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THE ROLE OF HUMAN BREAST MILK MUCUS AND MUCINS IN HIV-AIDS

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TO MY HUSBAND MFUNDO, WHO ALWAYS ENCOURAGED ME TO REACH MY FULL POTENTIAL. TO MY DAD, WHO NEVER STOPPED BELIEVING IN ME AND ALWAYS WORKED HARD TO SEE ME SUCCESSFUL. TO MY MOM, THANK YOU FOR ALL YOUR PRAYERS.

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6. Mthembu, Y.H. (2010) Presented: The role of human breast milk in HIV/AIDS. Department of Surgery Research Day, University of Cape Town.

DECLARATION OF PLAGIARISM

I, **Yolanda Mthembu**, 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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PREFACE

This study was done from February 2010 to December 2011 under the supervision of Professor Anwar Suleman Mall, in the Department of Surgery, Faculty of Health Sciences, University of Cape Town, South Africa.

The studies in this thesis represent the original work by the author and have not been submitted in any other form to another University. Where use has been made of the work of others, it has been acknowledged in the text.

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ABSTRACT

HIV in South Africa is transmitted predominantly through heterosexual sex, with mother-to-child transmission being the other main route of infection. According to the data that was published in 2010 by Statistics South Africa, an estimated 40,000 children in South Africa are infected with HIV each year, reflecting poor prevention of mother-to-child transmission.

Human breast milk through breast-feeding is an essential source of nutrition and it also offers protection from respiratory and gastrointestinal infections. Breastfeeding has been shown to lower the incidence of other infectious diseases during the first year of life. Breast-feeding also maintains the maternal-fetal immunologic link after birth which may favour the transmission of immuno-competence from the mother to her infant.

Milk molecules such as mucins, antibodies, bactericidal enzymes like lysozymes and fatty acids that lyse bacteria, viral particles and bacterial peptides, offer anti-microbial activity in milk. Despite human breast milk being rich in anti-microbial substances, such as mucin, that protect against pathogens and viruses, it remains a significant route of HIV transmission from mother to child. This leaves the question of the ability of the breast milk and its mucin components to protect against the human immunodeficiency virus.

The objectives of the study were to isolate, purify, identify and investigate the anti-HIV-1 activity of crude breast milk particularly the human milk fat globule material (MFGM) and its purified mucin components, in HIV positive patients (n = 20) compared with those who are not infected (n = 20). This study also tested the effect that heat (80°C, 10 min) might have on breast milk which might release the milk mucins and consequently have an inhibitory effect on HIV-1.

Breast milk was extracted and solubilised by stirring in 6M guanidinium chloride and a cocktail of protease inhibitors in a ratio of 5 parts breast milk and 1 part guanidinium chloride. The milk fat globule membrane was obtained after the human breast milk was defatted by centrifugation at 3 000g for 1 hour at 4°C. Following Sepharose CL-4B gel filtration, the identity of mucins MUC1 and MUC4 was confirmed by Western blotting. MFGM-I peaks from both groups were further purified by isopycnic density gradient centrifugation in caesium chloride. Amino acid composition on the purified milk mucins of the MFGM-I was by high performance liquid chromatography.

In order to determine whether human breast milk mucin had any variation of MUC1 and MUC4, breast milk mucin glycoproteins were separated based on charge using the HPLC ion-exchange chromatography before applying the fractions to a 0.7% agarose gel electrophoresis, and then blotted onto the nitrocellulose which was probed with antibodies against MUC1 and MUC4. The anti-HIV activity of the milk and purified mucins was determined by an *in vitro* HIV-1 neutralisation assay that exploits the use of “pseudo-viruses”.

The SDS-PAGE gels stained with PAS for carbohydrates from the non-purified HIV positive MFGM and HIV negative MFGM showed the presence of mucin bands on top of the stacking gel as well as those that have entered the running gel. The mucins that have entered the running gels for both groups seem to vary in intensity of staining and are in the region of molecular weight ($M_r \sim 100\text{-}220\text{kDa}$). The HIV positive non-purified MFGM seemed to have more intense staining than the HIV negative MFGM.

When mucins were isolated from the MFGM by Sepharose CL-4B gel filtration, the elution profiles for the HIV positive group and HIV negative group showed that the MFGM material have two mucin rich peaks. The first peak MFGM-I eluted in the void volume (V_o) whilst the second peak MFGM-II eluted in the included volume (V_i) of the column. The HIV positive samples had two large distinct peaks while the HIV negative ones showed one small peak and one large peak. When the peaks were separated on SDS-PAGE, the MFGM-I peaks from both groups showed more intensely stained mucins than the MFGM-II peaks. In both groups, MFGM-I peaks had less contaminant proteins than MFGM-II.

Western blot analysis of the semi-purified material eluting from the Sepharose 4B gel filtration column confirmed the presence of MUC1 and MUC4 in samples from both HIV positive and HIV negative groups. The staining on the blots was more intense for the V_o (MGFM-1) than the V_i (MGFM-II) material for both MUC1 and MUC4, again for both HIV positive and negative groups. Overall there was more detectable material on the Western blots for mucins from the HIV positive group than that for the HIV negative group. However, except for intensity of staining, no clear difference was seen between the HIV positive and negative groups, but rather an inter-individual variation within each group with respect to the electrophoretic behaviour of MGFM-1 and MGFM-II material.

The MFGM-I from both groups were further purified by caesium chloride isopycnic density gradient centrifugation. Both purification profiles showed a clear separation between the lower density proteins and the higher density mucin fractions which were assayed by PAS. To confirm purification, the fractions with the highest mucin peaks were separated on SDS-PAGE gel before staining with PAS which showed that the mucins were present in both groups at the top of the running gel. The SDS-PAGE gel was also stained with Coomassie Brilliant Blue R-250 which showed detectable proteins, again at the top of the running gel. The absence of bands within the running gels shows that the mucins were successfully purified.

There was no difference in the content of amino acid between the two groups. For the HIV positive group, the amount of serine threonine and proline added up to 41% of the MFGM-I, while they added up to 37% of the MFGM-I for the HIV negative.

Possible changes in the glycosylation of milk mucins in the two groups, namely the HIV positive and negative groups were investigated by using a combination of anion-exchange HPLC and agarose gel electrophoresis. The PAS profile for the HIV positive MFGM fractions from the HPLC shows evidence of two major population groups and one other minor population that is not as distinct. These fraction samples from the HPLC were subjected to agarose gel electrophoresis before blotted onto a nitrocellulose membrane then probing with anti-MUC1 and anti-MUC4 antibodies. When probed with anti-MUC1, the HIV positive fractions showed the presence of three distinct populations of MUC1. Two visible electrophoretic patterns were observed on the Western blots, the first pattern confirms population I, while the second pattern verifies the presence of population II. The minor population III was observed as smears of MUC1. The results showed a variation in amounts, charge difference and electrophoretic mobility for MUC1 in the HIV positive group compared to the HIV negative group.

When probed with anti-MUC4 antibody, the HIV negative fractions confirmed the presence of MUC4 to be present in population I and III. However, the variation seen in MUC1 in the HIV positive group was not as marked for MUC4 in this group.

The PAS profiles of the HIV negative MFGM fractions shows that the mucins do not vary as much as those of the HIV positive. There was a single distinct population I observed on the

PAS profile, population II was observed as a minor peaks present. Another minor peak was observed which could be another potential population. When probed with anti-MUC1, the HIV negative fractions showed that MUC1 was detected on the fractions of population I. When the blot was probed with anti-MUC4 antibody, MUC4 was detected on fractions which formed population I. There seemed to be faint traces of MUC4 detected on fractions of the slight peak observed on the PAS profile which could potentially form population II. Neither MUC1 nor MUC4 were detected in the third minor peak.

The anti-HIV activity of the crude breast milk, purified milk mucins, and heated milk from the HIV positive (n=16) and HIV negative (n=16) groups were tested against the Du422.1 pseudovirus using the reporter gene assay in TZM-bl cells. The crude breast milk samples showed 60% neutralisation for the HIV positive samples, while the HIV negative milk neutralised 40%. The purified milk mucins from the HIV positive group neutralised 50% of the viral activity, while the HIV negative mucins had much lower neutralisation efficacy with 35% neutralisation. Increased neutralisation of viral activity was seen for the heated milk samples with neutralisation of 80% for the HIV positive samples and 70% for the HIV negative samples.

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ABBREVIATIONS

Aa	Amino acid
AMPS	Ammonium persulphate
AIDS	Acquired immunodeficiency syndrome
BIS	NN'-methylenebisacrylamide
BSA	Bovine serum albumin
CsCl	Caesium chloride
CHAPS	3-((3-cholamidopropyl)-dimethyl-ammonio)-1-propane sulfonate
CO ₂	Carbon Dioxide
dH ₂ O	Distilled water
°C	Degrees Celsius
1D AgPAGE	1 Dimensional Agarose Poly acrylamide-gel electrophoresis
DC-sign	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DEAE	Dextran diethylaminoethyl ether of dextran
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemilluminescence
EDTA	Ethylenediaminetetra- acetic acid
FBS	Fetal bovine serum
g	Gram
GuHCl	Guanidinium chloride
GM	Growth media
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immuno-deficient virus
HPLC	High pressure liquid chromatography
HRPO	Horse radish peroxidase
Kb	Kilo-base
kDa	Kilo-Dalton
µl	Microliter
MFGM	Milk fat globule membrane

Mbar	Milibars
Min	Minute
ml	Milliliter
MUC	Mucin
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
M _w	Molecular weight
Na ₂ CO ₃	Sodium carbonate anhydrous
NaH ₂ PO ₄	Sodium dihydrogen orthophosphate
Na ₂ C ₄ H ₄ O ₆	Sodium tartrate
Na ₂ PO ₄	Disodium hydrogen orthophosphate anhydrous
NaCl	Sodium Chloride
NaN ₃	Sodium azide
NaOH	Sodium hydroxide
Neg	Negative
NEM	N-ethylmaleimide
NH ₄ HCO ₃	Ammonium bicarbonate
PAS	Periodic acid schiff
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonylfluoride
Pos	Positive
RT	Room temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Poly acrylamide-gel electrophoresis
SNF	Supernatant fluid
SSC	Sodium chloride/ tri-sodium citrate buffer
TAE	Tris-acetate-EDTA
TBS	Tris buffered Saline
TBST	Tris buffered saline-tween
TEMED	<i>N, N, N, N'</i> -tetramethylethylenediamine
TRIS	(hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
V	Voltage
VNTR	Variable number of tandem repeats

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

In South Africa alone in 2004, between 4.68 and 7.03 million people were living with HIV/AIDS, of which 55% were females (Habte *et al.*, 2008). An estimated 1.9 million [1.6 million–2.2 million] people were newly infected with HIV in sub-Saharan Africa in 2008, bringing to 22.4 million [20.8 million–24.1 million] the number of people living with HIV (UNAIDS, 2009). HIV in South Africa is transmitted predominantly through heterosexual sex, with mother-to-child transmission being the other main route of infection. The national transmission rate of HIV from mother to child is approximately 11%. An estimated 40,000 children in South Africa are infected with HIV each year, reflecting poor prevention of mother-to-child transmission (PMTCT) (UNGASS, 2010 and statistics South Africa, 2010). Therefore, whilst there is an urgent need for the development of a safe, natural, and affordable vaccine, other initiatives to develop therapies and preparations are of utmost importance.

Previous work in our laboratory (Habte *et al.*, 2007, Habte *et al.*, 2008) has shown that the purified mucin component of breast milk (MUC1 and MUC4) inhibits HIV-1 virus in an *in vitro* p24 assay, whilst the crude milk preparation does not. This finding could explain why infants acquire the virus through mother-child-transmission during breast feeding. This study will undertake to statistically verify these findings using an HIV-1 neutralization assay. Although this study did not look at the mechanism of inhibition, there is a suggestion that the mucins aggregate the virus purely by physical entanglement through a charge interaction with carbohydrate side-chains considering that mucins have a high density of negative charges through sialic acid and sulphate residue (Habte *et al.*, 2007). This hypothesis, however, has not yet been proven, and thus requires further investigation.

In this study, a comparison of the potential inhibitory role of mucins in breast milk of HIV positive individuals has also been made and did not show any differences in the inhibition from the HIV negative group. However, variation in the electrophoretic behaviour of the mucins from the HIV positive group and HIV negative groups was noted. Our laboratory has also previously reported studies of salivary mucus and mucins and showed that whilst normal

salivary mucins inhibit the HIV-1, salivary mucus and mucins from HIV positive individuals do not (Habte *et al.*, 2010).

Breastfeeding has been reported as an important factor in protection against enteric infection with various pathogens. Numerous studies have recorded that children who are breast fed undergo substantially fewer total episodes of gastroenteritis compared with infants who are bottle fed (Yolken *et al.*, 1992). Whilst human breast milk is rich in components, such as mucin, that have been reported to protect against pathogens and viruses, there is still a need to balance the protective effect of breast feeding against other competing causes of infant mortality and the low but ongoing risk of infant HIV acquisition via breast milk.

1.2 Human breast milk

As an essential food for newborn mammals, milk is a natural emulsion in which lipids are present in the form of small droplets called fat globules (as shown on Figure 1.1) (Lopez *et al.*, 2010) with diameters of 1 to 10 μ m (Schroten *et al.*, 1992) that are coated with a protective layer generally known as the milk fat globule membrane (MFGM) and which represents 3-4% of the total volume of milk (Wilson *et al.*, 2008). Human breast milk contains protein, fat, carbohydrates and minerals. The milk also contains numerous biologically active compounds such as lactoferrin, lysozyme, secretory leukocyte protease inhibitors, β -casein, oligosaccharides, glycolipids, glycoproteins, mucins and glycosaminoglycans, which protect breast-fed infants from bacteria, viruses and toxins (Habte *et al.*, 2007).

The milk lipids are secreted out from the mammary gland cells and then enclosed by the MFGM in the apical-cell membranes of mammary secretory cells (Shimizu *et al.*, 1986), where they are released, together with the membrane bound proteins (i.e. mucin glycoproteins, including the transmembrane mucins) from the cells by budding (Wilson *et al.*, 2008).

The milk lipids contain fat soluble vitamins, as well as essential and non-essential fatty acids and cholesterol for construction of membrane throughout the body (Buchheim *et al.*, 1988). The milk lipids are natural vehicles that deliver the triglycerols (~98% of total lipids and an important source of energy) and biologically active molecules such as polar lipids and proteins essential for nutrition and good health (Lopez *et al.*, 2010).

The MFGM consists of a complex mixture of glycoproteins (20-60%), glycerophospholipids (15-33%), sphingolipids, glycolipids, triacylglycerols, sterols (0.3% of milk lipids), enzymes and other minor components (Lopez *et al.*, 2010). This membrane prevents flocculation and coalescence of lipid droplets and protects the fat against lipolysis (Vanderghem *et al.*, 2008). The glycoproteins in MFGM act as specific bacterial and viral ligands which contribute to the prevention of pathogenic organisms from attaching to the intestinal mucosa in the stomach of infants (Wilson *et al.*, 2008).

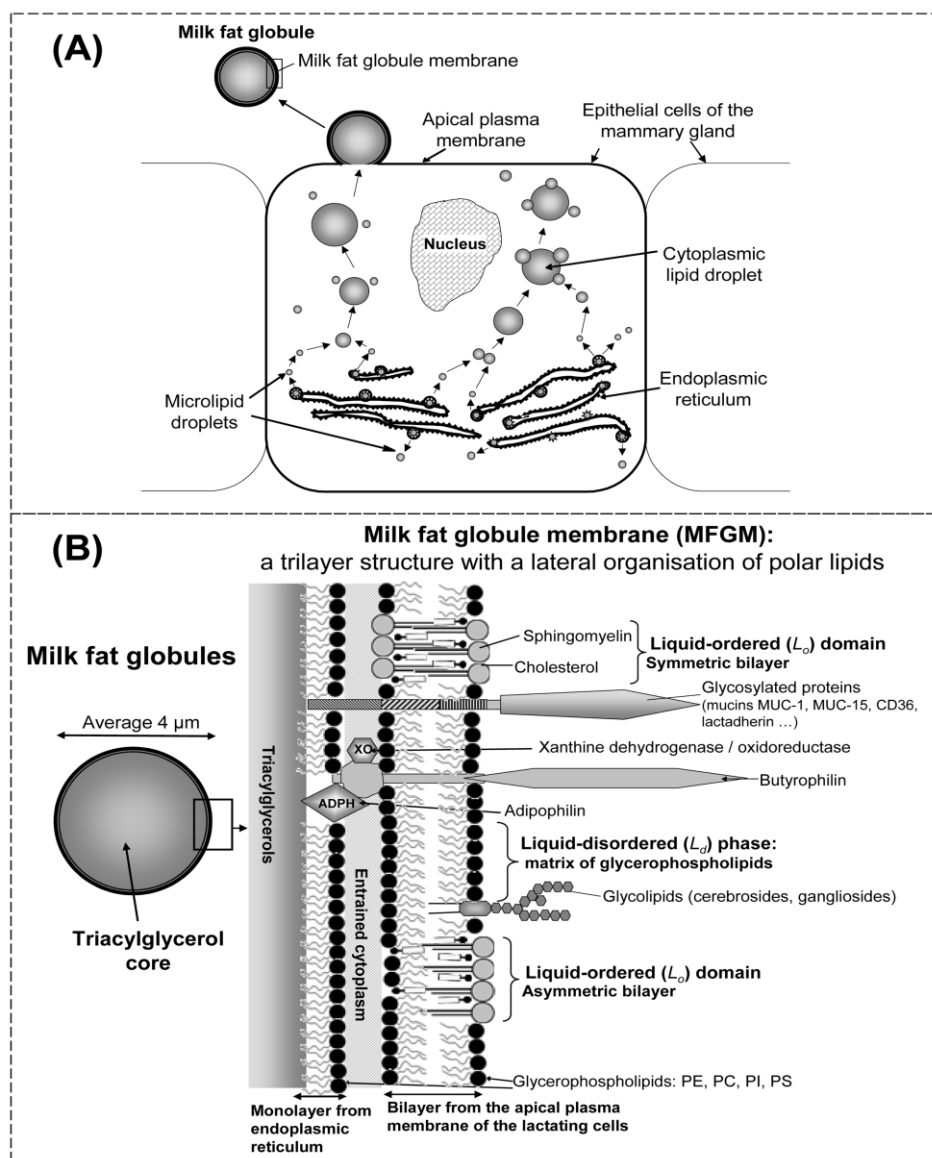


Figure 1.1 Origin and structure of the milk fat globule membrane (MFGM) reproduced from Lopez *et al.*, 2010 (A) Schematic representation of the pathways for the intracellular origin, growth and secretion of milk fat globules which are surrounded by the MFGM. (B) Structure of the MFGM showing the lateral segregation of sphingomyelin and cholesterol in L_o domains surrounded by the fluid matrix composed of glycerophospholipids. The drawing is highly schematic and the sizes are not proportional.

1.3 Mucus

Mucus is a complex, viscous and sticky secretion synthesized by specialized goblet cells of the columnar epithelium that lines the major internal tracts of the body that are exposed to the external environment. This includes the respiratory, reproductive and oculo-rhinotolaryngeal tracts. Mucus is the major organic secretion in the stomach and duodenum where it protects against corrosive gastric juices (Allen, 1983). Malfunctioning of mucus has been implicated in the etiology of many diseases, such as peptic ulceration, ulcerative colitis, bronchitis, infertility and external disorders of the eye (Allen, 1983).

The gel-like mucus shows properties both of a liquid (spontaneous flow by gravity, annealing or reclosure of cuts and interruption) and a solid (deformation is resisted, elasticity, adherence) (Gevers, 1987). The sliminess and stickiness of the mucus is dependent on the mucous glycoproteins (mucins) (Carlstedt *et al.*, 1983). Crude mucus is composed primarily of 95% water, but also contains glycoproteins, salts, lipids such as fatty acids, phospholipids and cholesterol. It also contains proteins such as lysozyme, immunoglobulins, defensins, growth factors and trefoil factors which serve a defensive purpose (Gipson *et al.*, 1997). However, the main component responsible for its viscous and elastic gel-like properties is the mucous glycoprotein (mucin) (Bansil *et al.*, 2006).

The crude mucus layer on epithelia ensures the hydration of the superficial epithelium. Mucus gels are versatile and have different roles in various parts of the body. For example, mucus gels in the stomach protect from strong acid and from potent proteases. It facilitates the movement of food and hard faecal pellets through the gastrointestinal tract. It protects the alveoli by trapping dust particles and other objects in the inhaled air in the tracheo-bronchial tubes. Mucus in the gall bladder protects the underlying epithelium against a concentrated mixture of surface-active chemicals. Saliva mixes with chewed food and aids in its homogenization and lubrication.

Mucus is the first barrier against pathogens. Nutrients and enteric drugs must interact and diffuse through mucus which acts as a permeable gel layer, in order to be absorbed and gain access to the circulatory system and their target end organs (Bansil *et al.*, 2006). In addition to its protective functions, mucus is also involved in many disease processes.

1.4 Mucus renewal and viscosity

Mucus is continuously produced from the epithelial surface in the gut, and lost to the lumen during renewal (Sababi *et al.*, 1995). Under normal conditions, the mucus blanket is maintained and renewed by the slow, continuous release of mucin from individual goblet cells throughout the epithelium. The secretion occurs by conventional exocytosis: the intermittent fusion of a single mucous granule membrane and the apical plasma membrane (Specian *et al.*, 1980).

The mucus gel is maintained by multiple interaction types (Taylor *et al.*, 2003) and the mucin structure affects the potential inter-molecular interaction. However, there is no information as to how structural and compositional differences in the component mucins relate to the rheology (physical) and functional properties of the mucus gels (Taylor *et al.*, 2004).

The erosion of the mucus gel layer at its luminal surface by proteases and mechanical sloughing (from a motile gut, passage of faeces, etc.) is balanced by secretion of new mucus to maintain the thickness of the adherent gel layer seen *in vivo* (Allen *et al.*, 1998), thus maintaining a dynamic balance between synthesis and degradation of the epithelial surface.

1.5 Definition of Mucins

Mucins are mucous glycoproteins that are found in the crude mucus gels that line the epithelial surfaces of the internal tracts of the body or at the cell surface of epithelial cells in the glandular ducts and other epithelia (Desseyn *et al.*, 2008) and are heavily O-glycosylated. They differ structurally and chemically from serum glycoproteins and connective tissue proteoglycans. These glycoconjugates contain galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid, but no uronic acid and only traces of mannose (Allen, 1983).

These large polymeric molecules contribute to the viscoelastic gel properties of mucus (Andersch-Björkman *et al.*, 2007). The high M_r glycoprotein mucins contain at least one and sometimes multiple protein domains that are sites of extensive O-glycan attachment (mucin domains). Thus, the composition of these glycoproteins is dominated by carbohydrates, which can total in some cases to as much as 80% of the weight of the molecule (Thornton *et al.*,

2004). The mucin linear flexible macromolecules have a M_r of approximately 2×10^6 and a mucin subunit has a M_r of 5×10^5 kDa consisting of a heavily glycosylated backbone with one or two “naked” regions of peptide with little or no glycosylation at either end (Kornelia *et al.*, 1997, Andersch-Björkman *et al.*, 2007). These mucin subunits are linked by disulphide linkages that are susceptible to reductive disruption by thiols such as 2-mercaptoethanol or dithiothreitol (DTT). The regions on the subunit that have no glycosylation are prone to proteolysis by enzymes such as trypsin, and the products of digestion are referred to as T-domains. T-domains make up a subunit and subunits make up the polymeric mucin (Kornelia *et al.*, 1997). These T- domains have a high content of serine, proline and threonine and are resistant to further proteolysis, the protein core being shielded by carbohydrates.

