The impact of HIV exposure status and maternal feeding practice on the concentration of SLPI and E/tr-2 protein in infant saliva and maternal breast milk.

By

Nobomi Dontsa

(DNTNOB001)

Research Thesis: M.Sc. (By dissertation)

In the

Division of Immunology, Department of Pathology,

Faculty of Health Sciences,

University of Cape Town

South Africa

Supervisor: Prof Clive Gray

Co-Supervisor: Dr Heather Jaspan

2019

ACKNOWLEDGEMENTS

This thesis owes its existence to the help, support and inspiration of several people. Firstly I would like to express my appreciation and gratitude to my supervisor Prof Clive Gray and co-supervisor Dr Heather Jaspan for their patience, guidance and advice during the course of my research. It is through their support that this thesis exists.

Secondly to my former colleague and friend Anthony Rapulana for training me on doing the ELISA assay thank you mate. To Mr Agano Kiravu and Dr Sonwabile Dzanibe for assisting with the analysis of my results and reviewing my entire thesis; your support is really appreciated. I have no words to express how grateful I am for your efforts and professional guidance.

I would also like to thank the entire INFANT study team, all the participants who made this study possible. If it wasn't for you, this research would not be possible.

I would like to thank my friends on and off campus for always lending an ear when I needed to offload. To my late grandmother Nomawethu Dontsa, how I wish you were here to see my efforts in life, I love you Mambhele. May you continue to rest in eternal peace. To my prayer warrior Busisiwe Dontsa, my mother, I will always strive to make you proud. To my sisters and brother through you I know the meaning of LOVE I don't know what I would be without you. To Trishana Nundullal, my jack of all trades, I am glad that our paths crossed. Thank you for your never-ending support and kindness. To Nicole Prins, Yamkela Qumbelo and Bokani Nleya, thank you for being the good friends you are to me.

Finally to my Husband, Siyabonga Mdlangu, your love, kindness, encouragement and your care is tremendously appreciated.

DECLARATION

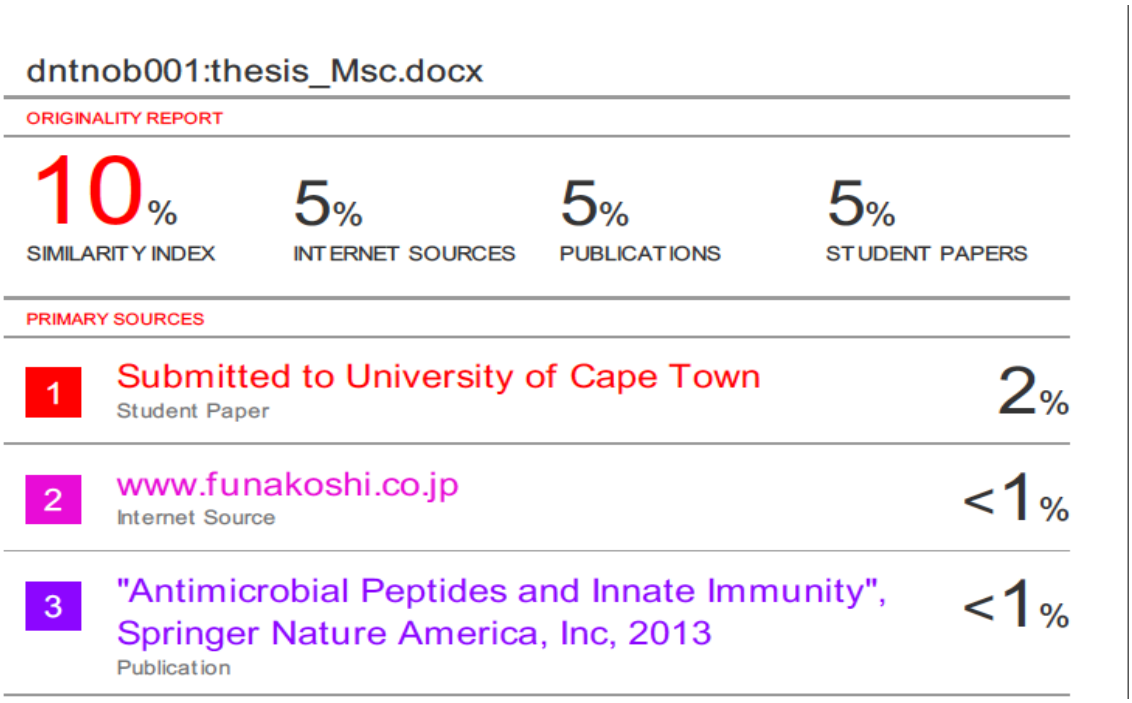
I, Nobomi Dontsa, hereby declare that the work on this thesis is my original work (except where acknowledgement indicates otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature

Signed by candidate

Date: 06 May 2019



dntnob001:thesis_Msc.docx

ORIGINALITY REPORT

10%	5%	5%	5%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to University of Cape Town Student Paper	2%
2	www.funakoshi.co.jp Internet Source	<1%
3	"Antimicrobial Peptides and Innate Immunity", Springer Nature America, Inc, 2013 Publication	<1%

TABLE OF CONTENTS

ACKNOWLEDGEMENTS 2

ABBREVIATIONS **7**

LIST OF FIGURES **12**

LIST OF TABLES **14**

ABSTRACT **15**

1.1. INTRODUCTION **18**

1.2. HIV EPIDEMIC **20**

1.2.2. HIV STRUCTURE AND THE MECHANISM OF ACTION 21

1.2.3. HIV TRANSMISSION 24

1.2.3.1. MOTHER TO CHILD HIV TRANSMISSION *24*

1.3. INFANT IMMUNE SYSTEM **26**

1.4. BREAST MILK **28**

1.4.2. BREAST MILK BIOACTIVE COMPONENTS 28

1.5. THE ORAL MUCOSAE AND IMMUNOLOGICAL PROTECTION AGAINST PATHOGENS 30

1.5.2. THE ORAL MUCOSA 30

1.5.3. PROTECTIVE IMMUNOLOGICAL PROPERTIES IN ORAL SECRETIONS 30

1.6. HIV AND INNATE FACTORS IN SALIVA AND BREAST MILK **32**

1.6.2. WHEY ACIDIC PROTEINS (WAP) 33

1.6.2.1. SECRETORY LEUKOCYTES PROTEASE INHIBITORS (SLPI) *33*

1.6.2. PROPERTIES OF SLPI AND ELAFIN/TRAPPIN-2 36

1.6.2.1. ANTI-MICROBIAL AND ANTI-INFLAMMATORY ACTIVITY *36*

1.6.3. TISSUE REMODELLING AND WOUND HEALING 36

1.6.4. ANTI-VIRAL ACTIVITY 37

1.6.5. ANTI-HIV MECHANISMS OF SLPI AND E/TR-2 37

7.1. STUDY HYPOTHESIS **39**

7.1.1. STUDY AIMS AND OBJECTIVES 39

2. MATERIALS & METHODS **40**

2.1. STUDY DESIGN **41**

2.1.1. STUDY SITE 41

2.1.2. STUDY PARTICIPANTS 41

2.2. CLINICAL ASSESSMENTS **42**

2.3. FEEDING PRACTICE **43**

2.4. ETHICAL CONSIDERATIONS **43**

2.5. SAMPLE COLLECTION AND PROCESSING **43**

2.6. INFANT SALIVA **44**

2.7. MATERNAL BREAST MILK 45

2.8. SANDWICH ELISA USED TO MEASURE INNATE PROTEINS 46

2.8.1. HUMAN SLPI QUANTIKINE ELISA 46

2.8.2. E/TR-2- HUMAN TRAPPIN-2/ELAFIN DUO SET ELISA KIT 47

2.9. STATISTICAL ANALYSIS 48

2.10. OPTIMIZATION OF ELISA METHODOLOGY 49

2.10.1. SPIKE AND RECOVERY 50

2.10.2. LINEARITY TESTING 51

2.11. LEVY-JENNINGS QUALITY CONTROL CHART 52

3. RESULTS 54

3.1 ASSAY OPTIMIZATION RESULTS 55

3.2. MUCOSAL FLUIDS CONTAIN MEASURABLE CONCENTRATIONS OF SLPI AND E/TR-2 57

3.3. INNATE FACTOR MEASUREMENTS FALL WITHIN THE LIMITS OF INTER-PLATE VARIABILITY 59

3.4. STUDY PARTICIPANTS CHARACTERISTICS ACCORDING TO HIV STATUS 61

3.5. THE CONCENTRATION OF INNATE PROTEINS CHANGES OVERTIME IN BREAST MILK AND INFANT SALIVA. 63

3.5.1. CONCENTRATIONS OF SLPI AND E/TR-2 IN MATERNAL BREAST MILK DECREASE IN THE FIRST 36 WEEKS POSTPARTUM. 63

3.5.2. SLPI AND E/TR-2 CONCENTRATIONS IN INFANT SALIVA DO NOT SHOW A CONSISTENT TREND IN THE FIRST 36 WEEKS OF LIFE 65

3.5.3. MATERNAL HIV STATUS DOES NOT IMPACT THE CONCENTRATION OF SLPI AND E/TR-2 IN MATERNAL BREAST MILK. 66

INFANT EXPOSURE TO MATERNAL HIV DIFFERENTIALLY IMPACTS THE CONCENTRATION OF SLPI AND E/TR-2 IN INFANT SALIVA. 68

3.6. THE IMPACT OF FEEDING PRACTICE ON THE CONCENTRATION OF SLPI AND E/TR-2 IN INFANT SALIVA 70

3.6.1. THE CONCENTRATION OF SLPI IN EBF INFANT SALIVA INCREASES OVER TIME COMPARED TO EFF INFANTS. 71

3.6.2. MATERNAL FEEDING MODE DOES NOT IMPACT SLPI AND E/TR-2 CONCENTRATION IN INFANTS. 73

3.7. ASSOCIATION OF SLPI AND E/TR-2 LEVELS IN MATERNAL BREAST MILK WITH INFANT SALIVA 75

3.7.1. MATERNAL BREAST MILK E/TR-2 CONCENTRATIONS DO NOT CORRELATE WITH EXPRESSION IN INFANT SALIVA 75

3.7.2. MATERNAL BREAST MILK SLPI CONCENTRATIONS DO NOT CORRELATE WITH CONCENTRATIONS IN INFANT SALIVA 77

4. DISCUSSION 81

THE CONCENTRATION OF INNATE PROTEINS IN INFANT SALIVA AND MATERNAL BREAST MILK OVER TIME **82**

THE IMPACT OF HIV EXPOSURE STATUS ON THE CONCENTRATION OF INNATE PROTEINS IN MATERNAL BREAST MILK AND INFANT SALIVA **83**

THE EFFECT OF MATERNAL FEEDING MODE ON THE CONCENTRATION OF INNATE PROTEINS IN INFANT SALIVA **85**

5. APPENDICES **87**

APPENDIX I **88**

APPENDIX II 90

APPENDIX III **92**

APPENDIX IV **94**

REFERENCES **102**

ABBREVIATIONS

µ Micro

^{0}C Degree(s) Celsius

(A)

AIDS Acquired immunodeficiency syndrome

APC Antigen presenting cells

ART Antiretroviral therapy

ARV Antiretroviral

(B)

BAS baseline

BM breast milk

(C)

CCR5 Chemokine receptor 5

CD3 Cluster of differentiation 3

CD4 Cluster of differentiation 4

CD40 Cluster of differentiation 40

CD54 Cluster of differentiation 54

CD80 Cluster of differentiation 80

CD81 Cluster of differentiation 81

CD86 Cluster of differentiation 86

C-terminal carboxyl-terminus

CXCR4 C-X-C Chemokine Receptor 4

CXCR5 CXC Chemokine Receptor 5

(D)

D4-7 day 4-7

dH_2O Distilled water

dil. Factor Dilution factor

(E)

EFV Efavirenz

E/Tr-2 Elafin/Trappin-2

EBF Exclusively breast fed

EFF Exclusively formula fed

ELISA Enzyme-linked immunosorbent assay

Env Viral envelope protein

env Viral gene encoding the Envelope protein

E. coli Escherichia coli

(F)

FTC Emtricitabine

(G)

g Gram(s)

GCP Good clinical practices

Gp120 120kDa Envelope glycoprotein

Gp160 160kDa Envelope glycoprotein

Gp41 41kDa Envelope glycoprotein

(H)

h Hour(s)

HE HIV Exposed

HEU HIV exposed uninfected

HIV Human Immunodeficiency Virus

HIV +ve Human Immunodeficiency Virus positive

HIV –ve Human Immunodeficiency Virus negative

HLA-DR Human leukocyte antigen-DR isotype

HRP Horseradish peroxidase

HU HIV unexposed

(I)

IFN interferon

IgA Immunoglobulin A

IgM Immunoglobulin M

IL-1 Interleukin 1

IL-10 interleukin 10

IL12 Interleukin 12

IL-6 Interleukin 16

IQR Interquartile range

IRB Institutional review board

(K)

kDa Kilodalton

(L)

LCL lower control limit

LOD limit of detection

LPS Lipopolysaccharide

(M)

MF Mixed feeding

MG-1 a mucin with a high molecular weight

MG-2 a mucin with a low molecular weight

MHC-1 major histocompatibility molecule class 1

MHC-2 major histocompatibility molecule class 2

MIP1- α Macrophage inflammatory protein 1-alpha

MIP1- β Macrophage inflammatory proteins-beta

MOU Midwife obstetric unit

MSM Men who have sex with men

M-TB-Mycobacterium tuberculosis

MTCT Mother to Child HIV Transmission

(N)

NEBF Non-exclusively breast fed

NEFF Non-exclusively formula fed

NH2 terminal amino terminus

Ng/ml nano grams per mil

(P)

PRRs pathogen recognition receptors

(R)

RD reagent diluent

RNA Ribonucleic acid rpm revolutions per minute s second(s)

RSV respiratory syncyntial virus

(S)

sIgA Secretory immunoglobulin A

SLPI Secretory leukocyte protease inhibitors

STD Dev. Standard deviation

SV saliva

(T)

TDF Tenofovir Disoproxil fumerate

TGF- β transforming growth factor beta

TH-1 T helper cell 1

TNF tumour necrotic factor

(U)

UCL Upper control limit

(W)

WAP Whey acidic proteins

WFDC1 WAP four-disulfide core domain protein 1

WHO World Health Organization

Wk36 week 36

Wk15 week 15

(Y)

Yrs. Years

LIST OF FIGURES

Literature review

Figure 1.2.1. Global map depicting the prevalence of HIV around the world by country.

Figure 1.2.2.1. Image depicting the structure of HIV.

Figure 1.2.2.2. Schematic ddepicting viral entry into host cell.

Figure 1.2.3.1: three major modes of HIV transmission from mother to their infants in the absence of interventions.

Figure 1.6.2.1. SLPI schematic structure.

Figure 1.6.2.2. E/tr-2 schematic structure.

Materials and methods

Figure 2.6. Saliva collection method.

Figure 2.7. Whole breast milk before and after spinning at 4^0C.

Figure 2.8: A sandwich ELISA schematic illustrating the principle of the assay.

Figure 2.10: Sample preparation and serial dilutions for spike and recovery and linearity testing (breast milk and infant saliva).

Results

Figure 3.1.1. Standard calibration curve used to determine the concentrations of SLPI in infant saliva and maternal breast milk.

Figure 3.1.2. A standard calibration curve used to determine the concentrations of E/tr-2 in infant saliva and maternal breast milk.

Figure 3.3(a). Quality control charts for SLPI ELISA assays.
Figure 3.3(b). Quality control charts for E/tr-2 ELISA assays.

Figure 3.5.1. Kinetics of SLPI and E/tr-2 protein levels in breast milk.

Figure 3.5.2. Kinetics of SLPI and E/tr-2 protein levels in infant saliva.

Figure 3.5.3. The impact of HIV exposure on the concentration of SLPI and E/tr-2 protein levels in maternal breast milk.

Figure 3.5.4. The impact of HIV exposure on the concentration of SLPI and E/tr-2 protein levels in infant saliva.

Figure 3.6.1. Changes in SLPI and E/tr-2 protein levels in HIV Exposed EBF/NEBF and HIV Exposed EFF/NEFF infant saliva.

Figure 3.6.2: The impact of feeding on the concentration of SLPI and E/tr-2 in HIV Exposed Uninfected infant saliva.

Figure 3.7.1: Spearman correlation between E/tr-2 levels in breast milk and infant saliva from all groups.

Figure 3.7.2.1: Spearman correlation plot of SLPI levels between breast milk vs. infant saliva from all groups.

Figure 3.7.2.2. Spearman correlation plot of SLPI comparing the two variables in HIV +ve and HEU at week 15.

Figure 3.7.2.3. Spearman correlation plot of SLPI comparing the two variables in HIV +ve and HEU.at week 36.

LIST OF TABLES

Table 2.1 Participant Selection Criteria

Table 3.2.1: Spike and recovery results for the optimization of SLPI and E/tr-2 ELISA assay

Table 3.2.2: Linearity testing results for SLPI protein tested in saliva and breast milk

Table 3.2.3: Linearity testing results for E/tr-2 protein tested in saliva and breast milk

Table 3.4.1 Maternal and infants characteristics

ABSTRACT

Background

Among infants, postnatal HIV transmission occurs via the oral route when ingesting breastmilk containing HI-virions (Kuhn *et al*., 2007). HIV acquisition via this route is however very low, possibly due to the presence of innate proteins in the oral secretions. Such innate proteins with anti-HIV activity include secretory leukocyte protease inhibitors (SLPI) and Elafin/trappin-2 (E/tr-2). At physiological concentrations SLPI and E/tr-2 have been shown to inhibit *in vitro* HIV replication in human monocytes and epithelial cells, respectively.

Study Aims

1) To determine the impact of HIV infection/ exposure on the concentration of SLPI and E/tr-2 in maternal breast milk and infant saliva; 2) To investigate the impact of of feeding modes on the concentration of innate proteins in infant saliva.

Methods

This study compared the concentrations of SLPI and E-tr2 among three groups of maternal-infant pairs to address the study aims. Saliva was collected from HIV unexposed breast fed (UBF, n=135), HIV exposed breast-fed (EBF, n=151) and HIV exposed formula fed (EFF, n=141) infants together with breast milk from HIV negative (HIV-ve, n=144) and HIV positive (HIV+ve, n=165) mothers. SLPI and E/tr-2 concentrations were measured in samples collected at birth, 15 and 36 weeks of infant age using ELISA.

Results

Breast milk concentrations of SLPI and E/tr-2 significantly decreased over time in both HIV+ve and HIV-ve women. The salivary SLPI concentration in HEU infants significantly increased over time. In the HU infants, significantly high SLPI concentrations were observed at birth, however the concentration of the analyte was significantly decreased at week 15 and then increased again significantly at week 36.

The concentration of E/tr-2 in infant saliva remained the same at all-time points. No significant differences were observed in breast milk SLPI and E/tr-2 concentrations between HIV+ve and HIV-ve mothers at birth and 15 weeks of infant age. However, breast milk SLPI concentrations were significantly higher in the HIV-ve mothers compared to the HIV+ve mothers at 36 week of infant age.

Salivary SLPI concentrations were significantly higher in the HU infants at birth, however, at 15 weeks of age an inverse was observed where HEU infants had significantly high SLPI concentrations compared to the HU infants. Additionally, salivary SLPI concentration was significantly higher in HIV exposed formula fed infants compared to HIV exposed breast fed infants at birth. However these differences dissipated with advancing age. No differences were observed for E/tr-2 concentration for both breast fed and formula fed infants. Lastly, no correlation was observed between maternal breast milk and infant salivary SLPI and E/tr-2 concentration at all-time points.

Discussion

The production of SLPI and E/tr-2 in infants is possibly due to inflammation as a results of bacterial LPS or other factors and not breastfeeding as originally hypothesized. In our cohort, we observed that elevated or low concentrations of SLPI in maternal breast milk is independent of HIV infection. However, in infant saliva HIV exposure played a significant role. An interesting finding in our study was that infants who received breast milk at birth had significantly low SLPI concentrations compared to those who were exclusively formula fed. However, at 15 and 36 weeks of infant age the concentration of SLPI increased probably due to ongoing exposure. It was also notable that we did not see any changes in E/tr-2 concentrations over time nor differences at all-time points.

Conclusion

The data suggest that HEU infants produce sufficient SLPI concentrations capable of blocking HIV infection at 15 weeks of life, as previously shown (Mcneely *et al.,* 1995, Bacqui *et al*., 2002). Further studies are required to investigate the ability of this protein to block HIV infection *in vitro* using human saliva.

