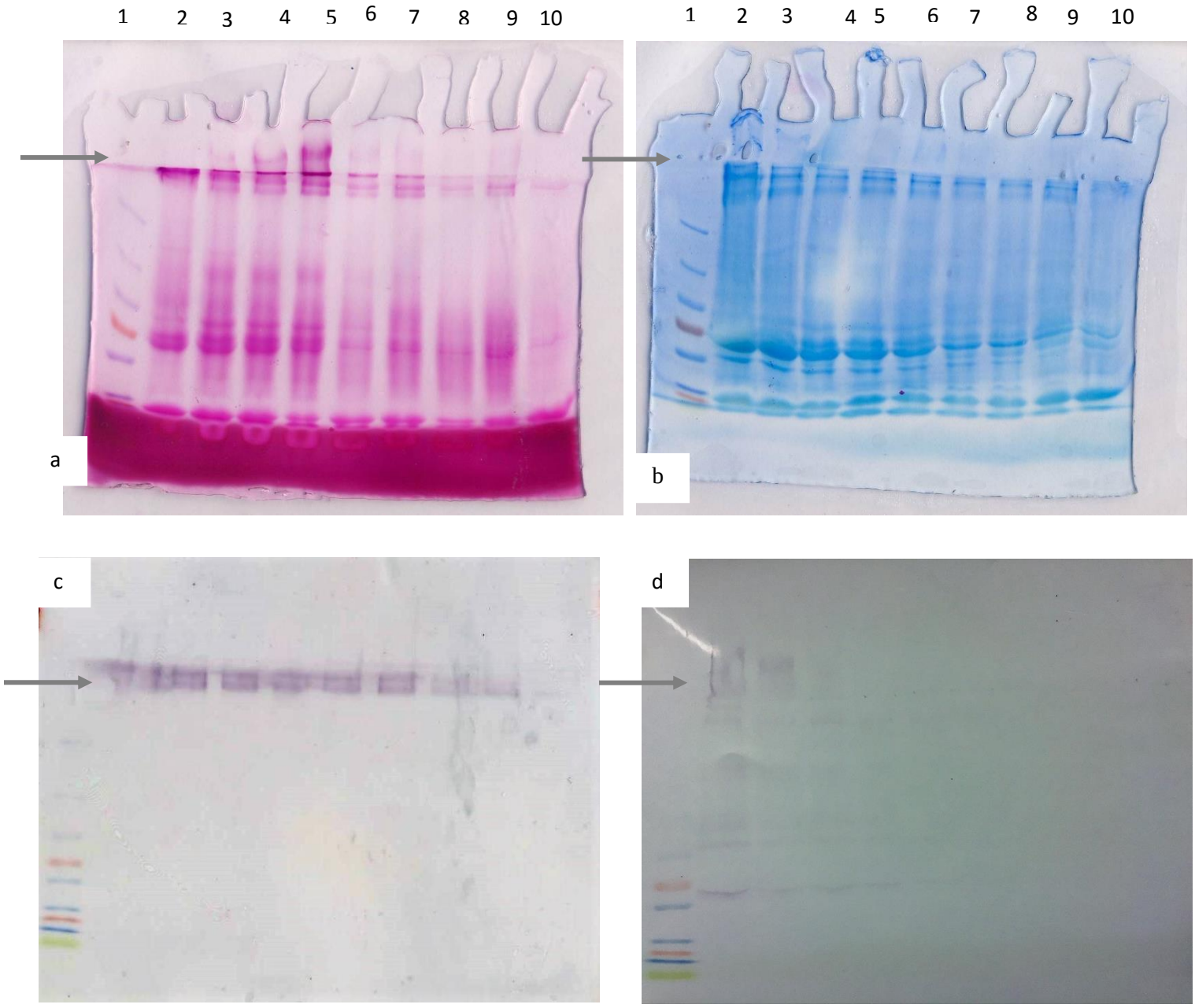

Figure 3.2. Investigation of the nine freeze dried fractions from the CsCl density gradient ultracentrifugation spin for TNC purification. The nine fractions were subjected to 4-20% SDS-PAGE analysis and stained with a) PAS staining and b) Acqua staining. A Western blot analysis was also performed for c) MUC1 and d) TNC. Lane 1 is the molecular weight marker and lanes 2-10 are the nine fractions from the CsCl density gradient ultracentrifugation spin in ascending order. The grey arrows indicate the area of interest for both the 4-20% SDS-PAGE and the Western blot.

3.1.2 Gel filtration

The first and second skim milk fractions from the CsCl density gradient ultracentrifugation spin were pooled and dissolved in 15 ml of 0.2 M NaCl and 0.02% NaN_3. The sample (6 ml) was then chromatographed on a Sepharose CL-2B column and eluted with 0.2 M NaCl and 0.02% NaN_3 to collect 0.5 ml fractions. The sample was chromatographed twice (6 ml each) due to the total volume. Thereafter, a PAS and Bradford assay was performed on the fractions to determine the glycoprotein and protein profiles of the material. Figure 3.3 is a representation of the Sepharose CL-2B column in which the first peak is the void volume (V_0) and the second peak is the included volume (V_i). For both the V_0 and V_i, a distinct glycoprotein and protein positive peak was noted. The respective peaks were pooled, dialysed and freeze dried for subsequent analysis.

To investigate the contents of both the V_0 and V_i, SDS-PAGE and Western blot analysis was performed (Figure 3.4). The protein content of both the V_0 and the V_i was substantial indicating that both peaks contained a large amount of contaminant proteins, which were not of interest to this study (Figure 3.4a lane 2 and 3). A Western blot was performed and probed for both MUC1 and TNC with the respective primary antibodies, to establish whether the V_0 and V_i contained TNC and any residual MUC1. The V_0 and V_i stained positive for MUC1 and TNC, with a lower intensity of staining for both MUC1 and TNC noted in the V_i (Figure 3.4b lane 3 and 3.4c lane 3). A strong MUC1 band was present (Figure 3.4b lane 2, blue box) with some corresponding TNC staining in the same area as MUC1 (Figure 3.4c lane 2, blue box). Stronger staining noted in the V_0 for both MUC1 and TNC. MUC1, a highly glycosylated glycoprotein, showed low electrophoretic mobility which is a characteristic of the movement of glycoproteins (Wu et al. 1994). A TNC band was present at ±250 kDa correlating with the molecular weight of TNC, again with stronger staining noted in the V_0 (Gulcher et al. 1991) (Figure 3.4c lane 2, red box). Therefore, the V_0 was chosen for subsequent experiments to purify TNC and co-purify MUC1 and TNC using gel extraction.

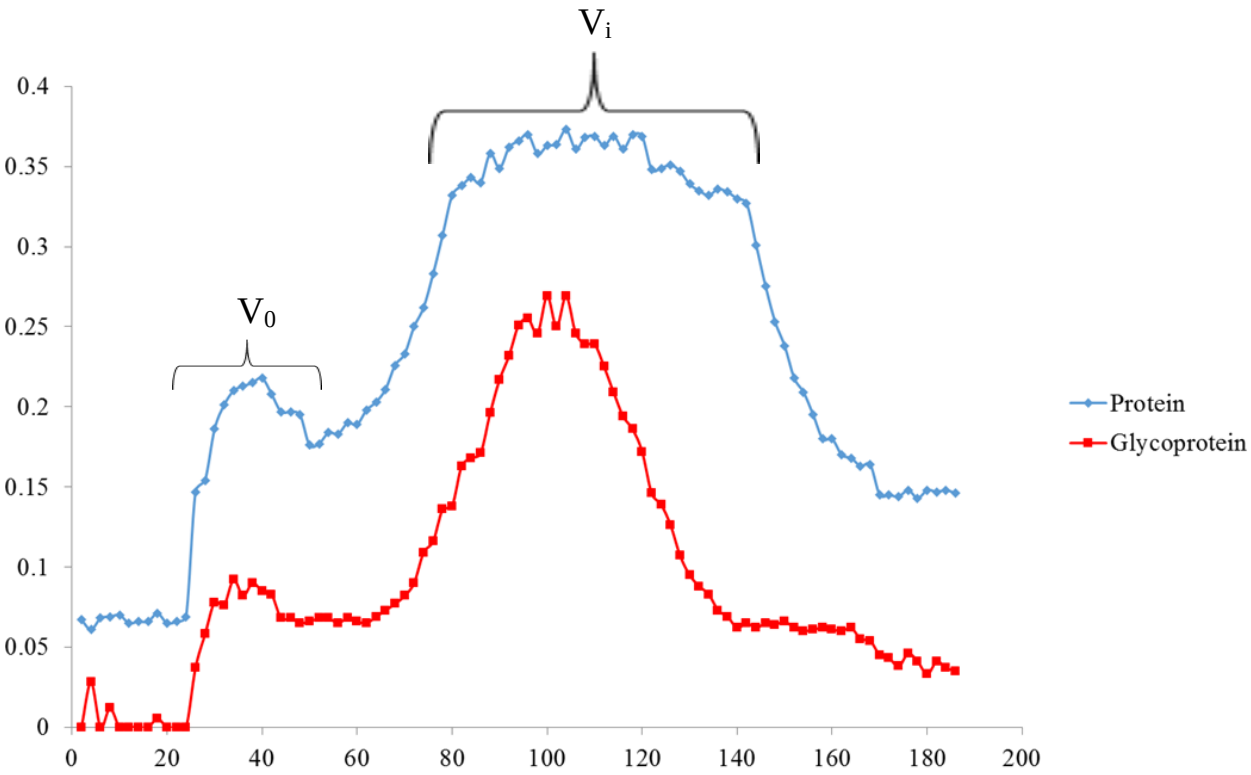

Figure 3.3. A representative Sepharose CL-2B seperation of skimmed breast milk. Every second fraction was tested for both the glycoprotein (red) and protein (blue) content. The V_0 and V_i are indicated and the corresponding fractions were pooled.

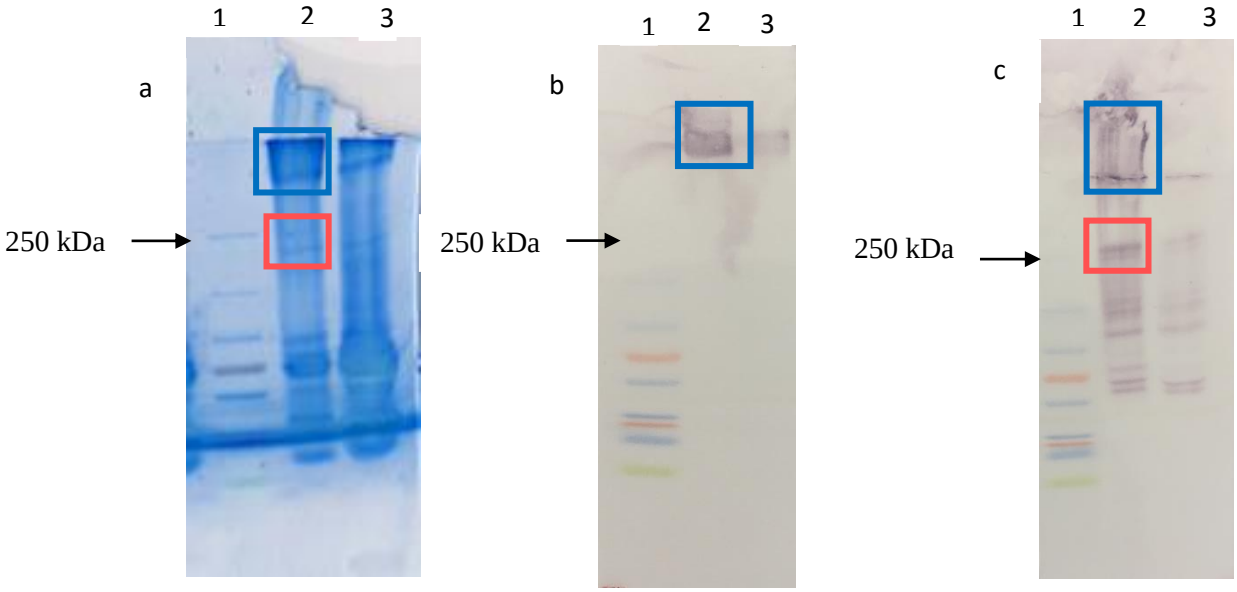

Figure 3.4. The investigation of the contents of the V_0 and V_i from the Sepharose CL-2B column on a 4-20% SDS-PAGE gel. The protein content of the V_0 and V_i stained with a) Acqua stain and the corresponding Western blots b) MUC1 and c) TNC. Lane 1 is the molecular weight marker, lane 2 is the V_0 and lane 3 is the V_i.