1.6 Mucin protein backbone (Apomucin)

The apoprotein is the protein core without the carbohydrate of the mucin which makes up 20% of the molecular mass, and is made up of two regions. The central glycosylated mucin domain, also called the PTS domain, is made up of tandem repeats (TR) which are rich in serine, threonine and proline (STP repeats). These TR domains make up more than 60% of the total amino acid content. Serine and threonine provide a high number of attachment sites for *O*-linked carbohydrates (Andersch-Björkman *et al.*, 2007), with N-acetylgalactosamine (GalNAc) the first sugar to be attached (Verma and Davidson, 2004). The second region of the apomucin (the protein core without the carbohydrate) is located at the amino (N) and carboxyl (C) terminals which have few *O*-glycosylation and N-glycosylation sites; this region has a high proportion of cysteine. These domains are important for the disulfide-mediated polymerization of mucins (Sheehan *et al.*, 2004).

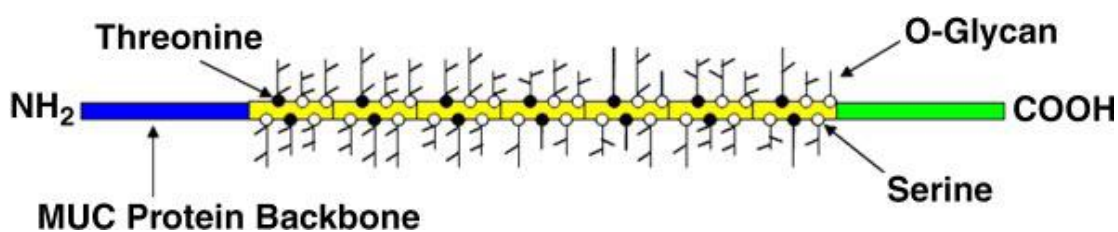


Figure 1.2 A secretory mucin glycoprotein depicting a MUC protein backbone and its *O*-glycans. Made up of the NH_2 -terminal domain (blue), central domain with tandem repeat containing serine, threonine and proline, where *O*-glycans are attached (yellow). A $COOH$ -terminal domain (green) (reproduced from Rose *et al.*, 2006).

1.7 Mucin O-glycans

Mucin-type O-linked glycosylation is initiated in the *cis* to *trans* golgi apparatus by the post translational addition of N-acetylgalactosamine (Gal-NAc) to the hydroxyl group of serine and threonine (Rottger *et al.*, 1998) catalyzed by peptidyl Gal-NAc transferase. The incorporation of Gal-NAc gives rise to the simplest known O-glycan structure which serves as a scaffold for these O-glycans. The attachment of the sugar residues to the initial Gal-NAc results in the formation of the core structures, which can then be elongated to form complex structures (Beum *et al.*, 2003).

Oligosaccharide side chain can be elongated by the sequential addition of the sugars such as galactose, sialic acid, fucose, traces of mannose and sulphates (Rottger *et al.*, 1998), via various core structures, each reaction being catalyzed by specific glucosyltransferases (Burchell *et al.*, 1999). Terminal sugars such as sialic acid and sulphates that are attached to Gal-NAc impart negative charges to the mucins, whereas fucose imparts hydrophobicity (Forstner and Forstner, 1994). O-glycans can be classified on the basis of their core structures which differ based on the sugars that are attached to the protein-bound N-acetylgalactosamine residue (Fakuda, 2002). These glycans transport water so that the aqueous phase surrounding mucins is viscous (Patton *et al.*, 1999).

1.8 Mucin genes and their classification

Approximately 12 mucin genes (designated MUC) had been identified, cloned and partially sequenced in humans by the year 2000 (Vinall *et al.*, 2000) and the number of identified genes had increased to 19 by the year 2006 (Bansil *et al.*, 2006). Only three MUC genes (MUC1, MUC2 and MUC5B) have been totally sequenced due to the difficulty of accurately assembling the large size central tandem repeats (Bansil *et al.*, 2006).

Based on their amino acid sequence and chromosomal localization and form, human mucins are classified into three classes; gel-forming secreted, secreted but non-gel-forming and trans-membrane mucins. The families of four genes on chromosome 11p15.5 (MUC2, MUC5AC, MUC5B, and MUC6) are thought to encode the major gel-forming mucins. These secreted mucins impart gel forming properties to crude mucus gels that adhere to the epithelium of the intestinal tract and other major tracts of the body (Allen, 1981). MUC1, MUC3A, MUC3B,

MUC4, MUC11, MUC12, MUC13, MUC16 and MUC20 glycoproteins are membrane associated and have a dual function of protection as well as signaling, cell-to-cell contact and possibly differentiation. MUC7, MUC8 and MUC9 are secreted mucins that are non-gel forming (Mall *et al.*, 2008, Vinall *et al.*, 2000). These MUCs are packed into mucous granules and secreted to the surface for the formation of a mucous layer that protects the epithelial tissues from both physical and mechanical elements.

1.8.1 Large, secreted and gel-forming mucins

Secreted mucins are large (≈ 2 MDa). They polymerise through disulphide, ionic and hydrophobic interactions with proteins as well as other mucins. The genes of several secreted mucins (MUC2, MUC5AC, MUC5B and MUC6) clustered on chromosome 11p15.5 contain domains with significant homology to von Willebrand factor D domains. This is because of their high content of cysteines which acts as sites for oligomerization (Callaghan *et al.*, 2001). The cysteines in the C-termini of these mucins induce polymerisation via disulphide bonds which contribute to their large size and gel-forming properties (Van Seuning *et al.*, 2000).

1.8.2 Small, secreted and non gel-forming mucins

Non gel-forming secreted mucins (MUC7, MUC8 and MUC9) are small and have a simpler structural organisation (Moniaux *et al.*, 2001), which differ from the gel-forming mucins as they lack cysteine rich domains. *MUC7* is found on chromosome 4q13.3 and *MUC8* on chromosome 12q24.3 (Shanker *et al.*, 1997). There is little information on MUC8 and MUC9 glycoproteins. However, MUC7 has been well characterised, particularly in saliva.

1.8.3 Membrane-bound mucins

Membrane-tethered mucins (MUC1, MUC3, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20) (Mall *et al.*, 2008), which contain short cytoplasmic tails in association with the cytoskeletal and cytosolic adaptor proteins are bound to cells by an integral trans-membrane domain (Rose *et al.*, 2001, Pallesen *et al.*, 2002). MUC1, MUC4 and MUC16 are the best characterised transmembrane mucins with MUC16 being the least well characterised of the three, owing to the difficulty in analysing a protein of its enormous size. The other described tethered membranes are uncharacterized (Hattrup *et al.*, 2008).

1.9 Biosynthesis of mucins

Mucin biosynthesis is reported to start in the nucleus where the mucin or MUC gene expression is activated by the transcription factors and translated into MUC proteins in the ribosomes (Dekker and Strous, 1990). Mucin monomers are synthesized in the ribosome and are N-glycosylated in the endoplasmic reticulum and form disulphide-linked dimers.

Once in the golgi complex, dimeric mucin precursors are O-glycosylated, sulphated and assembled into disulphide-bonded oligomers/multimers. Post glycosylation, these dimers are polymerized to form mucins (Sheehan *et al.*, 1999, 2000), which get packaged in a condensed manner, into secretory granules (Perez-Villar *et al.*, 2005).

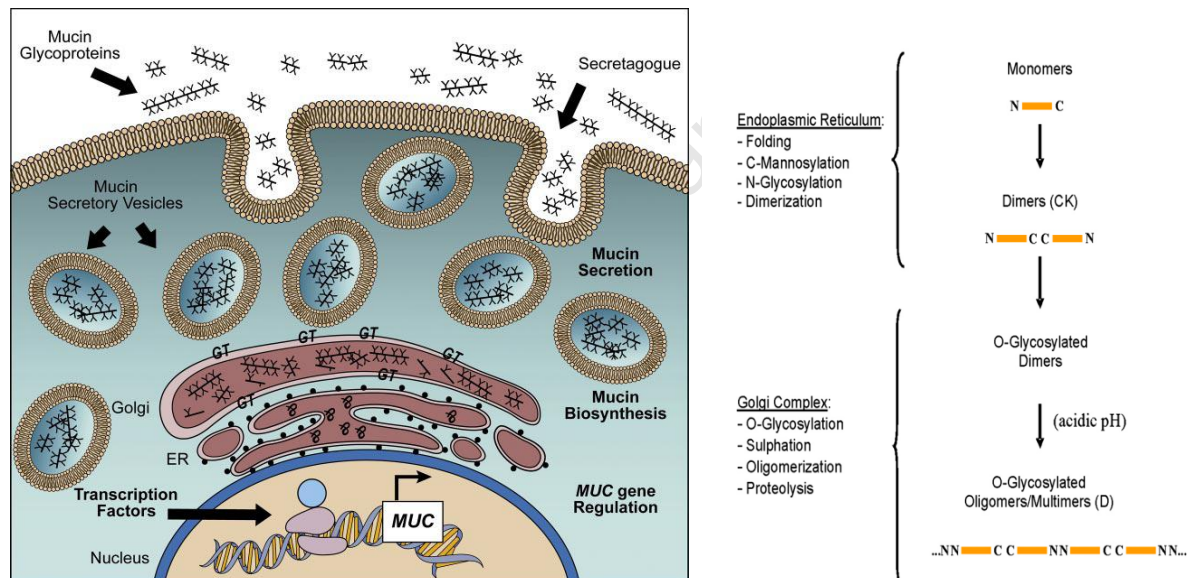


Figure 1.3 The Model of biosynthesis and secretion of mucin glycoproteins in a goblet or mucus cell. Rose *et al.*, (2006) and Perez-Villar (2007). Mucins are produced by both goblet cells in the surface epithelium and mucous cells in the submucosal glands (Svitacheva *et al.*, 2001). In the goblet cells of epithelium, mucins are synthesized and stored in cytoplasmic membrane-bound granules. Upon appropriate stimulation, the granule translocates to the cell periphery, where the granule membrane fuses with the plasma membrane and the contents of the granule (mucin) is released into the lumen via the process of exocytosis (Park *et al.*, 2008, Perez-Villar *et al.*, 2007).

Transmembrane mucins apoprotein undergoes a proteolytic cleavage event after translation, generating two cleavage products. These form a tight hetero-dimer complex that is composed

of the large repeat array-containing extracellular domain, linked by non-covalent, SDS-sensitive bonds to the much smaller protein molecule, which include the trans-membrane and cytoplasmic domains (Baruch *et al.*, 1999).

1.10 Breast milk mucins

Reports indicated that there are two human milk mucins, namely MUC1 which is best characterised and the second milk mucin previously called MUCX which is more heavily glycosylated and has a higher apparent molecular weight (Rossi *et al.*, 1996).

Reports have demonstrated MUCX to be MUC4 (Zhang *et al.*, 2005). About 70% of mucins are present in the fat globule membrane (cream fractions) and the rest associated with the skim milk (Habte *et al.*, 2007). The milk mucin is an integral membrane component, unlike those secreted by goblet cells in the lining of the intestine, airways and reproductive tract (Habte *et al.*, 2007). Both MUC1 and MUC4 in milk are reported to have a potential role in pathogen adhesion (Wilson *et al.*, 2008). The peptide core of the MUC1 extracellular domain ranges in size from 120-300kDa, which can more than double with full glycosylation whilst MUC4 is at roughly 900kDa (Hattrup and Gendler, 2008).

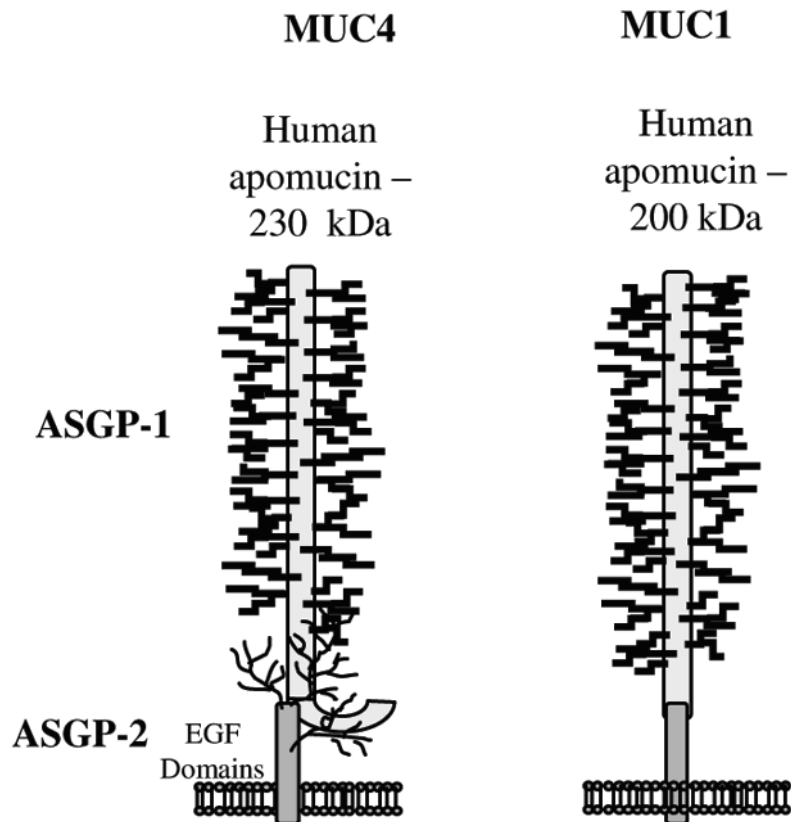


Figure 1.4 Structures of the human milk fat globule membrane mucins (Wilson *et al.*, 2008).

1.10.1 MUC1

MUC1 is an integral membrane protein rather than a simple secreted mucin that has been studied the most as it is the first mucin to be cloned (Govindarajan and Gipson, 2010). MUC1 is the only mucin that characteristically is expressed by normal breast epithelium (Diaz *et al.*, 2001). MUC1 and MUC4 are expressed in large quantities in both the late pregnant and lactating mammary glands, whereas the virgin mammary glands produce little or no MUC1. MUC1 is also found in the uterus where it offers protection against pathogens.

1.10.1.1 Structure of MUC1

Full length MUC1 is synthesized as a single polypeptide chain which undergoes an early proteolytic cleavage in the endoplasmic reticulum, creating heterodimer fragments that remain associated during its post-translational processing and transport to the cell surface (Ligtenberg *et al.*, 1992).

MUC1 has a molecular weight between 130-300 kDa. The full length cDNA encodes a protein that has a large extracellular domain (1000-2200 amino acids) (Gendler, 2001) which is extensively glycosylated with a variable number of tandem repeats. The larger fragment has the N-signal sequence and the VNTR domain of 20-100 repeats of the human sequence GSTAPPAHGVTSAPDTRPAP (Hattrup and Gendler, 2008). In addition to O-linked glycosylation, MUC1 contains five consensus sites for N-linked glycan addition in a non-repetitive sequence adjacent to the single trans-membrane domain (Altschuler *et al.*, 2000).

MUC1 also has a smaller fragment that is collectively called MUC1-CT. The MUC1-CT consists of three parts, an extracellular stem region of 58 residues (with multiple O- and five N- glycosylation sites), a short transmembrane domain, and the 72 amino acids CT (Hattrup and Gendler, 2008) which contain conserved phosphorylated tyrosines that bind to kinases (Lacunza *et al.*, 2009). The core peptide of the three combined domains is 14kDa, which increases in size to 25-30kDa post glycosylation and phosphorylation (Hattrup and Gendler, 2008).

When MUC1 is characterised with respect to its amino acid and carbohydrate composition, it is found to be rich in serine, threonine and proline. It is also estimated to contain 50% carbohydrate, 30% of which is sialic acid (Patton *et al.*, 1995).

In MUC1 oligosaccharide, galactose is added to the initial GalNAc to form a core 1 structure. In normal breast epithelial cells, core 1 is converted to core 2 by the addition of N-acetylglucosamine. This reaction is catalysed by the enzyme core 2 β 1,6-N-acetylglucosaminyltransferase. Core 2 is then extended by the addition of polylactosamine units. In breast carcinomas however, conversion of core 1 to core 2 is reduced, resulting in the O-glycans on the tumour associated MUC1 being shorter and less complex (Burchell *et al.*, 1999).

1.10.1.2 Functions of MUC1

MUC1 is a tethered transmembrane which can act as a second line of defence. It acts as a sensor of any environmental disturbances and signals the information to the interior of the cell (Gendler, 2001). This mucin has also been reported to be an anti-adhesive protein as its large

and extended conformation may block cell-cell interaction. It has also been shown to have the ability to bind bacteria in human breast milk (Gendler, 2001).

MUC1 has long polylactosamine chains in lactating mammary glands, which hinder the binding of peptide reactive antibodies. MUC1 plays a crucial role in regulating many cellular functions, including cell proliferation and apoptosis of T-cells. The membrane bound MUC1 is involved in cell signalling, immunoregulation and inhibition of cell–cell and cell–matrix adhesion (Rakha *et al.*, 2005).

In addition to mechanical functions, the MUC1 cytoplasmic tail has been shown to compete for or interact with β -catenin through a similar motif found in E-cadherin thus inhibiting the formation of E-cadherin/ β -catenin complex (Rakha *et al.*, 2005). MUC1 may participate in tumour cell detachment, invasion and metastases and may be associated with aggressive tumour behavior and poor prognosis. Through its cytoplasmic domain, phosphorylated MUC1 associates with other signal molecules such as Grb2/SOS which are signaling mediators of tyrosine kinase receptors. This suggests a potential role for MUC1 in cell signaling (Pandey *et al.*, 1995, Baruch *et al.*, 1999). In addition, co-localization and interaction between MUC1 and EGFR family members have also been reported (Rakha *et al.*, 2005).

1.10.1.3 MUC1 as a pathological marker

Modifications of human mucins have been associated with certain diseases. Diseases such as cystic fibrosis, malignancies of the gastrointestinal or respiratory tracts are associated with dramatic changes in mucin composition and production (Bolscher *et al.*, 1995). The rate and level of MUC1 production in human breast milk is known to be an important marker in metastatic breast cancer progression (Sekine *et al.*, 1985). It has been reported that aberrant expression of MUC1 in patients with ductal hyperplasia with atypia and especially in ductal carcinoma *in situ* of the breast are at higher risk for subsequent development of invasive breast carcinoma (Mommers *et al.*, 1999).

Furthermore, the expression of MUC1 in colorectal carcinoma is a valuable indicator of poor prognosis in Caucasian patients as opposed to those of African American descent (Manne *et al.*, 2000). A strong MUC1 expression in the stomach has been shown to give a negative

prognostic impact (Sakamoto *et al.*, 1997). Other studies have reported MUC1 to be a marker for some carcinomas such as those of the pancreas (Rhaka *et al.*, 2005).

1.10.2 MUC4

MUC4 is a transmembrane mucin that is expressed in the ciliated and goblet cells of the trachea and bronchus. Human MUC4 is the homolog of the rat sialomucin complex (SMC) (Price-Schiavi *et al.*, 1998). The human MUC4 and rat SMC differs from each other in that the SMC lacks the 16 amino acid repeat on the Ascites sialoglycoprotein-1 (ASGP-1) which is the defining characteristic of MUC4.

Beyond the respiratory tract, MUC4 is expressed in the epithelial tissues of the stomach, breast, endocervix, colon (Damera *et al.*, 2006), uterus, ovary, salivary gland, prostate, thyroid, mammary gland, esophagus, testis and placenta (Zhang *et al.*, 2005). MUC4 is also found in secreted fluids like tears (Govindarajan and Gipson, 2010) and has been proposed to be MUCX described in human milk (Zhang *et al.*, 2005).

1.10.2.1 Structure of MUC4

Human MUC4 was originally cloned and sequenced from an airway cDNA library. MUC4 is similar to MUC1 in that it is a hetero-dimeric multi-functional glycoprotein encoded by a single gene. Structurally, it consists of a large 850kD membrane bound MUC4 α subunit which is O-glycosylated and it is also a homolog of ASGP-1, which is tightly, but noncovalently bound to a smaller 80kD trans-membrane MUC4 β subunit which is N-glycosylated and is also known as ASGP-2 (Damera *et al.*, 2006, Wilson *et al.*, 2008). The larger extracellular domain of the MUC4 α contains an N-terminal region comprised of a sequence of imperfect repeats and a tandem repeat region of 16 amino acids. This extracellular domain also contains a cysteine-rich site, a nidogen homology sequence (NIDO), the adhesion associated domain, AMOP and a von Willebrand factor type D (VWD) sequence close to the transmembrane domain (Govindarajan and Gipson, 2010). The EGF-domains are found in the region rich in N-glycosylation near the transmembrane domain of MUC4.

1.10.2.2 Functions of MUC4

MUC4 binds to ErbB2 via its EGF-like domain. Post MUC4-ErbB2 binding, phosphorylation of p38 MAPK is stimulated. This promotes cell survival and differentiation. In cancer, it is hypothesized that this MUC4-ErbB2 complex protects cancer cells from undergoing apoptosis (Govindarajan and Gipson, 2010). MUC4 also has a possible function as a growth regulator due to the fact that ASGP-2 can bind receptor tyrosine kinase p185^{neu} (Rossi *et al.*, 1996). ASGP-1 provides anti-recognition and anti-adhesive properties which may be important to the high metastatic capacity of certain cancers in ascites cells (Price-Schiavi *et al.*, 1998).

1.10.2.3 MUC4 as a pathological marker

As previously stated (Bolscher *et al.*, 1995), mucins can potentially influence pathological classification, diagnosis and prognostication of neoplasia. For example, it has been confirmed that MUC4 is absent in normal pancreas and chronic pancreatitis, but is highly raised in human pancreatic cancers which are on the increase worldwide with a survival rate ranging from 4 to 6 months (Andrianifahanana *et al.*, 2001). MUC4 is a potential candidate marker for early diagnosis of epithelial ovarian carcinoma which is detected in the early-stage tumor samples and may be utilized in combination with MUC16 to achieve greater sensitivity for the detection of late stage tumours (Chauhan *et al.*, 2006).

1.11 Anti-infective properties breast milk and milk mucins

Human breast milk is an essential source of nutrition and it also offers protection from respiratory and gastrointestinal infections. Molecules found in milk such as antibodies, bactericidal enzymes like lysozyme and fatty acids that lyse bacteria and viral particles and bacterial peptides offer anti-microbial activity. While breast milk might offer many benefits, it should be noted however that there is a significant risk of mother-to-child transmission of HIV-1 through breast-feeding (Nicoll *et al.*, 2000). Lactoferrin, but not casein or casein peptides, has been shown to be inhibitory to HIV virus (Berkhout *et al.*, 2002), while the undigestible fucosylated and sialylated oligosaccharides conjugated with protein (mucins) may inhibit the adhesion of diarrheal pathogens such as the Rotavirus to the host receptor. The mucin fraction of the milk also inhibits the adherence of S-fibriated *E.coli* to their receptors (Newburg *et al.*, 1995). Although human breast milk may contain components of

HIV, studies by Newburg (1995) have shown that milk also contains glycosaminoglycan moieties that prevent the binding of HIV envelope glycoprotein. Another study demonstrated that MUC1, which is another Lewis-X factor abundantly present in milk, specifically bound to DC-sign (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) on DC and effectively blocked DC-sign mediated HIV-1 transmission to T-cells (Saeland *et al.*, 2009). Cytokines are known to play a role in immune-modulation and immune-protection of breast milk (Chirico *et al.*, 2008).