1. LITERATURE REVIEW

1.1. INTRODUCTION

Children, especially infants, are at high risk of infectious diseases. In 2017, 5.4 million deaths in children under the age of 5 years were recorded globally (WHO, 2017). Infectious diseases such as diarrhoea, pneumonia and malaria have contributed about 30% of these deaths (WHO, 2017). Increased risk of infection during infancy is mostly attributable to either functional immaturity of the immune system (Velilla *et al.* 2006) or possibly active suppression of immunity at birth. In developing countries, particularly sub-Saharan Africa, the rate of infant mortality is exacerbated by overburdened health care systems, malnutrition, poor access to clean drinking water, and exposure to HIV, *M.* tuberculosis and malaria infections (Reidpath & Allotey 2003).

The innate immune system is the first line of defence against infections. This line of defence in infants is, however, impaired, making them more vulnerable to infections (Maródi 2006). Infants possess lower proportions of neutrophils compared to adults, making them more susceptible to bacterial infections (Maródi 2006). Infants' adaptive immune response is also inefficient in clearing pathogens partly due to defects in T-cell activation (Zaghouani *et al.* 2009). Infants have impaired TH-1 mediated responses compared to adults (Gervassi & Horton, 2014) due to skewed cytokine production. Infants were also shown to have upregulated anti-inflammatory cytokines such as IL-10 which hinders TH1 mediated response (Zaghouani *et al.* 2009).

In addition to diarrheal and respiratory infections, infants in low resource countries where there is high prevalence of HIV infection among women of child bearing age are at increased risk of acquiring HIV postnatally (Shapiro *et al.* 2007). Approximately 1.4 million infants are born to HIV infected mothers annually (Diallo et al. 2016) and the major route of HIV acquisition in infants is through the ingestion of HIV infected breast milk (Farquhar et al. 2002). In the absence of ART, above 70% of HIV exposed infants remain HIV uninfected despite repeated exposure through breast feeding. This constitute one of the major paradoxes of HIV transmission via breast milk (Wahl *et al,* 2012).. The lack of HIV transmission through breast feeding (Farquhar *et al.* 2002), despite repeated exposure suggests that this secretion is composed of immune factors with anti-HIV activity (Farquhar & Steward, 2003). In developed countries, replacement feeding has played a significant role in the reduction of HIV mother to

child transmission (MTCT). Exclusive formula feeding has reduced postnatal HIV transmission rates to less than 2% (European Mode of Delivery Collaboration, 1999). However, in countries where there are limited resources, replacing breast milk with formula is associated with increased risk of death (Onyango-Makumbi, 2010). Furthermore, breastfed infants benefit from bioactive immunological components in breast milk that confer passive protection (Farquhar & Steward, 2003). Maternal breast milk is rich in bioactive immunological components including innate factors such as defensins, lactoferrin, SLPI and immunoglobulin's, especially secretory IgA (Planer et al. 2016). However, this protection may be transient as mature breast milk contains lower concentration of these immunological factors (Farquhar et al. 2002), (Castellote et al. 2011), (Gregory & Walker 2013)

HIV infected mothers are encouraged to exclusively breast feed (EBF) their infants until 6 months of age (Bland *et al.* 2008) Exclusive breast feeding (EBF) decreases the risk of postnatal HIV transmission compared to mixed feeding (Kuhn *et al.* 2007). These findings have been corroborated by Charurat et al, 2009, which showed that mix feeding increases the risk of postnatal HIV transmission, and the effects of mix feeding in HIV transmission can be seen at early as 7 days after birth. Mix feeding was shown to damage the already delicate and permeable infant gut wall, thus allowing the virus to be easily transmitted (Coovadia *et al*, 2007). However, in exclusively breastfed infants, the rate of HIV transmission was reduced to less than 4% among women who exclusively breastfed their infants for ≥ 6 months of age and who initiated ART early during pregnancy (Bispo et al. 2017). EBF is defined as feeding infants with only breast milk during the first six months of life with no other liquids or solids except for prescribed medicines (Duncan *et al.* 1993). The mechanism by which EBF lowers the rate of HIV transmission is yet unknown, but may be due to increased concentration of innate factors in breast milk.

Breastfed infants acquire HIV by ingesting the virus in maternal breast milk (Coutsoudis *et al.* 1999), although this route of infection is inefficient. Even in the absence of antiretroviral therapy, more than 60% of infants born to HIV infected mothers did not acquire the virus despite repeated exposure through breast feeding (Ahmed *et al.* 2016) Improved coverage of antiretroviral (ARV) treatment and exclusive breast feeding have significantly reduced post-natal HIV transmission. Moreover, the

presence of innate immunological factors in oral secretions may also contribute to the low rates of HIV transmission via the oral route (Mcneely *et al.* 1995).

This study aimed to measure the concentration of key innate antiviral proteins in maternal breast milk and infant saliva, by comparing the concentration of these factors between HIV-infected and HIV-uninfected maternal breast milk, in HIV-exposed and HIV-unexposed infant saliva. The study will also investigate the impact of different feeding modes on the concentration of these innate factors in infant saliva.

1.2. HIV EPIDEMIC

As CD4+ T cells are also HIV target cells (UNAIDS WHO, 2007), the ability of the adaptive immune system to fight infections is impaired. This causes infected individuals to be more susceptible to acquiring infections. If untreated, the infection eventually develops into Acquired Immune Deficiency Syndrome (AIDS), which results from a combination of symptoms and illnesses that develop as a results of advanced HIV infection (UNAIDS, 2007).

HIV infection is a global health concern. An estimated 36.9 million people are currently living with HIV infection (UNAIDS, 2018) and more 940 000 of these people have died from AIDS-related causes. Two thirds of people living with HIV reside in sub Saharan Africa, and more than 43% of new HIV cases have been recorded in Eastern and Southern Africa (UNAIDS, 2018). Figure 1.2.1 depicts global HIV prevalence, showing Africa as the most affected continent (UNAIDS, 2018). This includes more than 160 000 children who are less than 15 years of age, who were infected either during pregnancy, childbirth and/or breast feeding (WHO, 2016).

Global prevalence of HIV in adults (2017)

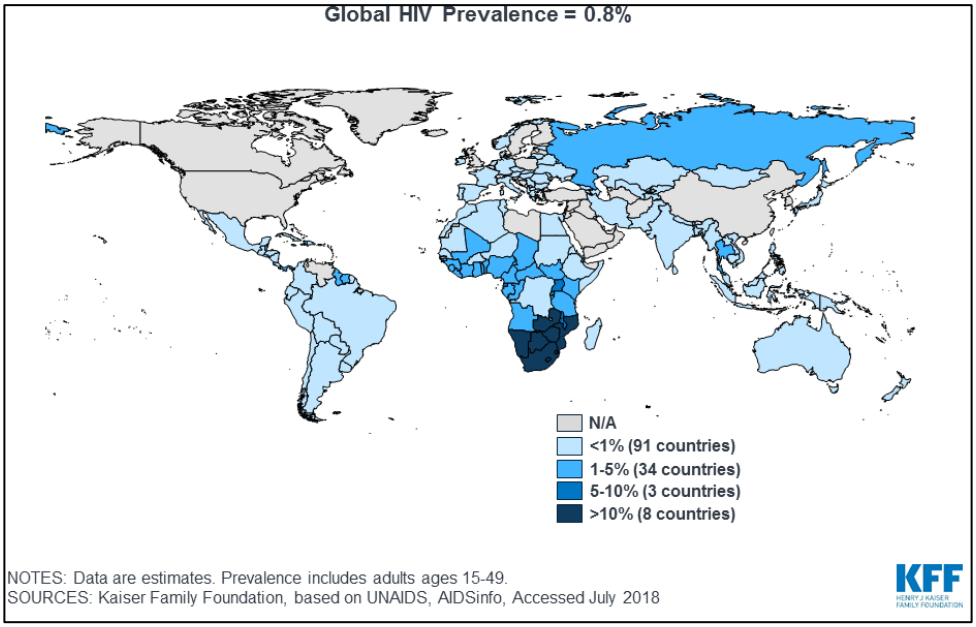


Figure 1.2.1. **Global map depicting the prevalence of HIV around the world by country**. As depicted in the map HIV infection is more prevalent in African countries compared to other continents in the world (UNAIDS, 2018).

1.2.2. HIV STRUCTURE AND THE MECHANISM OF ACTION

HIV is an RNA virus belonging to the Lentivirus genus. It is composed of a 100nm icosahedral structure consisting of 72 external spikes (figure 1.2.2.1). These spikes are produced by two viral envelope glycoproteins gp120 and gp41 (Briz *et al.* 2006). The glycoprotein gp120 is composed of five conserved domains (C1-C5) and five variable loops (V1-V5). The variable loops occur at the surface of gp120 and play a major role in binding to host cell receptors (Scheib *et al.* 2006).

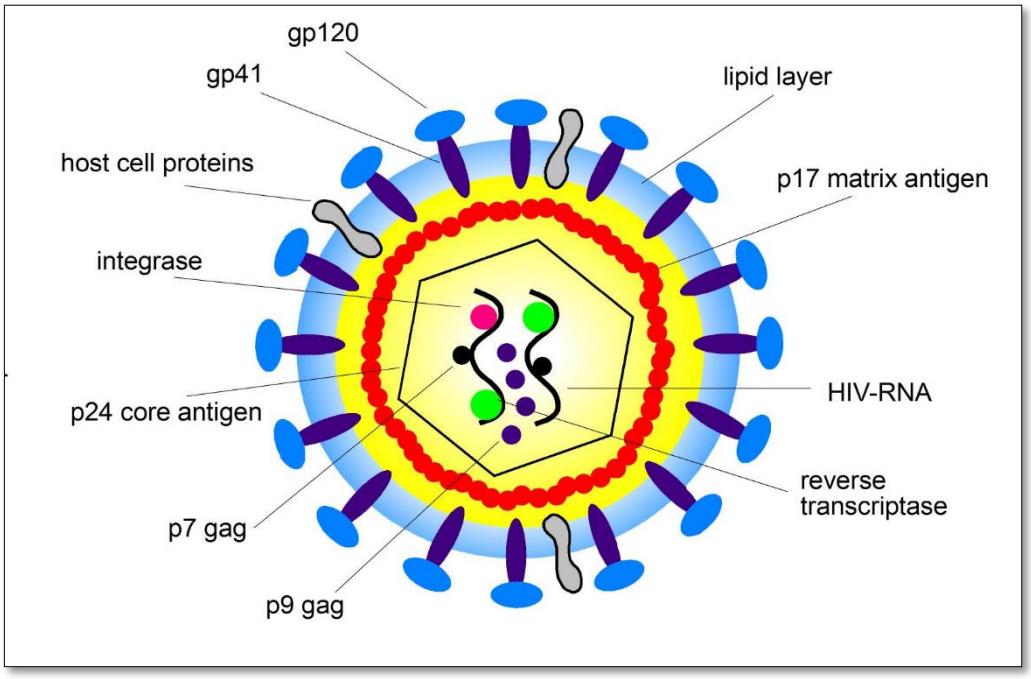


Figure 1.2.2.1. **Image depicting the structure of HIV**. HIV utilises gp120 to gain entry into the host cell by binding to CD4 T cells. The binding of gp120 to CD4 cells induces conformational changes in gp120 exposing a co-receptor binding site on gp120. Thereafter, gp41 initiates membrane fusion followed by viral entry (Rubbert *et al.* 2011).

HIV uses envelope glycoproteins to gain entry into host cells. It achieves that by attaching to the CD4 molecule expressed on the surface of T helper cells, dendritic cells, macrophages, monocytes and other cell types in the host (Wilen *et al.* 2012). However the virus can gain entry into the cells using other routes other that the CD4 cells. These includes the use of C-type lectin reseptors DC-SIGN, a dendritic cell receptor that is utilised by the virus to gain entry in to the host cell (Turville *et al*. 2003). In addition to DC-SIGN (Geijtenbeek *et al.* 2000) (Kwon *et al.* 2002), langerin is utilised by HIV virus to gain entry into langerhan cells (Turville *et al*. 2003), however the mechanism utilised by these C-type lectin receptors to initiate infection is yet to be identified. CD4 proteins are members of immunoglobulin family that function by enhancing T-cell receptor mediated signalling (Wilen *et al.* 2012). The interaction of envelope gp120 with CD4 results in rearrangement of gp120 viral loops and the formation of bridging sheets that play a critical role in co-receptor engagement (Wilen *et al.* 2012).

The virus utilises coreceptors CXCR4 and CCR5 to gain entry into CD4+ T cells (Bleul *et al.* 1997). Viral tropism is defined by the coreceptor it utilises to bind to CD4+ T cells; R5 viruses use CCR5, X4 viruses use CXCR4 and viruses that utilise both the coreceptors are referred to as R5X4 or dual-tropic (Berger *et al.* 1998). T helper cells and some macrophages and dendritic cells are susceptible to HIV infection because they express CD4 receptors and either CCR5 or CXR4 on their surface (Clapham & ,McKnight, 2001).

Binding of gp120 to host cell receptors causes changes in gp41 leading to viral and host cell membrane fusion. The fusion causes the release of viral RNA into the host cell cytoplasm (Wilen *et al.* 2012) as depicted in figure 1.2.2.2. The RNA released consists of genetic code for synthesis of viral capsid, integrase, reverse transcriptase and accessory proteins. Once the viral RNA is in the cytoplasm, reverse transcription is initiated to convert the RNA into double stranded DNA (Freed 2001). The double stranded DNA is then transported to the host cell nucleus, where the virus is incorporated into the transcriptional sites of the chromosome, leading to viral integration into the host cell DNA (Freed 2001).

HIV integration, assembly and budding

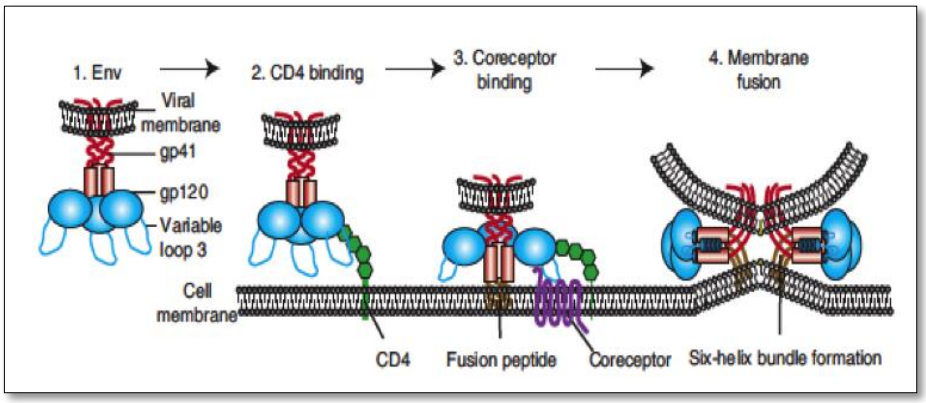


Figure 1.2.2.2. Schematic depicting viral entry into the host cell. (1) Gp120 and gp41 attaches to the host cell by binding to CD4. (2) This causes conformational changes of envelope glycoproteins resulting in co-receptor binding. (3) The next step is membrane fusion achieved by the fusion of gp41 peptides into the targeted membranes. (4) Membrane fusion (Wilen *et al.* 2012).

1.2.3. HIV TRANSMISSION

The major mode of HIV transmission in adults is via sexual intercourse *(Supervie et al.* 2010). However, HIV transmission can also occur via intravenous drug use, through contact with infected blood, *in utero* and via breast feeding from an HIV infected mother. Sexually, HIV can be transmitted through penile-vaginal intercourse, the major route of transmission in women (Baggaley *et al.* 2009) and (Kalichman, Simbayi & Jooste, 2009), penile-anal intercourse (Beyrer et al. 2012) and via oral sex, however, infection via this route is extremely low (Farquhar *et al.* 2003). There are various factors that may increase the risk of sexual transmission of HIV that include concurrent infections such as gonorrhoea, chlamydia, syphilis and *Trichomonas* (McClelland *et al.* 2001) and (Quinlivan *et al.* 2012)). Douching, partner HIV viral load and the general health of the mucosal barrier are among other factors that can influence HIV transmission (Muessig *et al.* 2012), (Rodger *et al.* 2014).

1.2.3.1. MOTHER TO CHILD HIV TRANSMISSION

Perinatal Mother to Child Transmission (MTCT) accounts for about 35-40% of HIV-infections in children ≤ 15 years of age in the absence of intervention (Lehman & John-Stewart *et al*. 2009). Approximately 130 000 new HIV infections were recorderd in infants globaly (UNAIDS, 2017). The transmission of HIV from mother to child can happen in three major ways as depicted in figure 1.2.3. *In utero* HIV transmission occurs via the placenta (Spector 2001). Intra-partum occurs via infant swallowing infected maternal blood during delivery, but can also be due to medical complications that include chorioamnionitis or micro breaches in the placenta during labour.

Postpartum transmission occurs via the ingestion of HIV infected maternal breast milk, this is the major route of HIV transmission in infants (Mcneely et al. 1995). In the absence of ART, HIV transmission through breastfeeding is estimated to affect more than 20% of infants in developing and under developing countries (Page-Shafer *et al.* 2006). However, the risk of transmission is dependent on the duration of breast milk (AVERT, 2015), maternal viral load, CD4 count (WHO, 2015) as well as maternal and infant factors (Wahl *et al*. 2012). This is a paradox because breast milk was also

shown to inhibit HIV transmission in vitro (Wahl et al. 2012). The inhibitory effect exerted by breast milk is through a variety of innate factors disseminated in mucosal secretions, explained in detail in section 1.4 (Farquhar et al. 2002). The initiation of Option B i.e. ART treatment initiated in all HIV positive women, until one week after cessation of breast feeding as well as option B+ i.e. initiating lifelong combination of ART for all pregnant women (WHO, 2012), has also significantly reduced MTCT to less than 5% (AVERT, 2016).

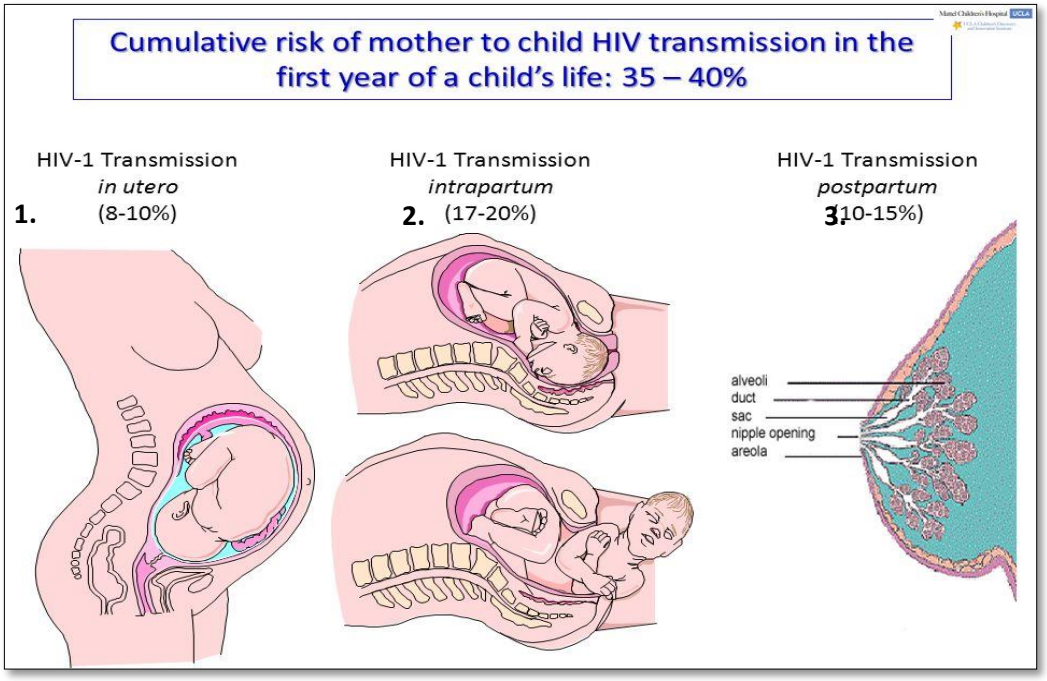


Figure 1.2.3.1: Three major modes of HIV transmission from the mother to their infant in the absence of interventions. (1) HIV transmission during gestation, (2) HIV transmission during delivery and (3) HIV transmission postpartum during breast feeding (Nielson, 2016).