3.1.3 Gel extraction of MUC1 and TNC from the V_0 peak

Due to the protein contamination and the low, freeze dried yield in the V_0, gel extraction was utilised to purify TNC, the ±250 kDa glycoprotein indicated by a red box in Figure 3.4c. The MUC1 and TNC band at the top of the running gel (Figure 3.4 lane 2), indicated by the blue box, was also purified using gel extraction as a single band. The freeze dried V_0 was solubilised in sample application buffer (1 mg/100 µl) and subjected to SDS-PAGE for approximately 1.5 hours at 200 V. The band at the top of the running gel containing both MUC1 and TNC (Figure 3.4a blue box) was cut out of the gel, gently crushed and placed in a gel extraction buffer in a centrifuge tube at room temperature overnight. Thereafter, the contents of the tube was centrifuged at 3 000 rpm for 20 minutes and the supernatant was removed, dialysed and freeze dried. To purify TNC, the ±250 kDa band was extracted from the SDS-PAGE gel using the same method as described (Figure 3.4a red box) and subsequently dialysed and freeze dried.

In order to assess the purity of the MUC1/TNC and TNC after extraction, the following experiments were performed: SDS-PAGE gel stained for the glycoprotein and protein content and a slot blot to confirm the presence of MUC1 and TNC. The MUC1/TNC band was previously aggregated and restricted to the top of the running gel before gel extraction (Figure 3.4a lane 2, blue box). The two glycoproteins were subsequently separated using the gel extraction method and an increased electrophoretic mobility of both glycoproteins can be noted (Figure 3.5a lane 2, grey arrow and 3.5b lane 2, grey arrow). One band was noted at the top of the running gel (MUC1) (Figure 3.5a lane 2) and another above 250 kDa (TNC) with no other contaminating proteins (Figure 3.5b lane 2). The blue streaking in the gel is due to the 4-20% gradient of the gel which picked up some of the Acqua stain. The slot blot indicated that both MUC1 and TNC were present (Figure 3.5c and 3.5d).

The TNC extracted from the V_0 was assessed using the above mentioned method. The band present at ±250 kDa on the SDS-PAGE gel confirms the presence of purified TNC (Figure 3.6a lane 2, red box). The smearing observed in the SDS-PAGE gel with some potential degradation might be due to the glycosylation of the glycoprotein which was not observed previously in the V_0 due to the presence of multiple other proteins. The slot blot confirmed that this glycoprotein band was TNC (Figure 3.6b).

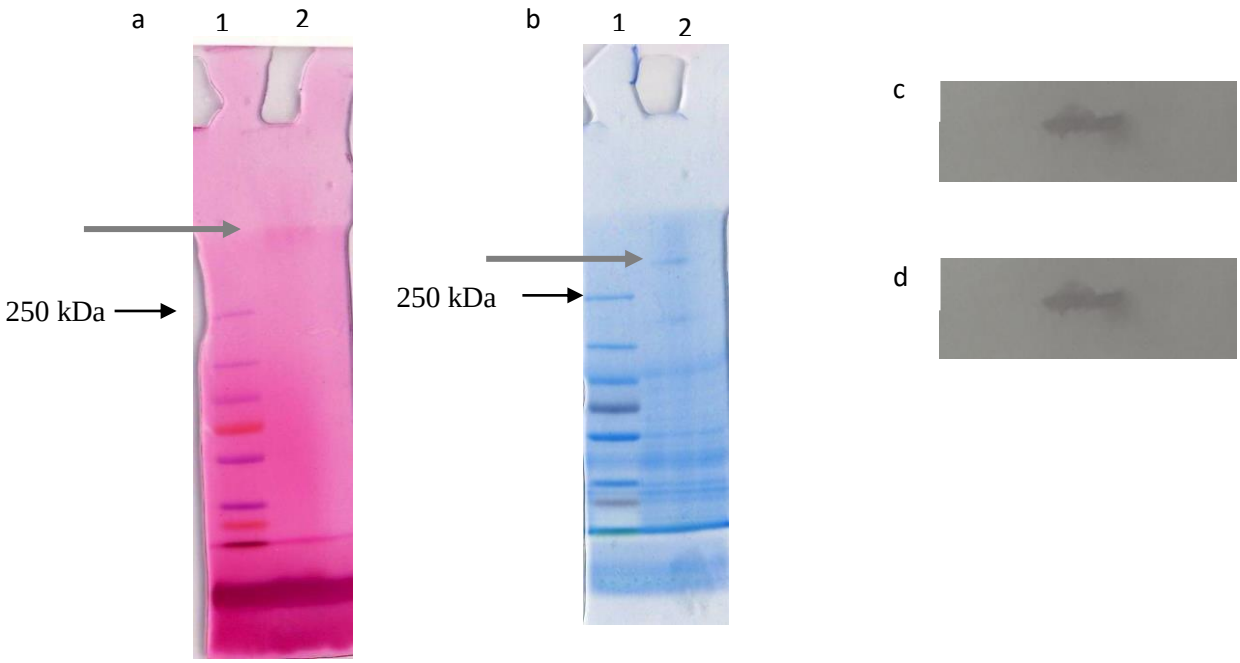

Figure 3.5. SDS-PAGE (4-20%) and slot blot analysis of the MUC1/TNC gel extraction purification from the V_0 of the skim milk chromatographed on a Sepharose CL-2B column. The a) glycoprotein and b) protein content was stained with PAS and Acqua stain respectively. The presence of c) MUC1 and d) TNC was confirmed using a slot blot with anti-MUC1 and anti-TNC primary antibodies respectively. In Figure a and b, lane 1 is the molecular weight marker and lane 2 is the purified MUC1/TNC. The grey arrows indicate the purified MUC1 (a) and TNC (b).

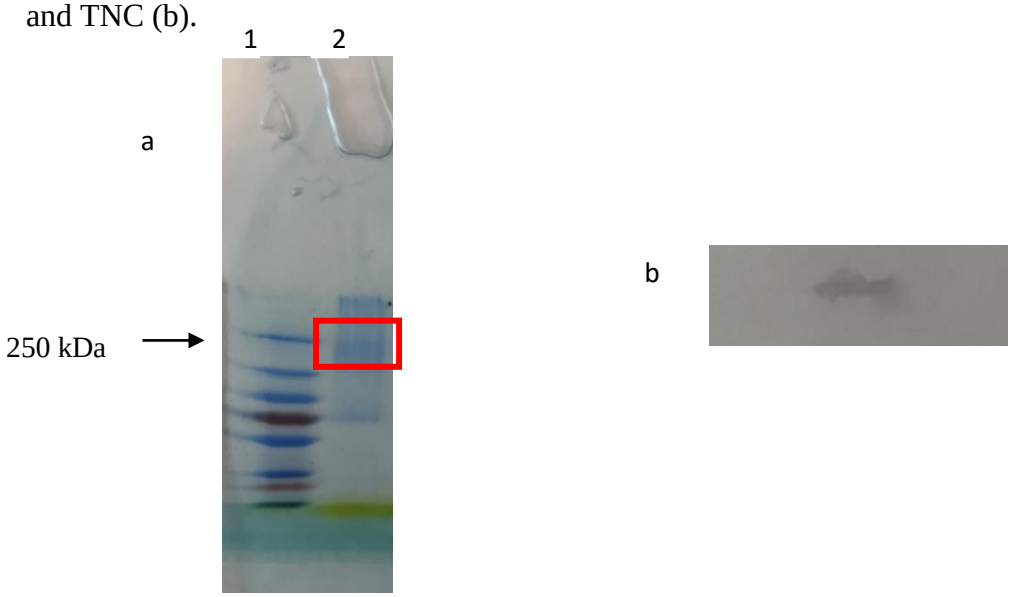

Figure 3.6. The presence of TNC after gel extraction purification was confirmed with 4-20% SDS-PAGE stained with a) Acqua stain and b) a slot blot using an anti-TNC primary antibody. The red box indicates purified TNC. Lane 1 is the molecular weight marker and lane 2 is the purified TNC.

3.2 Extraction of breast milk mucins MUC1 and MUC4

The milk fat obtained from the CsCl density gradient ultracentrifugation spin was thawed at room temperature and diluted in sample application buffer (1 mg/100 µl). The milk fat samples were subjected to SDS-PAGE for approximately 1.5 hours at 200 V. A template for the gel extraction of MUC1 and MUC4 was created by staining the 4-20% SDS-PAGE gel with Acqua stain and the molecular weight marker was used as a guide for subsequent gel extraction. A number of clear bands were noted throughout the gel indicating the milk fat globule membrane proteins in the sample (Figure 3.7). A clear band, stained with both PAS and Acqua stain, was situated at the top of the running gel which was suspected to be MUC1 (Figure 3.7a lane 2 and 3.7b lane 2, red box). A very faint PAS stained band was noted around the 72 kDa size range correlating to the band stained with Acqua stain around this size range which was suspected to be MUC4 (the β subunit weighing approximately 80 kDa) (Figure 3.7a lane 2 and 3.7b lane 2, blue box). As MUC1 and MUC4 are glycoproteins, the two bands stained with PAS were of interest for gel extraction. Furthermore, MUC1 is a large glycoprotein that is heavily glycosylated and was therefore expected to have low electrophoretic mobility and thus would be situated at the top of the gel as seen in Figure 3.7a, lane 2. MUC4 with two subunits, a large and small subunit, is present in the milk fat globule membrane (Zhang et al. 2005; Moniaux et al. 2007; Chaturvedi et al. 2008). Therefore, it was suspected that the band around the 80 kDa could be the smaller subunit of MUC4. These two bands, highlighted in red box (MUC1) and blue box (MUC4) in Figure 3.7a and 3.7b (lane 2), were used separately for subsequent gel extraction for the milk fat samples using approximately eight 4-20% SDS-PAGE gels per sample.

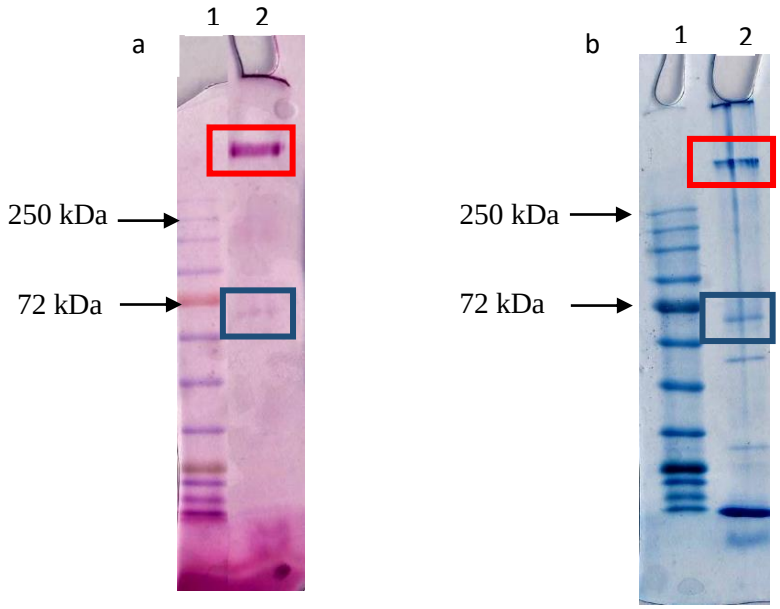

Figure 3.7. The milk fat extraction templates from a 4-20% gradient SDS-PAGE gel utilised for the extraction of breast milk mucins stained for both the a) glycoproteins and b) proteins. MUC1 was extracted from the top of the running gel, indicated by the red box, and MUC4 was extracted around the 72 kDa band, indicated by a blue box. MUC4 was not successfully purified. Lane 1 is the molecular weight marker and lane 2 is the milk fat from the breast milk.