1.12 HIV-1 in South Africa

Human immunodeficiency virus type 1 (HIV-1) is an enveloped virus that was first isolated from a patient in Paris in 1983. This virus infects primarily human CD4-positive T cells and macrophages (Sakomoto *et al.*, 2003). HIV gains entry into host cells via interaction of the viral envelope protein gp120 with at least one co-receptor expressed on the surface of the host cell membrane (Jenkinson *et al.*, 2003). Post viral exposure, HIV-1 infection is characterised by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in the blood. P24 antigen is one of the components of the virus which forms the major internal structural protein of HIV-1. HIV p24 antigen tests employ ELISA technology with modifications to detect the antigen. HIV viral tropism is determined by the gp120 expressed on the virus and the chemokine receptors present on the cells. Macrophage tropic (M-tropic) HIV-1 strains tend to use CCR5 while the T lymphocytic-tropic (T-tropic) HIV-1 strains utilize CXCR4 as their co-receptors. The Dual tropic strains utilize both CCR5 and CXCR4 (Hong *et al.*, 1999). When gp120 binds with CD4 and the appropriate co-receptor, conformational change and unmasking of a second viral envelope called gp41 occurs. The interactions of gp41 with the host cell membrane, results in the fusion of the viral envelope with the cell. This allows for the transfer of viral genetic information into the host cell and continuation of viral replication (Jenkinson *et al.*, 2003). Infection with HIV- 1 results in progressive deterioration of the immune system due to qualitative and quantitative defects of CD4⁺ T-lymphocytes (Sakomoto *et al.*, 2003).

South Africa is reported to have more human immunodeficiency virus infected people than in any other country in the world, with a national HIV prevalence of 11.4% (Jaspan *et al.*, 2006). The first national population-based HIV survey was conducted in 2002 and showed that 9.3% of persons between the ages of 15 and 24 years were HIV positive and that females were more

likely to be infected than males (Jaspan *et al.*, 2005). HIV-1 subtype C is the predominant HIV-1 genotype in Southern Africa and is rapidly spreading throughout the world (Ndung'u *et al.*, 2006) and accounts for approximately 50% of pediatric HIV-1 infections (Zhang *et al.*, 2006).

1.13 Breast-feeding and HIV transmission

Women of child bearing age constitute nearly half of the 30 million adults currently living with HIV/AIDS in the world (Dabis *et al.*, 2000). According to a report by UNAIDS, approximately 1 million women give birth each year in South Africa, with more than 250 000 of these infants being born to HIV-infected mothers (Jaspan *et al.*, 2005).

Breast-feeding maintains the maternal-fetal immunologic link after birth which may favour the immuno-competence transmission from the mother to her infant. It is also considered an important contributory factor to the neonatal immune defence system which is not well developed at birth (Chirico *et al.*, 2008). Breast-feeding has been shown to lower the incidence of other infectious diseases during the first year of life. A large number of studies have shown that breast-fed infants have a lower incidence of gastrointestinal and respiratory system infections than do bottle-fed infants (Peterson *et al.*, 1998). The with-holding of breast milk can have serious consequences for infants living in areas of the world where enteric disease is endemic, for both HIV-infected and HIV-uninfected infants (Newburg *et al.*, 1995).

Human breast milk is rich in mucin. However, mother-to-child transmission of HIV-1 through breast feeding accounts for the majority of HIV-1 infections among infants as high as 40% (Habte *et al.*, 2008 and Schrotten *et al.*, 1992). Proteins present in milk such as lactoferrin, lysozyme and secretory leukocyte protease inhibitor have been shown to possess anti-HIV properties (Naarding *et al.*, 2005).

1.14 Questions and Objectives of the study

Human breast milk is rich in components, such as mucin, that have been reported to protect against pathogens and viruses (Habte *et al.*, 2007). The question is that despite all these protective components in breast milk, HIV-1 transmission from mother to child is still a huge risk? The biological objectives of the study are to isolate, purify, characterise and determine the anti-HIV-1 activity of crude breast milk and its purified mucin components, namely

MUC1 and MUC4, in HIV positive patients compared with those who are not infected. This study will also test the effect that heat might have on breast milk which we think can release the milk mucins from the MFGM and consequently have an inhibitory effect on HIV-1.

University of Cape Town

CHAPTER 2

MATERIALS AND METHODS

2.1. Ethics

This study was approved by the University of Cape Town Health Sciences Faculty Research Ethics Committee: REC REF 077/2010 and all patients gave written informed consent.

2.2. Materials

Guanidinium chloride (GuHCl), agarose, bovine serum albumin (BSA) and caesium chloride (CsCl) were obtained from the Sigma Company, St Louis, USA. Dialysis tubing and pure nitrocellulose membranes (0.22 μ m) were purchased from Argon Laboratory Services, RSA. ULTRA CLEARTM centrifuge tubes (16 x 76 mm) were supplied by Beckman International. Ammonium persulphate (AMPS), Folin-ciocalteu phenol reagent, acrylamide, N,N'-methylenebisacrylamide (Bis), N-ethylmaleimide (NEM), sodium metabisulphite, sodium dodecyl sulphate (SDS), periodic acid and 2-mercaptoethanol were supplied by the British Drug House, Dorset, UK. Phenylmethylsulfonyl fluoride (PMSF), N,N,N,N'-tetramethylethylenediamine (TEMED), Coomassie Brilliant Blue-250, sodium carbonate anhydrous, disodium hydrogen orthophosphate anhydrous and sodium dihydrogen orthophosphate were from SAARCHEM (Merck). Monoclonal mouse anti-MUC1 antibody was from Novocastra (Newcastle, UK). Monoclonal mouse anti-MUC4 was from Santa-Cruz. The HRPO linked rabbit anti-mouse secondary antibody was from Dako-Cytomation. The syngene G: Box was from Vacutech. The 293T cell line was from the American Type culture collection, catalogue No CRL 11268. TZM-bl cell lines were from the NIH AIDS Research and Reference reagent program (catalogue No 8129). Alcian Blue was from Sigma-Aldrich Corp, ST Louis, MO). The AG50WX8 cation-exchange resins were from Bio-Rad. The 100 kDa molecular weight cut-off spin filters were from Millipore, Bedford, MA. The negative ion LC-ESI-MS and MS/MS (ion trap LCQ DECA system) was from Thermo Electron Corp., Waltham, MA). HyperCarb porous graphitized carbon columns (5 μ m HyperCarb, 0.32 \times 150 mm) were from Thermo Hypersil-Keystone, Bellefonte, PA. Surveyor auto-sampler was from Thermo-Finnigan, San Jose, CA. The high-pressure liquid chromatography column for amino acid analysis was from Waters Associates, Medford, Mass., USA. Sterile DMEM with L-

glutamine, sodium pyruvate, glucose and pyridoxine (catalogue No 41966029); fetal bovine serum (FBS) (catalogue No 10106-169); Sterile trypsin-EDTA (catalogue No 25200-056); sterile PBS (catalogue No 10010015) was from Gibco BRL life technologies. DEAE dextran, hydrochloride (catalogue No D9885) were from Sigma.

2.3. Sample collection

2.3.1. Collection of breast milk from HIV positive and HIV negative patients

Human breast milk samples were collected from forty HIV negative and thirty HIV positive, lactating mothers from the post-natal and neonatal wards at Groote Schuur Hospital (Cape Town, South Africa). The milk samples were collected into 5ml 6M guanidinium chloride (GuHCl), 1mM phenylmethylsulfonylfluoride (PMSF) and 10mM ethylenediaminetetra-acetic acid (EDTA) at a ratio of approximately 1:5, immediately frozen at -20°C and stored. In preparation for the inhibition assay, milk was thawed, dialysed overnight at 4°C against three changes of distilled water and then freeze-dried.

2.4 Heat treatment of milk

Crude milk samples were heat treated in order to determine the effect of heat on the milk. Crude milk (1ml) samples from each individual were aliquoted, dialysed and freeze dried before reconstituting with 10% DMEM and then heating at 80°C for 10 min in preparation for the neutralization assay.

2.5 Preparation of milk fat globule membrane

Milk fat globule membrane was prepared according to the method of Schroten *et al* (1992). Frozen milk samples were thawed at room temperature in the presence of 1mM PMSF, 10mM EDTA and 6M GuHCl. Milk was defatted by centrifugation at 3000g for 1 hour at 4°C . The cream at the top of the tube appeared as a yellow fraction which was removed and resuspended in 0.2M sodium chloride (NaCl), 0.02% sodium azide (NaN_3) containing 10mM EDTA and then kept at 4°C for 1 hour. This suspension was placed on a shaker at room temperature overnight until butter formed. The butter mixture was then incubated at 40°C in a water bath for 30 min to release the milk fat globule membrane. The membrane was collected

as a yellow pellet by ultracentrifugation for 30 min at 35,000 g at 4°C. The yellow membrane pellet was resuspended in 0.2M NaCl, 0.02% NaN₃ containing 10mM EDTA, then subjected to homogenization, using a Junkel Ultra-Turrax for 1 min at 9500 rev at room temperature to disaggregate it and increase its solubility. Insoluble materials were removed by centrifugation at 4400g for 10 min at 4°C.

2.6 Sepharose CL-4B gel filtration

Sepharose CL-4B gel filtration was used to separate MFGM material as peaks I and II. The method is used to separate the biological material according to size, between a continuous size phase and the smaller interior gel pore size (Brooks *et al.*, 2000). The column was equilibrated and samples eluted using 0.2M NaCl, 0.02% NaN₃ containing a cocktail of protease inhibitors. Following periodic acid Schiff (PAS) and protein A₍₂₈₀₎ assays, the void and included volume fractions were pooled separately, dialysed against three changes of distilled water overnight at 4°C and then freeze dried.

2.7 Analytical determination

2.7.1 Lowry Assay

The protein assay was done to determine the amount of total protein present in the MFGM samples. Protein was estimated according to the method of Lowry *et al* (1951). The two basic steps for this method are; the formation of a copper ion complex with amide bonds, resulting in reduced copper in alkaline solution and the reduction of Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate) by tyrosine and tryptophan residues. This reduction of Folin-Ciocalteu reagent results in a blue colour formation detected at 690nm. Despite its variation in colour with respect to different proteins and interference by different laboratory chemicals such as detergents, carbohydrates, glycerol, EDTA, caesium chloride, mercaptoethanol and Triton X-100, it is more sensitive than UV detection of proteins at 280nm. For this study, 10ul of MFGM sample, 10µl NaOH and 200ul solution containing (anhydrous sodium carbonate, copper sulphate and sodium tartrate) was incubated for 10 min at 25°C before the addition of 20µl Folin's solution. The mixture was then incubated for a further 30 min at 25°C. The assay was read at 690nm in the microtitre plate reader. A standard

curve with bovine serum albumin of known concentration was included in every assay to determine the concentration of protein in the crude milk samples.

2.7.2 Total protein concentration determination in crude milk (Bradford assay)

Protein quantification was also performed using the modified Bradford assay (Bradford, 1976). This method involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. The dye exists in three forms: cationic (red), neutral (green), and ionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form. However, when the dye binds to protein, it is converted to a stable un-protonated blue form, detected at 595nm in the assay using a spectrophotometer or microtitre plate reader. BSA was used as the standard to generate the calibration curve. Using the microtitre plate, 5µl crude milk sample was added to the 250µl dye reagent, then incubated at room temperature and read after 5 min but not longer than 1 hour. The absorbance was measured at a wavelength of 595nm.

2.7.3 PAS Assay

Glycoproteins in mucins were estimated by the Periodic Acid Schiff (PAS) procedure of Mantle and Allen (1978). This method involves oxidation of the hydroxyl (-OH) group to aldehyde (-CHO) by periodic acid. Subsequently, the aldehyde group reacts with Schiff reagent to form a red-purple product. In the case of the mucins the periodic acid/Schiff reagent stains the vicinal diol groups of the peripheral sugars and sialic acids (Thornton *et al.*, 1996). Briefly, 20µl sample was incubated at 37°C for 1 hour with periodic acid solution, and then 200µl decolorized Schiff reagent was added before incubating for 30 mins at room temperature. Absorbance was read at 555nm on a microtitre plate reader.

2.8 Dialysis of samples

Prior to PAS, Lowry, SDS-PAGE and Western blotting analysis, GuHCl and caesium chloride (CsCl) were removed from the samples by dialysis as they interfere with these assays. Dialysis is based on the principle of osmosis and concentrates proteins in their native state. However, substantial loss of mucins may occur during dialysis due to their sticky characteristics (Bolscher *et al.*, 1995). In this study, dialysis tubing was prepared by boiling in

distilled water in the presence of EDTA and sodium carbonate. Samples were dialysed overnight at 4°C against three changes of distilled water with continuous stirring.

2.9 Freeze-drying

Freeze-drying was done to remove water from the samples in order to determine the total weight of the dry sample. Prior to freeze-drying, samples were placed into plastic containers with holes in their lids and frozen at -80°C overnight. Thereafter, samples were placed into suitable vacuum flasks for freeze drying. This was done by using the Christ ALPHA 1-5 freeze drier from Laboratory and Scientific Company.

2.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done in order to determine the presence and purity of the mucins and to determine their size. Breast milk mucins were prepared in reducing gel loading buffer containing 2% SDS, 10% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol and placed in boiling water for 1 min prior to loading. Electrophoresis was performed according to the method of Laemmli (1970) in a 10% (w/v) running gel and a 4% (w/v) stacking gel using the Hoeffer Mighty small mini-electrophoresis system. Briefly, the running gel was made as follows: 3.67 ml 30% Bis/Acrylamide, 3.90 ml 0.1% SDS, 3.60 ml running buffer, 100ul ammonium persulphate (AMPS), 5ul N,N,N',N'-Tetramethylethylenediamine (TEMED). The running gel was poured between two gel plates; water was added on top to prevent the gel from drying. The gel was left to set. The stacking/spacer gel was made as follows: 0.5ml 30% Bis/Acrylamide, 2.8ml spacer buffer, 73µl AMPS and 5 ml TEMED. The water was poured off the running gel and the spacer gel was poured on the set running gel. A comb was inserted into the gel to make wells until the stacking gel had set. The gel was run at 20mAmps, until the bromophenol blue had reached the bottom of the gel. The gel was stained with PAS for glycoproteins or Coomassie Brilliant Blue R-250 for proteins.

2.11 Dot Blotting

Dot blotted samples were used for beta elimination analysis of O-linked oligosaccharides as well as for densitometric scanning of the mucins from PAS.

A Bio-rad blotting apparatus was used for the dot blots for beta elimination analysis and the Schleicher and Schuell Minifold II slot/dot blot apparatus was used for the dot blots for glycoform determination. The PVDF (beta elimination analysis) and nitrocellulose (densitometric scanning of the mucins from PAS) membranes was soaked in methanol for 2 min and then into distilled water (dH₂O) for another 2 min. A precut filter was also soaked in dH₂O for 2 min. The wet filter paper was placed on the gasket support plate before placing the wet membrane on top of the filter. The dot blot apparatus was assembled according to the manufacturer's instructions.

Using a narrow-mouth pipette tip, 40µl of sample was loaded onto the nitrocellulose membrane at the center of the grid. The sample was blotted onto the nitrocellulose membrane using a vacuum pump attached to the dot blot apparatus. The dot blots were either stained with Alcian blue (or direct blue) for beta elimination analysis or with PAS for densitometric scanning of the mucins.

2.12 Periodic acid Schiff gel staining

The SDS-PAGE gels were stained with PAS to determine the presence and size of the mucins. The gels were stained according to the method of Dubray and Bezard (1982). Briefly, the SDS-PAGE gels were placed for staining in 50% ethanol for 30 mins, and then washed in distilled water for 10 min. The gel was placed in 1% periodic acid made up in 3% acetic acid for 30 mins, then washed in distilled water for 30 mins or overnight. The gel was placed in 0.1% sodium metabisulphite in 10mM hydrochloric acid for 2 x 10 mins, then in Schiff reagent in the dark for 60 mins. The gel was then placed in 0.1% sodium metabisulphite in 10mM hydrochloric acid for 60 mins in the dark and then photographed.

2.13 Coomassie Brilliant Blue R-250 gel staining

SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 for the detection of proteins. In this method the dye-anion is electrostatically attached to the NH₃⁺ group of the protein in a slightly acidic solution. This dye-protein bond is fixed but can be fully reversed by changing pH (Fazekas de St *et al.*, 1963). In this present study gels were stained overnight with 0.1% Coomassie Brilliant Blue R-250 in 30% methanol and 10% acetic acid and destained in 20% methanol and 10% acetic acid until the required bands were clearly visible. The gels were stored in distilled water and then photographed.

2.14 Agarose gel electrophoresis

In this method gels are prepared by dissolving agarose in warm electrophoresis buffer to give a specific concentration (0.7%) of agarose. Once it has solidified, the agarose gel forms a matrix, the density of which depends on the concentration of the agarose. During electrophoresis, mucins, which are negatively charged at a neutral pH, migrate towards the anode. The mobility of the proteins is influenced by the agarose concentration and sample size with the smallest molecules migrating the longest distance (Boyer, 1993). The mucins were added to the sample loading buffer containing 40% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol in 1x Tris-acetate buffer and boiled for 2 min prior to loading onto the gel. Electrophoresis was carried out in a 0.7% (w/v) agarose gel prepared in running buffer containing 40mM Tris-acetate buffer, 1mM EDTA and 0.1% SDS pH 8.0. The agarose was boiled in a microwave until it completely dissolved and cooled down to approximately 50°C before pouring into the Bio-Rad DNA sub-cell gel apparatus. Upon polymerization the apparatus was filled with running buffer (TAE containing 0.1% w/v SDS) and electrophoresis was performed at 65 V, for 3 hours at room temperature.

2.15 Western blotting analysis

The identity of the mucins was performed by western blotting. This method involves agarose gel separation and vacuum transfer of samples to nitrocellulose membrane for their recognition by antibodies (Hughes *et al.*, 1988). Subsequent to blocking of the free binding sites on the membrane with non-fat milk powder, antigens are accessible to bind with their corresponding primary and secondary antibodies. The secondary antibodies which are specific to the primary antibodies are labeled with enzymes such as horse radish peroxidase, which react with substrates to form an insoluble coloured product.

Equal amounts of mucins (400µg) were resolved separately on 0.7% agarose gels. After agarose gel electrophoresis, the mucins were transferred to a nitrocellulose membrane (Nitrocellulose, 0.22 Micron) using a vacuum-blotting unit at 40mBars for 1 hour. The transfer buffer used was 4x SSC. After vacuum-blotting, non-specific binding was blocked by incubating the membranes for 30 min in 5% (w/v) low-fat milk powder in Tris-buffered saline (20 mM Tris-HCl, pH 7.2, 150 mM NaCl), 0.05% Tween-20 (TBST) at room temperature.

The membranes were then washed with TBST for 3 x 5 min before incubating overnight with 1:100 diluted mouse anti-MUC1 NCL monoclonal antibody (specific against the carbohydrate epitope of the human MUC-1 glycoprotein) diluted in 5% (w/v) low fat milk powder in TBST or with 1:200 diluted human MUC4-specific 1G8 monoclonal antibody (it recognizes an epitope in the central portion of the ASGP-2 between the 53aa at the N- terminus and the trans-membrane domain). The membranes were washed for 3 x 5 min with TBST and incubated for 30 min with HRPO linked rabbit anti-mouse secondary antibody diluted in 5% (w/v) low-fat milk powder in TBST at 1: 1000 concentrations. After another TBST wash (3 x 5 min), the membranes were treated with LumiGLO Reserve Chemiluminescent Substrate for 1 min and detected using the syngene-G: Box.

2.16 HPLC Ion-Exchange Chromatography

This study was performed in the laboratory of Professor David Thornton in the department of Biochemistry and Molecular Biology, University of Manchester, United Kingdom under the kind auspices of the South African National Research Foundation. The high- M_r glycopeptides were chromatographed on a Pharmacia Mono Q HR 5/5 ion-exchange column using a 2150 LKB titanium-head pump connected to a 2152 LKB controller and a 2040-203 LKB mixing valve according to the method described by Thornton *et al.*, (1991). Before chromatography, freeze dried samples of the MFGM-I (6mg) were resuspended into 6M urea. Because the samples were still poly-dispersed, samples were further resuspended into 4M urea. Gradients were formed at the low-pressure side and samples were loaded with a Pharmacia V-7 injector. The column was eluted at a flow rate of 0.5 ml/min, first isocratically for 10 min with 10 mM-piperazine/perchlorate buffer, pH 5.0 (buffer A), then with a linear gradient (60 min) to 0.25M-lithium perchlorate/10mM-piperazine/perchlorate buffer, pH 5.0 (buffer B). Fractions (0.5 ml) were analyzed for neutral sugars using a PAS assay (Mantle & Allen, 1978).

2.17 Amino acid analysis

The amino acid content of the breast milk mucins was determined by amino acid analysis. This involves four basic steps namely: the hydrolysis of the protein to its individual amino acid content, labelling of amino acid with a detectable marker, high pressure liquid chromatography (HPLC) separation of mucins and data interpretation. The amino acid content of purified breast milk mucins was analyzed using an HPLC system according to the methods

of Klapper (1982). The samples were vacuum-dried and placed in a hydrolysis vessel containing some constantly boiling hydrochloric acid (HCl) and 1% (v/v) phenol. The vessel was purged with nitrogen gas and sealed under vacuum. The samples were then hydrolyzed in the gas phase at 110°C for 24 hours. Following hydrolysis, the vessel tip was cooled and vacuum dried to remove the residual HCl. The dried samples were re-dissolved in citrate buffer pH 2.2 and injected into a high-pressure liquid chromatography column packed with a cation exchange resin (sulfonated polystyrene cross-linked with divinylbenzene) and eluted with a series of buffers ranging from a low (0.25M trisodium citrate, pH 3.05) to high (0.25M sodium nitrate, pH 9.5) pH. Detection was carried out using post-column derivatization with o-phthalaldehyde, a fluorescent reagent that reacts with all the amino acids except proline. For proline detection, the samples were treated with sodium hypochlorite ahead of post column derivatization with OPA. The relative ratios of the individual amino acids for each sample were determined and compared.

2.18 Growing TMZ-bl cells from frozen stocks

TZM-bl cells were used in the pseudoviral neutralisation assay. Frozen TZM-bl cells were thawed quickly by placing the tube in a 37°C water bath. Cells were removed from the cryotube with a 1ml pipette and placed in a 15ml sterile tube. The DMSO (toxic) in the cells was diluted out by adding 9ml culture media drop by drop to prevent the production of stress protein due to shock, and gentle swirling often. The 15ml tube was centrifuged at room temperature 1200rpm for 7 min. The supernatant was removed by aspiration. The pellet was re-suspended with 12ml culture media and placed into labelled plates (first re-suspend with 5ml media before adding the rest to make up 12ml). The plates were incubated at 37°C in a CO₂ incubator.

2.19 Maintenance of TZM-bl cell lines

TZM-bl cells were cultured with 5% DMEM media (containing DMEM, FBS, HEPES and gentamycin, NEAE) until the cells were 60-80% confluent. The plates were examined under the microscope to check that the TZM-bl cells were approximately 80-90% confluent and that there was no fungal or microbial contamination. The media was removed by aspiration and discarded. PBS (5ml) was added on to the plate and the plate was “washed” by gently swirling. Using a sterile 2ml Pasteur pipette, the PBS was removed by aspiration and

discarded. Trypsin-EDTA (1ml) was added to the plate allowing the trypsin to cover the entire surface of the plate to which the cells were attached. The cells were allowed to incubate at 37°C in CO₂ incubator for 4 min. Cells were detached from the surface by tapping the plate gently before examining under the microscope. Culture media (5ml) was added into the plate to neutralise the trypsin. The suspension was centrifuged (1200 rpm for 5min) to pellet the cells. The supernatant was removed by aspiration and cells were re-suspended in 5ml media. The re-suspended cells (1/3 volume) were added to a fresh plate containing 10ml culture media. The plate was placed in 37°C incubator with CO₂.