1.3. INFANT IMMUNE SYSTEM

Infants are more susceptible to viral and bacterial infections such as respiratory syncytial virus (RSV), disseminated herpes simplex virus, Mycobacterium tuberculosis and plasmodium falciparum (causative agent of malaria) (Nizet & Esko, 2009). Impairment of functional T-cells and defects in neonatal antigen presenting cells (APCs) accounts for a large proportion of the increased susceptibility to infections in early life compared to adults (Velilla *et al.* 2006).

Infants are known to express low levels of TCR/CD3, a complex involved in antigen recognition and T cell activation(Boćko & Frydecka 2003). Antigen presenting cells in neonates are defective in producing IFN-α which is essential for the upregulation of MHC- class I molecules (Basha *et al.* 2014). Moreover, dendritic cells in infants express low levels of IFN-γ and IL-12 upon stimulation by bacterial lipopolysaccharides, hence hampering TH-1 mediated immune responses (Raundhal *et al.* 2015). Monocytes and dendritic cells of neonates express low levels of CD80, CD86 and CD40 which are co-stimulatory signalling molecules needed for T cell activation. Furthermore, neonatal T cells were shown to be more vulnerable to apoptosis resulting in increased susceptibility to infection (Adkins 1999).

The expression of cell surface receptors such as HLA-DR, an MHC class II protein required for antigen presentation, is downregulated in infants (Velilla *et al.* 2006). This results in defects in the function of antigen presenting cells resulting in limited ability to recognise invading pathogens (Velilla *et al.* 2006). It is also recorded that infant monocytes demonstrate limited response upon stimulation by LPS, leading to reduced capacity to differentiate into macrophages (Maródi 2006).

Neonates have lower proportions of neutrophils compared to adults and neonatal neutrophils express limited selectin and β-integrin which are important adhesion molecules. The low expression of these molecules may result in limited tissue recruitment of neutrophils (Garvy & Harmsen 1996). In addition to being quantitatively less, neonatal neutrophils have reduced antimicrobial activity which increases their susceptibility to infection (Antoniades *et al.* 2014).

Neonatal immunity demonstrates reduced ability to mount effective immune responses against invading pathogens due to inadequate production of essential immune factors such as proinflammatory cytokines TNF-α This results in increased susceptibility to infections by invading bacterial and viral pathogens and therefore relying heavily on maternal breast milk for immune protection. Breast feeding also facilitates neonatal immune development because of the bioactive components that are found in breast milk.

1.4. BREAST MILK

Maternal breast milk provides infants with passive immunity against infections (Andreas et al. 2015). This protective effect is a result of maternal immune components such as immunoglobulins, leukocytes and innate proteins, discussed in detail in section 1.4.1 (John-Stewart et al. 2004). Bioactive components in breast milk act as vehicles of communication between the maternal immune system and that of the infant (Yolken *et al.* 1992). Breast milk also serves to direct and educate immunity, metabolism and microbial communities in infants (Wright *et al.* 1998). Breast milk does not only protect infants against infection, but also facilitates immune development and tolerance (Wright *et al.* 1998), (Field 2005), (Walker 2010).

Breast milk can prevent gut pathology and allows for the establishment of a healthy gut microbiome in infants (Dieterich *et al.* 2013). This is thought to happen by increasing the acidity in the gut, thus lowering the number of harmful bacteria present (Dieterich *et al.* 2013). Replacing breast milk with formula alters the acidity of the gut environment, promoting the presence of harmful bacteria (Planer *et al.* 2016). Furthermore, mixed fed and formula fed infants have higher susceptibility to viral infections, respiratory infections and diarrhoea compared to exclusively breastfed infants (Kuhn *et al.* 2007).

1.4.2. BREAST MILK BIOACTIVE COMPONENTS

Breast milk consists of a diverse mixture of bioactive molecules that influence and modulate the immune status of an infant. It contains a high concentration of secretory IgA (sIgA) antibodies that are specific to pathogens previously encountered by the mother (Planer *et al.* 2016). These antibodies neutralize pathogens in the infant gut thus preventing invasion of the mucosa (Hocini *et al.* 1997). Secretory IgA exhibits an ability to survive in gastrointestinal mucosal and respiratory membranes, therefore can act against infectious agents in these sites while reducing tissue damage that would result from inflammation (Belyakov & Ahlers 2012). Colostrum, the initial milk that the mother produces after birth (Walker 2010), is highly concentrated with leukocytes such as macrophages and neutrophils (Uruakpa *et al.* 2002). Presence of these immune

cells aid in destroying invading pathogens. In an experimental mouse model, it has been shown that breast milk contains lymphocytes such as natural killer cells, B and T cells that can survive passage through the infant's gastrointestinal systems (Van De Perre *et al.* 2012). These lymphocytes are ingested by an infant through the act of breast feeding to facilitate the development of immunity (Van De Perre *et al.* 2012).

Breast milk also contains antimicrobial peptides such as lysozyme, defensins, and lactoferrin (Henrick *et al.* 2017). Lysozymes inhibit bacterial growth by disturbing bacterial proteoglycan layer (Henrick *et al.* 2017). Lactoferrin is a glycoprotein belonging to the transferrin family (De Almeida *et al.* 2008). It is composed of single polypeptide chains folded into two symmetrical globular lobes required for metal binding (White, 2009). Lactoferrin has a high affinity for iron and therefore hinders the proliferation of iron dependent pathogenic bacteria (Spadaro *et al.* 2008). Other innate proteins found in maternal breast milk include elafin/trappin-2 (E/tr-2) and secretory leukocyte protease inhibitors (SLPI) whose properties and functions are detailed in section 1.6.1. (Baqui *et al.* 1999) .

1.5. THE ORAL MUCOSAE AND IMMUNOLOGICAL PROTECTION AGAINST PATHOGENS

1.5.2. THE ORAL MUCOSA

The oral mucosa consists of heterogeneous structures and tissues working in synergy (Yoshizawa *et al.* 2013). Salivary fluid constituents include various proteins, immunoglobulins, mucosal glycoproteins, peptides and bacteria all essential in maintaining a healthy oral environment (De Almeida *et al.* 2008).

Saliva plays a significant role in the digestive process of ingested food. Enzymes such as amylases and maltase break down lipids and starches into maltose. Acinar cells in the salivary glands secrete saliva which drains into small salivary ducts and is released into the oral cavity (De Almeida *et al.* 2008). The salivary secretions consist of variable proteins that have immunological and non-immunological properties. Some of these proteins are involved in preventing calcium and phosphate ion precipitation (De Almeida *et al.* 2008).

1.5.3. PROTECTIVE IMMUNOLOGICAL PROPERTIES IN ORAL SECRETIONS

Saliva consists of various immunological factors essential in fighting against microbial and viral infections. Secretory IgA is the most abundant immunoglobulin in saliva, and is essential for viral, bacterial and enzymatic toxin neutralization *(Wood et al.* 2013). sIgA is important in coating bacteria therefore hindering their attachment to the oral tissue (Walker, 2010). IgG and IgM are also present in saliva however they are available in low concentrations.

Saliva is also composed of mucins, which are sulphated polysaccharides. These polysaccharides are mucus building blocks important in protecting the host against invading pathogens (Frenkel & Ribbeck 2015). They are present in oral secretions in two forms distinguished based on their molecular weight; high (MG1) and low (MG2) molecular weight. Modifications in the expression of these polysaccharides increases the host susceptibility to ulcerative colitis and asthma (Frenkel & Ribbeck 2015). The

unique structure of mucins characterized by (Desseyn *et al.* 1997) enables host protection against invading pathogens.

Included in oral secretion are also histatins, peptides that have antimicrobial activity against gram negative bacteria. Histatins also possesses antifungal activity against *Candida albicans* growth (Kavanagh & Dowd 2004). These peptides exert their antimicrobial activity by neutralizing the LPS of bacterial external membranes (Kavanagh & Dowd 2004).

1.6. HIV AND INNATE FACTORS IN SALIVA AND BREAST MILK

Components of the innate immune system are located at epithelial barriers, including the complement system, cells with phagocytic and antigen presenting properties including macrophages and dendritic cells (Janeway & Medzhitov 2002). These cells operate at both soluble and cellular levels using pathogen recognition receptors (PRRs). PRRs play a central role in innate immune defence because of their ability to recognise evolutionarily conserved structures of pathogens (Janeway & Medzhitov 2002). The innate immune system functions as a bridge between pathogen encounter and the delayed, antigen specific adaptive immune response (Iwasaki *et al.* 2010). Although the innate immune system are essential in clearing HIV infections, they can also facilitate infection.

Among the innate factors with anti-HIV activity, defensins are small cationic peptides (2-6KDa) produced by epithelial cells. These peptides have anti-viral properties against a wide range of viruses. In HIV infection, defensins exert their antiviral function through direct interaction with the viral envelope (Daher *et al.* 1986). Although defensins have anti-HIV properties, they also have an immunomodulatory role by acting as chemoattractants for T lymphocytes, monocytes and dendritic cells, and as regulators of cell activation and cytokine production (Lahey *et al.* 2012). Thus defensins may also promote HIV acquisition by recruiting HIV target cells to mucosal sites (Lahey *et al.* 2012).

As discussed in Section 1.4.1, Lactoferrin is an important mucosal innate glycoprotein. *In vitro* analysis demonstrated that Lactoferrin exhibits anti-viral activity against a variety of viruses including Influenza and HIV (Van der Strate *et al.* 2001). Anti-HIV activity is attributed to the binding of Lactoferrin to viral gp120 glycoproteins that are required for viral entry into the host (Kazmi *et al.* 2006). Therefore the protein acts against HIV in the early stages of infection probably during the adsorption of the virus into the host cell (Kazmi *et al.* 2006). Additionally Lactoferrin hinders HIV infection when it is in an iron-bound and iron-free state which concurrently exist in human saliva (White *et al.* 2009).

Another component of the innate immune system demonstrated to have anti-HIV activity is Interleukin-16 (IL-16). In-vitro, IL-16 was shown to hinder HIV replication in infected T cells (Idziorek *et al.* 1998). The mechanism by which IL-16 blocks infection of T cells with HIV is through direct binding to the CD4 receptor (Baier *et al.* 1995).The presence of anti-inflammatory cytokines such as IL-10 were also shown to suppress HIV replication in macrophages and monocytes by their anti-inflammatory properties (Saville *et al.* 1994).

Other components of the innate immune system known to play an active role in protecting the host from pathogens include secretory leukocyte protease inhibitors (SLPI) and elafin/trappin-2 (E/tr-2). These low molecular weight proteins exert their protective functions through their Whey acidic protein **(**WAP) domain that is unique with every species.

1.6.2. WHEY ACIDIC PROTEINS (WAP)

WAP, also known as whey/four disulphide core (WFDC), are small proteins that play a pivotal role in innate immunity. The amino acid composition of WAP is enriched with cysteine residues that are linked by disulphide bonds (Doumas *et al.* 2005). In humans, the WAP are encoded by a locus present on chromosome 20 which is also known as the WFDC locus (Bingle 2011). Secretory leukocyte protease inhibitors and elafin/trappin-2 are members of the WAP family, whose properties and functions are well studied (Bingle 2011). SLPI and E/tr-2 are multifunctional proteins with wound healing properties, antimicrobial and antiviral activities. They are expressed by epithelial cells at various mucosal surfaces (Moreau *et al.* 2008). In saliva and vaginal fluid, the presence of these proteins is associated with lower HIV transmission rates (Moreau *et al.* 2008).

1.6.2.1. *SECRETORY LEUKOCYTES PROTEASE INHIBITORS (SLPI)*

SLPI is a small 11.7kDa single-chain, non-glycosylated, soluble antimicrobial protein consisting of a cysteine rich peptide sequence with 107 amino acids. The SLPI gene is located on chromosome 20q1213.2 and consists of 4 exons and 3 introns that span

approximately 2.6kbp (Doumas *et al.* 2005). The protein has high affinity for neutrophil elastases and cathepsin G, which are serine proteases (Moreau *et al.* 2008). SLPI consists of two WAP domains that are homologous to elafin *(Moreau et al.* 2008) and distinguished by an NH_2-terminal involved in stabilising the protease-antiprotease complex (Mcneely *et al.* 1995.). The C-terminal domain of SLPI has the antiprotease activity (Doumas *et al.* 2005). Each domain consists of 53 and 54 amino acids respectively as depicted in figure 1.6.1.1 (Moreau *et al.* 2008).

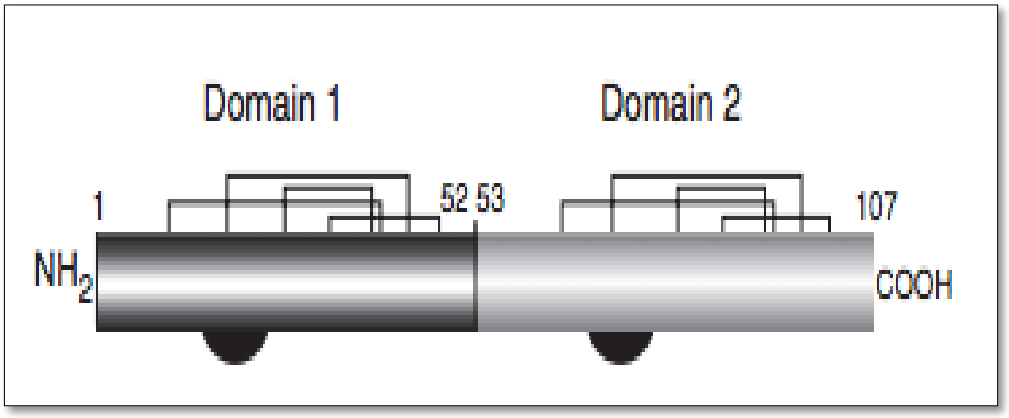


Figure 1.6.2.1. SLPI schematic structure. The plain lines represent the disulphide bond topology, half disc represent the protein inhibitory loops (Moreau et al. 2008).

1.6.2.1.1. SLPI SECRETION AND REGULATION

SLPI is produced by various epithelial cells located at different mucosal surfaces (Moreau *et al.* 2008), including the oral mucosa (Shugars 2002). vaginal mucosa (Moriyama *et al.* 1999) and the lungs. It is found in high concentrations in parotid (Thompson et al. 1986) and cervical fluid (Moriyama *et al.* 1999) and in the sputum of patients with chronic obstructive pulmonary diseases (Baqui *et al.* 1999). In the lungs, SLPI is produced by serous cells of tracheal and bronchial sub-mucosal glands and by non-ciliated bronchial epithelial cells (Doumas *et al.* 2005). The expression of SLPI is regulated by anti-inflammatory cytokines such as IL-10 and TGF-β (Py *et al.* 2009).

1.6.2.2. *ELAFIN/TRAPPIN-2*

Trappin-2 (Tr-2) is a serine protease inhibitor with anti-inflammatory properties at mucosal surfaces (Ghosh, Shen, *et al.* 2010). It is secreted as a cationic, non-glycosylated protein that is 9.9kda in size and consists of two WAP domains with different functions (Moreau *et al.* 2008). The cleavage of trappin-2 N-terminal domain reveals the C-terminal region that contains the WAP domain known as Elafin. Elafin/trappin-2 N-terminal domain acts as a substrate for transglutamase, an enzyme involved in the modification of proteins after gene translation (Doumas *et al.* 2005). It allows for the binding of elafin/trappin-2 to extracellular matrix components leading to the formation of elafin polymers (Drannik *et al.* 2012). Similar to SLPI, the C-terminal domain of Elafin blocks tissue proteolysis by neutrophils (Moreau *et al.* 2008). E/Tr-2 are pleotropic molecules in nature and have anti-proteolytic, immunoregulatory and antimicrobial properties (Drannik *et al.* 2012). The protein has inhibitory properties against human neutrophil elastase and proteinase-3 (Thomson & Saunders., 1986), (Walter *et al.* 1996). It has also been shown to inhibit the growth of fungi, gram-positive and gram-negative bacteria (Moreau *et al.* 2008). Similar to SLPI the protein contains multifaceted immune-regulatory properties that target binding, recognition and mounting of innate inflammatory responses against bacterial and viral antigens.

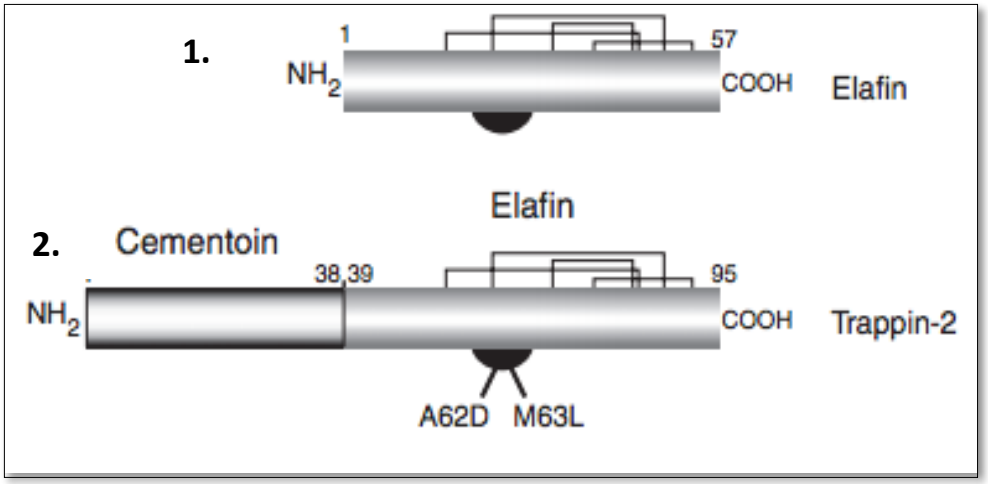


Figure 1.6.2.2. E/tr-2 schematic structure. Pre- elafin and the mature elafin/trappin-2 structure (Moreau *et al.* 2008).

1.6.2.2.1. E/TR-2 SECRETION AND REGULATION

Elafin/trappin-2 (E/tr-2) was first isolated from sputum in patients suffering from inflammatory lung disease and in scales of patients with psoriasis ((Zeeuwen *et al.* 1997). It was later discovered to be expressed at various mucosal surfaces prone to exposure to inflammatory stimuli (Ghosh, Shen, *et al.* 2010). E/tr-2 is found in tissues such as lungs, skin, colon, salivary glands, female genital tract, and in the placenta and foetal membranes (Shaw & Wiedow 2011). The protein is upregulated at sites that experience physical trauma, by conditions such as the psoriasis and by pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-a).

1.6.2. PROPERTIES OF SLPI AND ELAFIN/TRAPPIN-2

1.6.2.1. ANTI-MICROBIAL AND ANTI-INFLAMMATORY ACTIVITY

Antimicrobial properties of SLPI and Elafin have been demonstrated both *in vitro* and *in vivo* (Skott *et al.* 2002). The N-terminal domain of SLPI and E/tr-2 is suggested to mediate the antimicrobial activity against *Eschericia coli*, *Staphylococcus aerus* and *Pseudomonas aeruginosa* (Hiemstra *et al.* 1996), by utilizing the cationic nature of the NH-2 domain in interacting with the anionic cell membrane of the bacteria therefore destabilising the membrane integrity (Sallenave *et al.* 1997).

1.6.3. TISSUE REMODELLING AND WOUND HEALING

SLPI and E/tr-2 play a significant role in tissue remodelling and wound healing (Doumas *et al.* 2005). E/tr-2 has been shown to be involved in cellular differentiation of foetal to adult submandibular glands (Henson *et al.* 2006). In the case of cutaneous injury in humans, high expression of SLPI and E/tr-2 have been noted (Williams *et al.* 2006). SLPI has also been shown to be an endogenous factor in cutaneous and oral mucosal wound healing (Williams *et al.* 2006). The SLPI protein was also shown to be an active mediator for the healing of dermal scarring (Henson *et al.* 2006). In an *in vitro* model, SLPI was shown to inhibit fibroblast-mediated collagen gel contraction (Henson *et al.* 2006).

1.6.4. ANTI-VIRAL ACTIVITY

Antiviral activity of SLPI during HIV infection has been well studied in saliva. McNeely et al. 1995 reported that SLPI salivary concentration of ≥10 μg/ml is capable of blocking HIV from replicating in human monocytes *in vitro* (McNeely *et al.* 1995). Confirming these findings is a study by Wahl et al., 1997 demonstrating that treatment of HIV infected monocytes with whole unstimulated saliva decreased HIV infection at concentrations that ranged from 1-10ug/ml (Wahl *et al.* 1997).