Once the gel extraction was completed, the milk fat samples were dialysed and freeze dried and subsequently a slot blot was done to confirm the presence of MUC1 and MUC4. SDS-PAGE analysis was used to confirm the purity of both MUC1 and MUC4. The PAS staining confirmed both the presence and purity of MUC1 from the milk fat (Figure 3.8a lane 2). The presence of MUC1 was confirmed by a MUC1 primary antibody in the slot blot (Figure 3.8b). The freeze dried yield of MUC4 was considerably lower than that of MUC1 and the detection of MUC4 was inconsistent across the samples. MUC4 was only detected in two of the samples with a MUC4 primary antibody (data not shown), but due to the low sample yield further experiments could not be performed using MUC4. The freeze dried MUC1 was stored at 4°C for the neutralisation and cell toxicity assay.

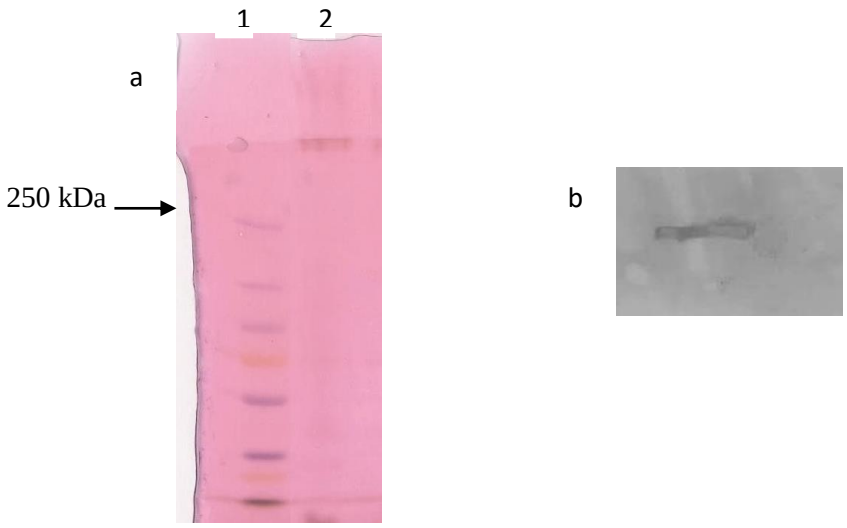

Figure 3.8. The presence and purity of MUC1 from the milk fat after gel extraction was confirmed using 4-20% SDS-PAGE stained for a) glycoprotein and b) a slot blot probed for MUC1. In Figure a, lane 1 is the molecular weight marker and lane 2 is the purified MUC1.

3.3 ELISA kits for MUC1, MUC4 and TNC

Elisa kits were purchased for MUC1, MUC4 and TNC and the methodology was performed as described as per the manufacturers guidelines. The absorbance values (450 nm) of the known, standard concentrations were then used to set up a standard curve, with the detection range for MUC4 and TNC ranging from 0.31-20 ng/ml, and for MUC1, the detection range was 0.16-10 ng/ml. The formula produced from the standard curve, with an R^2 value closest to 1.0, was used to calculate the concentration of either MUC1, MUC4 or TNC accounting for the dilution factor.

There was high variability in the MUC1 concentration in crude breast milk throughout the five samples, ranging from as low as 87.33 ng/ml to as high as 564.76 ng/ml. The remaining three samples had the following MUC1 concentrations: 510.77 ng/ml; 262.34 ng/ml and 114.05 ng/ml. Therefore the average concentration of MUC1 detected in these samples was 307.85 ng/ml. The concentration of MUC4 across all of the samples was below the detectable limit of the standard curve (0.31-20 ng/ml). Majority of the absorbance values (450 nm) for TNC were above the upper range of the standard curve and therefore the ELISA was saturated. The concentration of TNC in crude breast milk could not be accurately determined. Due to financial constraints the ELISA could not be repeated for MUC1, MUC4 or TNC.

3.4 Crude and heat treated breast milk

The crude and heat treated breast milk was freeze dried and applied to a 4-20% SDS-PAGE gel and subsequently stained with Acqua stain. The protein content is similar across the crude and the two heat treated groups (80°C and 62.5°C) (Figure 3.9).

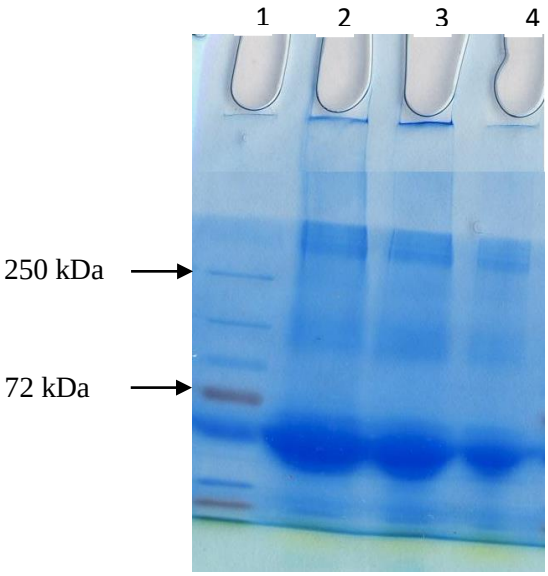

Figure 3.9. A 4-20% SDS-PAGE gel of the crude and heat treated breast milk stained with Acqua stain. Lane 1 is the molecular weight marker, lane 2 is the crude breast milk, lane 3 is the 80°C heat treated milk and lane 4 is the 62.5°C heat treated milk.

3.5 Pseudoviral assay

Crude and heat treated breast milk (n=15 for each group) as well as MUC1, TNC and MUC1/TNC (n=15 for MUC1, n=12 for TNC and n=14 for MUC1/TNC) were tested against the Du422.1 pseudovirus and the inhibitory potency was calculated at which 50% of HIV-1 infection (IC_{50}) in the TZM-bl/JC cells was reduced (Figure 3.12, Table 3.1 and Table 3.2). Any neutralisation values below 30%, including negative values, with the absence of increasing HIV neutralisation with ascending concentrations, were considered to show no anti-HIV-1 activity at these tested concentrations. Statistical analysis was performed across the three cohorts using a Kruskal Wallis test and a Mann-Whitney U test for post hoc analysis. The logIC_{50} were used during the analysis and samples which showed no inhibition were assigned an arbitrary IC_{50} value of 2 000 µg/ml, which was the log transformed. This arbitrary value did not affect the outcome of the analysis as the tests utilised were non-parametric. Unreliable samples were not included in the statistical analysis.

3.5.1 Crude and heat treated breast milk

From the data for the crude breast milk, eight of the 15 samples showed no inhibition at the tested concentrations with the data for one sample not available (Table 3.1). A further four samples had an IC_{50} below 500 µg/ml (ranging between 28.50 and 345.10 µg/ml) with sample 10 showing exceptional potency with an IC_{50} of 28.50 µg/ml. The remaining samples had an IC_{50} of above 500 µg/ml thus indicative of a lower anti-HIV-1 potency.

Of the heat treated milk at 80°C for 10 minutes, six of the samples showed no inhibition and the remaining nine samples showing large variation in the IC_{50} values (Table 3.1). Sample 2 indicated good HIV-1 neutralisation with an IC_{50} of 19.51 µg/ml. There were six samples with an IC_{50} below 500 µg/ml (range of 19.51 - 397.7 µg/ml). Three samples, with a wide range of IC_{50} values, had an IC_{50} above 500 µg/ml suggesting a lower HIV-1 neutralisation by these 80°C heat treated samples.

The anti-HIV-1 potency of the 62.5°C heat treated breast milk showed better HIV-1 inhibition when compared to the 80°C heat treated and crude breast milk. The range of IC_{50} values were narrower with 12 of the 15 samples having an IC_{50} below 500 µg/ml (Table 3.1). Sample 13 had an exceptional potency (IC_{50} 0.21 µg/ml) when compared to other samples within this cohort. The remaining two samples had an IC_{50} above this range (500 µg/ml) with one sample indicating no inhibition.

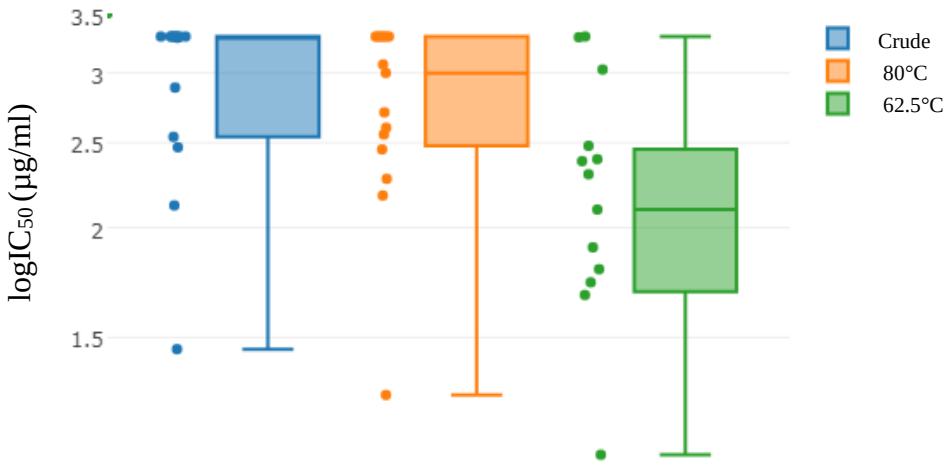

Figure 3.10. The anti-HIV-1 potency among the crude breast milk cohort and 80°C and 62.5°C heat treated breast milk cohorts.

The box plot indicated that the data across the crude and 80°C heat treated breast milk cohorts were left skewed (Figure 3.10) and therefore, non-parametric tests were chosen for the analysis. There was a statistical difference between the three cohorts (Kruskal Wallis, p-value = 0.0023). There was no statistically significant difference in the HIV-1 neutralisation ability detected between the crude and 80°C heated treated milk cohorts (Mann-Whitney U, p-value = 0.44). A statistically significant difference in the HIV-1 neutralisation ability was detected between the crude and 62.5°C heat treated breast milk cohorts (Mann-Whitney U, p-value = 0.0021). Among the 80°C and 62.5°C heat treated breast milk cohorts, there was a statistically significant difference in the HIV-1 neutralisation (Mann-Whitney, p-value = 0.0033).

3.5.2 MUC1, TNC and MUC1/TNC

More than half of the samples from the MUC1 cohort showed exceptional potency with low IC_{50} values in which six samples were below 1 µg/ml (5.329×10^{-15} – 0.9441 µg/ml) and two samples ranging between >1 µg/ml and <10 µg/ml (1.58 µg/ml and 4.77 µg/ml) (Table 3.2). Three samples fell within the range of >10 µg/ml and <100 µg/ml, and the last two samples had IC_{50} values above 100 µg/ml with one sample demonstrating no inhibition and one unrealiable.

IC_{50} values from sample 12 and 19 from the TNC cohort were unreliable and sample 2 was deemed to show no inhibition (Table 3.2). The remaining samples showed good potency with six samples IC_{50} values <1 µg/ml, two samples IC_{50} values just above 1 µg/ml and one of the samples IC_{50} value falling within the 10-100 µg/ml range.

The MUC1/TNC cohort showed less potent neutralisation with data not available for three of the 14 samples (Table 3.2). In five samples IC_{50} values were greater than 100 µg/ml and two samples IC_{50} values were between 10 µg/ml and 100 µg/ml. Sample 6 and 15 showed exceptional potency (<1 µg/ml) and sample 1 and 2 indicated good potency (>1 µg/ml and <10 µg/ml).