2.20 Production and titration of HIV-1 Env pseudovirus

Stocks of single-round infection HIV-1 Env pseudovirus were produced as previously described (Montefiori, 2005) by co-transfecting 293T/17 cells (3 x 10⁶ cells per T75 flask) with 4µg of an HIV-1 *rev/env* expression plasmid and 8µg of an *env*-deficient HIV-1 backbone plasmid (pSG3 Env) using FuGENE transfection reagent. Pseudovirus-containing supernatant was harvested 48 hours following transfection and clarified by filtration through a 0.45 micron filter. Single-use aliquots (1.0ml) were stored at -80°C. The 50% tissue culture infectious dose (TCID₅₀) for each pseudovirus preparation was determined by infection of TZM.bl cells.

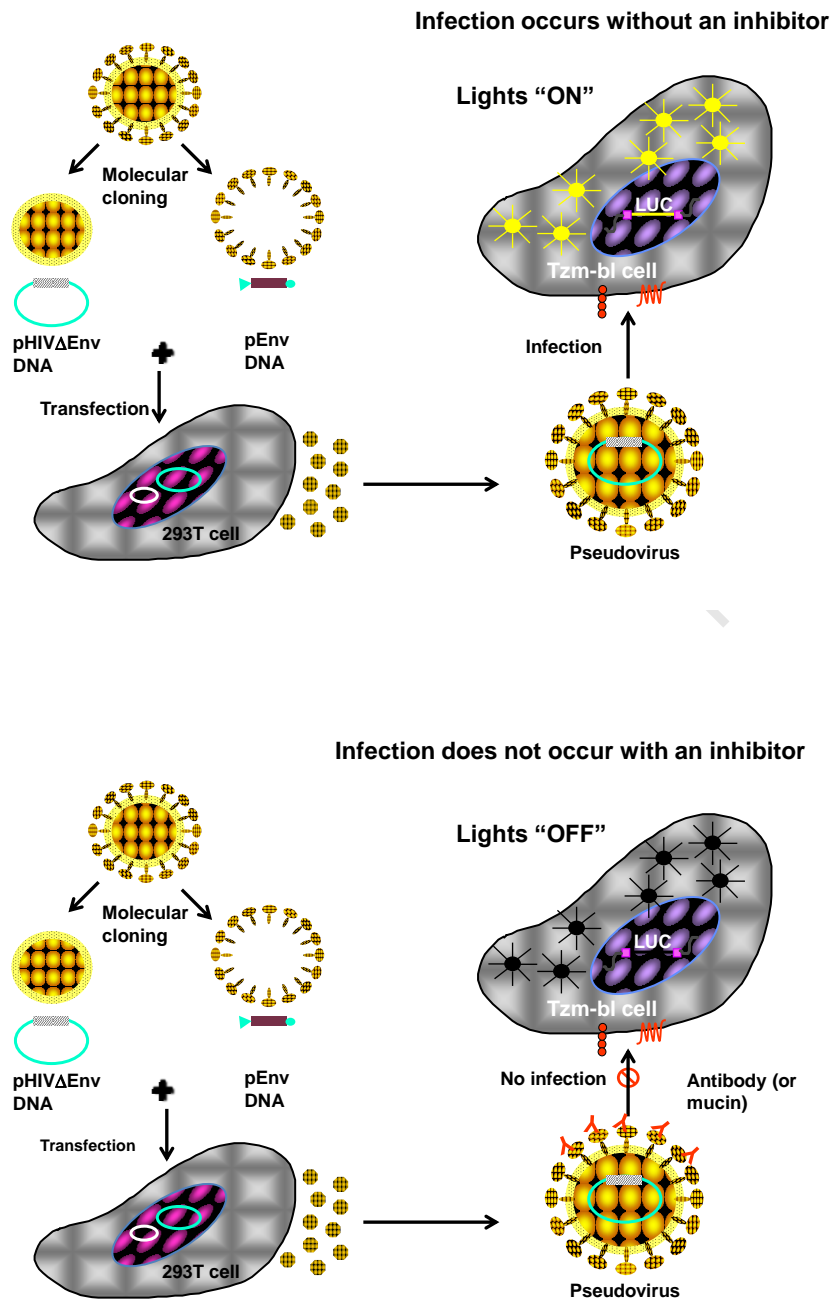


Figure 2.1 Sequential events in detection neutralization of Env-pseudotyped viruses in TZM-bl cells, reproduced from Montefiori (presentation) with slight modifications.

2.21 HIV pseudovirus neutralization assay using a luciferase reporter gene in TZM-bl cells

Virus neutralization was measured using a luciferase based assay in TZM-bl cells as previously described by Montefiori, (2005) with modification. This assay measures the reduction in luciferase reporter gene expression in TZM-bl cells following a single round of virus infection. Briefly, TZM-bl cells were added into a 96-well flat bottom microtitre plate (1×10^4 /well in a 50 μ l volume) in 10% DMEM growth medium containing DEAE-dextran at a final concentration of 7.5 μ g/ml. Assay controls included replicate wells of TZM-bl cells alone (cell control) and TZM-bl cells with virus (virus control). The TZM-bl cells were allowed to incubate for 26 hours before mucin samples and viruses were added. On a separate 96-well flat bottom microtitre plate, 3-fold serial dilutions of milk mucin samples (starting at 500 μ g/ml) were performed in duplicate in 10% DMEM growth medium (100 μ l/ well). An amount of diluted Du422.1 pseudovirus which would yield approximately 50000 RLU equivalent (50x dilution yields 50 000 RLU, viral dilution in each well is 1:4. dilution for virus and media that was added was 1:12.5) was added to each well in a volume of 50 μ l, and the plates were incubated for 1 h at 37°C. Then 150 μ l mucin and viral suspension was transferred into the 96-well flat bottom plate containing cells.

Following 48 hour incubation at 37°C, 100 μ l culture medium was removed from each well and 100 μ l of Bright-Glo luciferase reagent was added. The cells were allowed to lyse for 2 min in the dark, and then 100 μ l of the cell lysate was transferred to a 96-well black solid plate, and luminescence was measured using a Victor 3 luminometer. The 50% inhibitory dose (ID₅₀) titre was calculated as the mucin dilution that caused a 50% reduction in relative luminescence units (RLU) compared to the level in the virus control wells after subtraction of cell control RLU. All data were analyzed using Graph Pad prism version 5.0 (Graph Pad, La Jolla, USA) dose response inhibition.

2.22 MTT colorimetric assay

The rate of replication of each of the TZM-bl cell lines in the presence of human breast milk mucins was determined by lympho-proliferative assay using MTT dye [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], according to the method described by Mosmann (1983), Bounous *et al.*, (1992) and Carmicheal *et al.*, (1987) with slight

modifications. The assay was carried out as follows. TZM-bl cells were harvested from a plate, pelleted by centrifugation (1000rpm for 5min) and resuspended in approximately 5ml of fresh growth medium (containing DMEM, FBS, HEPES and gentamycin). The viable cells were counted by trypan blue dye (1:4 10ul cells: 30ul trypan blue) exclusion test and the cell count was adjusted to 1×10^5 cells/ml. In plate 1, a suspension of 50µl/well (1×10^4) cells in media was plated with 50µl growth media into each well. To serve as blank, all wells in column 1 (from A-H) were plated with only media. Column 2 wells (from A-H) were plated with media and cells to serve as the cell control. Other wells in column 3 had media, cells and solubilization reagent in order to serve as an example to show cell death. The cells were allowed to incubate for 26 hours before adding breast milk mucins. In a separate 96 well plate 2, each crude milk or purified milk mucin sample was done in triplicate. The initial volume of the samples was 250ul with a starting concentration of 250µg/ml in DMEM media. The samples were 2 fold serially diluted down in 125ul media. After the samples were diluted, 100µl of each diluted mixture from plate 2 was transferred to plate 1 which contained 50µl cells and 50µl growth media to make a final volume of 200µl. The blank (200µl GM), cell control (150µl GM, 50µl cells) and cells with solubilization agent (140µl GM, 50µl cells, 10µl solubilization agent) did not have mucins, but were each adjusted to have a final volume of 200µl. The plate was incubated after adding mucins at 37°C in 5% CO₂ for 44 hours before adding 20µl MTT dye (5 mg/ml of phosphate buffered saline (pH 7.2) stock, Sigma) to each well. The plate was incubated for another 4 hours before discarding 100µl from each well and then adding 100µl of dimethylsulfoxide (DMSO, Sigma) to all the wells to dissolve the formazan crystals. The plate was incubated overnight before reading the optical density (OD) at a test wave length of 490 nm and reference wavelength of 650 nm on UVmax kinetic microplate reader (Molecular Devices) replication index (RI) was calculated from the formula $RI = OD \text{ of SILs at 24 h} / OD \text{ of SILs at 0 h}$.

CHAPTER 3

THE ISOLATION, PURIFICATION AND IDENTIFICATION OF HUMAN BREAST MILK MUCIN

3.1 Introduction

This chapter describes the purification of milk mucins from the crude milk sample. The milk fat globule membrane (MFGM) was prepared from crude milk before the mucins could be isolated from it for characterization purposes. The MFGM was prepared by defatting the breast milk by centrifugation at 3 000g for 1 hour at 4°C. Milk mucins were isolated by Sepharose CL-4B gel filtration and purified by isopycnic density gradient centrifugation in 3.5M caesium chloride and 4M guanidinium chloride. The presence and the purity of milk mucins in the breast milk were assessed by SDS-PAGE gels which were stained for carbohydrate with PAS and for protein with Coomassie Blue stain R-250. The identity of MUC1 and MUC4 was confirmed by Western blotting and amino acid analysis.

3.2 Results

3.2.1 The SDS-PAGE analysis of crude MFGM material

The SDS-PAGE analysis of the prepared milk fat globule membrane (non-purified) was done in order to confirm the presence of mucins. The MFGM samples (50µl) from HIV positive and HIV negative patients were prepared in reducing gel loading buffer and subjected to 10% SDS-PAGE and the gels were stained with PAS, a carbohydrate stain and Coomassie Brilliant Blue, which stains for protein. The SDS-PAGE gels from the HIV positive MFGM (Figures 3.1 A) and HIV negative MFGM (Figures 3.1 B) showed the presence of high molecular weight PAS positive bands which were found on top of the stacking gel as well as some that have entered the running gel. The mucins that have entered the running gels for both groups seem to vary in intensity and appear as rather broad bands or even smears in the region of molecular weight ($M_r \sim 220\text{kDa}$, and spread as far down as 100kDa). The MFGM from the HIV positive group (Figures 3.1 A) seem to have more intense staining than the MFGM from the HIV negative (Figures 3.1 B), although there is an inter-individual variation from sample-to-sample within each of the groups. Whilst all the $M_r \sim 220\text{kDa}$ bands are visible for the HIV

positive group (Figure 3.1A lanes 2-9), the most prominent bands in this region for the HIV negative group are in lanes 2 and 8 (Figure 3.1B), with bands in the other lanes being of lesser intensity (Figure 3.1A, lanes 3-7). The Coomassie Brilliant Blue stained gel (Figure 3.2) shows that the MFGM material from both groups has other contaminant proteins other than the mucins of interest. However there is an inter-individual variation of size and smear-like material in the region of $M_r \sim 100-220\text{kDa}$, together with a distinct and separate band below this smear at roughly 50 kDa in some samples (Figure 3.2 lanes 4-7 and 9-12).

3.2.2 Sepharose CL-4B gel filtration of the crude milk fat globule membrane material

Sepharose CL-4B gel filtration was used for the separation of MFGM-I from MFGM-II. Twenty (20) ml of the MFGM material was chromatographed on a Sepharose CL-4B column (height 20cm, diameter 2cm and bed volume 100ml) and eluted with 0.2M NaCl, 0.02% NaN_3 at a flow rate of 2 min/tube. Figure 3.3 shows a profile of a representative sample from each group. The elution profiles for the HIV positive group (Figure 3.3A) and HIV negative group (Figure 3.3B) show that the MFGM material elutes as two peaks in both groups. The first peak eluted in the void volume (V_o) (MFGM-I) whilst the second peak eluted in the included volume (V_i) (MFGM-II). The HIV positive group (Figure 3.3A) had two large distinct peaks while the HIV negative group showed one small peak and one large peak. The MFGM-I and MFGM-II peaks from the HIV positive group showed more mucin which was assayed by PAS than the peaks from the HIV negative group as shown on the elution profiles. There was a background of protein (Bradford assay 490nm) in both groups.

The difference in the staining intensity of mucins between the two groups was confirmed by separating the freeze dried MFGM-I and MFGM-II under the peaks in both groups on a 10% SDS-PAGE after being dissolved in sample application buffer containing 0.2M 2-mercaptoethanol and staining with PAS for carbohydrates. The PAS staining was more intense in the HIV positive (Figure 3.4A, lanes 2-9) than in the HIV negative group (Figure 3.4B, lanes 2-7) for equal loading, in a region of about $M_r \sim 170\text{ kDa}$. The MFGM-I material from both groups (Figure 3.4) shows more intensely stained mucins than the MFGM-II material. In both groups, MFGM-I has less contaminant proteins than MFGM-II as shown in Figure 3.5, stained for protein by Coomassie Brilliant Blue. Again there is more intense staining in the HIV positive group (Figure 3.5A) than in the HIV negative group (Figure 3.5B).

3.2.3 Western blotting analysis of semi-crude (post gel filtration) MFGM-I and MFGM-II material

Western blotting analysis was done in order to confirm the identity of the mucins in the MFGM-I and MFGM-II samples. Freeze dried material from MFGM-I (400 μ g) and MFGM-II (400 μ g) from the HIV positive and HIV negative groups dissolved in sample application buffer containing 0.2M 2-mercaptoethanol were separated using 0.7% agarose gel electrophoresis and then transferred to a nitrocellulose membrane. The membranes were probed with mouse anti-MUC1 monoclonal antibody (Figure 3.6) and mouse anti-MUC4 monoclonal antibody (Figure 3.7).

The results showed that the MUC1 and MUC4 were detected in both groups. Figure 3.6; lane 1 is a bronchial lavage which was found to be positive for MUC1. Figure 3.6; lane 2 is a cervical mucin which is a positive control. Intense staining was seen for the V_o (MFGM-I) material for MUC1 in the HIV positive group (Figure 3.6 lanes 3, 5, 7) than the V_i (MFGM-II) material (Figure 3.6 lanes 4, 6, 8). Interestingly, the V_i material from one patient (Figure 3.6 lanes 10) was as intense but not as widely smeared as that from the V_o (MFGM-I) group (Figure 3.6 lanes 9). There was obvious inter-individual variation in both the HIV positive and the HIV negative groups for both peaks. The HIV negative group had far less material in both the V_o and V_i peaks with the V_i peaks hardly having material that was detectable.

MUC4 was also detected in both the HIV positive and the HIV negative group. Figure 3.7; lane 1 is a bronchial lavage which is positive for MUC4. Figure 3.7; lane 2 is a cervical mucin which is a positive control. There seemed to be more MUC4 in the samples from the HIV positive group (Figure 3.7; lanes 3-10) than the HIV negative group (Figure 3.7; lanes 11-18), and in both groups, the MFGM-I material (Figure 3.7; lanes 3, 5, 7, 9, 11, 13, 15, 17) seemed to have more MUC4 than the MFGM-II material (Figure 3.7; lanes 4, 6, 8, 10, 12, 14, 16, 18). There seemed to be no detectable MUC4 in the MFGM-II material in the HIV negative group (Figure 3.7; lanes 12, 14, 16, 18)

3.2.4 Purification of MFGM-I material from the Sepharose 4B gel filtration column by caesium chloride isopycnic density gradient centrifugation

The MFGM-I from the HIV positive and HIV negative groups had more mucins and less contaminant proteins and nucleic acid in comparison to the MFGM-II as shown on the gels and Western blots. The MFGM-I from both groups were further purified by caesium chloride isopycnic density gradient centrifugation. Representative profiles of purification are shown in Figure 3.8A for the HIV positive group and Figure 3.8B for the HIV negative group. These profiles show a clear separation between proteins (Bradford 595nm) and the mucins which were assayed by PAS (555nm).

In order to confirm purification, the purified MFGM-I samples from both groups were separated on 10% SDS-PAGE and stained with PAS and Coomassie Brilliant Blue R-250. Figure 3.9, lanes 2-5 shows the SDS-PAGE gel stained with PAS which shows that the mucins were present in both groups (lanes 2-3 from the HIV positive group and lanes 4-5 from the HIV negative group, arrow at the beginning of the running gel). The SDS-PAGE gel stained with Coomassie Brilliant Blue R-250 (Figure 3.9, lanes 6-9) showed proteins detected in the same region. Figure 3.9, lanes 6-7 are HIV positive samples and lanes 8-9 are samples from the HIV negative group.

3.2.5 Amino acid composition of the human breast milk mucins from the purified MFGM-I material

The identity of the MFGM-I from the HIV positive and HIV negative groups was further confirmed by amino acid analysis. Serine, threonine and proline amounted to a mean of 11.5%, 11.8% and 17.7% for the HIV positive (Table 3.1) and 11.2%, 11.0% and 14.8% for the HIV negative group mucins (Table 3.2). For the HIV positive group, the amount of serine threonine and proline added up to 41% of the MFGM-I, while they added up to 37% of the MFGM-I for the HIV negative group.

3.3 Discussion

The main objectives of this chapter were to isolate, purify and characterise mucins from the human breast milk of patients with and without HIV-1 infection. Crude breast milk samples were diluted with cold 6M guanidinium hydrochloride (GuHCl) buffer in a ratio of 1:5 GuHCl : milk. The 6M GuHCl contained a cocktail of protease inhibitors such as EDTA, NEM and PMSF to prevent endogenous proteolysis (Carlstedt *et al.*, 1983). EDTA chelates ions, PMSF is a serine proteinase inhibitor and NEM inhibits thiol proteinases (Carlstedt *et al.*, 1983).

The mucin apoprotein backbone has a glycosylated region of densely packed carbohydrate side chains which protects the protein core from proteolytic digestion and a naked non-glycosylated part which is susceptible to proteolysis (Snary and Allen, 1971). Guanidinium hydrochloride is a strong chaotropic agent that denatures the non-covalent bonds of mucin and renders it in its primary state (Francis and Bradford, 1976). Potential destructive methods such as sonication and high speed homogenization were avoided for solubilization of milk mucus.

The milk fat globule membrane was prepared from the cream fraction of the milk by defatting through centrifugation at 3000g for 1 hour at 4°C (Habte *et al.*, 2008). The MFGM had to be processed as the mucins in human breast milk are of the trans-membrane type and are embedded within the milk fat globule membrane as shown in Figure 1.1. Sepharose CL-4B gel filtration separated the MFGM samples into two peaks, which were labelled MFGM-I and MFGM-II respectively. MFGM-I had the mucin rich fractions while the MFGM-II contained some mucin but more associated protein. The mucins in the MFGM-I and MFGM-II were identified by Western blotting analysis, which confirmed that MFGM-I had increased amounts of MUC1 and MUC4 in comparison to MFGM-II, in both the HIV positive and negative groups. The MFGM-I from both groups was further purified by caesium chloride isopycnic density gradient centrifugation. Amino acid analysis of the MFGM-1 material with its characteristically high serine, threonine and proline content confirmed its identity as mucin for both groups (Andersch-Björkman *et al.*, 2007). However amounts of these amino acids were higher than those reported for mucins by Allen (1981) but comparable to those for mucin extracted in 6M GuHCl as shown by Mall (1988-PhD).

Before any further analysis could be performed, MFGM had to be prepared. This MFGM surrounds the lipid droplets in milk and it arises during a process in which intracellular lipid droplets are enveloped as they bud off from the apical membrane by apocrine secretion and are released into the milk. Mucin glycoproteins are the major constituents of these membranes. The presence of mucins in the MFGM samples were assayed by SDS-PAGE gels which were stained with PAS which stains the vicinal diol groups of the peripheral sugars and sialic acids (Thornton *et al.*, 1996). The SDS-PAGE gel results of the crude MFGM material from the HIV positive group (Figure 3.1A) showed the presence of mucins on the top of the stacking gel with the molecular weight >220 kDa and others that have entered the running gel with the molecular weight of >100 kDa but ≤ 220 kDa. The gel of MFGM samples from HIV negative group (Figure 3.1B) also presented with similar findings with the mucins on the stacking gel with a molecular weight of >220 kDa and others that have entered the running gel with a molecular weight of >100 kDa but ≤ 220 kDa. Previous reports suggest that the bands in the stacking gel represent MUC4 (which was previously known as MUCX) and the lower molecular band, MUC1 (Newburg *et al.*, 1990) is represented on the running gel.

There is an obvious variation in the staining intensity behaviour of the mucins between the HIV positive and the HIV negative group. The HIV positive group shows more intense PAS staining on the SDS-PAGE than the HIV negative group. Inter-individual variation in intensity was also noted within samples of the same group suggesting that there was no clear cut difference in the electrophoretic behaviour of mucins for these for HIV positive or negative groups. In some cases, the HIV positive (Figure 3.1A, lanes 2, 5, 9) and the HIV negative group (Figure 3.1B, lanes 2, 8, 9) had several bands that were more intensely stained than others. The Coomassie Brilliant Blue stained gel (Figure 3.2) shows that the MFGM material from both groups have other contaminant proteins other than the mucins of interest in the region of $M_r \sim 100$ -220kDa and a separate band below this smear at roughly 50 kDa in some samples

After the presence of mucins was confirmed in the MFGM samples from both groups, the MFGM material was semi-purified by Sepharose CL-4B gel filtration. Two peaks for both the HIV positive (Figure 3.3A) and HIV negative (Figure 3.3B) groups were observed, and these were named MFGM-I and MFGM-II peaks. The peaks from the HIV positive group (Figure 3.3A) were distinct, while the peaks from the HIV negative group (Figure 3.3B) had one small peak and one large peak, for reasons we cannot explain. In both cases increased levels

of mucins were isolated in the MFGM-I peak as shown on the SDS-PAGE gels stained with PAS (Figure 3.4). The MFGM-II peaks for both groups had more contaminant protein and less mucin as shown by the Coomassie Brilliant Blue stained gels (Figure 3.5).

The identity of the isolated mucins from the MFGM-I and MFGM-II was confirmed by Western blotting analysis to be MUC1 and MUC4. When separated on SDS-PAGE MUC1 is found on top of the running gel and it is smaller than MUC4 which is found on top of the stacking gel (Newburg *et al.*, 1990). The detection of MUC1 mucins by Western blotting analysis in both groups (Figure 3.6) appears to have larger smears which run close to the top of the gel, whilst the MUC4 mucins (Figure 3.7) have smaller smears and running lower in the gel. This might appear as if MUC1 is larger than MUC4 but it is not the case. The explanation for this is that the mouse anti-MUC1 monoclonal antibody that was used is specific to the carbohydrate epitope of the human MUC-1 glycoprotein which is between 130-300 kDa, while the human MUC4-specific 1G8 monoclonal antibody recognized an epitope in the central portion of the ASGP-2 between the 53aa at the N- terminus and the trans-membrane domain which is 80 kDa. The antibody used for MUC4 was not specific to the larger 850 kDa membrane bound ASGP-1 subunit. Both MUC1 and MUC4 were found in the MFGM-I in greater intensity (suggesting larger amounts) than for MFGM-II for both groups (Figure 3.6 and Figure 3.7). Despite their charge differences both MUC1 and MUC4 are large enough and share the same carbohydrate structure to elute in the excluded volume of the column (Patton *et al.*, 1995). Trace amounts of MUC1 and MUC4 was also observed in the MFGM-II material of the HIV positive group but even more difficult to detect in the HIV negative samples. It is possible that the elution of these mucins in the included volume, though large enough to be excluded on Sepharose 4B gel filtration, were a result of small amounts of mucin sticking to the Sepharose material and resisting elution during column equilibration and washing with buffer in between sample loadings. In this study we were also unable to separate MUC1 from MUC4 to test their inhibitory potential against HIV-1 because of time constraints. However further work has been planned in this regard.