E/tr-2 anti-HIV activity has been well studied in female genital tract. A study by Drannik *et al. 2012* proposed that the presence of E/tr-2 in cervicovaginal fluid may be induced by viral antigens in the female genital tract. The authors also demonstrated that rE/Tr-2 inhibited the attachment of HIV infected cells to epithelial cells and prevented transcytosis across human epithelial genital cells *in vitro*. Kenyan commercial sex workers that were highly exposed to HIV but remained uninfected had high concentrations of E/Tr-2 in their genital secretions (Iqbal *et al.* 2009), compared to HIV infected women and healthy women. Thus low HIV transmission across the cervicovaginal mucosa is correlated with high E/Tr-2 concentrations in female genital tract (Iqbal *et al.* 2009).

1.6.5. ANTI-HIV MECHANISMS OF SLPI AND E/TR-2

The mechanisms by which SLPI and E/tr-2 exert their anti-HIV activity are not fully understood. However, for SLPI, it is thought to be independent of direct binding to the virus or to viral proteins (Wahl *et al.* 1997). It is hypothesised that in macrophages, SLPI's anti-HIV activity involves blocking cellular annexin II, a cell surface cofactor that binds phosphatidylserine and promotes HIV entry by stabilising virus fusion beyond HIV receptor/co-receptor complex, therefore interrupting viral entry into host cells (Ma *et al.* 2004). Therefore SLPI's mechanism of action is thought to involve the inhibition of protease mediated event required for virus entry to vulnerable cells (Mcneely *et al.* 1997). A second study showed that SLPI, in its recombinant form, prevented HIV infection of primary T-cell by regulating the interaction of CD4 receptor with phospholipid scramblases (PLSCRS1 and PLSCRS4) (Py *et al.* 2009). PLSCRS1 and

PLSCRS4 are membrane proteins that facilitate movement of phospholipids between the inner and outer leaflets of the plasma membrane (Py *et al.* 2009). However, the mechanism employed by E/tr-2 in mediating anti-HIV activity is yet to be identified.

Exploring the impact of HIV exposure on the concentration of these innate proteins in infant saliva and maternal breast milk is pivotal to understand their regulation. The concentration of innate proteins in saliva of infants may be influenced by different feeding practices and maternal HIV status. There is a paucity of data on the role of SLPI and E/Tr-2 in salivary secretions and in the prevention of MTCT. This MSc dissertation explores the effect of intrauterine HIV exposure and feeding practices on the concentration SLPI and E/Tr-2 in the saliva of Exclusively Breast Fed (EBF), Mixed Fed (MF) and Formula Fed (FF) infants born to HIV positive and negative mothers.

7.1. STUDY HYPOTHESIS

Innate proteins in saliva and breast milk play a role in prevention of MTCT of HIV and are influenced by HIV status and feeding mode of infants.

7.1.1. STUDY AIMS AND OBJECTIVES

Aim 1: To determine changes in innate protein concentrations in mucosal fluids over time.
Objective 1: To quantify SLPI and E/tr-2 concentrations in HIV-infected and uninfected breast milk, in HEU and HU infant saliva over time.

Aim 2: To determine the impact of HIV infection/ exposure on the concentration of innate proteins in breast milk and infant saliva.
Objective 2: To compare SLPI and E/tr-2 concentration in breast milk and infant saliva in HIV-infected/ exposed and uninfected/ unexposed mother-infant pairs.

Aim 3: To determine the impact of feeding practice on the concentration of innate proteins in infant saliva.
Objective 3: To quantify and compare SLPI and E/tr-2 concentrations in the saliva of breast fed and formula fed infants.

2. MATERIALS & METHODS

2.1. STUDY DESIGN

2.1.1. STUDY SITE

A total of 350 mother-infant pairs were recruited at the Midwife Obstetric Unit (MOU) in site B Khayelisha for a larger parent study entitled, "*Innate, Adaptive and Mucosal Immune Responses in HIV-1 Exposed Uninfected Infants: A Human Model to Understand Correlates of Immune Protection"* (REC/REF 285/2012) aimed in identifying correlates of protection against HIV conferred by exclusive breastfeeding. Khayelitsha is an urban township with a high unemployment rate and informal housing consisting of about 400,000 people. The MOU is the largest of the two public sector delivery units and about 30-32% of deliveries at site B MOU are from HIV infected mothers. The MOU is situated 28 miles from the University of Cape Town where the study laboratories are located. All collected samples are stored at the same University for analysis.

2.1.2. STUDY PARTICIPANTS

Only participants who met the eligibility criteria (Table 2) were invited to participate in the study. Mothers self-selected their preferred feeding mode; the study participants were encouraged to exclusively breastfeed or exclusively formula feed for 6 months as supported by World Health Organization (WHO) guidelines (WHO, 1998). Trained counselors provided support and counseling to the mothers, including information pertaining to the risks related to HIV transmission. Prior to enrollment and all study procedures, all women and the infant's father (where applicable) signed a study informed consent form. The study has been implemented in conjunction with the routine pediatric follow up and the infant EPI vaccination programs to enhance its feasibility and acceptability to study participants.

Table 2.1 Participant Selection Criteria

Inclusion Criteria	**Exclusion Criteria**
Maternal Factors	**Maternal Factors**
1) Documented HIV status 2) Age of mother ≥ 18 yrs 3) Mother has elected to either exclusively breastfeed or formula feed her infant. 4) Mother is willing to do the follow up assessments 5) Mother is willing to sign the consent form	1) Complications during pregnancy and delivery such as chorioamnionitis and preeclampsia
Infant factors	**Infant factors**
1) Gestational age > 36 weeks 2) Birth weight >2.4kg	1) Hypoxic injury/seizures/sepsis/intrauterine growth retardation. 2) HIV infection

2.2. CLINICAL ASSESSMENTS

Clinical evaluations were undertaken for each scheduled study visit. Maternal baseline evaluations included a medical history, physical examination, HIV disease history (where applicable), ART treatment, most recent plasma HIV-RNA if available and CD4 count. In addition, obstetric information was abstracted from the medical charts. Infant baseline evaluations consisted of anthropometrics, physical examination, gestational age at birth and relevant birthing factors and complications. Both maternal and infant health status were monitored at each clinic visit using standardized forms. Specific health factors were evaluated at each visit, i.e. height, weight, co-infections, extensive feeding history, thrush (infant), and medications

including ART. In addition a breast exam was conducted at each visit to identify any clinical breast pathology (e.g. nipple cracks, mastitis).

2.3. FEEDING PRACTICE

Information on feeding practices was also collected using structured feeding questionnaires validated in similar settings (Kuhn *et al.* 2002). Importantly, research personnel administering the questionnaire were different from individuals that provided breastfeeding counselling to the mothers, in order to minimize bias.

2.4. ETHICAL CONSIDERATIONS

Prior to the implementation of study procedures, ethical approval was granted by UCT Human Research Ethics Committee (HREC) for the proposed research. Only participants who signed UCT Human Research Ethics Committee-approved consent forms were enrolled. Informed consent was obtained from the legal guardian in their preferred language. If maternal age was less than 18 years, they were ineligible to participate. All clinical research staff received GCP training. Breast milk volumes were well within the limits in this age group. Participants had an alternative of not participating in the study, and it was made clear that the choice of not to participate was not going to affect their care.

2.5. SAMPLE COLLECTION AND PROCESSING

Due to budget constraints, 427 infant saliva and 309 maternal breast-milk samples were analyzed at baseline (birth, d4-7), week 15 and week 36 for the purpose of this dissertation. The infant saliva was sampled from 29 HIV exposed-uninfected (HEU), 24 HIV unexposed (HU) and 24 HIV-exposed uninfected-formula fed (HEU-FF) infants. Maternal breast milk was sampled from 28 HIV infected (HIV+) and 22 HIV uninfected (HIV-) mothers.

2.6. INFANT SALIVA

As shown in Table 2.5, Infant saliva was collected at birth, week 15 and week 36. The collection was done using Salivette tubes (Sarstedt AG & Co, Numbrecht, Germany) by placing the swab into the infant's mouth, the infant was allowed to suck the swab for at least 60 seconds to stimulate salivation. After 60 seconds the swab was placed back into the salivette tube and the stopper was replaced. The salivette tube was immediately stored on ice and transported to the lab for processing. At the lab the salivette tube was centrifuged at 3000 rpm for 5 min, after which the collected saliva was pipetted into 2ml tubes and stored at -80°C until time of analysis (Figure 2.6). Of note infant saliva samples were collected at least 30 minutes after their last feeding to avoid saliva contamination by maternal breast milk.

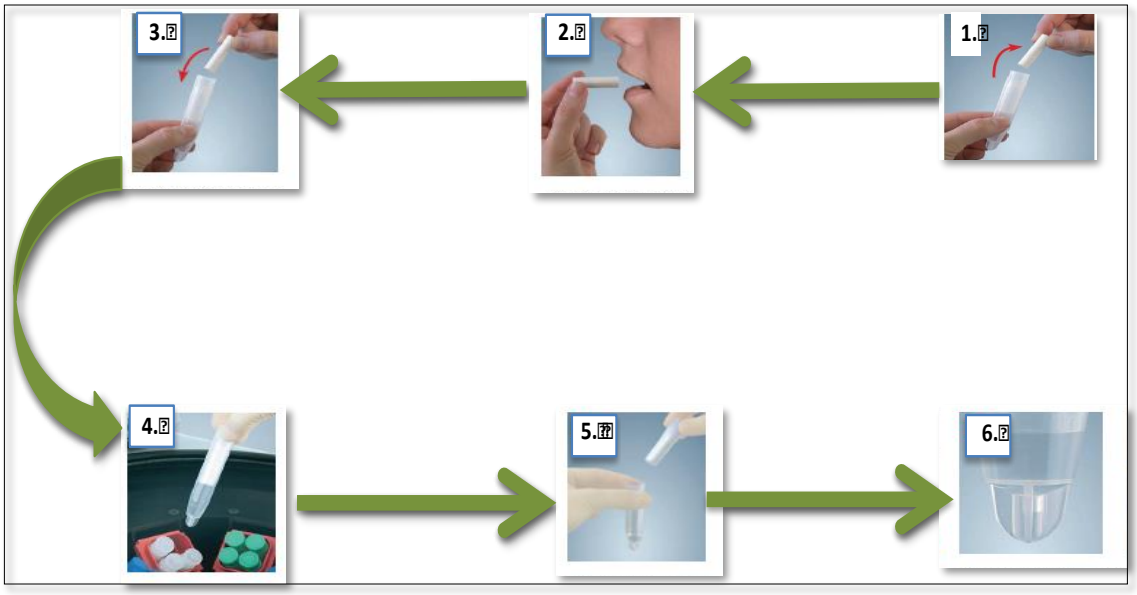


Figure 2.6: Saliva collection process 1. The swab is removed from the tube by clinic staff. 2. The swab is then placed into the infant's mouth and is left there for approximately 60 seconds. 3. After the 60 seconds the swab is removed from the infant's mouth and returned into the salivette. The stopper is also replaced; the salivette is placed into a cooler box with ice and is transported to the lab. 4 and 5. In the lab, the salivette is spun at 3000 rpm at 4 degrees for 2 minutes, after the 2 minutes lapse the closed insert containing the swab is removed and discarded. 6. The recovered saliva is then stored at -80oC until analysis (Sarstedt AG & Co).

2.7. BREAST MILK

Maternal breast milk was collected at between 4 to 7 days, and at weeks 4, 15, 36 postpartum (Table 2.2). Maternal breast milk was collected into 50ml sterile tubes (minimum of 5ml, maximum 15ml per breast), transported to the lab on ice and processed within 4 hours of collection. Breast milk was centrifuged at 1500rpm for 15 minutes at 4°C. After centrifugation the acellular aqueous and the lipid layer were aliquoted separately into 2ml tubes and were stored at -80°C until time of analysis (figure 2.7). The aqueous layer of breast milk was used to measure innate immunological proteins with anti-HIV activity in vitro.

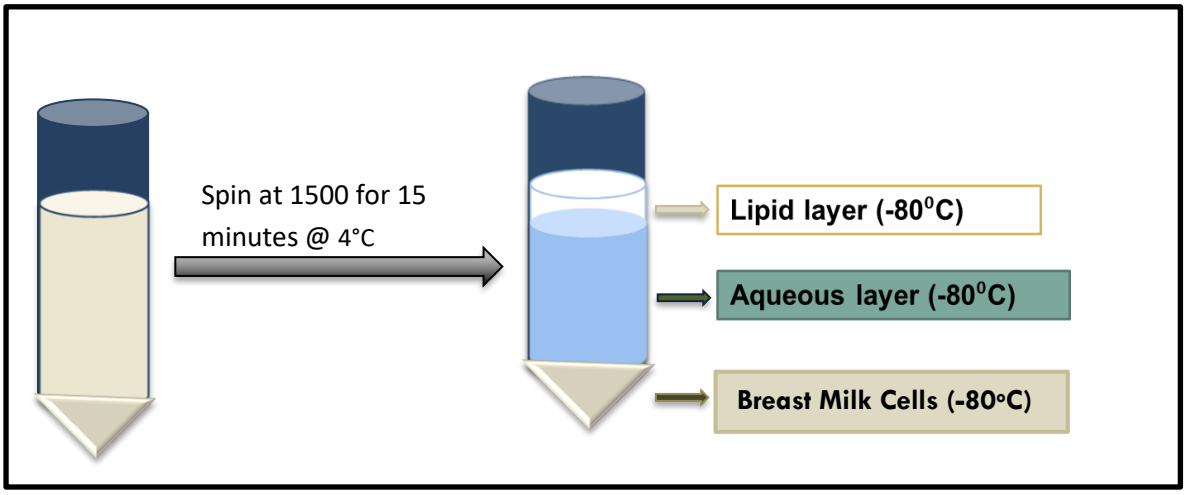


Figure 2.7: **Whole breast milk before and after spinning at 4°C**. Left tube depicts whole breast milk before processing. The tube on the right depicts three different layers after spinning breast milk at 4°C. The aqueous and the lipid layer were stored at -80^{0}C until time of analysis. The breast milk cells were stored at -80 then transferred to liquid nitrogen until time of analysis.

2.8. SANDWICH ELISA USED TO MEASURE INNATE PROTEINS

The enzyme-linked immune-absorbent assay (ELISA) assay was used to measure innate proteins of interest; SLPI and E/tr-2, in breast milk and infant saliva samples. The ELISA assay is a plate-based technique employed for measuring and detecting proteins in biological samples. There are two ways in which an ELISA can be used, to detect the presence of antibodies specific for an antigen or to detect the presence of an antigen recognised by an antibody (Sandwich ELISA). To achieve the aims of our study, a Sandwich ELISA was used; the capture antibody was bound to the plate, a sample with unknown amount of antigen was then added. Thereafter, detection antibody was added to the sample. The activity of the conjugated enzyme was assessed via incubation with a substrate. The important element of a Sandwich ELISA is antibody-antigen interaction (figure 2.8)

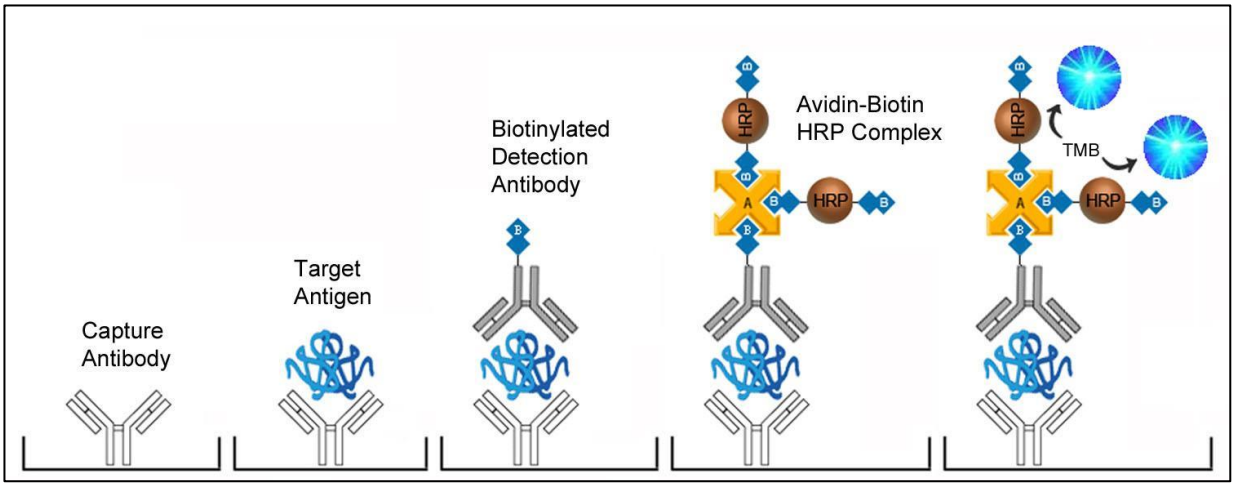


Figure 2.8: A sandwich ELISA schematic illustrating the principle of the assay.

2.8.1. HUMAN SLPI QUANTIKINE ELISA

To quantify SLPI, pre-coated 96 well plates were used (R&D systems). Using optimized dilutions of 1:80 (saliva) and 1:32 (breast milk) (see Appendix 3 for optimization), controls and samples were prepared. The standard was prepared according to the manufactures recommendation (Appendix 2). To measure SLPI in the study samples, 100ul of assay diluent RD1Q was added to each well. Immediately

after adding the assay diluent, 100ul standard, controls and samples were added to each well respectively. The plate was covered with adhesive strip and incubated for 2 hours at RT. After 2 hours, each well was washed with ~400ul of wash buffer, ensuring complete removal of liquid for each wash step. Any excess wash buffer was removed by inverting the plate and blot dried against a clean paper towel. 200µl of human SLPI conjugate was added to each well. The plate was covered with a new adhesive strip and incubated for 2 hours at RT. Aspiration/wash step was repeated as previously described. Substrate solution (200µl) was added to each well and the plate was incubated for 20 minutes at RT in the dark. Stop solution (50µl) was added to each well and the plate was incubated for 10 minutes at room temperature until a color change was observed i.e. blue to yellow. The optical density of each well was determined within 30 minutes, using a micro-titer plate reader set to 450 nm and 540nm. The SLPI concentration was derived from the linear regression of SLPI standard curve, where the minimum detectable concentration for the SLPI ELISA was 0.0625ng/ml.

2.8.2. E/TR-2- HUMAN TRAPPIN-2/ELAFIN DUO SET ELISA KIT

In order to determine the concentration of Elafin/ trapping 2 (E/tr-2) in infant saliva and maternal breast milk a Human trappin-2/elafin duo set ELISA kit was used (R&D systems). The following method was used. Capture antibody (100ul) was added to each well for overnight coating. The plate was sealed with adhesive strips and incubated overnight at RT. On the following day, the coating buffer was aspirated; the plate was washed using 400ul of wash buffer. The wash step was repeated twice for a total of three washes. After washing, 300ul of blocking buffer was added to each well and the plate was incubated at RT for 1 hour. After incubation, the blocking buffer was aspirated and the plate was washed three times as the previous wash step. Optimized dilution factors 1:128 (saliva) and 1:64 (breast milk) (Appendix 3) were used to prepare samples. Thereafter, 100µl of samples and controls were added to each well respectively. Standards were prepared according to the manufacturer's recommendations (Appendix 2) and added to their respective wells. The plate was covered with adhesive strip (Appendix 6) and incubated at RT for 2 hours. Thereafter, the aspiration/wash step was repeated. After washing 100µl of the detection antibody

was added to each well, the plate was covered with a new adhesive strip and incubated for 2 hours at RT, and the aspiration/wash step was repeated. 100µl of the working dilution of Streptavidin-HRP was added to each well, the plate was covered with new adhesive strips and incubated for 20 minutes at RT in the dark. After another wash, 100µl of Substrate Solution was added to each well. The plate was incubated for 20 minutes at RT in the dark. Then 50µl of Stop Solution was added to each well. To ensure thorough mixing the plate was gently tapped. The optical density of each well was determined immediately using a micro-plate reader set to 450 nm and 540. The E/tr-2 concentration was derived from the linear regression of E/tr-2 standard curve, where the minimum detectable concentration for E/tr-2 ELISA was 0.032 ng/ml.