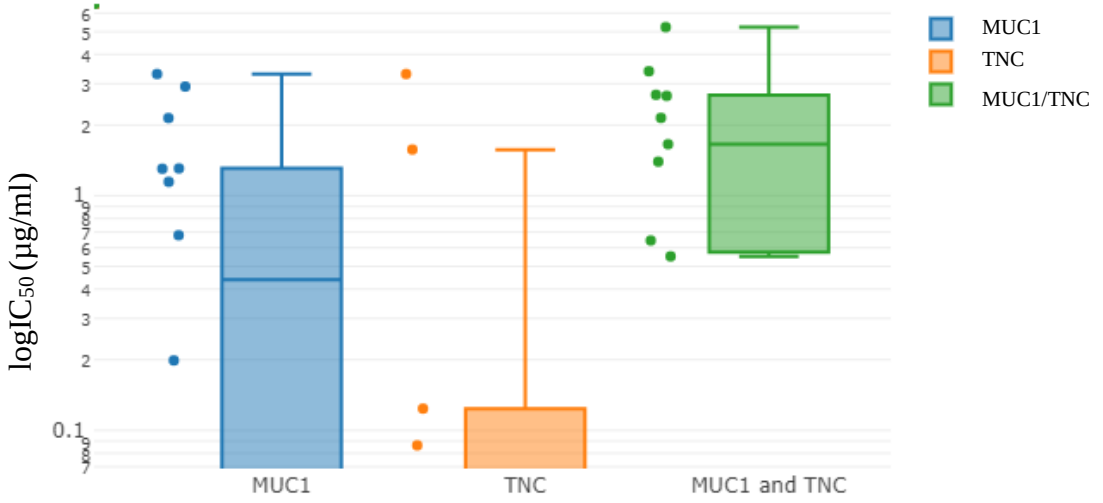

Figure 3.11. A comparison of the anti-HIV-1 potency among the purified glycoprotein cohorts (MUC1, TNC and MUC1/TNC).

From the box plot, it is evident that the data are right skewed and therefore non-parametric tests were utilised (Figure 3.11). There was no statistically significant difference in the HIV-1 neutralisation ability across MUC1, TNC and MUC1/TNC (Kruskal Wallis, p-value = 0.13). Therefore, despite the different ranges of IC_{50} values observed it was concluded that there was no statistical difference among these cohorts and no post hoc analysis was performed.

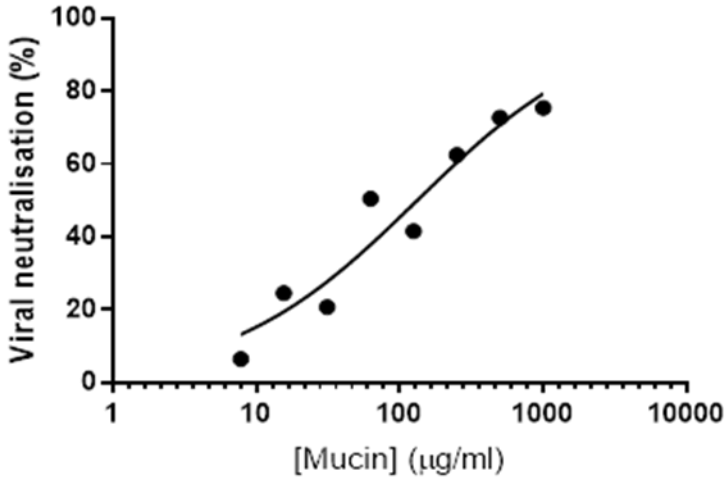

Figure 3.12. A representative HIV-1 neutralisation curve displaying the neutralisation of HIV-1 (Du422.1) by either crude and heat treated milk as well as the purified glycoproteins (MUC1, TNC and MUC1/TNC).

3.6 Cell toxicity assay

The cytotoxicity of the samples used in the pseudoviral assay were tested using an MTT assay. The cell viabilities were considered to be nontoxic if all of the cell viabilities were above 70% with no downward trend for the increasing concentrations of the sample. If these conditions were not met, the cell viability and correlating concentrations were imported into GraphPad Prism (La Jolla, CA, USA) to calculate the medium lethal concentration (LC_{50}) (Figure 3.13).

3.6.1 Crude and heat treated breast milk

The cytotoxicity of the crude breast milk showed variation with no detectable cell toxicity noted in seven of the samples. The remaining values showed a range of LC_{50} values. The data was deemed unreliable for sample 18. All of the 80°C heat treated samples had no detectable cell toxicity. Large variation of cytotoxicity was noted in the 62.5°C heat treated milk ranging from no detectable toxicity to LC_{50} values as high as 41 223 µg/ml and as low as 44.35 µg/ml.

3.6.2 MUC1, TNC and MUC1/TNC

The majority of the MUC1 cohort showed no detectable toxicity with the LC_{50} values for samples 5 and 7 being recorded as 519.80 µg/ml and 11 891 µg/ml respectively. No detectable cytotoxicity was noted in approximately half of the TNC and MUC1/TNC groups. The remaining TNC samples had LC_{50} values that showed large variation (241.30 – 2 235 µg/ml). High variability was noted in the other half of the MUC1/TNC samples in which the LC_{50} values ranged between 1.42 and 64 120 µg/ml.

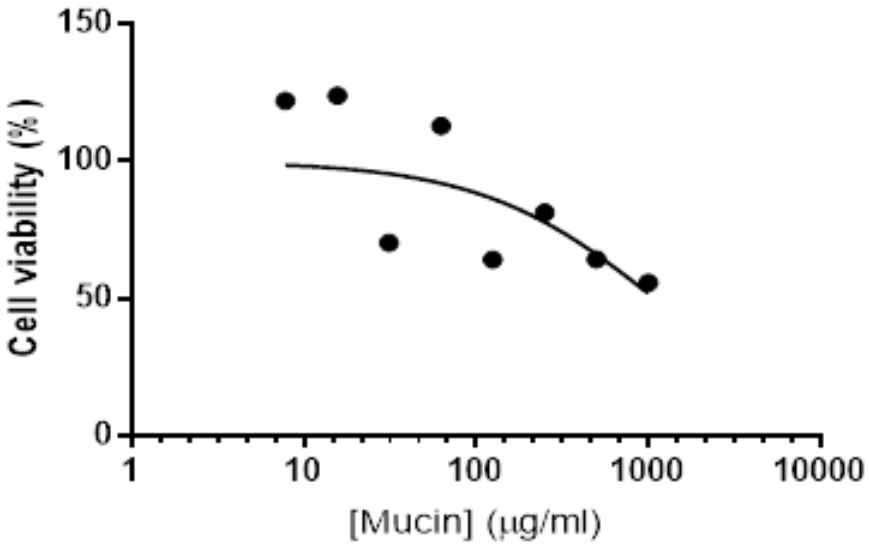

Figure 3.13. The cell viability of the TZM-bl/JC cells used to calculate the LC_{50} values (µg/ml) in samples that did not meet the criteria for no toxicity.

Table 3.1. The cytotoxicity (LC_{50}) and HIV-1 neutralisation (IC_{50}) of crude breast milk and 80°C and 62.5°C heat treated breast milk.

Sample	Crude		80°C heat treated		62.5°C heat treated	
	LC_{50} (µg/ml)	IC_{50} (µg/ml)	LC_{50} (µg/ml)	IC_{50} (µg/ml)	LC_{50} (µg/ml)	IC_{50} (µg/ml)
1			Not toxic	No inhibition	Not toxic	79.38
2	1 288	No inhibition	Not toxic	19.51	Not toxic	240.60
3			Not toxic	507.90	Not toxic	1 065
4			Not toxic	1 170		
5	75 474	No inhibition	Not toxic	187.70	3 003	199.90
6	10 305	No inhibition	Not toxic	397.70	44.35	0.42
7	Not toxic	131.90				
8	Not toxic	771.60	Not toxic	358	Not toxic	62.22
9	9 718	No inhibition	Not toxic	No inhibition	Not toxic	47.51
10	1 319	28.50	Not toxic	No inhibition	Not toxic	247.50
11	Not toxic	No inhibition				
12	1 094	294.50	Not toxic	1 001	41 223	No inhibition
13	Not toxic	345.10	Not toxic	No inhibition	Not toxic	0.21
14	Not toxic	No inhibition	Not toxic	No inhibition	969.5	125.20
15	Not toxic	1 948	Not toxic	285.50	Not toxic	300.90
16	1 113	No inhibition			Not toxic	54.12
17	Unreliable	No inhibition	Not toxic	150	Not toxic	1 972
18	Not toxic	Unreliable				
19			Not toxic	No inhibition	Not toxic	12.68

Table 3.2. The cytotoxicity (LC_{50}) and HIV-1 neutralisation (IC_{50}) of purified MUC1 and TNC and co-purified MUC1/TNC.

Sample	MUC1		TNC		MUC1/TNC	
	LC_{50} (µg/ml)	IC_{50} (µg/ml)	LC_{50} (µg/ml)	IC_{50} (µg/ml)	LC_{50} (µg/ml)	IC_{50} (µg/ml)
1	Not toxic	1.14×10^{-12}	Not toxic	0.03	Not toxic	4.41
2			Not toxic	No inhibition	12.58	3.56
3	Not toxic	13.99			Not toxic	Unreliable
4			1 699	0.06	Not toxic	139.70
5	519.80	0.05	Not toxic	0.96	2.898	2 459
6	Not toxic	0.94	Not toxic	1.33	42.27	2.04×10^{-7}
7	11 891	827.30			1.42	45.41
8	Not toxic	No inhibition	241.3	6.86×10^{-12}	35 031	459.90
9	Not toxic	Unreliable	Not toxic	0.00099		
10	Not toxic	0.002				
11	Not toxic	138.90			64 120	168 520
12			Not toxic	Unreliable	Not toxic	490.90
13	Not toxic	0.05				
14			Not toxic	0.09		
15	Not toxic	4.77	2 235	37.39	Not toxic	4.33×10^{-5}
16	Not toxic	5.33×10^{-15}	513.80	1.22	Not toxic	Unreliable
17	Not toxic	20.08			48.18	24.91
18	Not toxic	1.58				
19	Not toxic	20.26	255.20	Unreliable	Not toxic	Unreliable

3.7 Discussion

Previous studies in our laboratory have shown that crude breast milk does not inhibit HIV-1, whereas MUC1 individually and co-purified MUC1 and MUC4 successfully inhibit HIV-1 *in vitro* (Habte et al. 2008; Mthembu et al. 2014). These previous studies investigated both HIV positive and negative breast milk and the findings were the same for both groups. This study aimed to verify the previous results and explore the effect of pasteurisation on the anti-HIV-1 activity of breast milk. Furthermore, this study aimed to explore the anti-viral properties of TNC, a recently identified breast milk protein with anti-HIV-1 activity and compare that to the anti-viral abilities of breast milk mucins. Breast milk samples obtained for this study were HIV negative and their HIV status was confirmed by their medical records and nurses at the respective facilities.

The purification of both MUC1 and TNC was successful using the methodology previously described in this study. MUC1 was successfully purified from milk fat of breast milk and TNC was successfully purified from the skim milk. It is interesting to note that after the skim milk was chromatographed on a Sepharose CL-2B column, MUC1 was still present in the skim milk and it co-eluted with TNC in the V_0 fraction (Figure 3.4). From the Western blotting analysis, it was evident that TNC was present at the 250 kDa band and MUC1 and TNC had co-purified and stuck together in a separate band at the top of the running gel (Figure 3.4b and 3.4c). These glycoproteins might have disaggregated after gel extraction due to less contaminant proteins present thus allowing these glycoproteins to separate during SDS-PAGE. The reason for this co-purification is unclear as this phenomenon might have occurred during the purification process or might be present *in vivo.* This phenomenon could be explored in future research in order to determine if MUC1 and TNC aggregate in breast milk to alter the antiviral functions of breast milk.