Because almost all the mucin eluted as MFGM-I for both HIV positive and negative groups we considered it to be worthwhile to further purify the MFGM-I material. This purification was done successfully by isopycnic density gradient centrifugation in caesium chloride (Creeth and Denborough, 1970) and 4M guanidinium chloride (Carlstedt *et al.*, 1983) which gives mucins a buoyant density between 1.39-1.40g/ml for both groups. Purification profiles

are shown in Figure 3.8A for HIV positive MFGM-I and Figure 3.8B for HIV negative MFGM-I. The fractions with the highest PAS peak were recovered between the densities 1.32-1.41g/ml and 1.39-1.46g/ml respectively, and with the lowest protein levels. The extent of purification was monitored by SDS-PAGE analysis which showed an increased purity of the material, leaving large bands on the stacking and beginning of the running gels (Figure 3.9).

Mucin purity and identity was further confirmed by amino acid analysis of the MFGM-I material further purified by density gradient centrifugation. Although there was hardly a difference in the amount of serine, threonine and proline between the HIV positive and negative groups (41% and 37% respectively), the values obtained in this study for the percentage amounts of serine, threonine and proline per total amounts of amino acids are higher than previously reported (Allen, 1981). These amounts do vary and some researchers have reported higher percentages per total amino acids for serine, threonine and proline (Mall PhD thesis). This was due to the use of denaturing media in the extraction of mucins from crude mucus gels. It should also be noted that the studies on salivary mucin in HIV-AIDS in our laboratory, and the approach to their purification have, to our knowledge never been reported previously and the data here could well reflect the true amounts of serine, threonine and proline in MFGM-I material of saliva.

Essentially, MFGM-I and MFGM-II were separated by Sepharose 4B and showed two bands on SDS-PAGE which were stained with PAS, and were identified as MUC1 and MUC4 on Western blotting analysis.

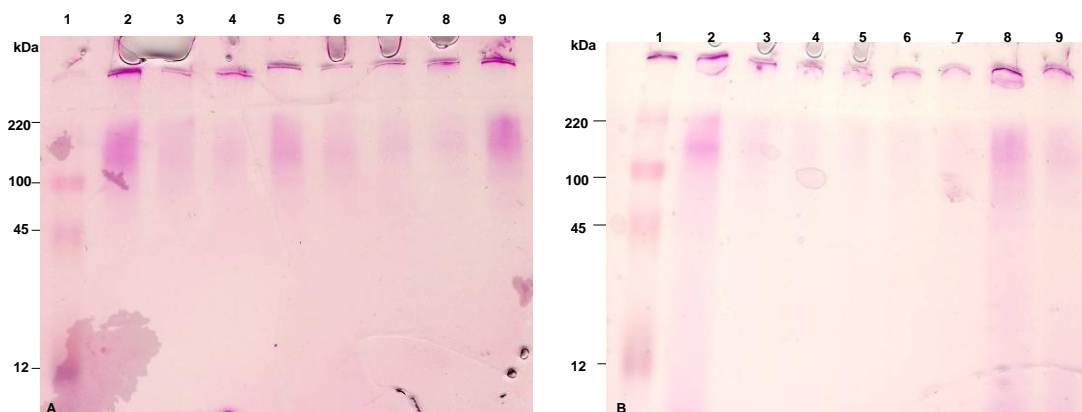


Figure 3.1 10% SDS-PAGE analysis of the non-purified milk fat globule membranes stained with PAS. Milk fat globule membrane (MFGM) samples (50ul) were prepared in reducing gel loading buffer and subjected to 10% SDS-PAGE and the gels were stained with PAS for carbohydrates. Figure A illustrates the presence of mucin glycoproteins in MFGM from HIV positive samples. Lane 1: protein molecular weight marker. Lanes 2-9: HIV positive MFGM samples. Figure B illustrates the mucins in MFGM from HIV negative samples. Lane 1: protein molecular weight marker. Lanes 2-9: HIV negative MFGM samples.

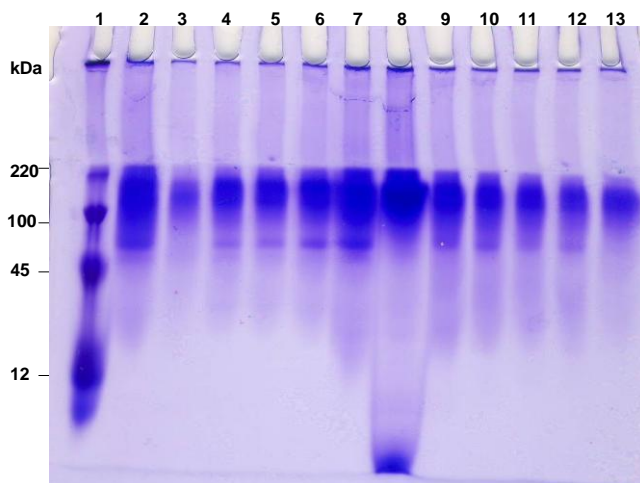
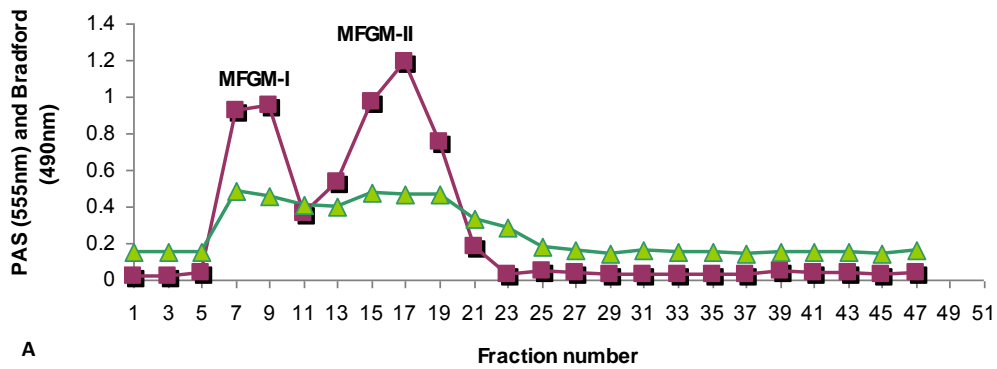
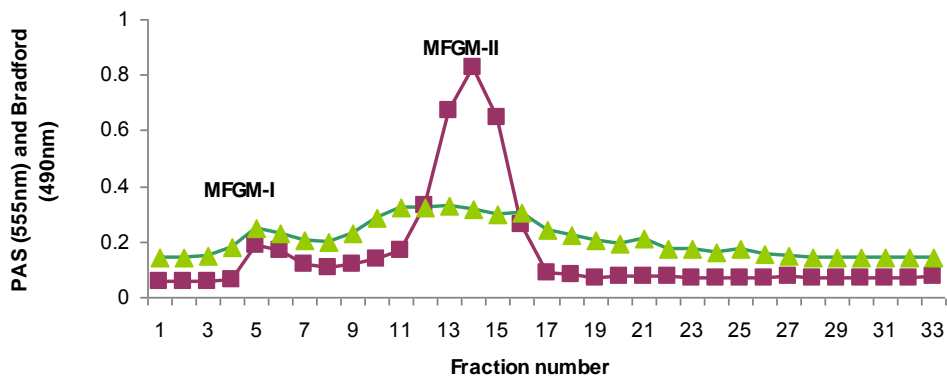


Figure 3.2 10% SDS-PAGE analysis of the non-purified milk fat globule membranes stained with Coomassie Brilliant Blue R-250. Samples of the milk fat globule membrane (50ul) were prepared in reducing gel loading buffer and separated on a 10% SDS-PAGE. The gel was stained for contaminant protein with Coomassie Brilliant Blue R-250. Lane 1: molecular weight marker. Lanes 2-7: HIV positive MFGM samples. Lanes 8-13: HIV negative MFGM samples.



A



B

Figure 3.3 Sephadex CL-4B gel filtration of the milk fat globule membrane (MFGM) material. An aliquot of MFGM (10ml) was chromatographed on a Sephadex 4B column and eluted with 0.2M NaCl, 0.02% NaN₃ containing protease inhibitors such as 10mM EDTA and 5 mM NEM, at the flow rate of 2 min/ tube at room temperature. The fractions were analysed for carbohydrates with PAS (red square) at 555nm and protein with Bradford (green triangle) assay at 490nm. Figures A and B are representative filtration profiles of MFGM from HIV positive and HIV negative groups respectively.

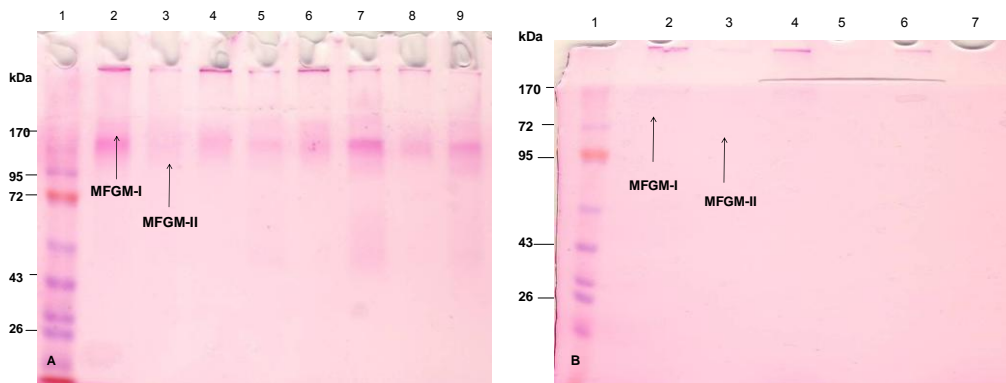


Figure 3.4 10% SDS-PAGE analysis of MFGM-I and MFGM-II milk mucins stained for carbohydrate with PAS stain. Freeze dried MFGM-I (100 μ g) and MFGM-II (100 μ g) respectively were prepared in reducing gel loading buffer and separated on a 10% SDS-PAGE. The gels were stained for carbohydrate with PAS stain. Figure A shows the presence of mucins detected from MFGM-I and MFGM-II from HIV positive samples. Lane 1: molecular weight marker. Lanes 2, 4, 6, 8: represents MFGM-I mucins from the HIV positive group. Lanes 3, 5, 7, 9: represents MFGM-II mucins from the HIV positive group. Figure B shows the presence and absence of mucins in the MFGM-I and MFGM-II respectively from the uninfected group. Lane 1: molecular weight marker. Lanes 2, 4, 6: represents MFGM-I mucins from the HIV negative group. Lanes 3, 5, 7: represents MFGM-II mucins from the HIV negative group.

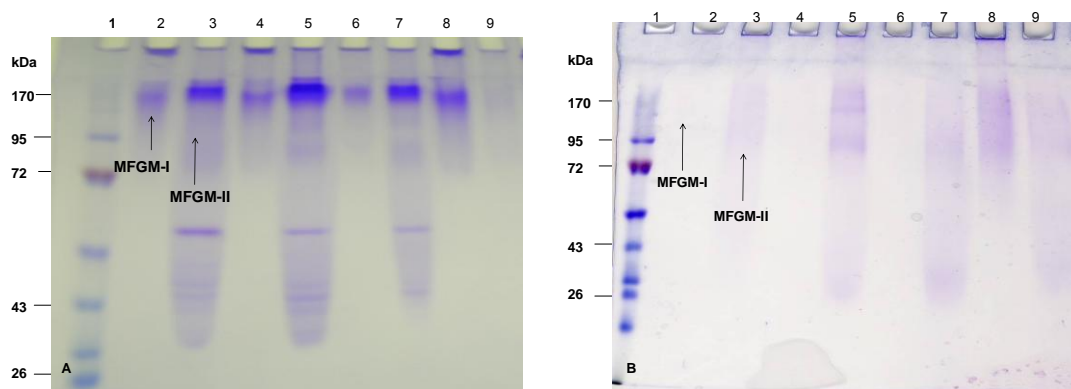


Figure 3.5 10% SDS-PAGE analysis of MFGM-I and MFGM-II milk samples stained for contaminant protein with Coomassie Brilliant Blue R-250. Freeze dried MFGM-I (100 μ g) and MFGM-II (100 μ g) respectively were prepared in reducing gel loading buffer and separated on a 10% SDS-PAGE. The gels were stained for protein with Coomassie Brilliant Blue R-250. Figures A and figure B both show the presence of proteins in MFGM-I and MFGM-II from the HIV positive (Figures A) and HIV negative (Figures B) sample groups. Figure A lane 1: molecular weight marker. Lanes 2, 4, 6, 8: represents contaminant proteins in the MFGM-I peak from the HIV positive group. Lanes 3, 5, 7, 9: represents contaminant proteins in the MFGM-II peaks from the HIV positive group. Figure B shows the presence and absence of mucins in the MFGM-I and MFGM-II respectively from the uninfected group. Lane 1: molecular weight marker. Lanes 2, 4, 6, 8: represents contaminant proteins in the MFGM-I peak from the HIV negative group. Lanes 3, 5, 7, 9 : represents contaminant proteins in the MFGM-II peaks from the HIV negative group.

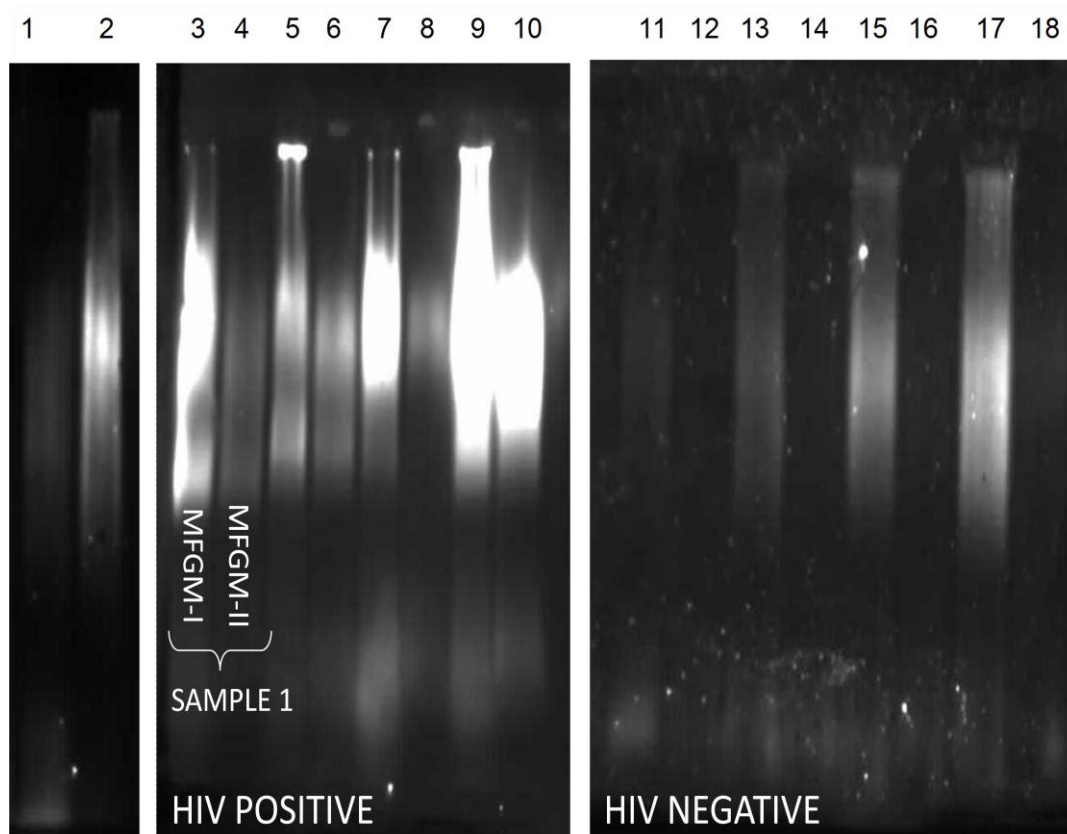


Figure 3.6 Western blotting analysis of MFGM-I and MFGM-II using mouse anti-MUC1 monoclonal antibody. Freeze dried MFGM-I (400 μ g) and MFGM-II (400 μ g) were separated on 0.7% agarose gel electrophoresis and then transferred to a nitrocellulose membrane before blocking overnight. Mouse anti-MUC1 monoclonal antibody was used as the primary antibody with rabbit anti-mouse horse radish peroxidase as the secondary antibody. Lane 1: MUC1 detected in bronchial lavage freeze dried sample. Lane 2: MUC1 detected in purified cervical mucins. Lanes 3-10 shows the detection of MUC1 in samples from HIV positive, while lanes 11-18 shows the presence of MUC1 in MFGM samples from HIV negative patients. Lanes 3, 5, 7, 9: represents MUC1 detected in MFGM-I from the HIV positive samples. Lanes 4, 6, 8, 10: represents MUC1 detected in MFGM-II from the HIV positive samples. Lanes 11, 13, 15, 17: represents MUC1 in MFGM-I from the HIV negative samples. Lanes 12, 14, 16, 18: represents MUC1 in MFGM-II from the HIV negative samples.

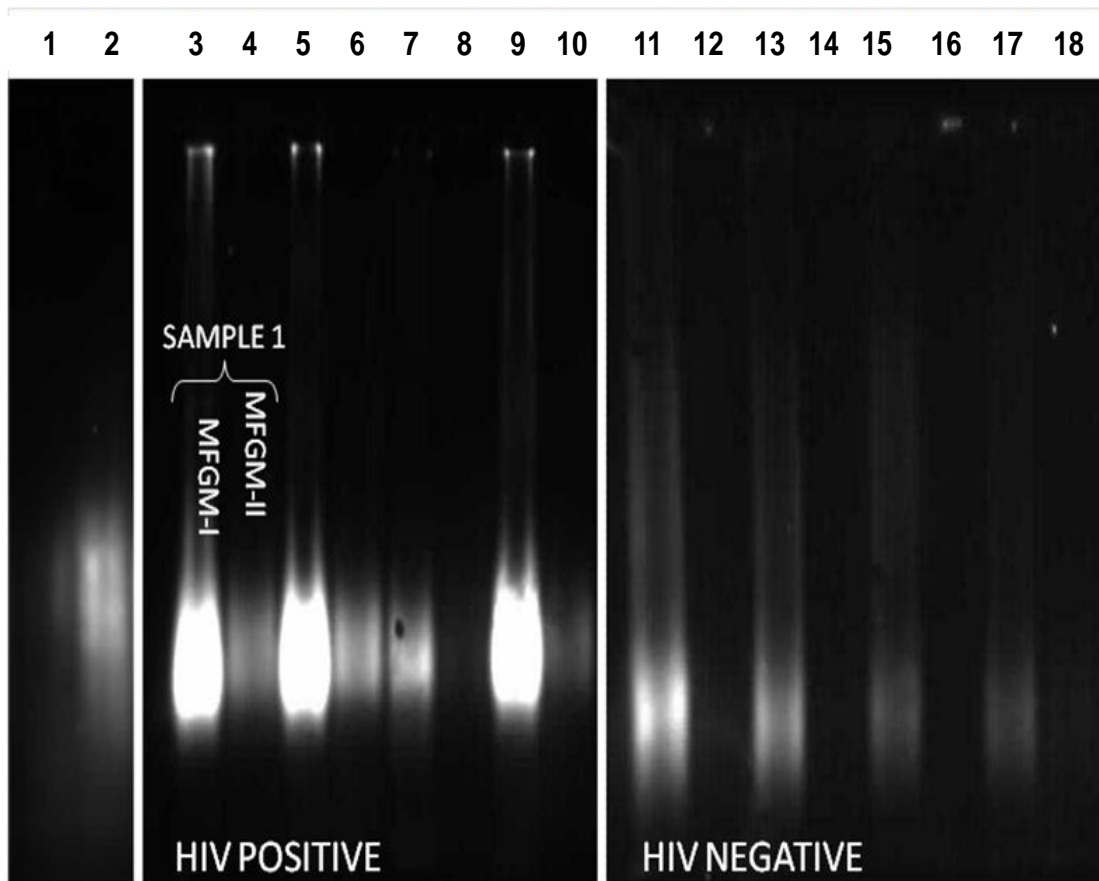


Figure 3.7 Western blotting analysis of MFGM-I and MFGM-II using mouse anti-MUC4 monoclonal antibody. Freeze dried MFGM-I (400 μ g) and MFGM-II (400 μ g) were separated on a 0.7% agarose gel electrophoresis and then transferred to a nitrocellulose membrane before blocking overnight. Mouse anti-MUC4 monoclonal antibody was used as the primary antibody with rabbit anti-mouse horse radish peroxidase as the secondary antibody. Lane 1: MUC4 detected in bronchial lavage. Lane 2: MUC4 detected in purified cervical mucins. Lanes 3-10 shows the detection of MUC4 in samples from HIV positive, while lanes 11-18 shows the presence of MUC4 in MFGM samples from HIV negative patients. Lanes 3, 5, 7, 9: represents MUC4 detected in MFGM-I from the HIV positive samples. Lanes 4, 6, 8, 10: represents MUC4 detected in MFGM-II from the HIV positive samples. Lanes 11, 13, 15, 17: represents MUC4 in MFGM-I from the HIV negative samples. Lanes 12, 14, 16, 18: represents MUC4 in MFGM-II from the HIV negative samples.

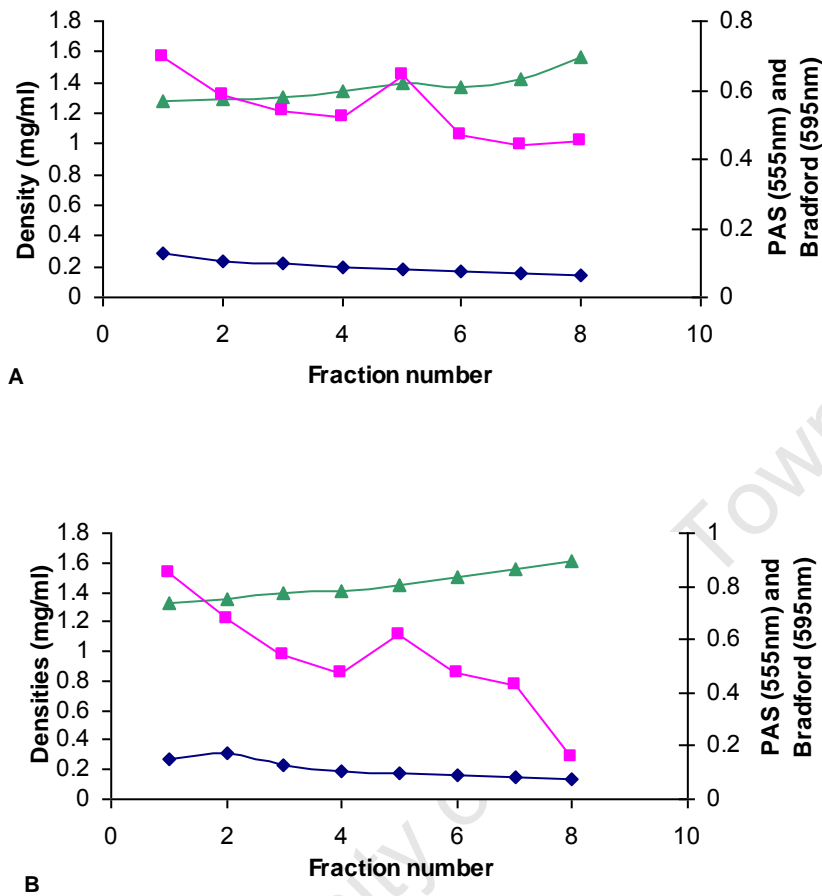


Figure 3.8 CsCl density-gradient centrifugation of HIV positive (A) and HIV negative (B) MFGM-I sample material. The purification of mucins after centrifugation step in a CsCl density gradient. Solid CsCl and 4M GuHCl containing 10mM EDTA, 5mM NEM and 0.05% CHAPS pH 6.5 were added to the semi-purified mucin rich MFGM-I obtained from the Sepharose CL-4B gel filtration to give a starting density of 1.39-1.40g/ml. After centrifugation the tubes were fractionated into 8 equal fractions and an aliquot of each fraction was used for density measurements and the estimation of mucin and protein. The figures A and B shows a clear separation of proteins detected by Bradford (◆), from the mucin rich fractions 4, 5 and 6 assayed by PAS (■) at density (▲) of 1.32 - 1.41g/ml from HIV positive and HIV negative groups.