2.9. STATISTICAL ANALYSIS

For data analysis that compared two groups, a non-parametric Mann-Whitney U t-test was used because the data did not follow Gaussian distribution. Median and interquartile ranges (25% and 75% respectively) were used to express the data. The rate of change per week was calculated using this formula ΔC/t

ΔC= differences in concentration at birth & week 15/36

t= number of weeks

All tests conducted to determine the strength and direction of association between two ranked variables was completed using the Spearman correlation which generated both the p and R values. A p value of <0.05 was considered to be statistically significant.

2.10. OPTIMIZATION OF ELISA METHODOLOGY

Prior to the measurement of SLPI and E/tr-2 concentration in maternal breast milk and infant saliva, the ELISA kits needed to be optimized on breast milk and saliva as the manufacturer had not validated the kits for these samples. Optimization was carried out to determine the correct dilution factor from the standard curve range that would be suitable for the study samples, so as to ensure accurate measurements and recovery of the analytes from the study samples after spike and recovery testing. The accurate measurement of a sample value is dependent on the interactions between the protein of interest with the ELISA antibodies and comparison of this interaction to a recombinant protein standard curve. The effect of factors such as buffer components, sample matrix and heterophilic antibodies can interfere with antibody binding in the natural sample resulting in the inaccuracy of the ELISA results (R&D systems). Samples that were below limits of detection (LOD) were assigned a value ½ LOD. To ascertain that the measured values are accurate, spike and recovery and linearity testing was done. Sample preparation for spiked and recovery and linearity testing is depicted in figure 2.10.

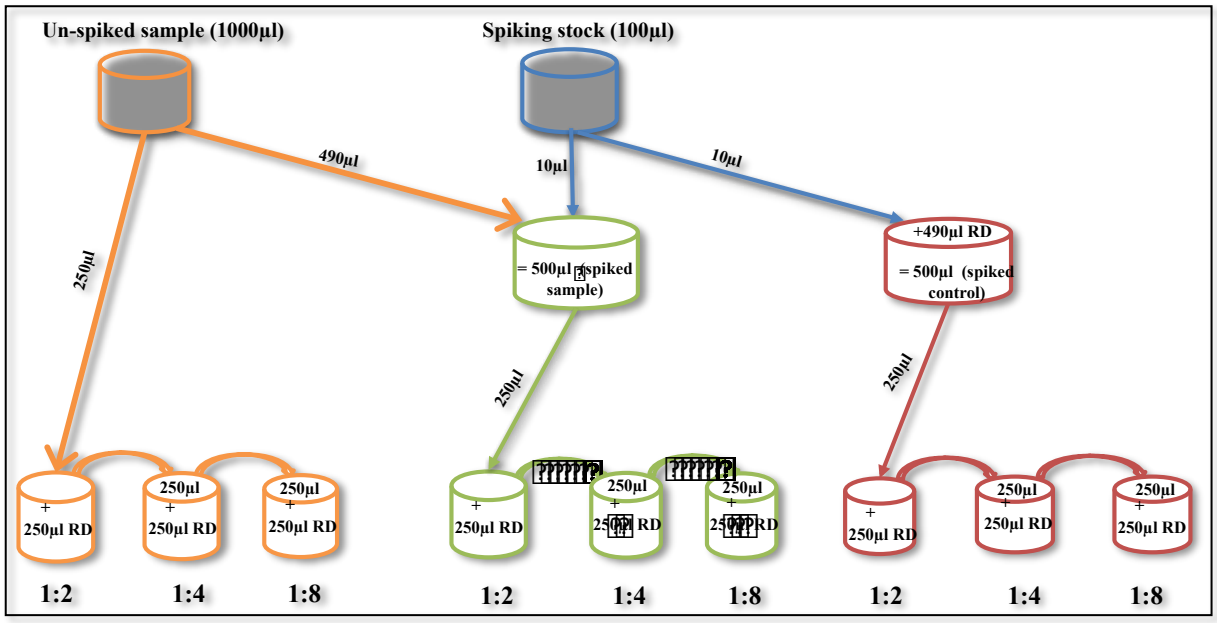


Figure 2.10: Sample preparation and serial dilutions for spike and recovery and linearity testing.

2.10.1. SPIKE AND RECOVERY

To do a spike and recovery test, a known amount of recombinant protein was spiked into either BM or saliva prior to the ELISA. If the resulting concentration of the spiked sample is similar to the input concentration, this demonstrates that the analyte can be measured accurately (R&D systems). However, if the recovered value significantly (i.e. +/-20%) differs from the expected amount, then factors in the sample matrix (BM and/ saliva) could be interfering with the correct measurement of the analyte (R&D systems).

Three types of samples were used to achieve the goals of spike and recovery test, a spiked, un-spiked sample and a control spike. To prepare SLPI spiking stock (40 000 pg/ml), lyophilized SLPI standard was reconstituted with 500ul reagent diluent. The spiked sample was prepared by adding 5ul of the spiking stock to 245ul sample (saliva and breast milk). A control spike was prepared by adding 5ul of the spiked solution to 245ul of reagent diluent and the un-spiked sample consisted of 250ul of neat sample (saliva and breast milk). All samples were respectively serially diluted as depicted in Figure 2.10.

For optimizing E/tr-2, a standard stock was prepared by reconstituting E/tr-2 standard with 500ul reagent diluent from the STD stock. 182ul was pipetted out and added to 818ul reagent diluent to prepare a spiking stock solution. The same method used for SLPI described above for spiked, un-spiked and control sample preparation was also used for E/tr-2. After plate reading a %recovery was calculated (Appendix 4) to determine if the values obtained were within acceptable range of 80-120% (R&D systems).

$$\% \text{ Recovery} = \frac{\text{Observed} - \text{Unspiked}}{\text{Expected}} \times 100$$

***Observed= spiked sample value**
Un-spiked= un-spiked sample value
Expected= amount spiked into a sample (calculated based on assigned concentration of spiking stock and volume spiked into sample).

2.10.2. LINEARITY TESTING

It is important that analytes of interest are not over- or under-diluted when measuring by ELISA (R&D systems). The linearity test was done to choose an optimal dilution of sample either saliva or breast milk which fell within the linear range of the standard curve shown in figure 3.1.1 and 3.1.2 in section 3. Linearity at different dilutions was calculated (Appendix 3). A linearity range of 80-120% demonstrated that the sample tested (BM and/saliva) exhibited natural linearity. Figure 2.8 shows how the methodology for determining linearity of samples.

$$\%\ recovery\ (1:2) = \frac{observed\ value\ \left(\frac{ng}{ml}\right) of\ 1{:}2\ dilution}{expected\ value\ \left(\frac{ng}{ml}\right) devided\ by\ 2}\ x\ 100$$

$$\%\ recovery\ (1:4) = \frac{observed\ value\ \left(\frac{ng}{ml}\right) of\ 1{:}4\ dilution}{expected\ value\ \left(\frac{ng}{ml}\right) devided\ by\ 4}\ x\ 100$$

$$\%\ recovery\ (1:8) = \frac{observed\ value\ \left(\frac{ng}{ml}\right) of\ 1{:}8\ dilution}{expected\ value\ \left(\frac{ng}{ml}\right) devided\ by\ 8}\ x\ 100$$

2.11. LEVY-JENNINGS QUALITY CONTROL CHART

A Levy Jennings plot is a quality control chart used to detect and limit errors in laboratory assays (Henry & Segalove 1952), this plot ascertain reproducibility and consistency in results obtained from laboratory assays. For the purpose of our study, two control samples were used, i.e. plasma (SLPI) and saliva (E/tr-2). Each control sample was from the same donor and was run in duplicates across multiple plates (n=9) for each of the innate proteins measured. The control sample mean concentration and standard deviation was determined across all experiments using the equation described below. The lower and upper control (UCL and LCL respectively) were applied based on the 2 standard deviations from the mean.

Standard deviation:

$$s = \sqrt{\frac{\sum_{k=1}^{n}\left(x_k - \bar{x}\right)^2}{n-1}}$$

Mean:

$$\bar{x} = \frac{\sum_{k=1}^{n} x_k}{n}$$

Lower and upper control limits:

$$\left(L_{low}, L_{high}\right) = \bar{x} \mp ms$$

To interpret the results obtained from the Levy Jennings chart, Westgard rules was applied. These set of rules were used to detect errors and to determine whether the results obtained from the laboratory assay passed or if a re-run was required. Westgard rules were also applied to determine assay performance limits, systematic and random errors (Et, 1981).

3. RESULTS

3.1 ASSAY OPTIMIZATION RESULTS

We measured the concentration of SLPI and E/tr-2 in infant saliva and maternal breast milk using ELISA as described in section 2.6. The kits used had high sensitivity; capable of detecting in the range 62.5-4 000 pg/ml (SLPI) and 31.25-2 000 pg/ml (E/tr-2). The results show that the SLPI can be quantified in infant saliva and maternal breast milk using 1:80 and 1:32 (Appendix 3) dilution factors respectively. The results also show that E/tr-2 can be measured in infant saliva and maternal breast milk using 1:128 and 1:64 (Appendix 3) respectively. These dilution factors were within the linear range of a standard calibration curve (figure 3.1.1 and 3.1.2)

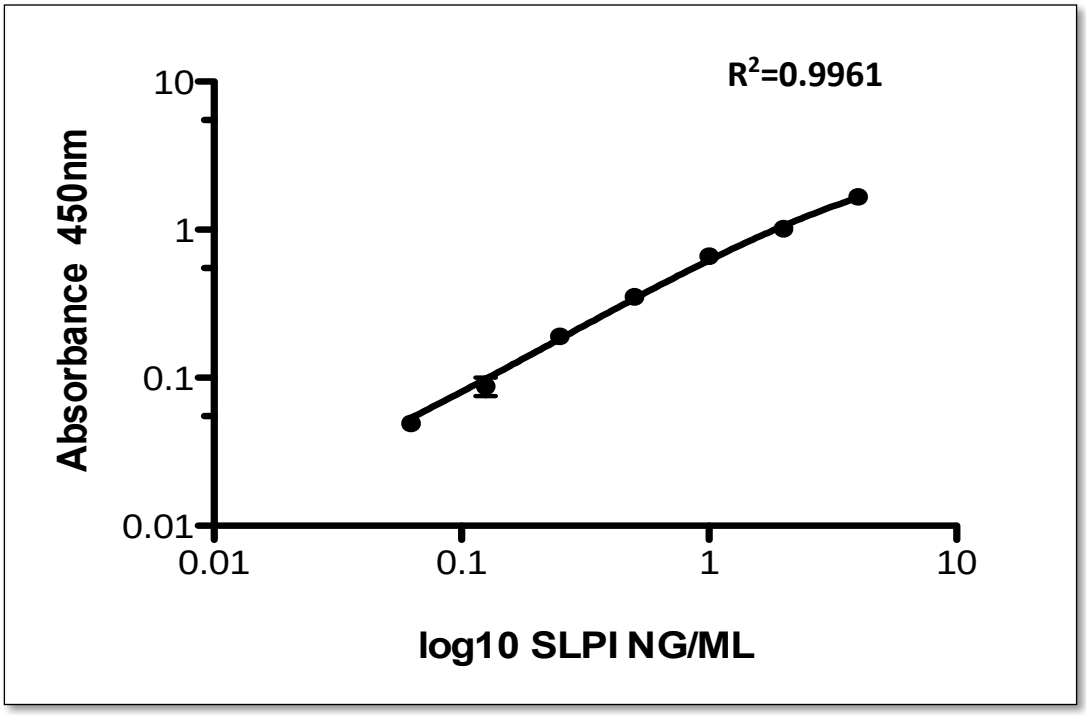


Figure 3.1.1. Standard calibration curve used to determine the concentrations of SLPI in infant saliva and maternal breast milk.

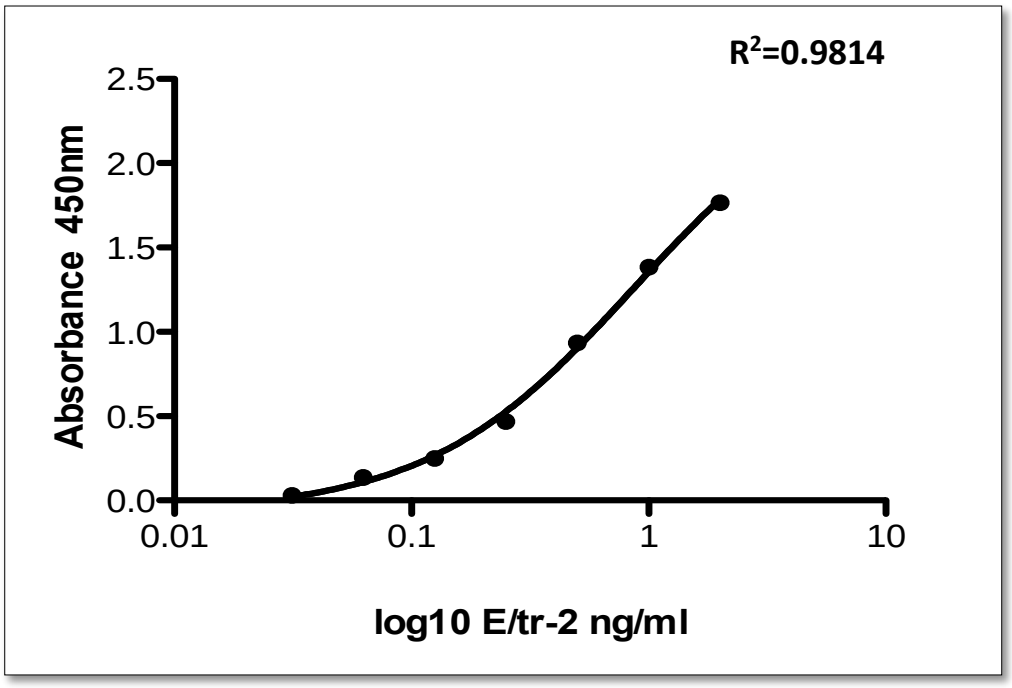


Figure 3.1.2. A standard calibration curve used to determine the concentrations of E/tr-2 in infant saliva and maternal breast milk.

3.2. MUCOSAL FLUIDS CONTAIN MEASURABLE CONCENTRATIONS OF SLPI AND E/TR-2

The results for spike and recovery show that SLPI detection range in infant saliva and breast milk was 106-120% and 102-111% respectively (Table 3.1.1). When the samples were tested for linearity, ranges of 84-119% for salivary SLPI and 91-94.8% for breast milk SLPI were obtained (Table 3.1.2). These experiments showed that both saliva and breast milk exhibited linearity at different dilutions and with no interference in the measurements. All results obtained were within the manufacturers recommended recovery and linearity range of 80-120% (R&D systems).

Likewise, recovery of E/tr-2 in infant saliva and maternal breast milk was between 88-102% and 94-97% respectively. When the samples were tested for linearity, the values obtained were 88-102% and 98-120% respectively (Table 3.1.3). These results suggest that there were minimal interfering molecules in the samples that would hinder accurate detection of E/tr-2. It was therefore concluded that the Human SLPI Quantikine ELISA and E/tr-2 Duo Set ELISA were accurate and sensitive enough for quantifying SLPI and E/tr-2 in infant saliva and maternal breast milk.

Table 3.2.1: Spike and recovery results for the optimization of SLPI and E/tr-2 ELISA assay (n=2)**.**

Analyte	Sample type	Spiked sample		Control	
		Ave %rec	Range %	Ave %rec	Range %
SLPI	Saliva	113	106-120	87.4	80-94,8
E/tr-2	Saliva	89.75	78-94	103.8	98,5-109
SLPI	BM	106.5	102-111	87.4	80-94,8
E/tr-2	BM	93.28	84-102	103.8	98,5-109

Table 3.2.2: Linearity testing results for SLPI protein tested in saliva and breast milk (n=1)

Analyte	Saliva		BM		Control	
	Dil. factor	**Spiked (%)**	**Dil.factor**	**Spiked (%)**	**Dil. factor**	**Spiked (%)**
SLPI	1:80	-	1:32	-	1:2	99
	1:160	94	1:64	96	1:4	96
	1:320	93	1:128	96	1:6	86
	1:640	84	1:256	91	1:8	92

Dil.factor= Dilution factor

Table 3.2.3: Linearity testing results for E/tr-2 protein tested in saliva and breast milk (n=)

Analyte	**Saliva**		**Breast milk**		**control**	
	Dil. fcator	**Spiked (%)**	**Dil. factor**	**Spiked (%)**	**Dil. factor**	**Spiked (%)**
E/tr-2	1:128	-	1:64	-	1:2	96,9
	1:256	91	1:128	98	1:4	102
	1:512	97	1:256	94	1:6	98,5
	1:1024	96	1:512	100	1:8	109

3.3. INNATE FACTOR MEASUREMENTS FALL WITHIN THE LIMITS OF INTER-PLATE VARIABILITY

The concentrations measured for SLPI and E/tr-2 from each of the control samples across all plates were all within two standard deviations (Upper Control Limit (UCL)) and -2 standard deviation (Lower Control Limit (LCL)) of the mean (Figures 3.3(a) and 3.3(b)). The mean concentration for SLPI was 41.37 ng/ml, UCL= 58.37 ng/ml, LCL= 24.36 ng/ml, STD Dev. = 8.5 ng/ml and E/tr-2: mean=42.3 ng/ml, UCL= 50.001 ng/ml, LCL= 34.60 ng/ml and STD Dev.=3.85 ng/ml. Therefore all samples were above the +/-2 STD Dev. control limits set for the assay. In conclusion, the Levy Jennings quality control results show no excessive inter-plate variation. Both ELISA kits were now used to measure concentrations of SLPI and E/tr-2 in maternal breast milk and infant saliva samples.

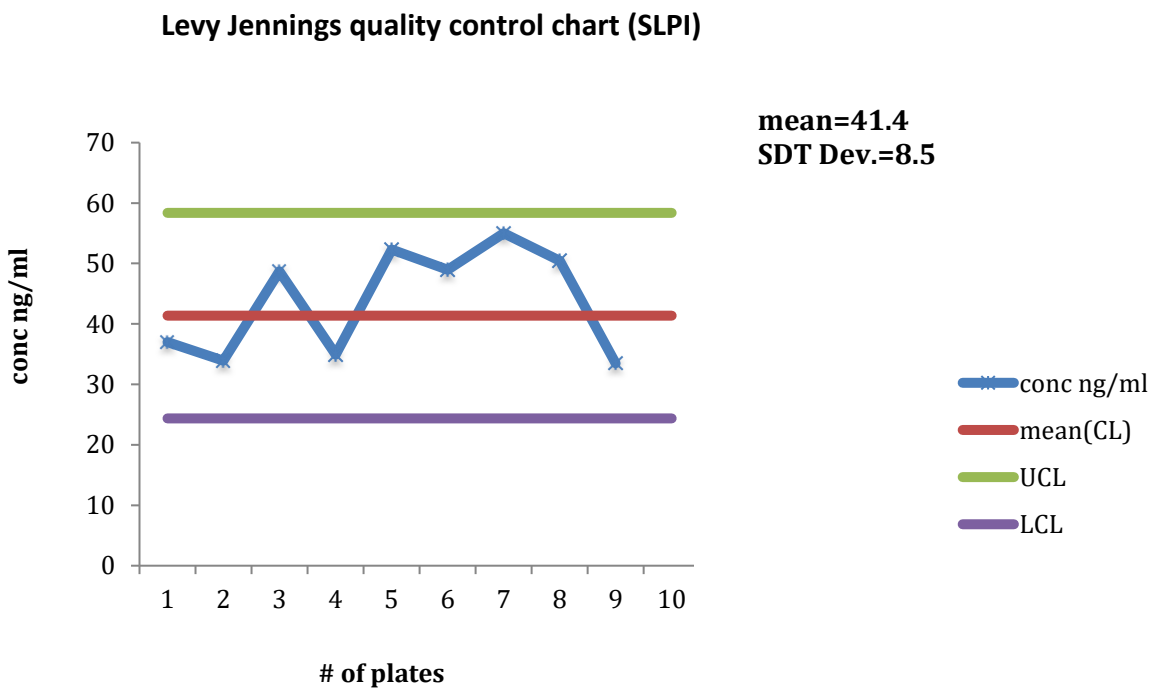


Figure 3.3(a). Quality control charts for SLPI ELISA assay. The horizontal bars represent the +2 Standard Deviations (STD Dev.) in green, the -2STD Dev. in purple and the mean in red. The analyte concentration is represented by the blue line.