From the ELISA data, it was clear that the concentrations of MUC1 was substantial and thus, purification for this glycoprotein was not an issue. From the preliminary data, it is suggested that the average concentration of MUC1 in crude breast milk is 307.87 ng/ml with a range of 87.33 - 564.76 ng/ml. The concentration of TNC could not be accurately determined due to the ELISA being saturated with majority of the absorbance values lying above the upper limit of the standard curve. This does however suggest that the concentration of TNC in crude breast milk might be higher than the highest concentration of the standard curve, 20 ng/ml. The ELISA experiment for MUC1 or TNC could not be repeated due to financial constraints.

Therefore, this would need to be repeated with more dilute samples to validate the concentration of TNC in crude breast milk. Due to the limited number of samples available to test, the large variability observed in the range of concentrations of MUC1 and no conclusive findings concerning both MUC4 and TNC, this should be further investigated in future research. The purification of both MUC1 and TNC was successful using this study methodology. This observation correlates with these two glycoproteins detected in lactating breast tissue (Chapter 4).

The concentration of MUC4 was below the detectable limit of the ELISA kit suggesting that the concentration was so low in our cohort that purification of MUC4 was not possible using the methodology employed. The MUC4 ELISA could not be repeated due to financial constraints. Interestingly, MUC4 was detected in two of the samples (data not shown) using an anti-MUC4 1G8 monoclonal primary antibody during Western blotting. This clone of antibody is specific to the MUC4β transmembrane subunit, which is analogous to ASGP-2 (Zhang et al. 2005; Chaturvedi et al. 2008). This clone of MUC4 primary antibody recognises and binds to the MUC4β subunit between 53 amino acids at the N-terminal and the transmembrane domain (approximately 80 kDa). This is in line with our postulation that MUC4 was present around the 80 kDa band seen in the SDS-PAGE analysis of the milk fat (Figure 3.7). Another primary monoclonal MUC4 antibody (8G7 clone) utilised, specific for the MUC4 peptide and MUC4 native to human tissues (Kitazono et al. 2014), did not detect anything across the milk fat samples. This is interesting as this data suggests that the MUC4β subunit might be predominantly expressed in breast milk secretions. This finding is further substantiated by the immunohistochemistry results in Chapter 4. The 1G8 clone of MUC4 was again utilised for the detection of the MUC4β subunit, and a strong expression was noted across all six cases.

Human breast milk mucins have been implicated in protection against the rota virus, the pox virus and HIV-1 (Yolken et al. 1992; Habte et al. 2007; Habte et al. 2008; Mthembu et al. 2014). Crude and pasteurised milk are often used in daily life by both HIV positive and HIV negative mothers, and therefore it is interesting to note their effect on HIV-1 neutralisation. From these data (Table 3.1), it is evident that the majority of the crude breast milk samples (53.33%) did not show HIV-1 neutralisation (eight of the 15 samples), with the remaining samples exhibiting varying degrees of HIV-1 neutralisation. One of these samples showed exceptional neutralisation with an IC_{50} of 28.50 µg/ml. The remaining six samples showed HIV-1 neutralisation at higher IC_{50} values (131.90 – 1 948 µg/ml), thus indicating the ability of the crude breast milk samples to neutralise HIV-1 to a certain extent over a wide range. But

it is important to note that the majority of the crude breast milk samples did not show inhibition with a further one sample (sample 15) having an IC_{50} value much higher than that of the other samples. These results are not consistent with previous studies in our laboratory done on crude breast milk. These studies concluded that crude breast milk does not inhibit HIV-1 in an *in vitro* assay across all samples (Habte et al. 2008; Mthembu et al. 2014). Furthermore, these results are inconsistent with findings from Kazmi et al. (2006) who demonstrated that crude breast milk samples showed medium to high anti-HIV-1 activity in 70% of their subjects. This variation in results could be due to the variability in samples obtained from across the study population and potentially the maturity of the breast milk at the time of collection. The crude breast milk samples that did exhibit HIV-1 neutralisation may be due to the presence of breast milk proteins implicated in HIV-1 neutralisation, including lactoferrin, glycosaminoglycan moieties and milk oligosaccharides with fucose residues and Lewis epitopes (Berkhout et al. 2002; Newburg et al. 1995; Hong et al. 2008). These milk oligosaccharides in breast milk compete with HIV-1 gp120 and bind DC-SIGN with a higher affinity thus reducing the binding of HIV-1 gp120 thereby reducing mother-to-child transmission (Hong et al. 2008). It is interesting to note that the inhibition of HIV-1 by crude breast milk is not always seen *in vivo* and a possible explanation may be due to the high concentration of viable virus (240 – 8 100 copies/ml) present in crude breast milk which would prevent the endogenous antiviral factors from preventing mother-to-child transmission in all infants (Kazmi et al. 2006). This reason may account for the proportion of infants who do acquire HIV-1 from their HIV positive mothers. A more detailed studied would be required to determine why some breast milk inhibits and others do not. Importantly, mother-to-child transmission via HIV positive breast milk is low with approximately 85% of infants who do not acquire HIV-1 from the breast milk from their HIV positive mothers (WHO et al. 2008). This therefore suggests that crude breast milk has antiviral properties which protects the majority of infants from acquiring HIV-1 *in vivo*. The discrepancy in our study results with this established fact might be due to the fact that it has previously been suggested that HIV positive breast milk contains a higher concentration of antiviral factors, thus potentially allowing HIV positive milk to be more effective at inhibiting HIV-1 (Mthembu et al. 2014). Our study only utilised HIV negative breast milk.

Both of the pasteurised milk cohorts showed HIV-1 neutralisation in the majority of the samples. The 80°C heat treated milk showed no neutralisation in approximately a third of the samples, and the remaining samples exhibited neutralisation ranging from 19.51 µg/ml to 1 170 µg/ml (Table 3.1). The range of IC_{50} values is slightly narrower than those demonstrated by

crude breast milk, indicating that 80°C heat treated, for 10 minutes, was more effective at neutralising HIV-1. This increased inhibition by 80°C heat treated milk seen in 60% of the subjects is similar to a previous study in our laboratory, which found that HIV-1 inhibition in these heat treated samples was more effective across all samples. The variability in results in terms of these samples showing both inhibition and no inhibition in this study could potentially be attributed to the difference in samples in terms of timing of milk collection, inter patient variability and the method. The previous study in our laboratory used freeze dried milk resuspended in 10% DMEM which was then heat treated at 80°C for 10 minutes before being tested on the pseudoviral assay. In this present study, crude breast milk was first heat treated at 80°C for 10 minutes and then brought up in 10% DMEM in preparation for the pseudoviral assay. This change in methodology might be more reflective of the variation of the ability of heat treated milk to neutralise HIV-1 in reality. The sample size would need to be increased across different populations in order to verify this. In the samples that showed HIV-1 inhibition, it is possible that the breast milk mucins could have been dislodged from MFGM potentially allowing these mucins to interact with HIV-1 and neutralise the transmission (Habte et al. 2007; Buchheim et al. 1988). The activity of other antiviral proteins not contained in the MFGM, such as lactoferrin and sIgA, could potentially be lowered by the effect of heat. This phenomenon has been seen in Holder pasteurisation and therefore, the inhibition ability of the heat treated milk could be lowered (Czank et al. 2009; Lönnerdal 2003).

The HIV-1 neutralisation in the 62.5°C heat treated milk for 30 minutes was markedly increased when compared to that of crude and 80°C heat treated milk. HIV-1 neutralisation was demonstrated by 93.33% of the subjects with a much narrower range of IC_{50} values (0.21 – 300.90 µg/ml), with three potential outliers including 1 065, 1 972 µg/ml and no inhibition for one of these samples (Table 3.1). Therefore, this shows that this pasteurisation method increases the HIV-1 neutralisation of breast milk. Holder pasteurisation has previously shown to decrease the activity of sIgA, which has been implicated in HIV-1 neutralisation, by 70% (Czank et al. 2009). Another study showed that the protein profile of human breast milk remained unchanged after Holder pasteurisation in 70% of the samples. However, differences were noted in 30% of the samples (both colostrum and transitional milk), specifically in α- and β-casein, TNC, lactoferrin and immunoglobulin A (Peila et al. 2016). It is therefore suggested that the activity of breast milk glycoproteins and proteins in our samples were not affected by the heat allowing good HIV-1 potency. Antiviral proteins, including milk mucins and lactoferrin, could potentially not have been as greatly affected by the heat and therefore showed

exceptional potency against HIV-1. Samples showing less potency could have been more affected by the heat thus altering their glycoprotein and protein profile and subsequently affecting their HIV-1 neutralisation. Furthermore, this passive heat over a prolonged time period (30 minutes) could have released the breast milk mucins, other glycoproteins and proteins contained within the MFGM in order to allow them to interact with HIV-1 and neutralise HIV-1. This explanation was suggested by Buchheim et al in 80°C heat treated milk samples (Buchheim et al. 1988). The three samples which are considered outliers for this cohort, could potentially be due to inter patient variability within this study population.

From the range of IC_{50} values, it was evident that the pasteurised breast milk had a better anti-HIV-1 potency when compared to crude breast milk. Furthermore, from the data it can be concluded that 62.5°C heat treated breast milk had the better HIV-1 neutralisation ability. A statistically significant difference in the HIV-1 neutralisation was detected between the crude and 62.5°C heat treated breast milk cohorts (Mann-Whitney, p-value = 0.0021). Furthermore, there was a statistically significant difference in the HIV-1 neutralisation between the 80°C and 62.5°C heat treated breast milk cohorts (Mann-Whitney U, p-value = 0.0033). It is important to note that the storage and pasteurisation could potentially effect this anti-HIV-1 activity and these results would need to be validated *in vivo* to ensure the effectiveness of pasteurisation as a prevention strategy for mother-to-child transmission. A larger sample size would be required to increase the power of the statistical tests and to explore the clinical significance of the data.

MUC1 and TNC and a combination of these two glycoproteins showed good neutralisation overall, with better potency when compared to the crude and heat treated breast milk. The inhibition potency of MUC1 was exceptional with just over a third of the samples requiring less than 1 µg/ml to inhibit 50% of HIV-1 (Table 3.2). A further five samples had IC_{50} vales greater than 1 µg/ml but less than 20.26 µg/ml. Two samples demonstrated higher IC_{50} values (138.90 and 827.90 µg/ml). Therefore, purified MUC1 was able to block the transmission of HIV-1 at low enough concentrations *in vitro* suggesting it might have some effect *in vivo* when we examine the average concentration of MUC1 (307.85 ng/ml) in breast milk in this study. The exceptional potency of MUC1 is consistent with findings in our laboratory in which it was shown that MUC1 and MUC1 co-eluted with MUC4 were able to inhibit HIV-1 *in vitro* (Habte et al. 2008; Mthembu et al. 2014). Furthermore, the role of MUC1 could be more important in HIV-1 positive mothers as it has been suggested that more MUC1 is present in the breast milk of HIV positive mothers (Mthembu et al. 2014). MUC1 has been shown to block DC-SIGN mediated transmission of HIV-1 by specifically blocking DC-SIGN from transmitting HIV-1

from dendritic cells to CD4+ cells (Saeland et al. 2009). This mechanism of inhibition could be at work in this experiment due to the Lewis X structures within the MUC1 domain which are recognised and bound by DC-SIGN thereby blocking the binding of HIV-1 (Saeland et al. 2009). Due to the large size of MUC1 it is furthermore possible that the sugar moieties aggregate the pathogens such as HIV and physically block them from entering the host cells thereby preventing infection (Mthembu et al. 2014). This could result in MUC1 entangling the virus by an interaction with the negatively charged sialic acid and sulphate residues on the carbohydrate side chains of the mucin (Mthembu et al. 2014). The method in which MUC1 inhibits HIV-1 was not explored in this study and could be a focus for future research.