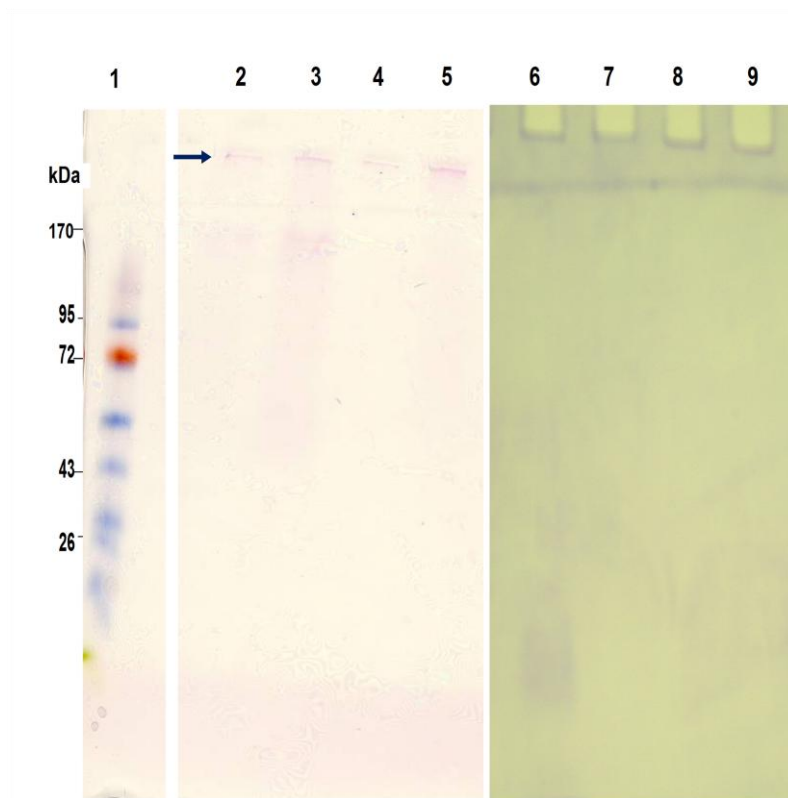


Figure 3.9 SDS-PAGE analyses of the purified milk mucins from MFGM-I from HIV positive and HIV negative samples. The gels were stained with PAS (lanes 1-5) or Coomassie Brilliant Blue (lanes 6-9). Lane 1(Molecular weight marker), lanes 2 and 3 (arrow indicates PAS stained mucins on the running gel from the HIV positive purified MFGM-I), lanes 4 and 5 (indicate PAS stained mucins on the stacking gel and from the HIV negative purified MFGM-I), lanes 6 and 7 (shows that there were no contaminant protein on gel after ultracentrifugation in CsCl density gradient purification from the HIV positive purified MFGM-I sample), lanes 8 and 9 (shows that no contaminant protein was detected on purified HIV negative MFGM-I peaks).

Amino acids	One letter	
	symbol	$\mu\text{mol } \%$
Aspartic acid	D	7.5
Threonine	T	11.8
Serine	S	11.5
Glutamic acid	E	8.8
Proline	P	17.7
Glycine	G	5.4
Alanine	A	8.0
Valine	V	5.1
Methionine	M	1.1
Isoleucine	I	2.8
Leucine	L	4.3
Tyrosine	Y	1.4
Phenylalanine	F	1.4
Lysine	K	1.4
Histidine	H	10.8
Arginine	R	1.1

Table 3.1 Amino acid composition of the HIV positive human breast milk mucins. The peptide bonds between the amino acid residues that are present in the mucin protein molecule were hydrolysed separated, detected by HPLC and the analysis of the resulting derivatised amino acids which allowed for the estimation of amino acid composition.

Amino acids	One letter symbol	$\mu\text{mol } \%$
Aspartic acid	D	6.8
Threonine	T	11.0
Serine	S	11.2
Glutamic acid	E	8.9
Proline	P	14.8
Glycine	G	5.8
Alanine	A	7.6
Valine	V	5.0
Methionine	M	0.9
Isoleucine	I	2.7
Leucine	L	4.1
Tyrosine	Y	1.2
Phenylalanine	F	1.2
Lysine	K	3.3
Histidine	H	14.6
Arginine	R	3.7

Table 3.2 Amino acid composition of the HIV negative human breast milk mucins. The peptide bonds between the amino acid residues that are present in the mucin protein molecule were hydrolysed separated, detected by HPLC and the analysis of the resulting derivatised amino acids which allowed for the estimation of amino acid composition.

CHAPTER 4

THE INVESTIGATION OF HUMAN BREAST MILK MUCINS BY HPLC

4.1 Introduction

The work described in this study was done at the University of Manchester in the laboratory of Professor David Thornton under the supervision of Dr Karine Rousseau. The aim was to investigate the possible changes in the glycosylation of milk mucins in the two groups, namely the HIV positive and negative groups. The samples were separated into fractions using anion-exchange HPLC which separates the mucin glycoproteins based on their charge. Samples of fractions from ion-exchange HPLC were blotted onto nitrocellulose membrane (0.20 μ m pore size) using a Schleicher and Schuell Minifold II slot/dot blot apparatus as described by Thornton *et al.*, 1991. Mucin glycoproteins on the slot blots were estimated by the periodic acid Schiff (PAS) procedure of Mantle and Allen (1978) and the blots were scanned densitometrically. Agarose gel electrophoresis and Western blotting analysis confirmed the presence or absence of any glycoforms as the mucins with increasing charge density will have an increase in electrophoretic mobility.

4.2 Results

4.2.1 The periodic acid (PAS) profiles and agarose gel electrophoresis of fractions from the anion-exchange HPLC Mono Q of milk fat globule membrane (MFGM) samples

Freeze dried samples of the MFGM-I were brought into solution with 6M and 4M urea before being chromatographed on a Pharmacia MonoQ HR 5/5 ion-exchange column. Aliquots of fractions (100 μ l) from ion-exchange HPLC were blotted on the nitrocellulose membrane (0.20 μ m pore size). Staining of the dot blots with PAS showed the fractionation profiles of the mucins from the HIV positive (Figure 4.1A) and negative (Figure 4.2A) groups on the MonoQ HR 5/5 chromatogram, which was scanned by densitometry.

MUC1 in the HIV positive group

The PAS profile for the HIV positive MFGM samples (Figure 4.1B) shows evidence of three major population groups of mucin in this group. The three distinct populations of mucins on the chromatogram confirmed the different charge densities of the mucins eluted from the MonoQ HR 5/5 column (Figures 4.1B). Population I is from fractions 7-12, population II from fractions 15-20 whilst possible population III could be from fractions 26-46 with a few small peaks that make population III not as distinct as the peaks that were observed for populations I and II (Figure 4.1B). The fractions obtained from the HPLC were subjected to agarose gel electrophoresis in order to determine the electrophoretic behavior of these 3 population groups for MUC1 in the HIV positive group. The gels were blotted onto a nitrocellulose membrane before probing with anti-MUC1 antibodies. Three visible electrophoretic patterns were observed on the Western blots (Figure 4.3A).

Fractions 8-12, that is population I on the chromatogram (Figures 4.1 B) showed bands of limited electrophoretic mobility (Figure 4.3A, fractions 6-12) compared to population II, (Figure 4.3A, fractions 16-20) which display far greater electrophoretic mobility. The Western blot analysis of fractions 26-46 (population III) (Figure 4.3A, fractions 26-46) appear as smears, quite different from the mucins of populations I and II. The Western blot analysis result confirms the finding from the PAS profile.

MUC4 in the HIV positive group

When the HIV positive blot was probed with anti-MUC4 antibody (Figure 4.3B, fractions 6-44), the presence of MUC4 was shown in fractions 6-12 which all form part of population I on the PAS profile (Figure 4.1B). MUC4 was also shown to be present on fractions 26, 38-40 (Figure 4.3B, fractions 26, 38-40) which could form part of population III. The results show that MUC4 is in Population I and III (Figures 4.1B and 4.3B), with electrophoretic mobility not as wide as that for MUC1, and with an absence of mucin in population II.

MUC1 in the HIV negative group

The PAS profiles of the HIV negative MFGM were again divided into 3 populations according to their chromatogram profile and elution from the MonoQ HR 5/5 column (Figure 4.2B). A single distinct population is observed on the PAS profile on fractions 7-16 (Figure 4.2A) corresponding to population 1 from the MonoQ HR 5/5 column (Figure 4.2B). There seem to be some minor peaks present on fraction 30-34, possibly population II, as well as

fractions 43-46 which could be population III (Figure 4.2B). However the Western blot analysis of MUC1 from the HIV negative group (Figure 4.4A) showed mucins clearly in population I, again of limited electrophoretic mobility, with hardly any detectable mucins for populations II and III in keeping with the chromatogram and MonoQ HR 5/5 profile (Figures 4.2A and B). The MUC1 mucins in this HIV negative group do not vary in their electrophoretic mobility (Figure 4.4A) as much as those of the HIV positive group (Figure 4.3A).

MUC4 in the HIV negative group

When the blot was probed with anti-MUC4 antibody (Figure 4.4B), MUC4 was detected on fractions 6-14 (Figure 4.4B, fractions 6-14), which form part of population I as shown on the PAS profile on figure 4.2B. There seems to be faint traces of MUC4 detected on fractions 30-34 (Figure 4.4B, fractions 30-34), which could possibly explain the slight peak observed on the PAS profile (Figure 4.2B, fraction 30-34). This peak could potentially form population II for the HIV negative samples. The explanation of the increased peak observed on fractions 43-46 on the PAS profile is not known, as neither MUC1 nor MUC4 were detected on those fractions by Western blot. This peak (Figure 4.2B, fractions 43-46) could contain some other trans-membrane mucin MUC16 that has been reported to be present in human breast milk (Hatstrup *et al.*, 2008).

4.3 Discussion

Anion exchange chromatography of mucins on a MonoQ HR 5/5 column has been a successful method to separate mucins from a single source into different populations on the basis of charge. The presence of different glycoforms of particular mucins such as MUC5B in asthma have been shown (Thornton *et al.*, 1997). This MUC5B glycoform has been found in the mucus obtained from a cadaver of a patient that died in status asthmaticus, suggesting that the tenacious plug of mucus that blocked this patient's airways could be due to the presence of that extra MUC5B glycoform. It is important therefore to separate mucins from individual samples according to their charge to determine any association between different populations of mucin and disease.

Using a combination of dot blots, anion-exchange HPLC and agarose gel electrophoresis followed by Western blotting, three populations for MUC1 in the HIV positive group have

been shown, whilst in the HIV negative group there was only one population for mucin detectable for MUC1. The three mucin populations in the HIV positive group as shown on the PAS profile (Figure 4.1) reflects the different levels of sialic acid and/or sulphate substitution of the oligosaccharide chains on the mucin (Thornton *et al.*, 1994). The significance of this variation of MUC1 in the HIV positive group is yet to be elucidated. However a hint of this variation was first seen in the crude preparations of MFGM-1 mucins in that there seemed to be larger amounts of MFGM-1 eluting on Sepharose 4B gel filtration for the HIV positive group (Fig 3.3A) compared to the HIV negative group (Figure 3.3B) and still more clearly on Western blots of HIV positive material for MUC1 in the HIV positive group (Figure 3.6 fractions 3, 5, 7 and 9) compared to the HIV negative group (Figure 3.6 fractions 11, 13, 15 and 17). Clearly there always was more detectable mucin (both MUC1 and MUC4 but especially MUC1) in the HIV positive group. Figure 3.6A shows very clearly the variation in intensity, size of smear and electrophoretic mobility of MUC1 in the MFGM-I material in the HIV positive group compared to the HIV negative group (Figure 3.6B). The results in this chapter are in keeping with these earlier findings in that MUC1 in the HIV positive group is divided into 3 populations, clearly seen by dot blot analysis (Figure 4.1A), the Mono Q column profile (Figure 4.1B) and Western blotting against an anti-MUC1 antibody (Figure 4.3A). This wide variation for MUC1 in the HIV positive group is not seen for the HIV negative group (Figures 4.2A and B and Figure 4.4A). Again the HIV positive mucins in the crude preparation generally seemed less in amounts (Fig 3.4B, 3.5B and Fig 3.6 fractions 11, 13, 15 and 17) and had less electrophoretic mobility in the gels.

MUC4 seems to be a less important player in breast milk mucins in HIV-AIDS. Clearly there was more of it in the HIV positive group (Figure 3.7 fractions 3, 5, 7 and 9) than in the HIV negative group (Figure 3.7, fractions 11, 13, 15 and 17). MUC4 eluted as populations I and somewhat III from the anion exchange column and showed some variation in the HIV positive group (Figure 4.3B) but hardly any such variation in the HIV negative group (Figure 4.4B).

All this suggests that in breast milk from HIV positive patients, there is an associated variation of MUC1 in terms of different charged populations of mucins and less so of MUC4. The HIV negative group has less mucin and even less variation. It is intriguing that this is so considering that our laboratory has shown that salivary mucins inhibit HIV-1 in an in vitro assay (Habte *et al.*, 2010). Clearly here larger amounts and greater variation of mucin

especially MUC1 in the HIV positive group, should be examined further to determine whether there is reduction in mother-to-child transmission of HIV-1. This would require a larger well planned study in which breast milk is obtained from HIV positive mothers whose offspring may or may not be HIV positive?

Changes in the glycosylation of MUC1 that are associated with cancer have been reported and includes the incomplete synthesis of carbohydrate chains, generating new antigens exploitable as laboratory diagnostic markers, among which are T(Gal β 1 \rightarrow 3GalNAc- α 1-O-ser/Thr), Tn3(GalNAc- α 1-O-ser/Thr) and sialyl-Tn antigens, in a variety of cancers (Kannan *et al.*, 2003). However, limited information is available about the changes in MUC1 glycosylation that are associated with HIV-1 infection. This is the first report of a finding that shows the presence of MUC1 populations that are associated with HIV-1 infection.

Glycans on the cancer-associated mucin differ from those found on MUC1 that is expressed by normal mammary epithelial cells. The normal mucin contains the extended core 2 based glycans that are formed when N-acetylglucosamine [GlcNAc] is attached to the GalNAc of core 1 [Gal β 1-3GalNAc], while on the cancer mucin the glycans can be shorter and core 1 based, with a high level of sialic acid (Taylor-Papadimitriou *et al.*, 2002). There is no data that has yet explained how MUC1 populations occur due to infection with HIV. This study is only reporting the presence of these populations.

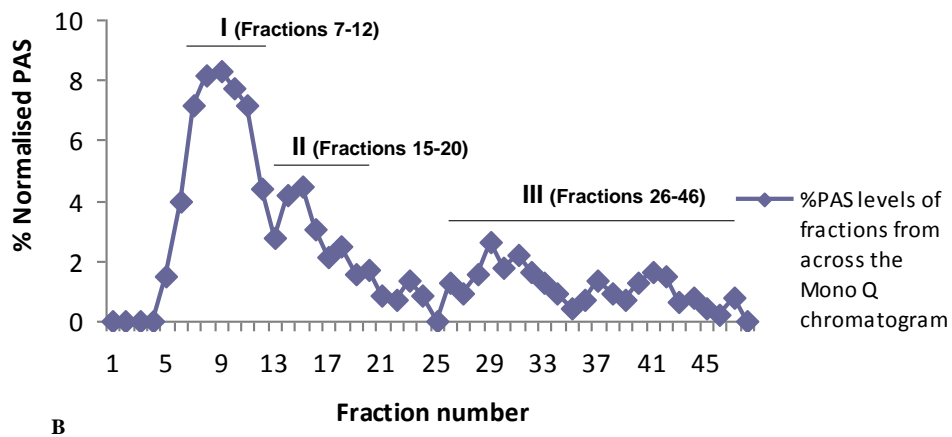
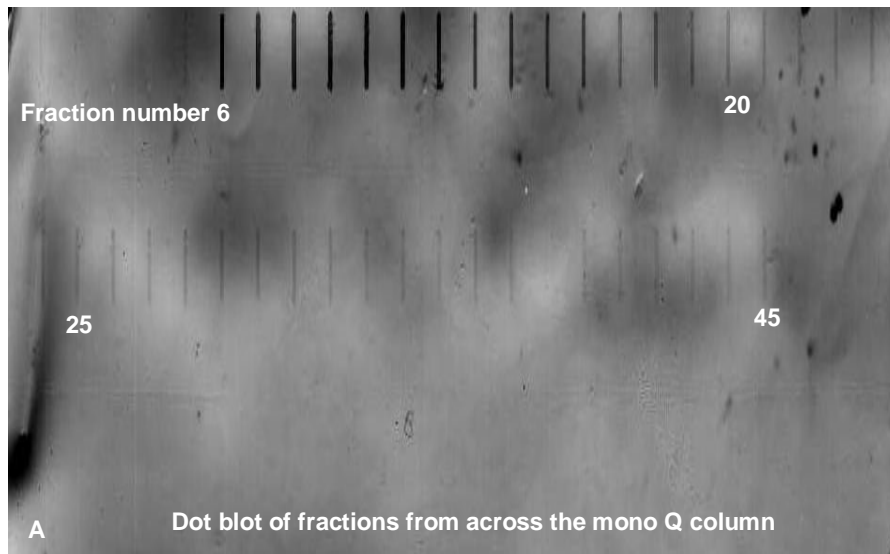


Figure 4.1 Anion exchange HPLC of MFGM-I from the HIV positive group. The high- M_r glycopeptides were chromatographed on a Pharmacia Mono Q HR 5/5 ion-exchange column. Samples of fractions (100 μ l) from ion-exchange HPLC were blotted on the nitrocellulose membrane (0.20 μ m pore size) (Figure A) before assaying the fractions for neutral sugars using the PAS assay (Figure B).

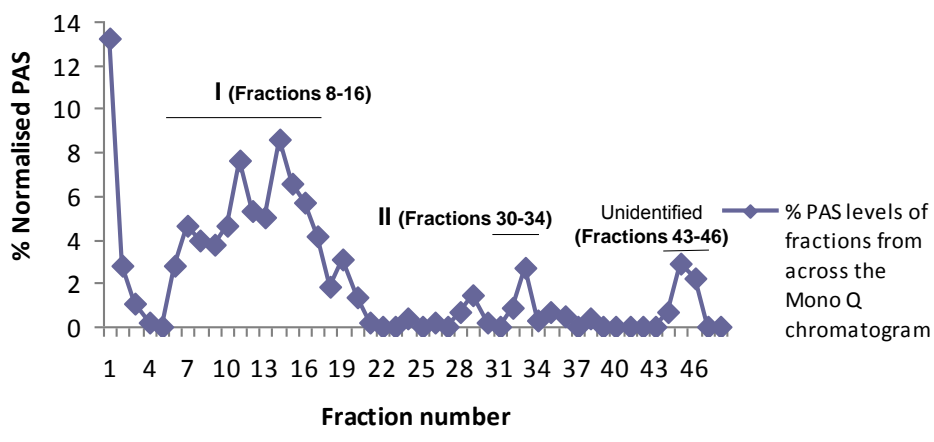
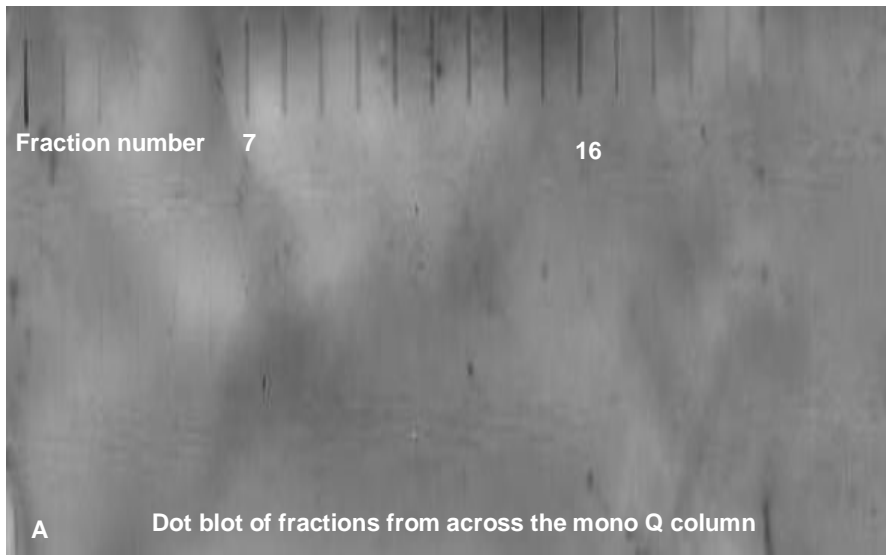


Figure 4.2 Anion exchange HPLC of MFGM from the HIV negative group. The high- M_r glycopeptides were chromatographed on a Pharmacia Mono Q HR 5/5 ion-exchange column. Samples of fractions (100 μ l) from ion-exchange HPLC were blotted on the nitrocellulose membrane (0.20 μ m pore size) (Figure A) before assaying the fractions for neutral sugars using PAS assay (Figure B).

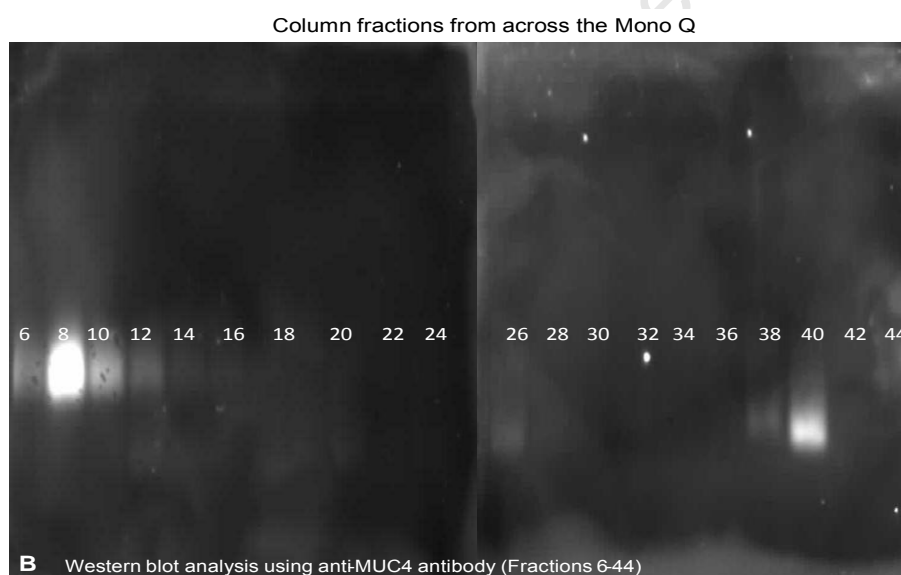
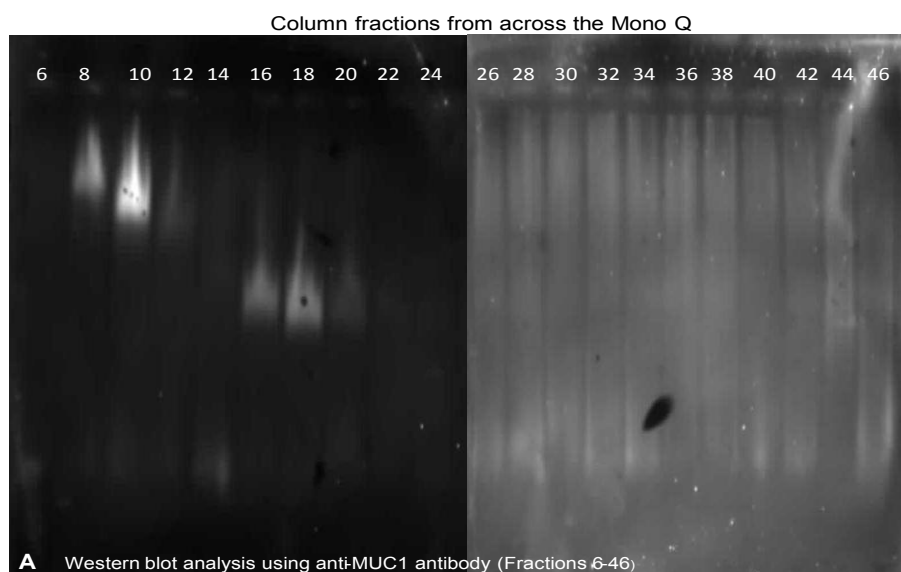


Figure 4.3 Agarose gel electrophoresis of fractions from across the Mono Q chromatogram of HIV positive MFGM mucins. Aliquots (150 μ l) of selected fractions from the HPLC were frozen by liquid nitrogen and the samples were freeze dried. The freeze dried samples were subjected to 0.7% (w/v) agarose gel electrophoresis and subsequently blotted onto the nitrocellulose membrane before blocking overnight. Mouse anti-MUC1 (Figure A fractions 6-46) and anti-MUC4 (Figure B fractions 6-44) monoclonal antibodies were used as the primary antibodies with rabbit anti-mouse horse radish peroxidase as the secondary antibody.