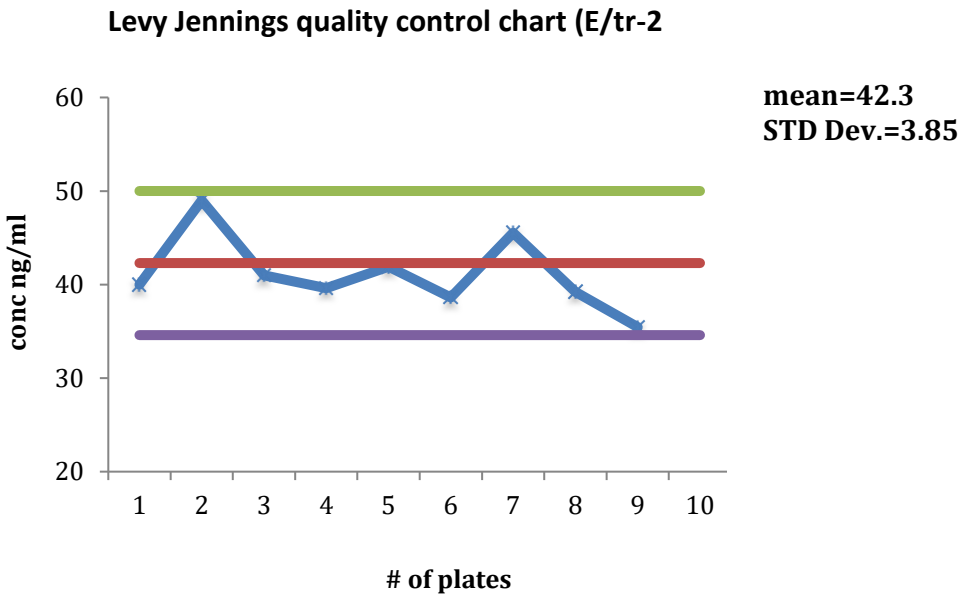


Figure 3.3(b). Quality control charts for SLPI ELISA assay. The horizontal bars represent the +2 Standard Deviations (STD Dev.) in green, the -2STD Dev. in purple and the mean in red. The analyte concentration is represented by the blue line.

3.4. STUDY PARTICIPANTS CHARACTERISTICS ACCORDING TO HIV STATUS

Breast milk samples were collected from 28 HIV infected mothers and 22 HIV uninfected mothers (median age 29 yrs, range 19-41 yrs vs. 28 yrs, range 19-39 yrs, p=0.3956). All HIV infected mothers in our cohort were receiving a combination ART treatment i.e. Efavirens (EFV), Emtricitabine (FTC) and Tenofovir (TDF). Saliva samples were collected from 29 HIV exposed Uninfected breast-fed infants (HEU-BF), 24 HIV exposed formula fed infants (HEU-FF) and 24 HIV unexposed breast-fed infants (HUE-FF). In addition, there was no significant difference in infant gestational age (median=37 wks, range= 36-41 wks, 39 wks, range=36-41 wks and 39 wks,range=36-41 wks, p=0.5550) between HEU-BF, HEU-FF and HU-BF infants respectively. All infants born to HIV infected mothers were given ARVs i.e. AZT for at least one month postpartum. More of participant characteristics are depicted in table 3.4.1.

3.4.1. Maternal and infant characteristics

Variables	HIV infection status		HIV exposure status		
	HIV +ve (n=28)	HIV -ve (n=22)	HEU-BF (n=29)	HEU-FF (n=24)	HU (n=24)
Maternal/gestational age					
Range	19-41yrs.	19-39 yrs.	36-41 weeks.	36-41 weeks	36-41 weeks
Median	29 yrs.	28 yrs.	37 weeks	39 weeks	39 weeks
Marital status			-	-	-
Married % (n)	35.71% (10)	36.36% (8)			
Living together % (n)	7.14% (2)	27.27% (6)			
Never married % (n)	57.14% (16)	31.81% (8)			
Highest education					
Primary school % (n)	21.43% (6)	0	-	-	-
Secondary school % (n)	78.57% (22)	100 (22)			

CD4 counts (cells/mm^3)					
Range	226-880	-	-	-	-
Median	470				

3.5. THE CONCENTRATION OF INNATE PROTEINS CHANGES OVERTIME IN BREAST MILK AND INFANT SALIVA.

The concentrations of SLPI and E/tr-2 in maternal breast milk were compared between HIV infected (HIV+ve) and HIV uninfected (HIV-ve) mothers, HIV Exposed Uninfected (HEU) and HIV Uninfected (HU) infant saliva. As described in section 2.1.2, mothers self-selected a feeding method for their infants. Only samples from mothers and their infants that chose to exclusively breast feed and exclusively formula feed their infants until 15 weeks of age were analysed for the purpose of this dissertation.

3.5.1. CONCENTRATIONS OF SLPI AND E/TR-2 IN MATERNAL BREAST MILK DECREASE IN THE FIRST 36 WEEKS POSTPARTUM.

SLPI and E/tr-2 concentrations in maternal breast milk during the first nine months postpartum were measured. A total of 309 breast milk samples from HIV infected (n=165) and HIV uninfected mothers (n=144) were assayed at the same time. In the HIV uninfected maternal breast milk, the median concentration of SLPI at baseline was 95.25 ng/ml (IQR= 64.68-138.3 ng/ml). The concentrations decreased over time to median 25.82 ng/ml, IQR= 12.55-39.21 ng/ml at week 15 and 35.91 ng/ml, IQR= 19.63-60.23 ng/ml at week 36; $p<0.0001$ for both groups, figure 3.5.1(a). Similarly, in the HIV-infected group, breast milk SLPI concentrations significantly decreased over time; baseline median = 77.40 ng/ml (IQR= 64.31 – 134.91 ng/ml), week 15 median = 28.15 ng/ml (IQR 13.46 - 58.62 ng.ml) and at week 36 median 16.79 ng/ml (IQR= 8.05 - 42.28 ng/ml), $p<0.0001$, figure 3.5.1(b). In addition, the rate of change in breast milk SLPI concentration was calculated per week. The results show no significant differences in change of analyte concentration between the two groups; HIV +ve (3.51 ng/ml/week) and HIV -ve (3.35 ng/ml/week) between d4-7 and week 15, and HIV +ve (-0.11 ng/ml/week) and HIV –ve (0.36 ng/ml) between week 15 and week 36.

A similar trend was observed for E/tr-2 concentrations when measured at baseline, 15 weeks and 36 weeks in the breast milk. HIV-infected women had median E/tr-2 concentrations of 70.32 ng/ml (IQR 19.31-210.22 ng/ml) at baseline, 22.07 ng/ml (IQR 9.45 - 44.26 ng/ml) at week 15, and 6.71 ng/ml (IQR= 3.30 - 26.11 ng/ml) at week 36, $p<0.0001$. HIV-uninfected women had median concentrations of 92.01 ng/ml, IQR =

9.15 - 173.14 ng/ml, 25.22 ng/ml, 9.55 - 57.15 ng/ml, and 13.21 ng/ml, 4.64 - 31.36 ng/ml respectively), p<0.0001, Figure 3.5.1(c-d). Furthermore, we calculated the rate of change between groups per week, and no significant differences were observed in the rate of change between d4-7 and week 15 post-delivery; HIV+ve (2.42 ng/ml/week) and HIV-ve (2.55 ng/ml/week). Similarly, no difference in the rate of change was evident between week 15 and week 36 for HIV+ve (-0.51 ng/ml/week) versus HIV-ve (-0.72ng/ml/week) mothers.

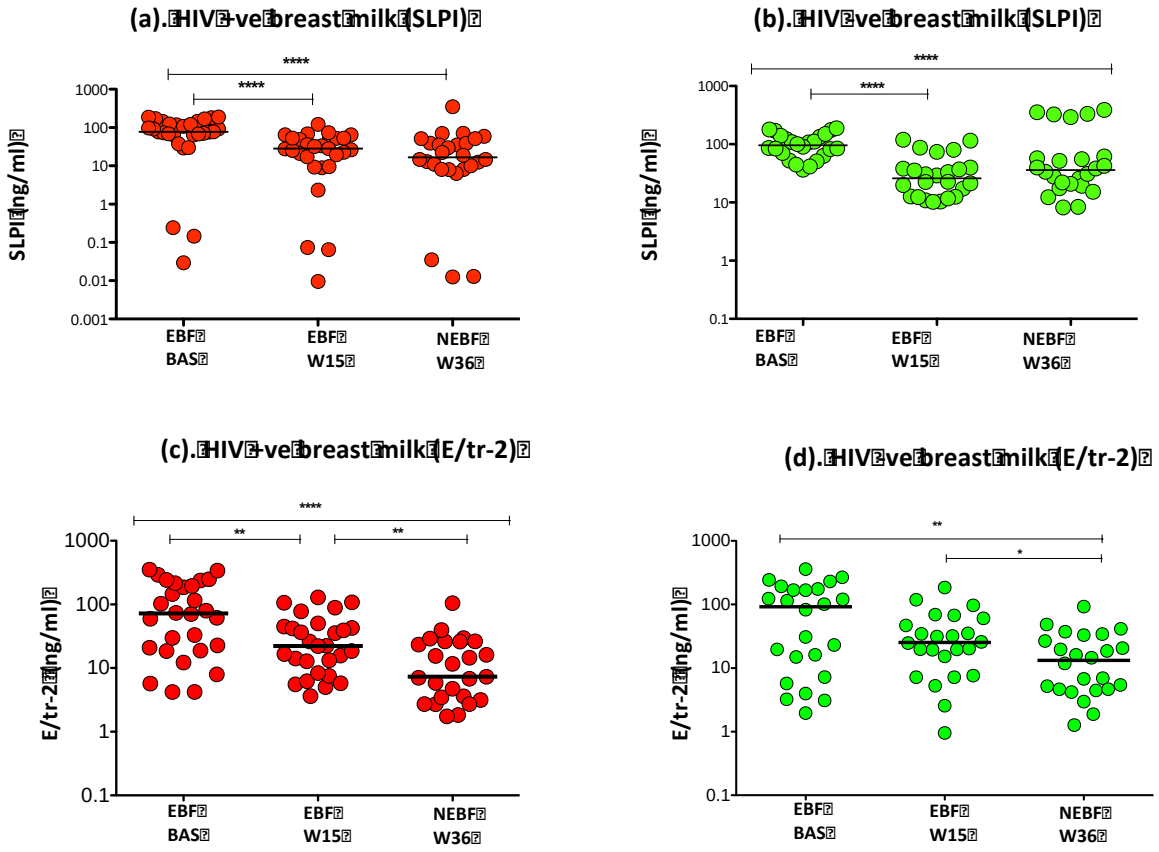


Figure 3.5.1. **Kinetics of SLPI and E/tr-2 protein levels in maternal breast milk**. Figure A and B represent SLPI concentration in HIV infected and HIV uninfected maternal breast milk respectively, while figure C and D represent E/tr-2 expression in HIV infected and HIV uninfected maternal breast milk respectively. The horizontal bars represent medians, red circles represent HIV+ and green circles HIV-ve. A Kruskal-Wallis test was used to test statistical differences over time and a Dunns post hoc test was used to correct for multiple comparisons (**** p<0.0001, ** p<0.01, * p<0.05). EBF-Exclusively Breast Fed and NEBF-None Exclusively Breast Fed.

3.5.2. SLPI AND E/TR-2 CONCENTRATIONS IN INFANT SALIVA DO NOT SHOW A CONSISTENT TREND IN THE FIRST 36 WEEKS OF LIFE

Next, SLPI and E/tr-2 concentrations were measured over 36 weeks in infant saliva from both HIV exposed uninfected (HEU) and HIV unexposed (HU) infants. A total of 286 saliva samples from HEU (n=151) and HU infants (n=135) were assayed simultaneously. Contrary to the decrease observed in breast milk, SLPI concentrations increased significantly with time in the saliva of HEU infants from a median of 58.71 ng/ml, IQR=48.27 -132.5 ng/ml at baseline, to a median of 133.9 ng/ml, IQR=91.42 - 171.5 ng/ml at week 15 and 169.9 ng/ml, IQR 91.14 - 272.5 ng/mlat week 36; $p<0.0060$, (Figure 3.5.2a). However in HU infant saliva, SLPI concentrations had no definite trend (median=172.23 ng/ml, IQR= 109.33 - 287.81 ng/ml at baseline, 85.20 ng/ml, (IQR= 62.21-105.36 ng/ml at week 15) and 206.5 ng/ml, (IQR = 141.40 - 257.06 ng/ml at week 36); $p<0.0001$ (Figure 3.5.2b). The rate of change in SLPI between the two groups was significantly different; the concentration of SLPI in HU infants increased at 6.83 ng/ml/week compared to HEU that decreased by 3.30 ng/ml/week, $p<0.0001$. However, the rate of change in salivary SLPI concentration between week 15 and week 36 was not significantly different (HEU: 3.57 ng/ml and HU: 2.63 ng/ml per week).

E/tr-2 concentrations in infant saliva remained similar over time for HEU from baseline (median =69.61 ng/ml, IQR= 36.86 - 111.7 ng/ml), to week 15 (97.85 ng/ml, IQR= 32.38 -153.9 ng/ml) and week 36 (83.93 ng/ml, IQR= 36.61-168.7 ng/ml), $p<0.8260$. Similarly for the HU group, baseline (median=88.26 ng/ml, IQR= 45.27 - 123.4 ng/ml), week 15 (78.39 ng/ml, IQR= 41.86- 219.8 ng/ml) and week 36 (40.75 ng/ml, IQR= 30.37- 83.70 ng/ml week 36) all had similar levels; $p<0.1582$ (figure 3.5.2c-d). In addition, the rate of change between the two groups per week showed no significant differences between birth and week 15 (HEU:-0.5945 ng/ml compared to HU: 0.05243 ng/ml), week 15 and week 36 (HEU: -0.03839 ng/ml compared to HU: 0.3005 ng/ml).

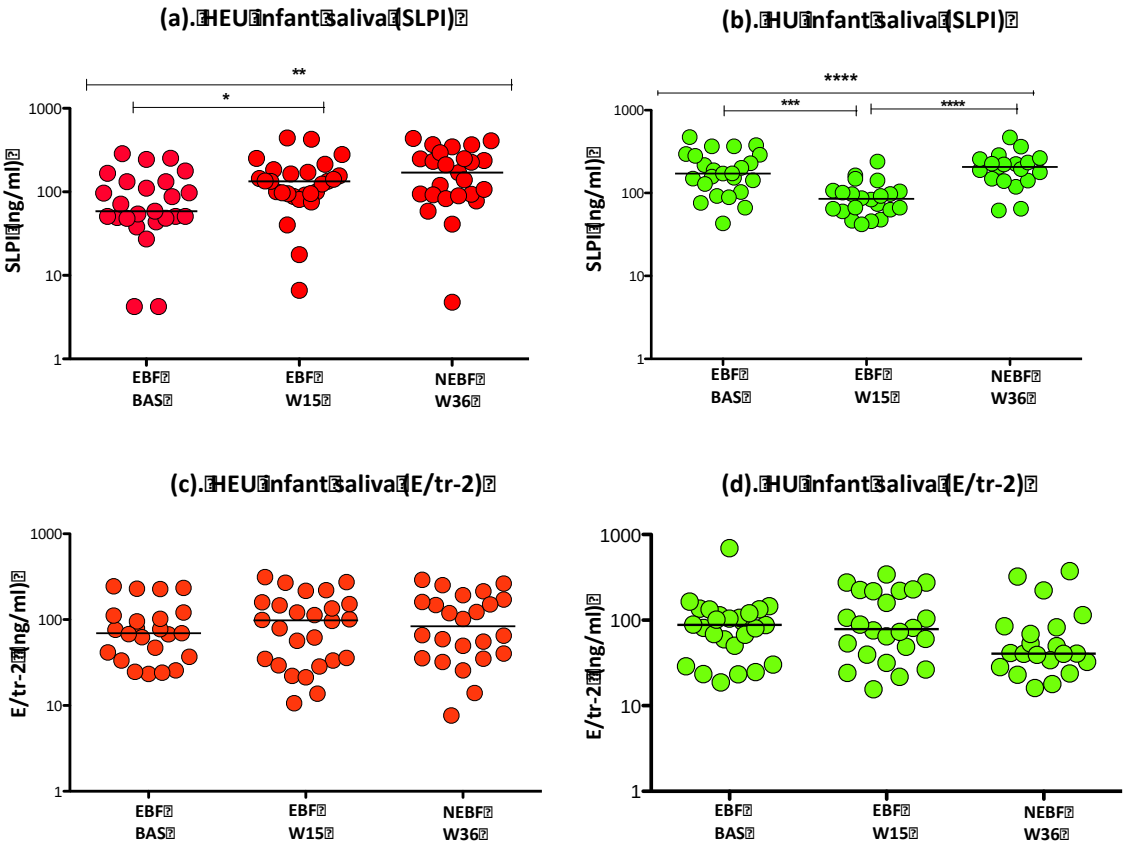


Figure 3.5.2. Kinetics of SLPI and E/tr-2 protein levels in infant saliva. Figure A and B represent SLPI concentration in HIV Exposed Uninfected (HEU) and HIV Unexposed (HU) infant saliva respectively, while figure C and D represent E/tr-2 concentration in HEU and HU infant saliva respectively. The horizontal bars represent medians, red circles represent HEU and green circles HU infant saliva. A Kruskal-Wallis test was used to calculate significant changes over time with a Dunn's post hoc test to compare different time points (**** p<0.0001, *** p<0.001, **p<0.01 and *p<0.05).

3.5.3 MATERNAL HIV STATUS DOES NOT IMPACT THE CONCENTRATION OF SLPI AND E/TR-2 IN MATERNAL BREAST MILK.

Next, we assessed whether the concentration of SLPI and E/tr-2 were influenced by maternal HIV status or infant HIV exposure. SLPI and E/tr-2 concentrations were compared between HIV-infected (HIV+) and HIV uninfected (HIV-) breast milk.

The median SLPI concentration in breast milk (ng/mL) was not significantly different between HIV+ (77.4, IQR=64.31-134.9 ng/ml) and negative women (95.24, IQR=64.86-138.3 ng/ml; p=0.49) at baseline. This was also true for week 15 with HIV+ median SLPI being 27.86 ng/ml (IQR= 9.51- 52.48 ng/ml) and HIV- (25.82 ng/ml, IQR=

12.55 -39.21 ng/ml; p=0.71) (Figure 3.5.3. However at week 36, breast milk from HIV positive women had significantly lower median SLPI concentration compared to that from HIV negative women (16.79 ng/ml, IQR= 8.05 -42.15 ng/ml vs. 35.91 ng/ml, IQR=19.63 ng/ml- 60.23 ng/ml, p=0.026) (Figure 3.5.3.(a)). However, this difference would not withstand multiple testing correction.

The impact of HIV exposure on the concentration of E/tr-2 was also assessed between HIV-infected and HIV-uninfected women. The levels of E/tr2 in breast milk did not differ by HIV status at all three measured time points (Figure 3.5.3(b)).

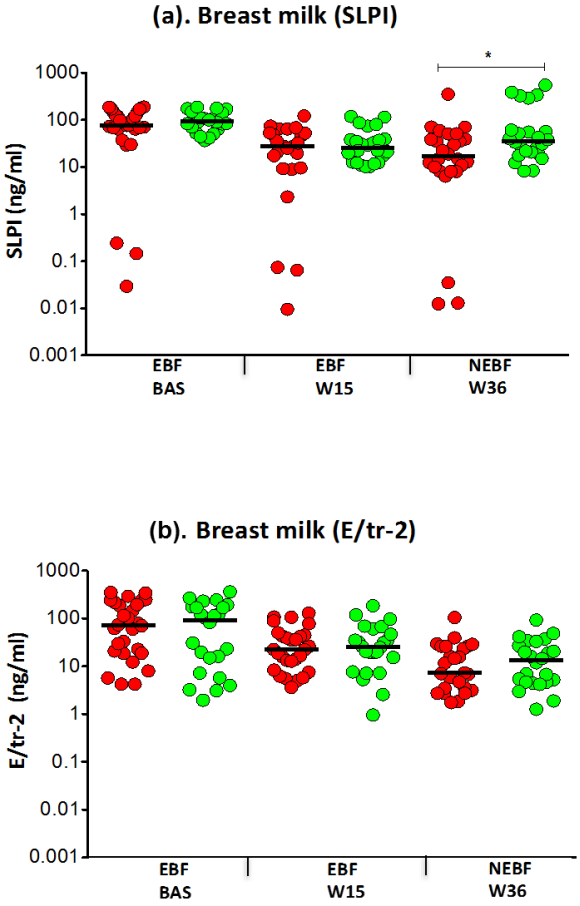


Figure 3.5.3 . The impact of HIV exposure on the concentration of SLPI and E/tr-2 protein levels in maternal breast milk. Figures a and b represent SLPI and E/tr-2 protein levels in the breast milk of HIV negative (green circles) and HIV positive (red circles) mothers. EBF - Exclusively Breast fed and NEBF -None Exclusively Breast Fed. The horizontal bars represent medians. P values were generated using the Man Whitney t test with a Dunns post hoc test to compare different time-points (*** p<0.0001, ** p<0.01, * p<0.05).