TNC, another glycoprotein implicated in HIV-1 neutralisation, demonstrated HIV-1 neutralisation in 75% of the subjects (Table 3.2). The IC_{50} values for those samples were lower overall when compared to those of MUC1, with 6 of the samples below 1 µg/ml and two samples just above 1 µg/ml (1.22 and 1.33 µg/ml). Only one sample in this cohort had an IC_{50} value as high as 37.39 µg/ml. Therefore, from this data it can be suggested that TNC is potentially more potent at inhibiting HIV-1 in an *in vitro* assay. Our findings are consistent with Fouda et al (2013) who demonstrated that TNC in breast milk is able to neutralise HIV-1. TNC prevents mother-to-child transmission by binding to a chemokine co-receptor site on the HIV-1 envelope protein (Fouda et al. 2013).

It was hypothesised that the co-eluted MUC1/TNC would demonstrate a higher potency than the two individual glycoproteins. However, this was not observed in the data (Table 3.2). From the data available, four of the samples showed exceptional HIV-1 potency with IC_{50} values below 10 µg/ml. Another two samples demonstrated good HIV-1 potency with IC_{50} values ranging between 24.91 – 45.51 µg/ml. The remaining five samples showed HIV-1 neutralisation with higher IC_{50} values, with three samples deemed unrealiable. The high variability seen in the IC_{50} values suggested that the neutralisation seen with these two glycoproteins combined was not as potent as the individual glycoproteins. This variability seen in the data might simply be due to inter-patient variability or the concentration of the individual glycoproteins in the breast milk at the time of the collection.

From this data, it is evident that purified MUC1 and TNC are able to neutralise HIV-1 with good to exceptional potency. However, there was no statistical difference detected among the three cohorts of purified glycoproteins (Kruskal Wallis, p-value = 0.13). The slight variability seen in the data might be due to inter-patient variability which may be reflective of the true

population. A larger sample size would be required to increase the power of the statistical tests and to determine which glycoprotein has a more potent effect on neutralising HIV-1. Furthermore, larger sample sizes would provide insight into the clinical significance of the study results.

A MTT assay was performed for all of the samples to test the cytotoxicity of the samples against the TZM-bl/JC cells. The samples were considered to be nontoxic if the cell viabilities were above 70% with no downward trend for the increasing concentrations of the sample. Overall, across all cohorts, the majority of samples were deemed to be nontoxic and therefore, validated the IC_{50} values and the HIV-1 neutralisation (Table 3.1 and 3.2). The LC_{50} values calculated for the remaining samples ranged from high to low. Higher LC_{50} values indicate that a high concentration of the sample would be required to result in 50% cell death *in vitro.* However, some of the LC_{50} values are considered to be very low across the cohorts, thus questioning the validity of the correlating IC_{50} values for those samples. Therefore, more samples would need to be used in the future in order to validate all results with an increased sample size.

Chapter 4: Histochemistry and immunohistochemistry

4.1 Histochemistry

Tissue sections from lactating breast tissue biopsies were stained with three histological stains including H&E, PAS/AB and HID. Different chemical components of breast tissue cells were identified using these stains. Normal breast tissue is not biopsied and therefore these tissue sections contain both normal and abnormal tissue, such as lactating adenomas. The histological stains showed differences across the six patients. Firstly, with H&E staining, the acini of the breast tissue showed wide, dilated lumina present in at least half of the patients (Figure 4.1a). Vacuolated cytoplasm was present in the majority of cases resulting in cytoplasmic blebs peaking off in certain areas of the tissue. Prominent vacuoles were noted as well as the staining of luminal secretions in some of the cases.

Breast tissue sections, rich in lipids, were mostly PAS positive and AB negative (Figure 4.1b). The luminal secretions, present in some of the tissue sections, stained PAS positive with one case staining PAS positive on the basement membrane. Therefore, the mucins present in these tissue sections were neutral. Less than 5% AB stained luminal material was noted in only one of the cases showing the presence of acidic mucins.

HID stained negative in all of the cases with the HID/AB stain. There was weak focal AB staining present in either the apical membranes or the luminal secretions indicating the presence of sialomucins in four of the breast tissue cases (Figure 4.1c).

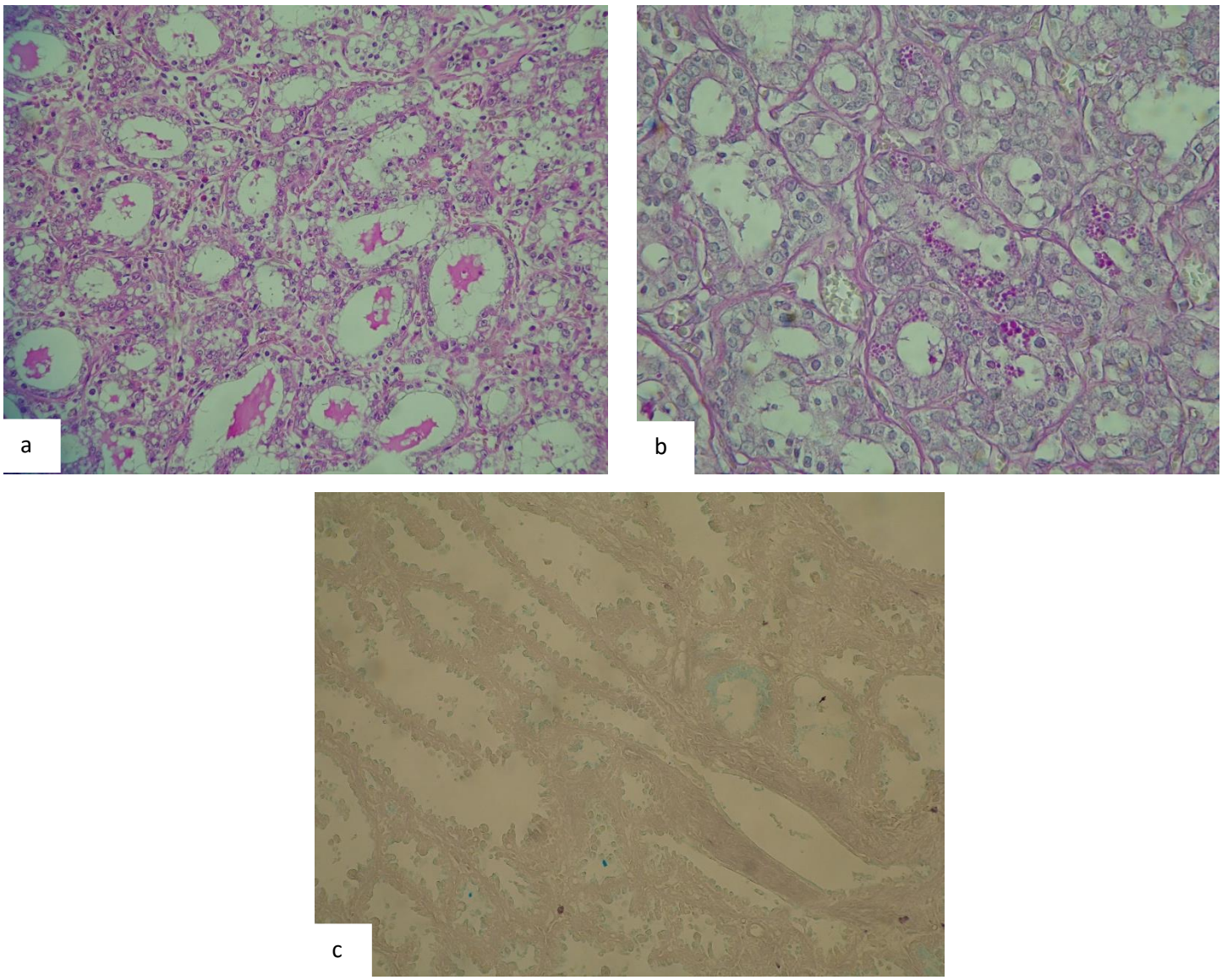

Figure 4.1. Histological stains of lactating breast tissue. a) Lactating breast tissue with acini showing dilated lumina and cytoplasmic vacuolation (H&E, objective magnification 20×). b) PAS positive cytoplasmic globules present in few acinar cells (PAS/AB, objective magnification 40×). c) Apical linear alcian blue staining of acinar cells (HID/AB, objective magnification 20×).

4.2 Immunohistochemistry

MUC1, MUC4 and TNC were detected in the majority of the tissue sections and the staining was graded by Professor D Govender (Table 4.1). MUC1, consistently expressed across all of the cases, was graded 4+. The staining was present in both the membrane and/or cytoplasm with varying degrees. The membrane staining was accentuated in some areas, whereas cytoplasmic staining was accentuated in other areas (Figure 4.2a and 4.2b). The cases with the luminal secretions stained positive for MUC1.

MUC4 positivity was noted across all six cases with only one case graded 3+ and the remaining cases graded 4+. Diffuse cytoplasmic staining of MUC4 was noted, with no membrane accentuation (Figure 4.2c).

TNC staining was inconsistent across the six cases. Four of the cases were graded 4+ with strong TNC staining whereas the remaining two cases were graded 1+ or negative respectively. TNC staining was localised to the cytoplasm, the luminal secretions and the connective tissue between the acini (Figure 4.2d). TNC positivity was noted around some of the bigger ducts and the blood vessels in the breast tissue sections.

Table 4.1. Immunohistochemical expression of MUC1, MUC4 and TNC in lactating breast tissue.

Stain	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
MUC1	4+	4+	4+	4+	4+	4+
MUC4	4+	4+	3+	4+	4+	4+
TNC	1+	4+	4+	Negative	4+	4+