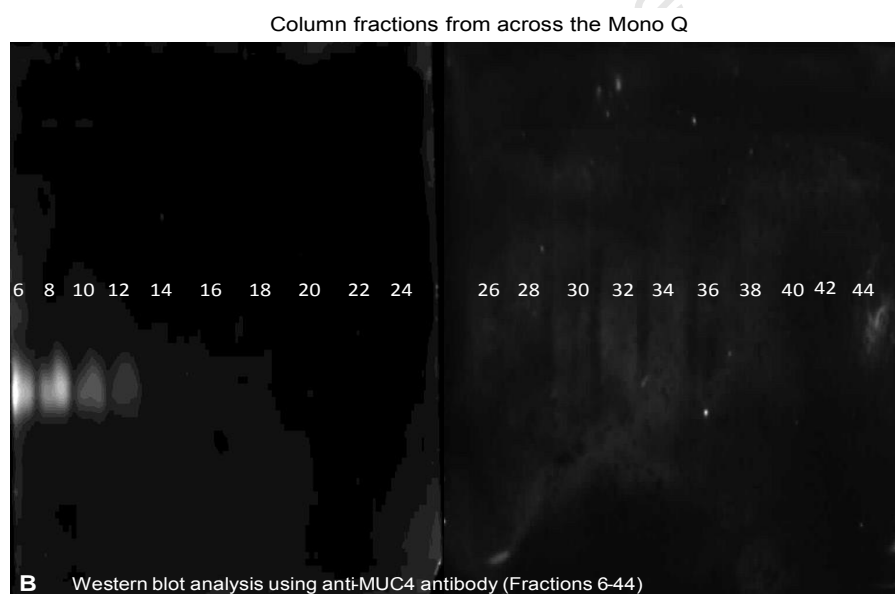
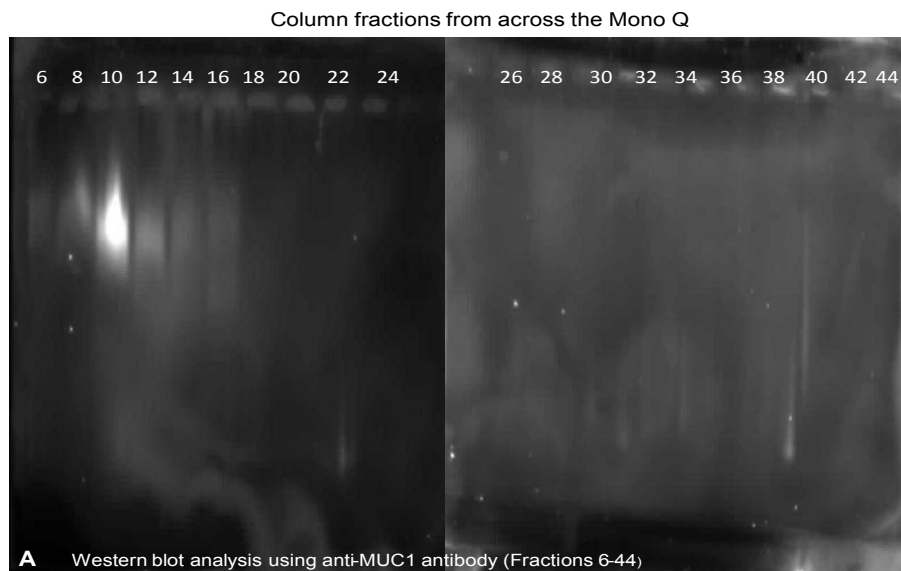


Figure 4.4 **Agarose gel electrophoresis of fractions from across the Mono Q chromatogram of HIV negative MFGM mucins** Aliquots (150 μ l) of selected fractions from the HPLC were frozen by liquid nitrogen and the samples were freeze dried. The freeze dried samples were subjected to 0.7% (w/v) agarose gel electrophoresis and subsequently blotted to the nitrocellulose membrane before blocking overnight. Mouse anti-MUC1 (Figure A fractions 4-46) and anti-MUC4 (Figure B fractions 4-46) monoclonal antibodies were used as the primary antibodies with rabbit anti-mouse horse radish peroxidase as the secondary antibody.

CHAPTER 5

THE ACTIVITY OF CRUDE BREAST MILK (WITH OR WITHOUT HEAT TREATMENT AT 80°C,10 MIN) AND PURIFIED MILK MUCINS AGAINST THE HIV PSEUDOVIRUS USING A LUCIFERASE REPORTER GENE ASSAY IN TZM-bl CELLS

5.1 Introduction



The purpose of the study described in this chapter was to determine the anti-HIV activity of crude breast milk and its mucin component as well as that of heat treated milk using a luciferase reporter gene assay in TZM-bl cells. The principle of the HIV-1 neutralisation assay used in this study exploits the use of “pseudoviruses” that incorporate molecularly cloned HIV-1 Env into defective virus particles that are capable of only a single round of infection. The 293T cells are used as a conduit to generate the pseudoviruses by the co-transfection with an env-mutated plasmid, along with the env clone of choice; this is then used to infect a transformed cell line which expresses the appropriate receptors (Polonis *et al.*, 2008). Post co-transfection, the generated pseudovirus particles are infectious but are unable to produce infectious progeny virions for subsequent rounds of infections. The env-mutated plasmid gets transcribed into viral genomic, RNA packaged by the pseudovirions for delivery of the *tat* gene to the TZM-bl cells. Soon after infection, luciferase reporter gene expression is induced by viral *tat* protein. Luciferase activity is then quantified as relative luminescence units (RLU) which is directly proportional to the number of infectious virus particles present in the inoculum over a wide range of values (Montefiori, 2009).

In this study, the TZM-bl cells were allowed to incubate at 37°C for 26 hours before mucin samples and viruses were added. The pseudovirus was allowed to incubate with dilutions of samples (crude breast milk with or without heat treatment at 80°C for 10 min or purified mucins) for an hour before adding this mixture to the cultured TZM-bl cells. The luciferase activity was measured after 48 hours of culture.

The possible cytotoxicity of crude milk and milk mucins against the TZM-bl cells was investigated. A study was conducted to determine the proliferative index of these cells by an *in vitro* MTT colorimetric assay.

5.2 Results

5.2.1 The activity of crude breast milk (with or without heat treatment at 80°C, 10 min) and purified milk mucins (MUC1 and MUC4) against the HIV pseudovirus Du422.1.

The anti-HIV activity of the crude breast milk (n=16 from each HIV positive and HIV negative groups), heated (80°C, 10 min) milk (n=10 from each group), and purified milk mucins (n=16 from each group) were tested against the Du422.1 pseudovirus using the reporter gene assay in TZM-bl cells. BNAB0044 is an antibody from an HIV infected serum sample  which was used as a positive control for neutralization (Fig 5.1-5.3). The results show that the positive control neutralized the virus in a dose response manner with 80% neutralization at a serum dilution of 1:100. Bovine serum albumin  was used as a negative control at a starting concentration of 195ng/ml which neutralised <25% of the viral activity. Figure 5.1 shows the representative neutralisation curves of the crude breast milk samples against Du422.1 pseudovirus. Crude breast milk from HIV positive donors showed 60% neutralisation of the virus, while the HIV negative donor's milk neutralised the virus at about 40%. Increased neutralisation of viral activity was seen for the heated (80°C for 10 min) milk samples (Figure 5.2) with neutralisation of 80% for the HIV positive samples and 70% for the HIV negative samples. The purified milk mucins (Figure 5.3) from the HIV positive group neutralised 50% of the viral activity, while the HIV negative mucins had much lower neutralisation efficacy (35% neutralisation).

5.2.2 Neutralization potency of the crude breast milk (with or without heat treatment at 80°C, 10 min) and purified milk mucins samples from the HIV positive and HIV negative groups against the pseudovirus Du422.1

The neutralization potency of individual samples from each category was assessed and summarized (Table 5.1). Non-linear regression using Prism Pad was used to generate the IC₅₀ values, which is defined as the concentration required to inhibit HIV activity by 50% (Walker *et al.*, 2011). For the HIV positive crude breast milk, 10 out of 16 (10/16) (Samples 1-8, 15, 16) showed exceptional potency with low IC₅₀ values of <10 ng/ml (0.24 -6.55 ng/ml) and 3 samples had IC₅₀ values that were a 100x fold higher (30.38-196.4 ng/ml). We were not able to generate the IC₅₀ values for samples 9-11 in the HIV positive group. For the HIV negative

crude milk samples, 6 samples (samples 1-2, 7, 10-11, 13) had IC₅₀ values of <10, while 3 samples had IC₅₀ between 17.88-51.70 ng/ml. The data for sample 12 was not obtained.

Among the HIV positive heated milk samples, only one sample (sample 5) elicited potent neutralization with an IC₅₀ of <10, and 2 other samples had higher values (>100ng/ml). IC₅₀ for samples 3-4, 7, 9 were either not obtained or the IC₅₀ value generated was 0 ng/ml. The IC₅₀ values generated for the 3/9 HIV negative heated breast milk were >10 but < 100 ng/ml while the other 6 samples had IC₅₀ values of >100 ng/ml.

Only 2 purified mucin samples (samples 2, 10) from HIV positive donors neutralized the virus at IC₅₀ <10. We were not able to obtain data for samples 5-6, 11-13. The IC₅₀ values obtained for the HIV negative pure mucins showed to have IC₅₀ of > 100 for samples 2, 6-7, 13-15, with no data obtained for samples 1, 4, 8, 9, 12.

5.2.3 MTT assay: Analyzing toxicity of crude milk with or without heat treatment (80°C, 10 min) and purified milk mucins.

The toxicity of the human breast milk and purified mucins against the TZM-bl cells was tested by an *in-vitro* MTT [3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay. The viable cells were counted by trypan blue dye (1:4 dilution) exclusion test and the cell count was adjusted to 1×10⁵ cells/ml. The crude milk, milk mucins and heated milk samples from both the HIV positive and HIV negative groups were shown to be non-toxic to the TZM-bl cells with viability of ≥ 80% (Figure 5.4). The TZM-bl cells were treated with 10% SDS in 0.01M HCl as a negative control and the number of metabolically active TZM-bl cells was 0% viable.

5.3 Discussion

Previous studies (Habte *et al.*, 2008) done in our laboratory showed that purified human milk mucins have inhibitory activity against HIV-1 whilst the crude breast milk does not. The work that is reported in this chapter sought to verify the findings of Habte *et al.*, (2008) by utilizing a high throughput and validated neutralisation assay that has a high degree of inter-experiment reproducibility. The work that was carried out by Habte *et al.*, (2008) had limitations in that the study only focused on the anti-HIV activities of the HIV negative breast milk. This study

(Habte *et al.*, 2008) also required the pooling of samples within specific groups (for example milk from normal subjects), to successfully purify sufficient amounts of mucin for analysis. Another limitation of this study was the absence of a well defined 'normal' group. Individuals who declared living a 'risk free' lifestyle were co-opted to form what we defined as 'normal' for these studies.

The work reported in this chapter looked at both the HIV positive and HIV negative groups with no pooling of sample. The HIV status of those individuals in the Maternity Clinic at Groote Schuur Hospital whose human breast milk samples were collected was confirmed by the clinicians.

Other studies showed that human breast milk mucins have the ability to protect against the rota virus (Yolken *et al.*, 1992) and pox virus (Habte *et al.*, 2007). When comparing the IC₅₀ between the 6 groups (Table 5.1), the HIV positive crude breast milk samples seem to have more potent neutralisation effect with IC₅₀ values of < 10ng/ml. Most of the samples within the same group exhibited differing degrees of overall neutralisation potency. The explanation of the higher pseudoviral neutralisation efficacy of the crude milk samples from the HIV positive samples could be as a result of the HIV Env-specific secretory IgA, secretory IgM and IgG antibodies that have been reported to be present in milk. However, the role of these antibodies in the prevention of infant HIV acquisition is still unclear (Fouda *et al.*, 2011). The crude breast milk from the HIV negative groups also showed increased neutralisation of the pseudovirus. This could be explained by the presence of the molecule lactoferrin in crude milk which has been shown to be inhibitory to HIV virus (Berkhout *et al.*, 2002). Crude milk also contains glycosaminoglycan moieties that prevent the binding of HIV envelope glycoprotein (Newburg *et al.*, 1995). Milk oligosaccharides that have multiple fucose residues and multiple Lewis epitopes have high binding affinity for DC-SIGN and compete with HIV-1-gp120 for binding to DC-SIGN (Hong *et al.*, 2008). Using ELISA based assay and cell based assay, studies by Hong *et al.*, (2008) showed that human milk oligosaccharides (500mg/l) reduced HIV-1-gp120 binding to DC-SIGN by more than 60% and 80% respectively.

Our results, with respect to the behaviour of crude breast milk are similar to the findings of Kazmi *et al.*, (2006), who found that crude breast milk has an HIV inhibitory role. The findings are different from those that were reported by Habte *et al.*, 2008 who found that crude breast milk does not inhibit the virus.

Heated (80°C, 10 min) milk samples showed to be even more effective at neutralising HIV-1 and the reason for this is not clear. The milk mucins (MUC1 and MUC4) are enclosed in milk fat globules (Schroten *et al.*, 1992; Habte *et al.*, 2007). One possibility of the increased viral neutralisation could be that these membrane bound mucins have physical contact with the virus once they are dislodged from the membrane. The mucins were dislodged from the membrane by heating the milk at 80°C for 10 min (as previously shown by Buchheim *et al.*, 1988), and thus were able to interact and possibly neutralise the virus.

Although the results showed that the purified mucins from both groups had far less neutralization potency in comparison to the crude milk sample (with or without heat treatment), this study also showed that the purified mucins from the HIV positive group had higher neutralisation potency than the mucins from the HIV negative group. The reason for this is unknown, but could be attributed to a higher content of mucins that were observed in the HIV positive samples after the separation with Sepharose CL-4B gel filtration and confirmed with Western blotting analysis. It could be that the HIV positive mucins had much more MUC1, which is another Lewis-X factor abundantly present in milk, specifically bound to DC-sign (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) on DC and effectively blocked DC-sign mediated HIV-1 transmission to T-cells (Saeland *et al.*, 2009). The concentration of mucins in the mucous is very low, about 0.5-1% (Creeth, 1978), this level of mucins might be suppressed in the HIV negative group and increased in the positive group and possibly as a defence mechanism.

There was inter-individual variation noted in the neutralization potency within the crude (with or without heat treatment) and purified sample groups. This is why representative neutralisation efficacy curves were selected. It should also be noted that invalid IC₅₀ values were obtained for some samples. There is a need to verify these findings in another study with increased study material numbers.

HIV-1 gains entry into the host cells by means of the CD4 receptor and at least one co-receptor that is expressed on the surface of the cell membrane. Viral gp 120 bind the CD4 and the appropriate co-receptor of the target cells which then results in a conformational change and unmasking of a second viral envelope protein called gp41. Viral gp41 interacts with the host cell membrane resulting in fusion of the viral envelope with the cell (Jenkinson *et al.*,

2003). Antibodies such as b12 antibodies have been reported to neutralise by binding to the CD4 binding site on the gp120 and therefore stopping the engagement of gp120 and CD4 on the cell surface. The blocking of CD4 engagement thus results in the halting of the plasma membrane fusion as well as entry into the cell through endocytosis in epithelial cells (Schaeffer *et al.*, 2004). The antibody BNAB0044 was used as a positive control in this study.

Certain assay parameters determined the outcome of the neutralisation assay. These include the density of the TZM-bl cells used as the target cells, the virus used, the viral/mucin dilution or concentration, length of culture time, volumes of components added, duration of pre-incubation of mucin samples with the virus, duration of infection and the endpoint measured (Polonis *et al.*, 2008). The pseudovirus that was used is called Du422.1 from Kwazulu-Natal and it is a clade C which is common in South Africa (Binley *et al.*, 2004). According to the rank-ordering analysis that was done, the Du422.1 pseudovirus exhibits a distinctively sensitive phenotype of neutralising antibody sensitivities as the virus was neutralised by each of the plasma pools that were tested. The clustering analysis is the method that is used to distinguish four subsets of viruses that are representative of distinct tiers of neutralization sensitivity. Viral isolates that demonstrate the greatest sensitivity to neutralisation form their own cluster called tier 1A, while those with above-average sensitivities have been termed tier 1B. The larger subgroup of tiers 2 viral isolates exhibit moderate sensitivity to neutralisation. The subgroup of tier 3 viruses is resistant to neutralisation (Seaman *et al.*, 2010). The cells that were used in this assay are called TZM-bl cells (also known as JC53BL-13). They are CXCR4-positive HeLa cell clones that are engineered to express CD4 and CCR5. These cells are further engineered to contain integrated reporter genes for luciferase and *Escherichia coli* β -Galactosidase under control of an HIV long terminal repeat sequence. The luciferase reporter allows for automated quantification of HIV infection while the β -Galactosidase reporter allows for direct enumeration of infectious viral units by counting β -Gal expression positive infected cell colonies under a microscope (Wei *et al.*, 2002). These TZM-bl cells are highly permissible to infection by most HIV strains; including the primary HIV-1 isolates (Montefiori, 2005).

In the majority of neutralisation assays, the virus and antibody are pre-incubated then added to the CD4+ target cells (Polonis *et al.*, 2008). In this experiment however, seeding cells 26 hours prior to adding virus and sample was a novel way to reduce the toxicity levels. The MTT assay was used to measure the toxicity of mucins against the TZM-bl cells. This assay is

based on the capacity of the mitochondrial enzyme, succinate dehydrogenase of living cells to convert MTT tetrazolium [3-(4, 5-dimethylthiozole-2yl)-2,5-diphenyltetrazolium bromide] into a blue coloured product (formazan) that was assayed 5 days post-infection, and is proportional to the number of living cells present (Saravanan *et al.*, 2003). The cytotoxicity studies were conducted with 1×10^5 cells per well. The result of the MTT assay showed activity in the TZM-bl cells, which is a reflection that the crude breast milk and milk mucins are non-toxic to these cells.

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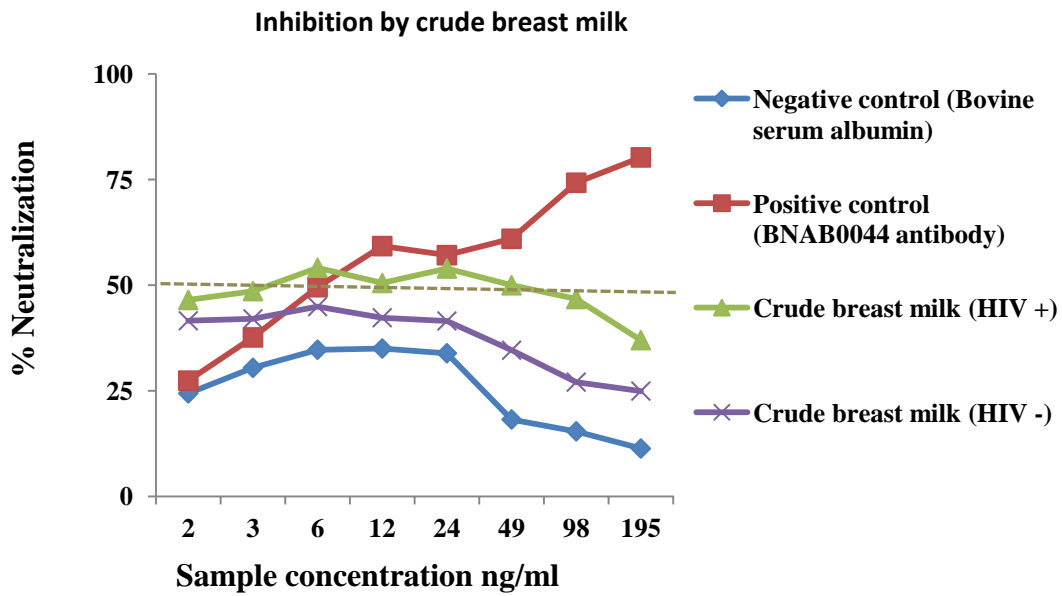


Figure 5.1 Representative neutralisation dose response curves against HIV-1 subtype C env pseudovirus Du422.1 with HIV positive crude breast milk and HIV negative crude breast milk at a starting concentration of 195ng/ml. The antibody BNAB0044 was used as a positive control at a dilution of 1:100 and bovine serum albumin was used as a negative control at a starting concentration of 195ng/ml. The 50% inhibition concentration point (IC_{50}) is indicated by the broken line.

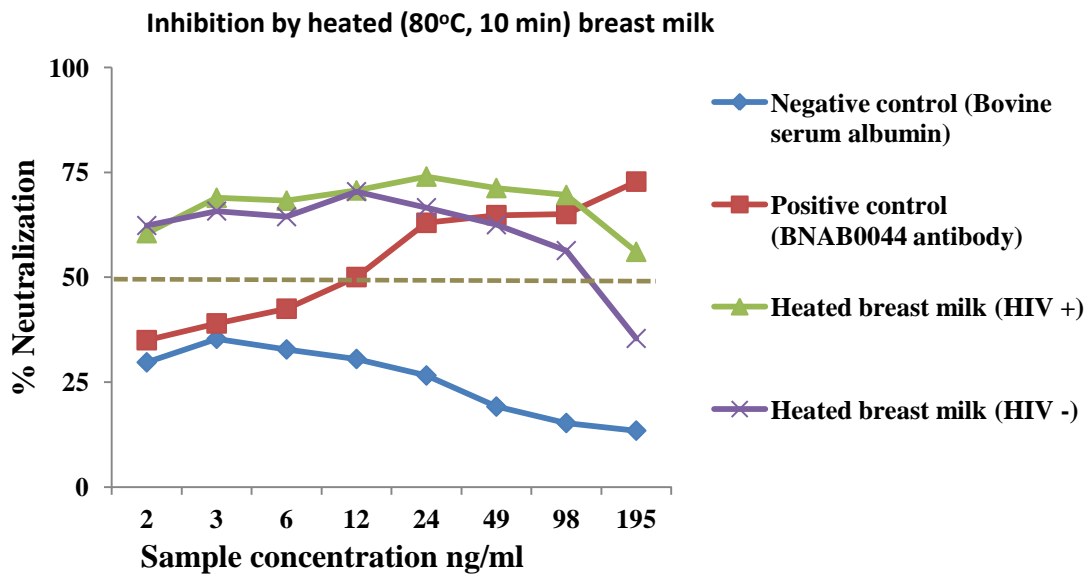


Figure 5.2 Representative neutralisation dose response curves against HIV-1 subtype C env pseudovirus Du422.1 with HIV positive heated breast milk and HIV negative heated breast milk at a starting concentration of 195ng/ml. The antibody BNAB0044 was used as a positive control at a dilution of 1:100 and bovine serum albumin was used as a negative control at a starting concentration of 195ng/ml. The 50% inhibition concentration point (IC₅₀) is indicated by the broken line.