3.5.4. INFANT EXPOSURE TO MATERNAL HIV DIFFERENTIALLY IMPACTS THE CONCENTRATION OF SLPI AND E/TR-2 IN INFANT SALIVA.

We next assessed whether changes in the concentration of SLPI and E/tr-2 in infant saliva were influenced by maternal HIV status or infant HIV exposure. SLPI and E/tr-2 concentrations were compared between HIV exposed-uninfected (HEU) and HIV unexposed (HU) infant saliva.

Median SLPI concentrations in infant saliva were higher in the HU group compared to HEU infants at baseline (median 172.2 ng/ml, IQR=109.3 -287.2 ng/ml vs. 58.71 ng/ml, IQR= 48.27 ng/ml-132.5 ng/ml respectively, p=0.0024). At week 15, however, an inverse relationship was observed as higher SLPI concentrations were detected in the saliva of HEU infants compared to HU infants (median=133.9 ng/ml, IQR=91.42 -171.5 ng/ml vs. 85.20 ng/ml, IQR=62.21 -105.4 ng/ml, p=0.0150). No significant differences were observed between HEU and HU infant saliva at week 36 (median=169.9 ng/ml, IQR=91.14 ng/ml-272.5 ng/ml vs. 206.51 ng/ml, IQR=141.4 ng/ml-257.1 ng/ml, p=0.5379), Figure 3.5.3. (a), although there was a trend towards higher SLPI concentrations among HEU infants.

No significant differences in E/tr2 concentrations were observed between HEU and HU infant saliva at any time point (Figure 3.5.3(b)).

These results suggest that the concentration of SLPI in maternal breast milk and infant saliva is affected by maternal HIV status/ infant exposure to HIV. The impact of maternal HIV infection is observed at 36 weeks in breast milk, with significantly high concentration observed in HIV-uninfected mothers. However, for infant saliva the HEU group have significantly low SLPI concentrations at baseline, but at 15 weeks an inverse is observed as the HU group has significantly low concentrations. It is important to note that, other factors other than HIV may also lead to the elevation of SLPI at these mucosal secretions. However, the concentration of E/tr-2 in infant saliva and in maternal breast milk does not appear to be impacted by maternal HIV or infant exposure.

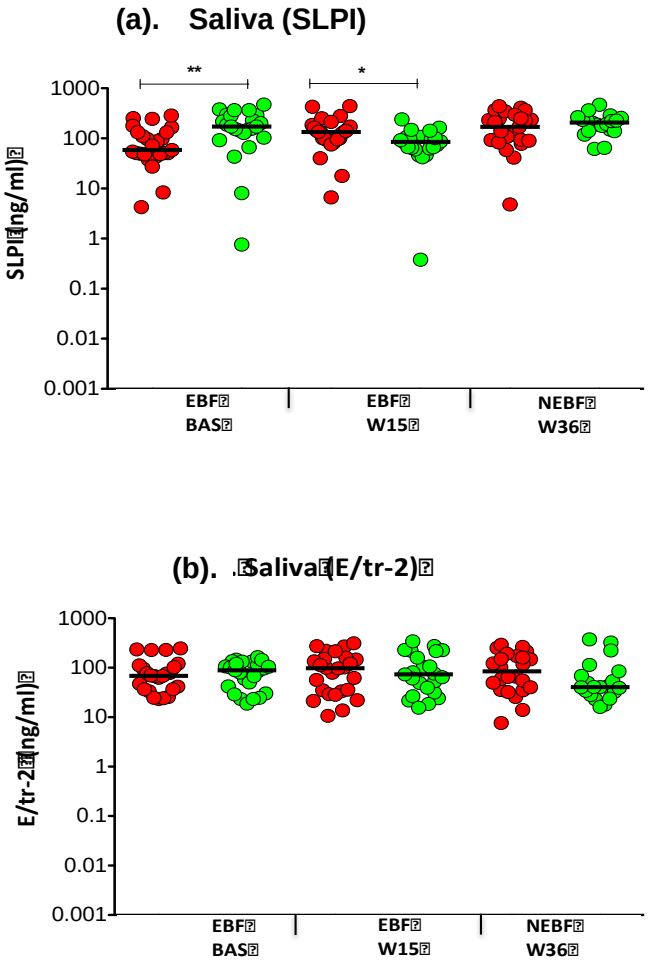


Figure 3.5.4. The impact of HIV exposure on the concentration of SLPI and E/tr-2 protein levels in infant saliva. Figures a and b represent SLPI and E/tr-2 respectively in the saliva of HEU (red circles) and HU (green circles) infant saliva. EBF -Exclusively Breast fed and NEBF -None Exclusively Breast Fed. The horizontal bars represent medians. P values were generated using the Man Whitney t test with a Dunns post hoc test to compare different time-points (*** p<0.0001, ** p<0.01, * p<0.05).

3.6. THE IMPACT OF FEEDING PRACTICE ON THE CONCENTRATION OF SLPI AND E/TR-2 IN INFANT SALIVA

The choice of feeding (breast, mixed or formula) plays an essential role in the development of the infant's immune system ((Gil & Rueda 2002) as well as their susceptibility to HIV infection via breast milk. Exclusively breast fed infants were shown to have reduced mortality and morbidity compared to formula and mixed fed infants (Natchu *et al.* 2012). Immune benefits provided by breast milk are responsible in part for the observed reduction in infant morbidity (Leon-Cava, Lutter, Ross & Martin, 2002). Breast milk contains essential molecules including innate factors that play an important role in protecting infants against infections (Leon-Cava, Lutter, Ross & Martin, 2002). Formula and mixed fed infants were shown to have weakened immune system causing them to be more susceptible to acquiring infections (Mead *et al*., 2008), (Coovadia & Coutsoudis *et al*. 2008). We hypothesized that exclusively breast fed infants would have higher concentrations of SLPI and E/tr-2 analytes in their saliva compared to formula fed infants.

A total of 292 samples from HIV exposed exclusively breast fed (EBF) (n=151) and exclusively formula fed infants (EFF) (n=141) were analysed. The SLPI and E/tr-2 protein levels were measured at baseline, week 15 and week 36 for both EBF and EFF infant saliva respectively. At baseline and week 15 all infants were exclusively breast fed (EBF) or exclusively formula fed (EFF). EBF was defined as receiving nothing else but breast milk or prescribed medicines, and EFF means the infants were given no other feeds except formula. However at week 36 both groups were receiving supplementary feeds i.e. the infants were given supplementary foods and liquids other than breast milk and/or infant formula, as is developmentally appropriate.

3.6.1. THE CONCENTRATION OF SLPI IN EBF INFANT SALIVA INCREASES OVER TIME COMPARED TO EFF INFANTS.

We analysed changes in SLPI and E/tr-2 concentrations in the saliva of EBF versus EFF infants' over time. Figure 3.5.1 shows that the concentration of SLPI in breast fed infant saliva significantly increases over time (Figure (3.6.1(a)), with levels of 58.71 ng/ml, IQR=48.27 - 132.5 ng/ml at baseline, 133.9 ng/ml, IQR=91.42-171.5 ng/ml at week 15 and 169.9 ng/ml, IQR= 91.14-272.5 ng/ml at week 36, p=0.0060. In formula fed infants saliva, there was a significant decrease in SLPI concentration between baseline (median= 160.8 ng/ml IQR=117-347.2 ng/ml) and week 15 (110.0 ng/ml, IQR=78.22-152.2 ng/ml),p<0.0466. However, no significant changes were observed between week 15 and week 36 (99.75 ng/ml, IQR=14.66-197.5 ng/ml), p=0.1172 (Figure 3.6.1(b)).

SLPI and E/tr-2 are constitutively expressed in mucosal fluids, therefore changes in E/tr-2 concentrations over time in breast fed and formula fed infant saliva was also investigated. The results obtained in figure 3.6.1(c-d) show no significant changes in exclusively breast fed salivary E/tr-2 concentration over time, with median levels at baseline of 69.61 ng/ml, week 15 of 97.85 ng/ml and at week 36 83.93 ng/ml. Similarly, no changes over time in E/tr-2 concentrations were observed in exclusively formula fed infants between baseline (median=58.87 ng/ml), week 15 (83.33 ng/ml) and at week 36 (130.0 ng/ml), p=0.1621.

These results suggest that at least in HIV-exposed infants, exclusive breast feeding significantly increases the concentration of SLPI over time. On the other hand, SLPI concentrations decreased over time in saliva of exclusively formula fed infants although not statistically significantly. However, E/tr-2 levels in saliva of HEU infants remain stable and are mostly unaffected by feeding modality.

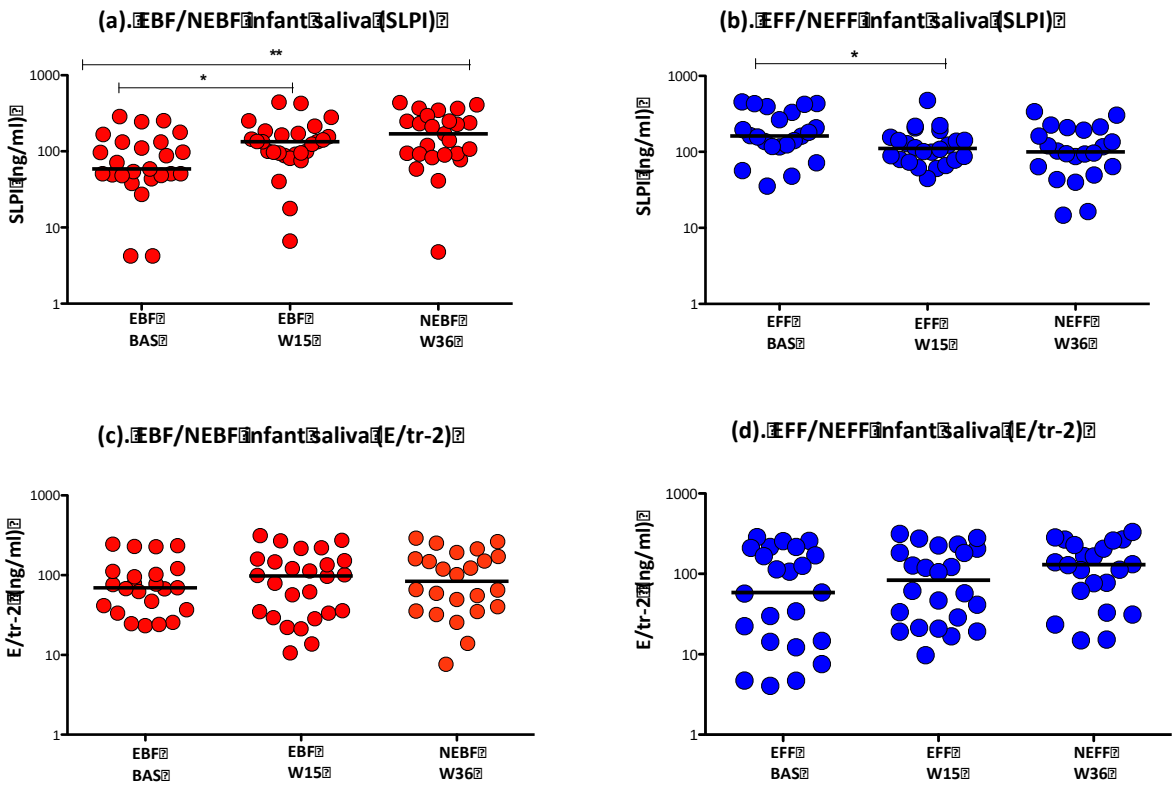


Figure 3.6.1. Changes in SLPI and E/tr-2 protein levels in HIV exposed Exclusively Breast Fed (EBF)/None Exclusively Breastfed and HIV exposed Exclusively Formula Fed (EFF)/None Exclusively Formula Fed (NEFF) infant saliva. Figure A and B represent SLPI concentration in EBF and EFF infant saliva respectively, while figure C and D represent E/tr-2 expression in EBF and EFF infant saliva respectively. The horizontal bars represent medians. P values were generated Kruskal-Wallis test (*** $p<0.0001$, ** $p<0.01$, * $p<0.05$).

3.6.2. MATERNAL FEEDING MODE DOES NOT IMPACT SLPI AND E/TR-2 CONCENTRATION IN INFANTS.

After observing changes in analyte concentrations in breast fed and formula fed infants saliva over time, it was next assessed whether the concentration of SLPI and E/tr-2 were influenced by exclusive breast feeding or exclusive formula feeding (Figure 3.6.2). The results showed that there was significantly higher SLPI concentration in EFF versus EBF infants at baseline (median=58.71 ng/ml vs. 160.8 ng/ml, p=0.0039, respectively; Figure 3.6.2a). At week 15, no significant difference were observed (median=133.9 ng/ml vs. 111.0 ng/ml, p=0.5150). At week 36, when all infants were supplementary feeding, no significant difference in the concentration of SLPI was observed between BF and FF infant saliva (median=169.9 ng/ml vs. 99.75 ng/ml, p=0.075; figure 3.6.2).

The concentration of E/tr-2 was not affected by maternal feeding choice. Our results show no differences between breast fed and formula fed infant salivary E/tr-2 concentrations at any time point, not at baseline (median=68.68 ng/ml vs. 57.77 ng/ml, p= 0.6781), week 15, (median= 97.85 ng/ml vs. 83.33 ng/ml, p= 0.9923) or week 36 (median=83.93 ng/ml vs. 130.0 ng/ml, p=0.3171).

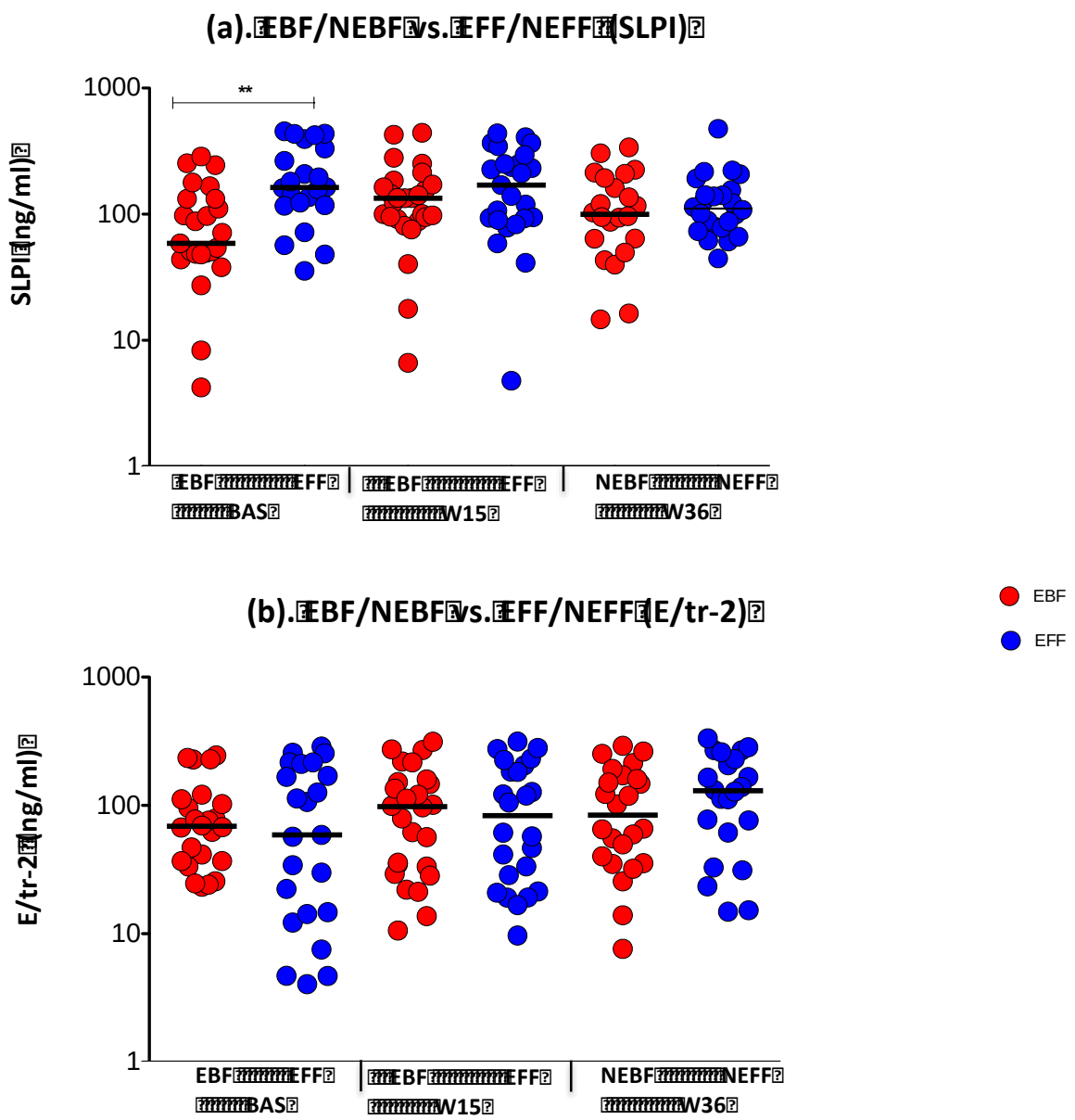


Figure 3.6.2. The impact of feeding on the concentration of SLPI and E/tr-2 in HIV exposed uninfected infant saliva. Figure (a) represent SLPI protein levels in breast fed and formula fed infant saliva. Figure (b) represent E/tr-2 protein levels in breast fed and formula fed infant saliva. The red circles represent Exclusively Breast Fed (EBF)/None Exclusively Breast Fed (NEBF) and the orange circles represent Exclusively Formula Fed (EFF)/None Exclusively Formula Fed (NEFF) infant saliva. The horizontal bars represent medians. P values were generated using Man Whitney t test (*** p<0.0001, ** p<0.01, * p<0.05).

3.7. ASSOCIATION OF SLPI AND E/TR-2 LEVELS IN MATERNAL BREAST MILK WITH INFANT SALIVA

Maternal transfer of immunological factors to the infant is essential for providing early protection against disease (Gil & Rueda, 2013). These immunological factors including innate proteins are transferred during gestation, delivery and post-partum via breast feeding (Kuhn *et al.* 2007). To assess whether the source of infant salivary factors is maternal breast milk, breast milk levels of SLPI and E/tr-2 were correlated with infant salivary levels of these factors. A total of 34 mother-infant pairs were analysed at baseline, week 15 and week 36 for SLPI and E/tr-2.

3.7.1. MATERNAL BREAST MILK E/TR-2 CONCENTRATIONS DO NOT CORRELATE WITH EXPRESSION IN INFANT SALIVA

Breast milk levels of E/tr-2 across all time points were correlated with matched infant saliva irrespective of maternal HIV status; as it was shown that E/tr-2 levels were not impacted by HIV status (section 3.5.3). To eliminate the possibility that the concentration of E/tr-2 in infant saliva was influenced by maternal breast milk, a relationship between E/tr-2 protein levels in maternal breast milk and infant saliva was examined at three different time points. The results obtained show no association between maternal breast milk and infant saliva levels of E/tr-2 at baseline ($p=0.3506$, $R= -01652$), weeks 15 ($p=0.0978$, $R=0.2886$) and 36 ($p=0.3946$, $r=0.1508$) (Figure 3.7.1 (a-c)).

To summarise, there was no evidence of an association between maternal breast milk and infant saliva E/tr-2 levels in the first 36 weeks of infant life. This lack of association suggests that E/tr-2 is not transferred to the infant via breast milk but rather produced by the infant.