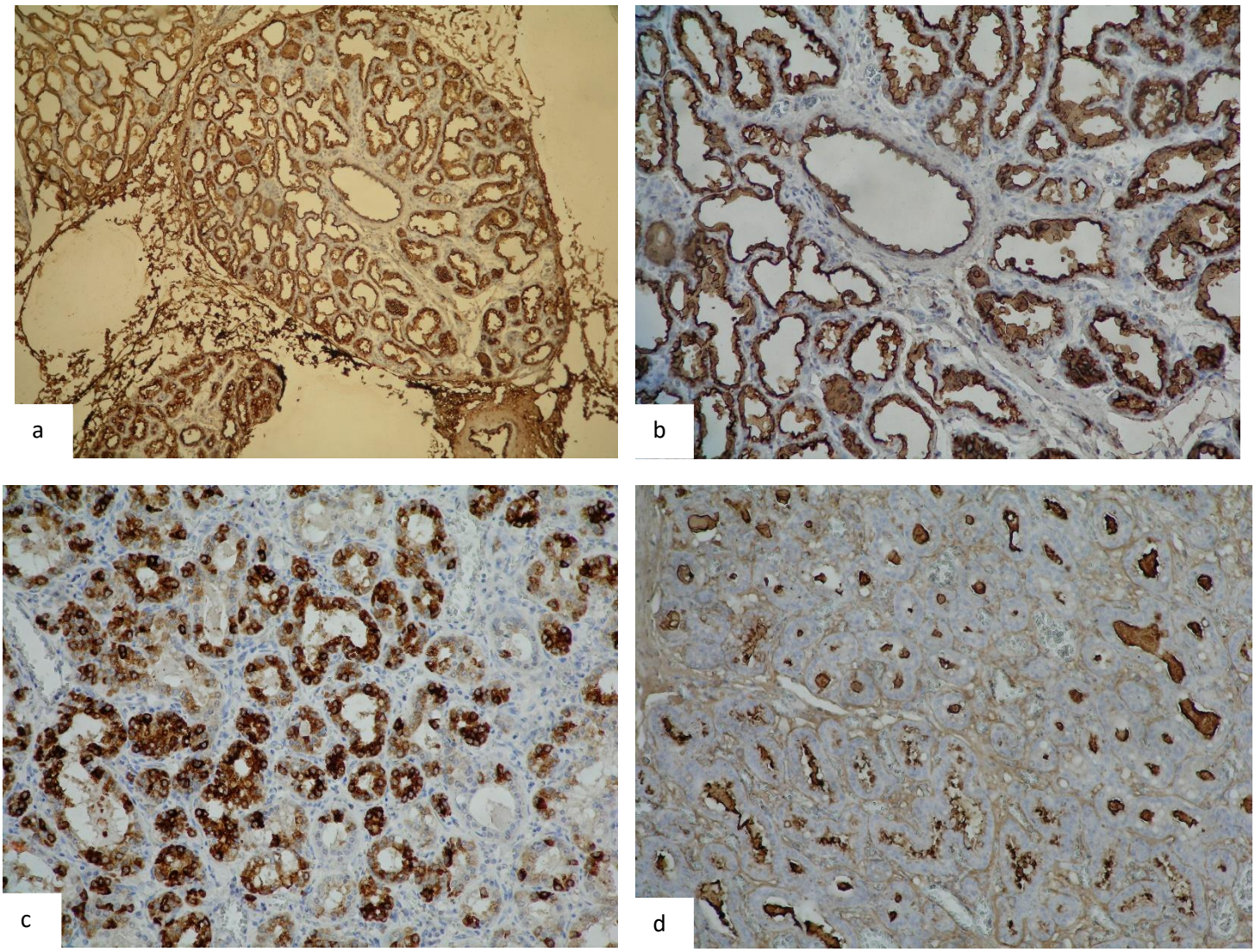

Figure 4.2. The expression of MUC1, MUC4 and TNC in lactating breast tissue. a) Diffuse and strong MUC1 membrane staining of lactating breast acini (MUC1, objective magnification 10×). b) Accentuated membrane staining for MUC1 in lactating acini (MUC1, objective magnification 20×). c) Strong cytoplasmic staining for MUC4 in lactating acini (MUC4, objective magnification 20×). d) Intense staining of luminal secretions and focal staining of apical cytoplasmic staining of the lactating acini (TNC, objective magnification 20×).

4.3 Discussion

The histochemical stains verified the morphology of the acini and ducts in the lactating breast tissue obtained. Normal breast tissue is not usually biopsied and therefore these cases contain normal tissue as well as palpable masses such as lactating adenomas which are tubular adenomas which occur due to hormones that cause lactational change (Magno et al. 2009). The H&E stain showed the acini (the basic functional unit of the lactating breast tissue) with wide, dilated lumina and vacuolated cytoplasm which was peaking off (Figure 4.1a). In some cases, luminal secretions and prominent vacuoles were noted. The luminal secretions, when present, mostly stained with PAS using the PAS/AB stain indicating the presence of neutral mucins in these secretions (Figure 4.1b). Focal AB staining (less than 5%) was noted in one of the six cases using the PAS/AB stain, suggesting that there was a small amount of acidic mucins present. No sulphated mucins were detected using HID/AB stain. Weak AB staining was noted in two thirds of the cases. Half of these cases showed apical membrane positivity and the other half showed AB staining in the luminal secretions (Figure 4.1c). Therefore, this suggests that siaolmucins are present in both the apical membrane of the breast tissue as well as the luminal secretions of lactating breast tissue. The AB staining was relatively weak when compared to the control tissue.

MUC1, MUC4 and TNC, was detected in the majority of the tissue sections, and has previously been reported within breast milk secretions (Mthembu et al. 2014; Fouda et al. 2013). The immunohistochemistry results indicated that MUC1 was consistently expressed across all six cases with the same intensity of staining noted throughout (Table 4.1 and Figure 4.2). MUC1 was present in both the membrane and cytoplasm. In certain areas of the tissue the presence of MUC1 was either accentuated in the membrane or cytoplasm. The luminal secretions, when present, also showed MUC1 positivity (Figure 4.2a and 4.2b). These results are consistent with the successful purification of this transmembrane mucin from the milk fat of the breast milk collected from lactating mothers (Chapter 3).

MUC4 was strongly detected in all of the cases, with a slightly lower intensity noted in one of the cases. The transmembrane mucin was predominantly noted in the cytoplasm of the cells (Figure 4.2c). The concentration of MUC4 was insufficient for purification from the milk fat, which differs from the tissue results of MUC4 (Chapter 3). From the ELISA results, MUC4 was found to be below the detectable limit of the kit suggesting a low concentration of MUC4 present in the breast milk at the time of collection. The samples used for purification and

immunohistochemistry were not from the same patients and could potentially explain these inconsistent results.

TNC was detected in five of the six cases, to varying degrees with one of the cases not staining for this glycoprotein. The staining was predominantly present in luminal secretions and the connective tissue with minimal cytoplasmic staining (Figure 4.2d). This TNC positivity is consistent with the successful detection and purification of TNC from the skim milk collected from lactating mothers.

Chapter 5: Summary

HIV currently effects the world population at large with approximately 2.1 million individuals infected annually, despite the effective reduction of HIV infections over the recent year (UNAIDS 2016). The most recent data suggests that 36.7 million people were living with HIV in 2015 with the Eastern and Southern African region accounting for 52% of these infections (UNAIDS 2016). More than half of these reported infections are noted in women. The HIV infections in children have decreased by 66% over a five year period (2010-2015), but the burden of disease is still high among children infecting approximately 56 000 children in 2015 (UNAIDS 2016). In 2015, the prevalence of HIV in South Africa was approximately 11.2% with an increased burden of disease noted in Gauteng, Kwa-Zulu Natal and the Eastern Cape (Statistics South Africa 2015). From this data, it is evident that the HIV epidemic continues to devastate societies.

Within lower income countries, HIV positive mothers choose to breast feed their infants (Ramjee & Daniels 2013). The risk of HIV transmission is outweighed by the important nutritional and immunological factors provided by breast milk (Semba et al. 1999; Wahl et al. 2015). This is supported by the World Health Organisation and they suggest that it is imperative for HIV positive mothers to breastfeed their infants for the first six months of life, but importantly this should be combined with maternal and infant ART (WHO & UNICEF 2011). A small percentage of mother-to-child transmission can be attributed to breastfeeding however the transmission rate is so low showing that approximately 85% of infants would not acquire HIV through breast milk from their HIV positive mothers (WHO et al. 2008). This suggests that there are antiviral factors present in breast milk which protect infants in the majority of cases (Hanson et al. 2003; Wahl et al. 2015).

This study aimed to determine the anti-HIV-1 activity of three breast milk glycoproteins (MUC1, MUC4 and TNC) previously implicated in HIV-1 neutralisation (Habte et al. 2008; Mthembu et al. 2014; Fouda et al. 2013). Furthermore, this study aimed to investigate the effect of two different pasteurisation methods on HIV-1 neutralisation when compared to crude breast milk.

It was found that 53.33% of crude breast milk subjects showed no HIV-1 inhibition, with one sample deemed unreliable (Table 3.1). The remaining six samples demonstrated HIV-1

inhibition over a range of IC_{50} values. It is suggested that this variability might be reflective of what occurs *in vivo*, in which both mother-to-child transmission and no transmission is noted. This data suggests that crude breast milk overall does not inhibit HIV-1. These results are consistent with previous studies in our laboratory which found crude breast milk did not inhibit HIV-1 (Habte et al. 2008; Mthembu et al. 2014). This does not correlate with the fact that approximately 85% of infants do not acquire HIV through breast milk (WHO et al. 2008). A possible explanation for this might be that HIV negative breast milk was utilised for this study. It is postulated that HIV positive breast milk has increased concentrations of antiviral proteins such as MUC1 (Mthembu et al. 2014). Therefore, it is suggested that a lower concentration of these antiviral proteins are present in HIV negative breast milk and this could account for the discrepancy of anti-HIV activity in crude breast milk. In the samples exhibiting HIV-1 neutralisation, it is suggested that proteins such as lactoferrin, glycosaminoglycan moieties and milk oligosaccharides decrease the invasion of HIV-1 thus providing protection to the infant (Berkhout et al. 2002; Newburg et al. 1995; Hong et al. 2008).

It was interesting to note that the HIV-1 neutralisation was increased when the breast milk was pasteurised, more so in the 62.5°C heat treated cohort. Within the 80°C heat treated (10 minutes) cohort, 60% of the subjects demonstrated HIV-1 neutralisation with high variability (Table 3.1). It is suggested that the heat could have dislodged breast milk mucins from the MFGM (Habte et al. 2007; Buchheim et al. 1988), allowing these mucins to interact and neutralise HIV-1 *in vitro*. The effect of heat might have lowered the activity of other antiviral proteins (Czank et al. 2009; Lönnerdal 2003) which could account for the observation of no inhibition noted in 60% of the subjects.

Holder pasteurisation (62.5°C for 30 minutes) was used in another cohort of samples to test their anti-HIV-1 activity. The heat was lower than the previous pasteurisation cohort and the time was increased. Overall, the HIV-1 neutralisation was deemed more potent within this cohort and this was evident by the narrower range of IC_{50} values noted in 93.33% of these samples (Table 3.1). One sample indicated no inhibition. This data suggests that the activity of glycoproteins and proteins in the breast milk were not greatly affected thus allowing them to exhibit good HIV-1 neutralisation. The lower heat over a prolonged period of time could have released the glycoproteins and proteins within the MFGM allowing them to interact with HIV-1 and block its transmission.

From this, it was concluded that Holder pasteurisation would decrease mother-to-child transmission and allow HIV positive mothers to decrease the risk of this in lower income countries. This would need to be verified in larger cohorts of pateints across different populations.

The purified glycoproteins, MUC1 and TNC, showed much better HIV-1 potency when compared to the crude and heat treated breast milk (Table 3.2). Overall, TNC demonstrated better HIV-1 neutralisation when compared to that of MUC1. MUC1 co-eluted and co-purified with TNC was expected to show better HIV-1 potency, but from the data, this was not the case (Table 3.2). The individual glycoproteins seemed to be more potent in the inhibition of HIV-1.

MUC1 showed exceptional HIV-1 potency in a third of the samples with the remaining samples demonstrating good HIV-1 potency with only two of those samples requiring a higher concentration (138.90 and 827.90 µg/ml) to inhibit 50% of HIV-1. From this data, it was suggested that purified MUC1 was able to aggregate HIV-1 due to the large molecular weight of the glycoprotein which is extensively glycosylated (Mthembu et al. 2014; Patton et al. 1995; Brayman et al. 2004). Another study suggested that MUC1 binds to DC-SIGN thereby blocking DC-SIGN mediated HIV-1 transmission (Saeland et al. 2009). The method in which MUC1 blocks HIV-1 in breast milk should be further explored. It is further suggested that MUC1 would show greater inhibition in HIV positive breast milk due to MUC1 increasing in the breast milk as a defence mechanism against mother-to-child transmission.

The neutralisation was deemed to be more potent in the purified TNC cohort due to overall lower IC_{50} values and HIV-1 neutralisation in 75% of the subjects. The manner in which TNC from breast is able to block the transmission of HIV-1 was investigated by Fouda et al (2013). This study showed that TNC binds to the HIV-1 envelop protein via a chemokine co-receptor thus blocking HIV-1 *in vitro* (Fouda et al. 2013). This mechanism is suggested to block mother-to-child transmission.

MUC4, present in the MFGM of breast milk, was not successfully purified across the samples. It was suggested that the concentration of MUC4 in crude breast milk was not sufficient for purification. This hypothesis was substantiated by the ELISA results which showed that the concentration of MUC4 in the tested samples was below the detectable limit of the kit. In future research, this matter could be further explored to ensure MUC4 is successfully purified.

The variability seen throughout the data may be due to inter-patient variability and the maturity of the breast milk at the time of collection. A larger sample size across the purified cohorts would provide more insight into the nature of the anti-HIV-1 activity of MUC1 and TNC.

The H&E staining verified the morphology of the lactating breast tissue showing the wide, dilated lumina and vacuolated cytoplasm (Figure 4.1a). The luminal secretions stained PAS positive overall with the PAS/AB stain indicating the presence of neutral mucins (Figure 4.1b). Sialomucins were detected in the apical membrane and the luminal secretions of the breast tissue using the HID/AB stain (Figure 4.1c).