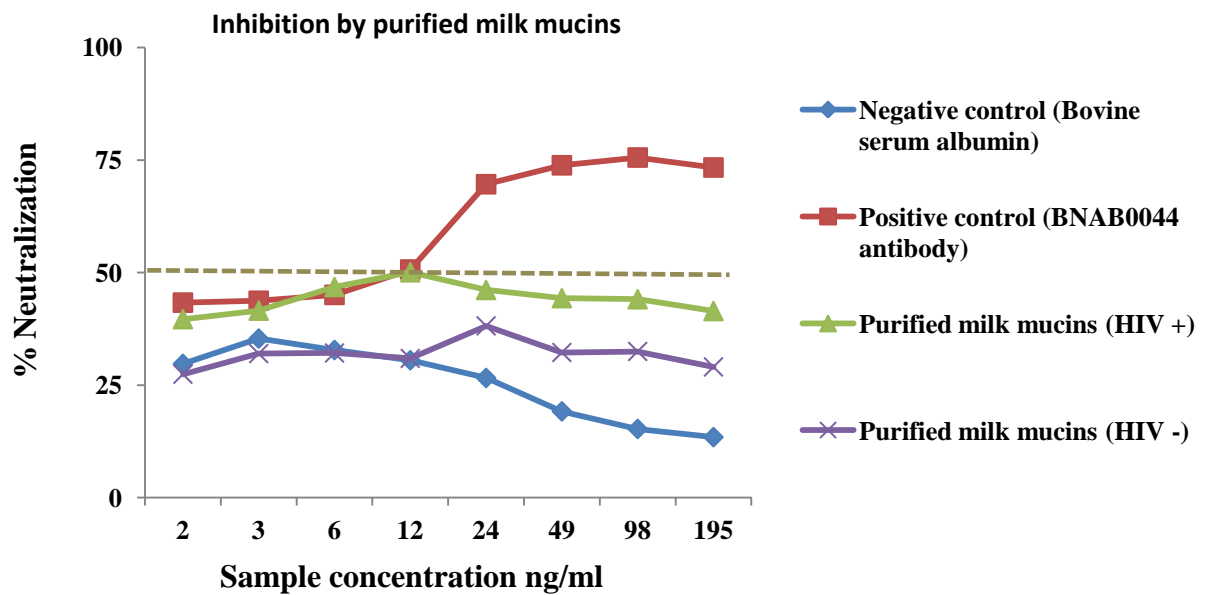


Figure 5.3 Representative neutralisation dose response curves against HIV-1 subtype C env pseudovirus Du422.1 with HIV positive purified milk mucins and HIV negative purified milk mucins at a starting concentration of 195ng/ml. The antibody BNAB0044 was used as a positive control at a dilution of 1:100 and bovine serum albumin was used as a negative control at a starting concentration of 195ng/ml. The 50% inhibition concentration point (IC₅₀) is indicated by the broken line.

IC ₅₀ (50% Inhibition of DU4221 pseudovirus)						
	Crude human breast milk		Heated (80°C, 10 min) breast milk		Purified milk mucins	
	HIV positive	HIV negative	HIV positive	HIV negative	HIV positive	HIV negative
Sample 1	0.83	6.5	183.2	932.3	91.75	
Sample 2	3.14	0.02	unavailable	162.1	0.03	516400000
Sample 3	5.52	96673	>195	30.47	25.27	unavailable
Sample 4	0.25	5567		97.3	>195	
Sample 5	0.72	458	0.8956	111.9	Interrupted	unavailable
Sample 6	0.04	unavailable	74	161.9	Interrupted	22558
Sample 7	1.77	1.03	>195	117.5	478.5	61083
Sample 8	0.93	113.2	394	93.49	48625	
Sample 9	Interrupted	17.88	0	515	70.52	
Sample 10	>195	0.02			2.12	30.41
Sample 11	Interrupted	0.02			unavailable	0.00000236
Sample 12	30.38	Not converged			>195	2
Sample 13	196.4	7.7				124887
Sample 14	170.6	44.95			2216	1205
Sample 15	0.24	51.7			15.21	1891
Sample 16	6.55	196.5			22.62	56.25

Table 5.1 IC₅₀ values of crude breast milk with or without heat treatment (80°C, 10 min) and purified milk mucins against HIV-1 subtype C env pseudovirus Du422.1. IC₅₀ <10 ng/ml, IC₅₀ >10 ng/ml and <100 ng/ml, IC₅₀ >100 ng/ml

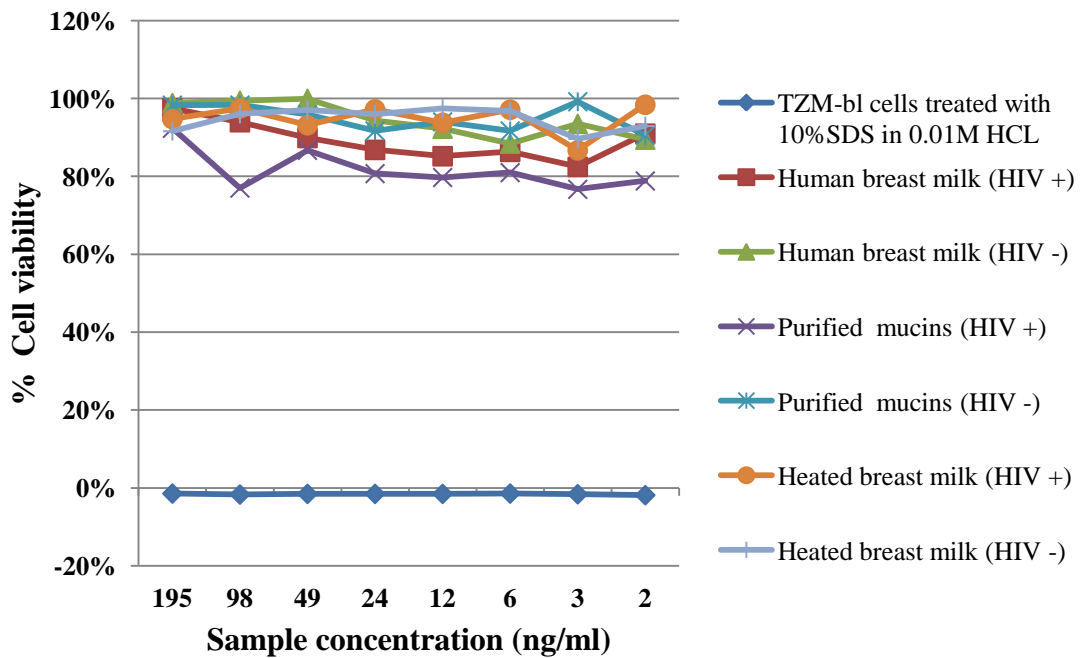


Figure 5.4 The response of TZM-bl cells to varying concentrations of crude breast milk with or without heat treatment (80°C, 10 min) and purified milk mucins from the HIV positive and HIV negative groups measured by MTT colorimetric assay

CHAPTER 6

SUMMARY

Mucus is synthesized by specialized goblet cells of the columnar epithelium that lines the major internal tracts of the body that are exposed to the external environment. The malfunctioning of the mucus has been implicated in the etiology of many diseases, such as peptic ulceration, ulcerative colitis, bronchitis, infertility and external disorders of the eye (Allen, A. 1983). It contains proteins such as lysozyme, immunoglobulins, defensins, growth factors and trefoil factors which serve a defensive purpose (Gipson *et al.*, 1997).

Mucin polymeric molecules contribute to the visco-elastic gel properties of mucus. Human breast milk contains numerous biologically active compounds such as mucins and glycosaminoglycans, which protect breast-fed infants from bacteria, viruses and toxins (Habte *et al.*, 2007). Reports indicated that there are two human milk mucins, namely MUC1 which is best characterised and the second milk mucin called MUC4. Both MUC1 and MUC4 are tethered trans-membrane mucins which can act as a second line of defence and are anti-adhesive proteins. They have a crucial role in regulating many cellular functions, including cell proliferation and apoptosis of T-cells and are involved in cell signalling and immunoregulation. These mucins also play a role as pathological markers. Both have a potential role in pathogen adhesion (Wilson *et al.*, 2008). MUC1 has been reported to effectively block HIV-1 transmission to T-cells (Saeland *et al.*, 2009; Habte *et al.*, 2008).

South Africa is reported to have more human immunodeficiency virus infected people than in any other country in the world, with a national HIV prevalence of 11.4% (Jaspan *et al.*, 2006). HIV-1 subtype C is the predominant HIV-1 genotype in Southern Africa and is rapidly spreading throughout the world (Ndung'u *et al.*, 2006) and accounts for approximately 50% of paediatric HIV-1 infections (Zhang *et al.*, 2006). Breast-feeding maintains the maternal-foetal immunologic link after birth which may favour the immuno-competence transmission from the mother to her infant. However, mother-to-child transmission of HIV-1 through breast feeding accounts for the majority of HIV-1 infections among infants as high as 40% (Habte *et al.*, 2008; Schrotten *et al.*, 1992).

The biological objectives of this study were to isolate, purify and identify mucins in human breast milk and to compare mucins from normal and infected breast milk with respect to their inhibition of the HI virus. This study investigated the possible changes in the glycosylation of milk mucins. The study also tested the anti-HIV-1 activity of crude breast milk with or without heat treatment, (80°C, 10 min), and its purified mucin components, namely MUC1 and MUC4, in HIV positive patients compared with those who are not infected.

Milk mucins were isolated from the prepared milk fat globule membrane by Sepharose CL-4B gel filtration column. The Sepharose CL-4B column elution profiles for the HIV positive and HIV negative groups showed that the MFGM have two mucin rich peaks. The first peak named MFGM-I was eluted in the void volume while the second peak named MFGM-II eluted in the included volume. The HIV positive elution profile had two large distinct peaks while the HIV negative elution profile showed one small peak and one large peak. When the MFGM-I and MFGM-II peaks were separated on SDS-PAGE and stained for carbohydrate by PAS, The SDS-PAGE gel from the HIV positive showed the presence of increased staining intensity of mucins in comparison to the HIV negative gel. The MFGM-I peaks from both groups showed more intensely stained mucins than the MFGM-II peaks.

The identity of the mucins in the MFGM-I and MFGM-II peaks was confirmed by Western blotting analysis to be MUC1 and MUC4. Both MUC1 and MUC4 were present in each MFGM-I and MFGM-II peaks from the HIV positive and HIV negative groups.

Intense staining was seen in the MFGM-I peak for MUC1 in the HIV positive group than the MFGM-II peak. The HIV negative group had far less material in both the MFGM-I and MFGM-II peaks with the MFGM-II peaks hardly visible at all.

MFGM-I peaks from the HIV positive and HIV negative groups were further purified by isopycnic density gradient centrifugation in caesium chloride and 4M guanidinium chloride. The purification profiles showed a clear separation between the lower density proteins and the higher density mucin fractions which were assayed by PAS. And the identity of mucins in MFGM-I from both groups was further confirmed by amino acid analysis which showed the presence of increased serine, threonine and proline. There were no differences between the amount of serine threonine and proline for the HIV positive group which added up to 41% of the MFGM-I, while these amino acids added up to 37% of MFGM-I for the HIV negative.

In order to investigate populations, MFGM samples were chromatographed on a Pharmacia Mono Q HR 5/5 ion-exchange HPLC. Samples of fractions from ion-exchange HPLC were blotted on the nitrocellulose membrane (0.20 μ m pore size) before assaying the fractions for neutral sugars using a solution PAS assay.

The PAS profile for the HIV positive MFGM-1 showed evidence of three populations. The agarose gel electrophoresis showed that MUC1 had two distinct population I and population II on the same fractions as the PAS profiles based on the two visible electrophoretic patterns that were observed on the blots. One other possible population III of MUC1 was also noted and appeared as smears on the Western blot. Varying populations for MUC4 mucins in the HIV positive samples were observed on the Western blot. MUC4 was present on fractions which formed part of population I on the PAS profile. It was also present on fractions which formed population III at a lower density.

The PAS profiles of MFGM-I from the HIV negative samples showed less variation in this group compared to those of the HIV positive group. A single distinct population I was observed on the PAS profile, as well as one other minor population II. A third minor peak was observed as well which could be another potential population. Agarose gels verified that MUC1 was detected on fractions of population I shown on the PAS profile. When the blot was probed with anti-MUC4 antibody, MUC4 was detected on fractions of population I. There seems to be faint traces of MUC4 detected on fractions of population II which explains the slight peak observed on the PAS profile. There is no explanation known to us of the third peak observed on the PAS profile as neither MUC1 nor MUC4 were detected on those fractions by Western blot.

The crude breast milk samples showed 60% neutralisation for the HIV positive samples, while the HIV negative milk neutralised at 40%. The purified milk mucins from the HIV positive group neutralised 50% of the viral activity, while the HIV negative mucins had much lower neutralisation efficacy with 35% neutralisation. Increased neutralisation of viral activity was seen for the heated milk samples with neutralisation of 80% for the HIV positive samples and 70% for the HIV negative samples.

The results in this study are intriguing in that firstly they differ from a previous study in our laboratory (Habte *et al.*, 2007) which showed that normal crude milk samples did not inhibit the virus while the purified mucins did. This work had the added advantage of comparing crude milk and purified mucin from normal and infected milk and further found that inhibition of HIV-1 was greater by infected samples, both crude and purified. This certainly needs further investigation together with the very crucial observation that milk heated prior to the assay had an increased inhibitory activity against HIV-1. This finding could have enormous implications for mother-to-child transmission of the virus through breast-feeding, a significant contributor to the pandemic in sub-Saharan Africa.

All in all this study has established the crucial role of mucins in the inhibition of the virus that causes AIDS. Whilst such a study requires more development in terms of numbers and size and a wider investigation, the implications of our study need to be considered.

Secreted mucins are known for their gel-forming properties and their contribution to forming unstirred crude gels on epithelial surfaces to which they afford protection and also keep these surfaces moist in the most hostile of environments (Allen 1981, Mall 2008). Considering that mucins inhibit the HI virus in an *in vitro* assay not only in breast milk but in saliva too (where work in our laboratory has pinpointed the crucial role salivary mucus and mucin play in preventing transmission of the virus by the exchange of oral fluids (Habte *et al.*, 2006), we ask whether mucins could be used as a basis for a formula that could be used as a vaginal and anal microbicide to prevent transmission of the virus through sexual intercourse.

Future work in our laboratory will be guided by such considerations as outlined above, whilst we continue to investigate in greater depth the characteristics of mucus and mucins from the saliva, breast-milk and gastrointestinal and cervical tracts (Habte *et al.*, 2008).

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APPENDIX

A CONSENT INFORMATION FORM

2010-02-08

Dear Patient,

We are doing a study on the mucus produced in your breast milk. Mucus is a sticky substance mixed with bodily secretions and is necessary for lubrication and protection of the tracts in the body. We wish to find out the role of mucus in the breast milk of HIV negative and positive individuals.

We need to take 200 ml of milk from you to use in an experiment which will determine the effect of mucus on the HI virus.

Please note:

1. We will need to take your folder number (NOT YOUR NAME) from which we will get details of your age, gender and origin. All this information will be kept **absolutely confidential** and we promise not to use your sample for any other purpose but that we have described.
2. You have a right to say that you do not wish to take part in this study. If you do not wish to be part of the study, this will not affect the treatment you receive.
3. If you agree to participate in this research project now and then decide at a later time that you do not wish to continue, that is also fine. That decision will once again not affect your right to treatment and care.
4. It is possible that we will publish the findings of this study but your name will not be mentioned.

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

Professor A Mall

Tel: 021-4066168/6227

C:\salivainfo

B. CONSENT LABORATORY FORM

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

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

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

Please fill in all the information requested:

Folder No. _____

Sex: M F

Date of Birth Year: _____ Month: _____ Day: _____ Ethnic Origin:

Contact Address: Hospital/Clinic where samples are taken

Town: _____ Fax: _____

Tel: _____

For Laboratory use only:

Date Received: YY _____ DD _____ Computer Index No.: _____

CONSENT FOR BREAST MILK SAMPLE

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.

ALL OF THE ABOVE HAS BEEN FULLY EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED

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

C. MUCIN ISOLATION, PURIFICATION AND CHARACTERIZATION

a. 10mM phosphate buffer(PBS):

A. 10mM Na_2PO_4 (Mw 141.96) 0.568gms/400ml

B. 10mM NaH_2PO_4 (Mw 119.98) 0.9598gms/800ml

Add A to B until pH 6.5 then store at 4°C

b. Salt azide buffer:

0.2 NaCl (Mw 58.44) 11.69gms/litre

0.02% NaN_3 (Mw) 0.2gms/litre

c. 6M Guanidinium hydrochloride in PBS pH 6.5

6M GuHCl (Mw 95.5) 573.0g/litre PBS pH 6.5

d. Protease inhibitors: (P.I.)

1mM Iodoacetamide 0.0925gms/500ml

5mM Benzamidine 0.437 gms/500ml

10mM EDTA 1.8612 gms/500ml

1mM PMSF 0.087gms/500ml

100mM Hexanoic Acid 0.656gms/500ml

1mg/litre Trypsin inhibitor 0.5mg/500ml

5mM NEM 0.3128gms/500ml

e. Periodic acid Schiff (PAS) assay for glycoproteins in mucin

10g Pararosaniline hydrochloride

1litre boiling distilled water

Stir continuously, and then cool to 50°C

Add 200ml 1M HCl

Allow to mature in the dark overnight

Add 30g activated charcoal

Stir for 5-60 minutes

Filter to remove charcoal

Add 30g activated charcoal

Stir for 5-60 minutes and filter again

The resulting red solution is stored at room temperature in a dark bottle.

Decolorized Schiff reagent (Make fresh for every assay)

100mg Sodium metabisulphite in 6ml schiff's reagent

Incubate at 37°C until the solution is colorless

Periodic acid solution

10ml 7% Acetic acid and 20ul periodic acid

Assay for a microtitre plate

20ul Sample/fraction

100ul Periodic acid solution

Incubate at 37°C for 1 hour

Add 200ul decolorized Schiff's reagent

Incubate at room temperature for 30 mins

Read absorbance at 555nm and plot graph

f. Lowry method for protein estimation

Solution A: 2% anhydrous sodium carbonate

2gms Na_2CO_3 in 100 ml 0.1 M NaOH

Make fresh

Solution B: (1) 0.25gms CuSO_4 in 25ml distilled water

(2) 0.5gms Sodium tartrate in 25 ml distilled water

Keep at 4°C and make as needed in 1:2 diltution to give working sol B

Solution C: 1ml working solution B to 50ml solution A made up just before using

Folins solution: Dilute 1: 2 with distilled water

Assay for a microtitre plate

10ul Sample

10ul 0.1M NaOH

200ul Solution C

Mix and incubate at 25°C for 10 min

20ul diluted Folins reagent

Mix and incubate at 25°C for 30 min

Read at 700nm

D. Gels

a. Buffers for SDS PAGE

BIS/Acrylamide: 30g Acrylamide

0.8 g BIS

Make up to 100ml with distilled water, store at 4°C in a dark bottle

Running gel buffer: 0.75M [(hydroxymethyl) aminomethane] Tris

0.2% SDS

In distilled water and pH 8.8 and store at 4°C

Spacer gel buffer: 0.25M Tris

0.2% SDS

Distilled water and pH 6.8 and store at 4°C

Tank/Reservoir Buffer: 0.025M Tris

0.19M Glycine

0.1% SDS

In distilled water and pH 8.8 and store at room temperature

Sample application buffer: 2% SDS

10% Glycerol

0.01% Bromophenol blue

Add 5% Mercaptoethanol for a reducing buffer, boil sample buffer for 1 min before loading

TEMED: use as it is

b. SDS PAGE

10% Running gel	Mini gel
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30% Bis/Acrylamide	3.67ml
--------------------	--------

0.1% SDS	3.90ml
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Running buffer	3.60ml
----------------	--------

AMPS	100ul
------	-------

TEMED	3ul
-------	-----

Spacer gel

30% Bis/Acrylamide	0.5ml
--------------------	-------

Spacer buffer	2.8ml
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AMPS	73ul
TEMED	5ul

c. Coomassie blue stain for protein on proteins

0.1% Coomassie blue

30% Methanol

10% Glacial acetic Acid

Method:

Place gel in Coomassie blue stain and shake gently overnight. Destain with 20% methanol acetic acid solution until gels are clearly visible. Wash in distilled water and photograph.

d. Periodic Acid Schiff (PAS) gel staining

Schiff's reagent:

Dissolve 1g pararosaniline in 200ml rapidly boiling distilled water, stirring constantly

Cool on bench to 50°C

Add 20ml 1M HCl

Cool to 25°C and add 1 g potassium metabisulphide and leave to stand for 12-24hrs in the dark

Add 2g activated charcoal

Mix for 1 min and then filter

Store at 25°C in a dark bottle

Method:

Place gel in 50% ethanol for 30 min

Wash in distilled water for 10 min

Place gel in 1% periodic acid made up in 3% acetic acid for 30 min

Wash in distilled water for 30 min or overnight

Place gel in 0.1 sodium metabisulphite in 10mM HCl for 2X10 mins

Place gel in Schiff's reagent in the dark for 60 min

Then in 0.1 sodium metabisulphite in 10mM HCl in the dark

e. Buffers for agarose gel electrophoresis and western blotting

50X TAE:

242g Tris

57.1g glacial acetic acid

100 ml 0.5M EDTA (18361g)
pH to 8.0 and make up to 1 litre with distilled water

Running buffer:

1X TAE containing 0.1% SDS (w/v)

20XSSC:

1402.4g sodium chloride
705.6g tri-sodium citrate
Ph to 7.0 and make up to 8liters with water

Transfer buffer:

4X SSC

Sample loading buffer:

40-50% glycerol
1X TAE
Smidgen Bromophenol blue

TBST

10mM Tris-HCl 1.21g
150mM NaCl 8.76g
0.05% Tween 20 0.5ml
pH to 8.0 and make up to 1liter with distilled water

Agarose Gel

0.7 % Agarose type V
1X TAE
0.1% SDS
Boil till dissolved, cool and then pour into gel apparatus with the required comb.

E. Pseudoviral neutralization assay

a. Complete growth media (50ml)

47ml DMEM

2.5 FCS or FBS

0.5ml 1M HEPES

50µl 10ng/ µl Gentamycin

F. Glycoform detection using anion exchange HPLC

a. 6M clean urea

Dissolve 720g urea in 2L distilled water

Add charcoal and stir

Filter- using Whatman paper (2 x big and 1 x small)

Add a couple of spatula of mixed bed resin

Filter through a 0.2µm membrane (1 x nitrocellulose, 1 x nylon together)

b. Buffer A for HPLC

10mM piperazine/perchlorate

c. Buffer B for HPLC

10mM piperazine/perchlorate

0.5M Lithium chloride

University of Cape Town