From the immunohistochemistry, the presence of MUC1, MUC4 (β subunit) and TNC was confirmed in lactating breast tissue (Figure 4.2). The presence of MUC1 and TNC correlates with the detection and purification of MUC1 and TNC from the breast milk collected. The MUC4 was strongly detected in the lactating breast tissue. This differs from previous findings in this study in which MUC4 was not successfully purified or detected across the majority of sample. A possible explanation could be the different samples were used for the purification and immunohistochemistry.

HIV continues to cause infection amongst children and adults as the innate immunity of individuals is not able to safe guard them against infection. Breast feeding infants provides nutritional and immunological factors which are imperative for healthy development. Therefore, methods to ensure that mother-to-child transmission is inhibited should be explored in order to completely protect infants during breast feeding. From this study, it is evident that pasteurising breast milk, especially using Holder pasteurisation, inhibits HIV-1 *in vitro*. Furthermore, both MUC1 and TNC could be purified, cloned and utilised as a factor to prevent HIV-1 infection across all modes of possible infection. For future research, it would be interesting to optimise the purification of MUC4 from human breast milk and test it against HIV-1. Furthermore, future work could include a combination of MUC1, MUC4 and TNC tested against HIV-1 to investigate if there is an additive HIV-1 neutralisation effect.

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Appendix 1: Buffers, reagents and solutions

Guanidinium hydrochloride in PBS, pH 6.5, with protease inhibitors

1. 10 mM PBS
    a. 0.568 g Na_2PO_4 in 400 ml dH_20
    b. 0.960 g NaH_2PO_4 in 800 ml dH_20

Add reagent 1 to 2 until pH 6.5 and store at 4°C

2. 4M GuHCl in PBS, pH 6.5
    a. 382 g GuHCl in 1 litre of 10 mM PBS, pH 6.5
3. Protease inhibitors
    a. 1 mM PMSF (0.174 g in 1 litre)
    b. 5 mM NEM (0.626 g in 1 litre)
    c. 10 mM EDTA (3.722 g in 1 litre)

Periodic acid Schiff (PAS) assay for glycoproteins

1. Schiff's reagent
    a. 10 g pararosanniline chloride dissolved in 1 litre boiling dH_2O, with constant stirring
    b. Cool solution to 50°C on bench and add 200 ml 1 M HCl
    c. Add 3 g activated charcoal, mix for 5 minute and filter to remove charcoal
    d. Add 3 g activated charcoal, mix for 5 minute and filter again
    e. Store at room temperature in a dark bottle
2. Periodic acid solution
    a. 10 ml 7% acetic acid
    b. 20 µl 50% periodic acid
3. Decolourised Schiff's reagent
    a. 100 mg sodium metabisulphite
    b. 6 ml Schiff's reagent
    c. Incubate at 37°C until colourless
    d. Prepared fresh for every assay

Bradford Assay

1. Bradford reagent is diluted 1:5 with dH_2O

4-20% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

1. Sample application buffer
    a. 2% sodium dodecyl sulphate (SDS)
    b. 10% glycerol
    c. 0.01% bromophenol blue

The reagents were dissolved in dH_2O and stored at room temperature.

2. 30% Bis/Acrylamide
    a. 30 g acrylamide
    b. 0.8 g bis

The reagents were dissolved in 100 ml dH_2O and stored in a dark bottle at 4°C

3. 1.5 M Tris-buffer with 0.1% SDS, pH 8.8
    a. 1.5 M Tris
    b. 0.1% SDS

The reagents were dissolved in dH_2O, adjusted to pH 8.8 and stored at 4°C

4. Spacer gel buffer
    a. 0.25 M Tris
    b. 0.2% SDS

The reagents were dissolved in dH_2O and adjusted to pH 8.8. This buffer was stored at 4°C

5. 10% AMPS
    a. 10% AMPS dissolved in dH_2O

Stored at 4°C

6. Tank buffer
    a. 0.025 M Tris
    b. 0.19 M glycine
    c. 0.1% SDS

The reagents were dissolved in 5 litres of dH_2O, adjusted to pH 8.8 and stored at room temperature

4-20% SDS-PAGE

1. 4% light solution
    a. 0.8 ml 30% Bis/Acrylamide
    b. 1.5 ml 1.5 M Tris-buffer with 0.1% SDS, pH 8.8
    c. 3.7 ml dH_2O

d. 30 µl 10% AMPS

e. 5 µl TEMED

2. 20% heavy solution

a. 4 ml 30% Bis/Acrylamide,

b. 1.5 ml 1.5 M Tris-buffer with 0.1% SDS, pH 8.8,

c. 0.5 ml dH_2O,

d. 30 µl 10% AMPS

e. 5 µl TEMED

Using a serological pipette, 2.3 ml of the 4% light solution is pipetted and thereafter 2.3 ml of the 20% heavy solution is pipetted into the same pipette. A single air bubble is passed through the solution to create the 4-20% gradient.

Periodic acid Schiff (PAS) gel staining

1. Schiff's reagent

a. 1 g pararosanniline hydrochloride dissolved in 200 ml boiling distilled water, with constant stirring

b. Cool solution to 50°C on bench and add 20 ml 1 M HCl

c. Cool to 25°C and add 1 g sodium metabisulphite and leave in the dark for 12-24 hours

d. Add 2 g activated charcoal, mix for 1 minute and filter

e. Store at 4°C in the dark

2. 50% ethanol

a. 50 ml ethanol

b. 50 ml distilled water

3. 1% periodic acid and 3% acetic acid

a. 50% periodic acid

b. 3% acetic acid

Slot blot

1. 20× SSC

a. 175.3 g sodium chloride

b. 88.2 g tri-sodium citrate

The reagents are added to 1 litre dH_2O, adjusted to pH 7 and stored at 4°C

2. 4× SSC

a. 200 ml 20× SSC, pH 7

b. 800 ml dH_2O

This buffer was stored at 4°C

Western blotting and slot blot

3. 1× TBST

    a. 1.21 g Tris-HCl

    b. 8.76 g sodium chloride

    c. 0.5 g Tween 20

The reagents are added to 1 litre dH_2O, adjusted pH 8 and stored at 4°C

4. 5% low-fat milk powder

    a. 10 g low-fat milk powder

    b. 200 ml 1× TBST

Salt azide buffer (0.2 M NaCl and 0.02% NaN_3)

1. 11.69 g NaCl
2. 0.2 g NaN_3

The reagents were dissolved in 1 litre of dH_20 and stored at room temperature

Histochemistry and immunohistochemistry

1. Mayer's Haematoxylin

    a. 1 g haematoxylin

    b. 50 g ammonium alum

    c. 0.2 g sodium iodate

    d. 1 g citric acid

    e. 50 g chloral hydrate

    f. 1 litre dH_2O

    g. Filtered and stored in the dark at room temperature

2. 1% eosin

    a. 1% eosin Y (1 g eosin Y dissolved in distilled water with thymol to prevent bacterial growth)

    b. 1% phloxine (1 g phloxine in 100 ml dH_2O)

c. 150 ml 1% eosin y and 75 ml 1% phloxine with 225 ml dH_2O

d. Filtered for use

3. Scott's tap water substitute

    a. 3.5 g sodium bicarbonate

    b. 20 g magnesium sulphate

    c. 1 litre dH_2O

4. Alcian blue (AB) solution

    a. 1 g alcian blue

    b. 100 ml 3% acetic acid

5. Periodic acid solution

    a. 1 ml periodic acid

    b. 100 ml dH_2O

6. De Thomasi Schiff's reagent

    a. 1 g pararosanniline hydrochloride dissolved in 200 ml boiling dH_2O with constant stirring

    b. Cool solution to 50°C on bench and add 20 ml 1 M HCl

    c. Cool to 25°C and add 1 g potassium metabisulphite and leave in the dark for 12-24 hours

    d. Add 2 g activated charcoal, mix for 1 minute and filter

    e. Stored at 4°C in the dark

7. High Iron Diamine (HID) solution

    a. 0.12 g N,N-Dimethyl-meta-phenylenediamine-dihydrochloride

    b. 0.02 g N,N-Dimethyl-para-phenylenediamine-dihydrochloride

    c. 50 ml dH_2O

    d. 5 ml ferric chloride

8. PBST

    a. 0.5 ml tween 20

    b. 1 litre PBS, pH 7.4-7.6

9. 1% H_2O_2

    a. 3 ml 30% H_2O_2

    b. 100 ml dH_2O

10. 0.01 M Citrate buffer, pH 6
    a. 2.1 g citric acid
    b. 1 litre dH_2O

5% and 10% DMEM

1. 5% DMEM
    a. 2.50 ml FBS
    b. 0.50 ml NEAA
    c. 0.25 ml of 200x PenStrep
    d. 0.5ml of 100x L-glutamine

The reagents are made up to 50 ml with a DMEM and L-glutamine solution

2. 10% DMEM
    a. 5.0 ml FBS
    b. 0.50 ml NEAA
    c. 0.25 ml of 200x PenStrep
    d. 0.5ml of 100x L-glutamine

The reagents are made up to 50 ml with a DMEM and L-glutamine solution

Appendix 2: Consent form and information sheet

PATIENT INFORMATION SHEET

Dear Patient,

We are doing a study on the mucus proteins produced in your breast milk.

The study will be conducted by Kathleen Kehoe under the supervision of Professor Anwar Mall in the Department of Surgery, Old Main Building, Groote Schuur Hospital.

Mucus is a sticky substance mixed with the bodily secretions and is necessary for lubrication and protection of the tracts in the body. We wish to find out the role of mucus and other proteins in the breast milk of HIV negative and HIV positive individuals in neutralizing HIV-1.

We would need 100 ml of breast milk from you to use in an experiment which will determine the effect of mucus and proteins on the HI virus. Once the milk has been collected from you, we will not require anything else from you. The collection of the breast milk involves no risks.

This study could have implications for mother-to-child transmission of the HI virus through breast-feeding, a significant contributor to the pandemic in sub-Saharan Africa.

Please note:

1. Informed consent will be taken before sample collection takes place.
2. We will need to take your folder number (**NOT YOUR NAME**) from which we will get the details of your age, $CD4^+$ count, and treatment status.
3. All this information will be kept **absolutely confidential** and we promise not to use your sample for any other purpose but that which we have described.
4. You have the right to say that you do not wish to take part in this study. If you do not wish to be part of this study, this will not affect the treatment you receive.
5. If you agree to participate in this research project now and then decide at a later date that you do not wish to continue, that is also fine. That decision will once again not affect your right to treatment and care.
6. It is possible that we will publish the findings of this study, but your name will **NOT** be mentioned.
7. We further emphasize that all samples donated to this study will not carry your name.

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

If there are any queries, please refer them to your clinician. If you think of any queries at a later date, please don't hesitate to contact the telephone number below.

Professor A. Mall

Tel: 021 406 6168/6227

REQUEST FOR BREAST MILK SAMPLE TO MEASURE THE EFFECT OF MUCUS AND PROTIENS ON THE HI VIRUS

HREC REF: 117/2016

Research Laboratory

Division of General Surgery

Old Main Building, Groote Schuur Hospital

UCT Medical School, Observatory 7925

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

Human Research Ethics Committee

Room E52

Old Main Building, Groote Schuur Hospital

Observatory 7925

Tel: (021) 406 6338

Please fill in all the information requested:

Folder No: ……

Date of Birth: …….....

$CD4^+$ Count: …….. …….

Treatment status: …….

Delivery Date: ……

For Laboratory use only:

Date Received: ……..

Computer Index No.: …….

CONSENT FOR BREAST MILK SAMPLE

Please note that your HIV status is not recorded on this form.

1. I give permission that my breast milk be taken for research purposes in the investigation of HIV AIDS.

2. I give permission that a portion of the sample be stored indefinitely 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 I understand and all my questions were answered.

Signature: _____