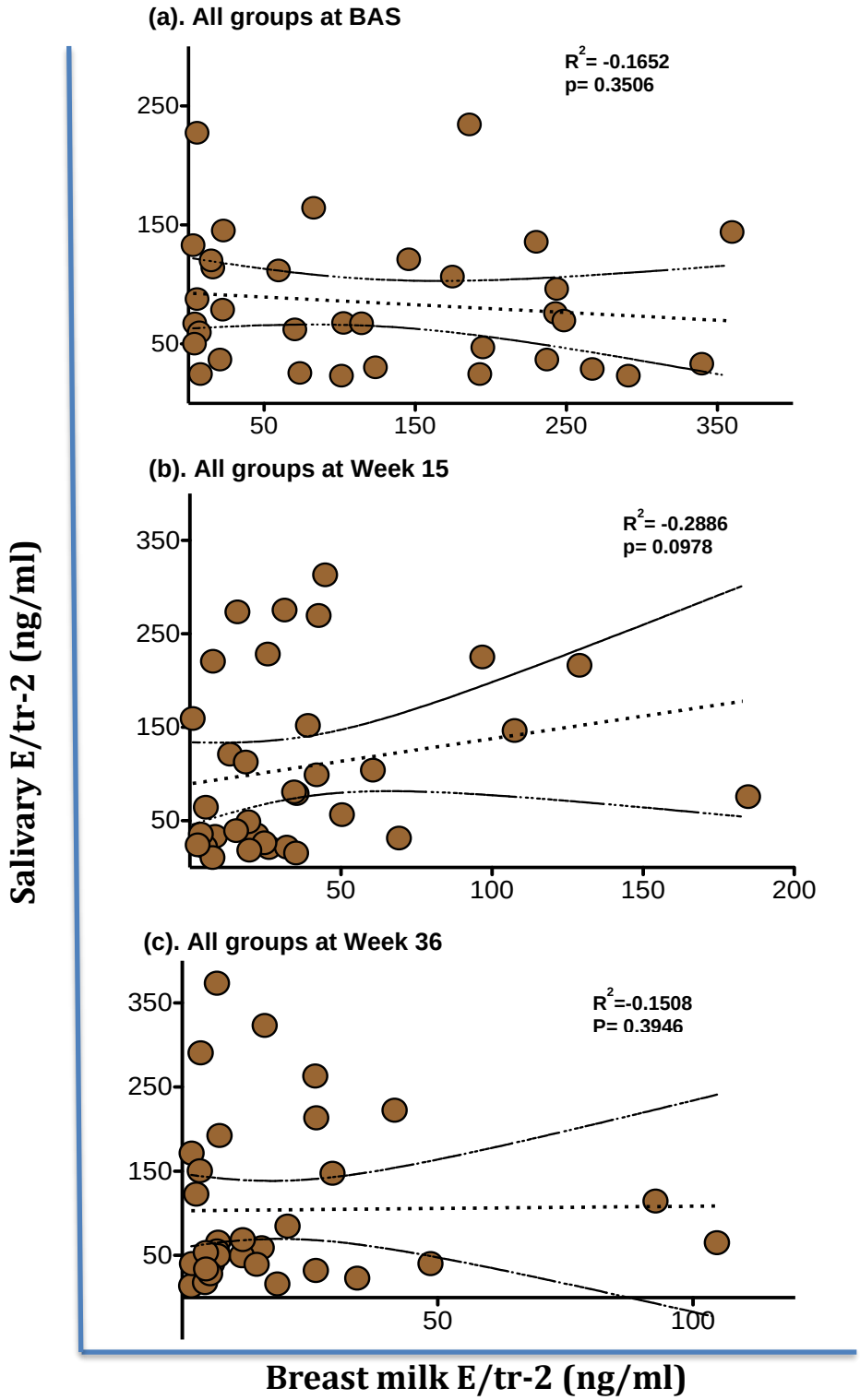


Figure 3.7.1. Spearman correlation between E/tr-2 levels in breast milk and infant saliva from all groups. (a) A Spearman correlation plot of E/tr-2 levels in breast milk vs. infant saliva at baseline. **(b)** A plot of E/tr-2 levels comparing the two variables at week 15. **(c)** A plot of E/tr-2 levels comparing the two variables at week 36. The three dotted line represent 95% confidence interval.

3.7.2. MATERNAL BREAST MILK SLPI CONCENTRATIONS DO NOT CORRELATE WITH CONCENTRATIONS IN INFANT SALIVA

Similar to the analysis of E/tr2 in section 3.7.1, maternal levels of SLPI in breast milk and infant saliva levels were correlated to determine whether there might be a direct transfer from mother to infant. Breast milk SLPI levels across all time points were first analysed with matched infant saliva irrespective of maternal HIV status and infant exposure status. The results showed no association between maternal breast milk and infant saliva SLPI levels at baseline (p=0.1707, R= 0.2369) (figure 3.7.2.1(a)), week 15 (p=0.8043, r=-0.04638) (figure 3.7.2.2(d)) or week 36 (p=0.5738, r= -0.1051) (figure 3.7.2.3(g)). However, because in section 3.5.3, we showed that there concentration of SLPI is affected by HIV exposure, we also analysed according to infant HIV exposure and maternal infection status. The results show no association in SLPI levels measured between HIV uninfected and HIV unexposed groups at baseline (p=0.4580, R=0.1868) (figure 3.7.2.1(b)), week 15 (p=0.2085, r=0.3585) (figure 3.7.2.2(e)) and week 36 (p= 0.2085, r=0.481) (figure 3.7.2.3(h)). Similarly, when the relationship of SLPI levels was assessed between HIV-infected maternal breast milk and HEU infant saliva at baseline (p=0.5474, r=0.1570) (figure 3.7.2.1(c)), week 15 (p=0.6394, r=-0.1225) (figure 3.7.2.2(f)) and week 36 (p=0.0907, r=0.4231) (figure3.7.2.3(i)).

Therefore, just like with E/tr-2, the results show no evidence of association between maternal breast milk and infant saliva SLPI levels in the first 36 weeks of infant life. This suggests that the concentration of SLPI in infant saliva is not influenced by maternal breast milk, regardless of HIV status but infants naturally produce the analyte.

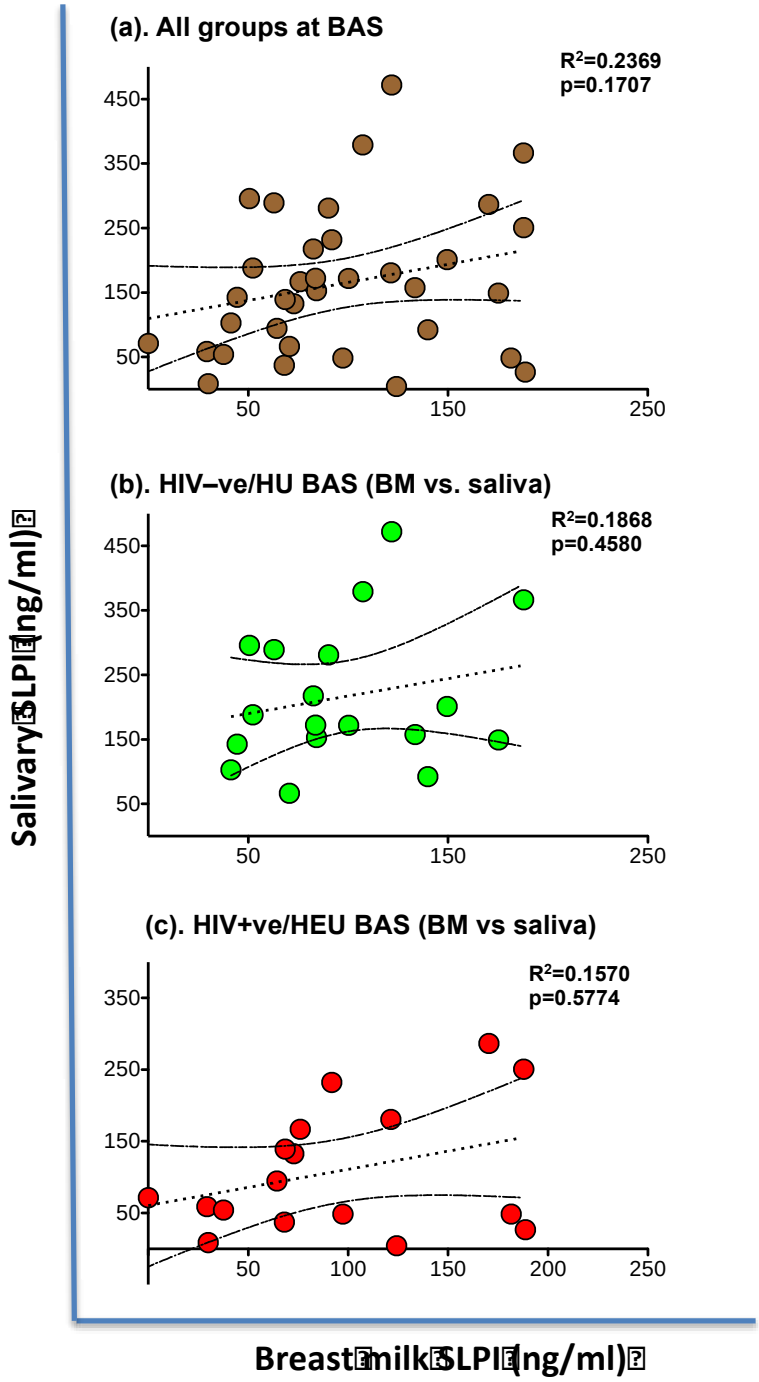


Figure 3.7.2.1. Spearman correlation plot of SLPI levels in breast milk vs infant saliva from all groups. (a) A plot of SLPI levels comparing the two variables between all groups at baseline. **(b)** and **(c).** The three dotted line represent 95% confidence interval.

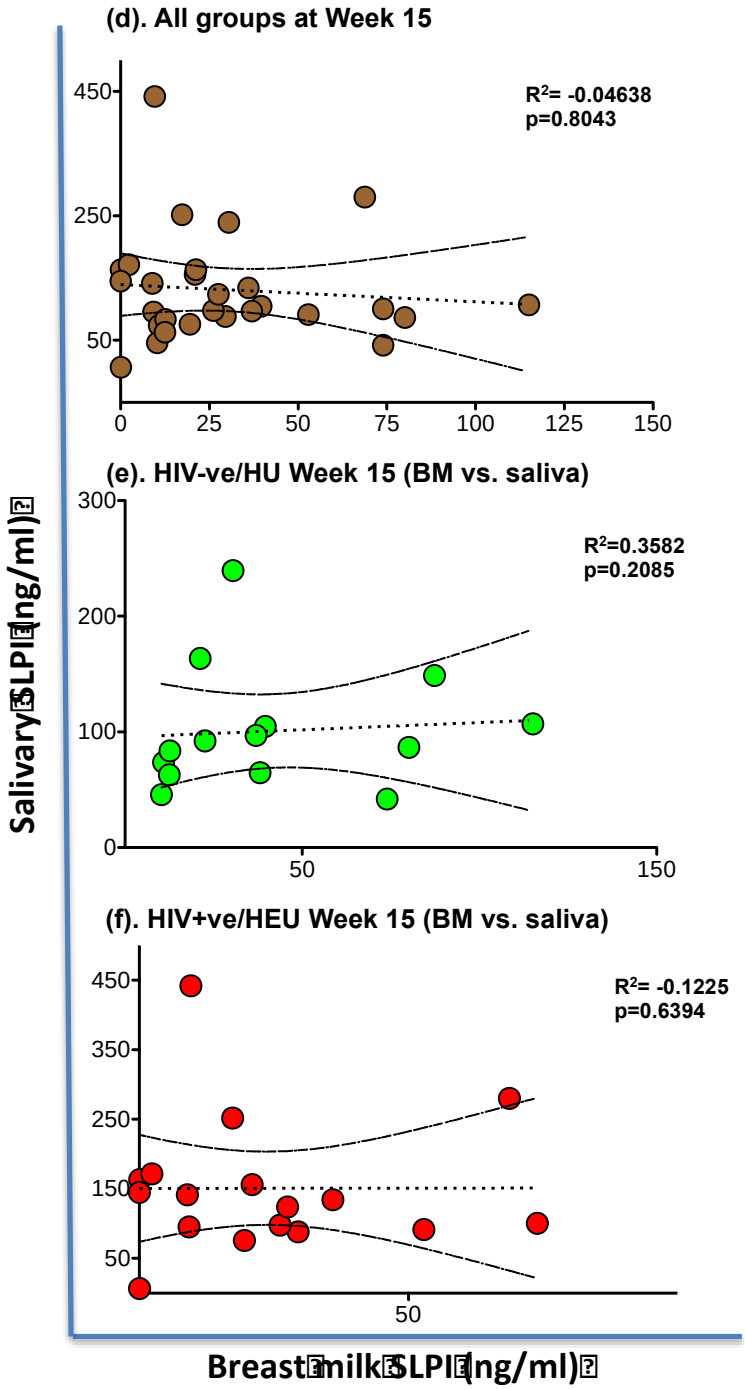


Figure 3.7.2.2. Spearman correlation plot of SLPI levels comparing the two variables in HIV +ve and HEU respectively at week 15. **(d)** A plot of SLPI levels comparing the two variables between all groups at week 15. **(e)** and **(f)** A plot of SLPI levels comparing the two variables in HIV +ve and HEU respectively at week 15. The three dotted lines represent 95% confidence interval.

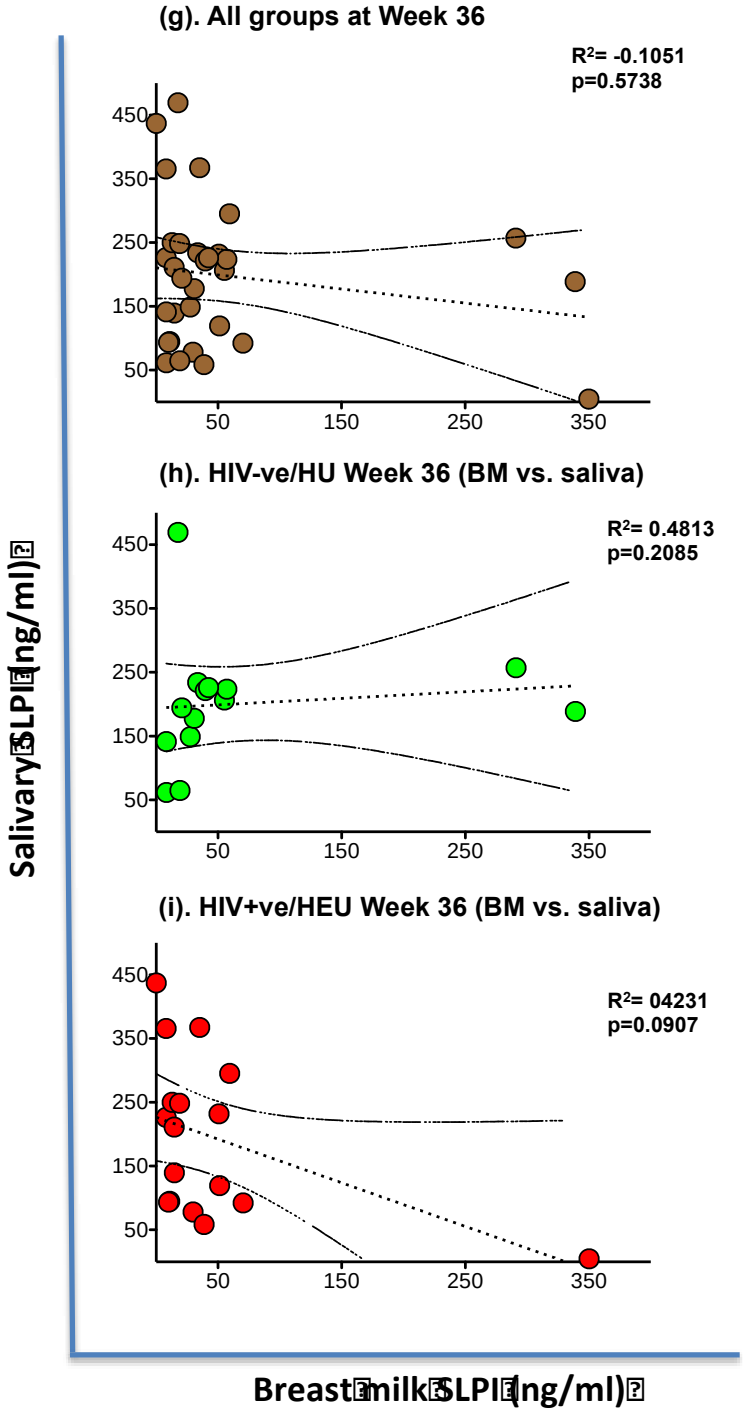


Figure 3.7.2.3. Spearman correlation plot of SLPI levels comparing the two variables in HIV +ve and HEU respectively at week 36. **(g)** A plot of SLPI levels comparing the two variables between all groups at week 36. **(h)** And **(i)** a plot of SLPI levels comparing the two variables in HIV +ve and HEU respectively at week 36. Spearman R and the P values were generated using a non-parametric spearman correlation. The three dotted lines represent 95% confidence interval.

4. DISCUSSION

Although infants born to HIV infected mothers may continue to be exposed to virus during breast feeding, more that 90% of these infants remain HIV uninfected even in the absence of ART (Coutsoudis *et al.* 1999). Human breast milk contains a variety of innate immunological factors that are associated with immunological protection (Hamosh, 2001). Amongst these innate proteins are SLPI and E/tr-2 whose presence in salivary and vaginal secretions is associated with reduced risk of HIV acquisition (Wahl *et al.* 1997). Since exclusively breastfed infants are relatively protected from HIV acquisition versus infants that are mixed fed, we hypothesized that innate proteins in saliva and breast milk play a role in prevention of MTCT and are influenced by HIV status and feeding mode. Therefore we sought to compare the concentration of SLPI and E/tr-2 in maternal breast milk (HIV infected and uninfected), in breast fed and formula fed infant saliva (HIV exposed and HIV unexposed).

THE CONCENTRATION OF INNATE PROTEINS IN INFANT SALIVA AND MATERNAL BREAST MILK OVER TIME

Firstly, the concentrations of SLPI and E/tr-2 in maternal breast milk and infant saliva were measured over time. The concentration of SLPI in maternal breast milk significantly decreased with time postpartum. This corroborates findings by (Becquart *et al.* 1999) and (Farquhar *et al.* 2002) who demonstrated that early breast milk had high SLPI concentrations that were later diluted in mature milk.

In acute lung injury, SLPI was shown to control and regulate inflammatory responses (Gipson *et al.* 1999). At physiological concentrations, SLPI can also disrupt cell membranes of *E.coli*, *S.aureus* (Hiemstra *et al.* 1996), and *M.tb* (Gomez *et al.* 2009) therefore destroying these bacteria. Therefore, we speculated that the presence of SLPI at high concentrations in early breast milk may confer protection against a variety of neonatal infections at physiological concentrations. However, the protection offered by this breast milk protein may wane with time. On the other hand, we observed that in HEU infants, salivary SLPI concentrations significantly increased with advancing age, suggesting that the infants' own innate immune system may play an important role in providing immunological protection against infections as they age. These findings suggest that the diminished protective effect

offered by mature breast milk is compensated for in infant saliva, through the production of high SLPI concentrations. The data in this dissertation contradicts findings by *(Farquhar et al.* 2002), who showed that salivary SLPI concentrations in HEU infants decreased with infant age. We speculate that variations in sample preparation may be the reason for such contradiction. In their study, Farquhar et al, added Protease inhibitor 4-(2-aminoethyl) benzenesulfluoride (AEBSF) in saliva samples before freezing. However in our cohort, whole saliva was used to measure SLPI levels in infant saliva. Therefore, we assume that the addition of this protease inhibitor may interfere with SLPI recovery. Another possible difference is that ours was a completely different cohort. It is most likely that only few mothers were on ART pre 2002, therefore mothers were sicker than those in our cohort.

To our knowledge, this study is the first to assess the concentration of E/tr-2 in breast milk and infant saliva from birth until 9 months of age. The data in this dissertation demonstrate that E/tr-2 is detectable in both breast milk and saliva, although there is a significant decrease in E/tr-2 concentrations in maternal breast milk over time. Conversely, similar concentrations of E/tr-2 were observed in infant saliva over time. These findings suggest that the presence of E/tr-2 in these mucosal secretions may have a potentially protective role against a variety of pathogens. E/tr-2, as with SLPI, is known for its role in protecting tissues against proteolysis by neutrophils (Moreau *et al.* 2008). Therefore detection of this protein in breast milk and saliva may suggest host protection from neutrophil-induced mucosal damage during infection. However, more studies are required to measure and compare the levels of this analyte at these mucosal fluids and to decipher the role of this analyte in saliva and breast milk.

THE IMPACT OF HIV EXPOSURE STATUS ON THE CONCENTRATION OF INNATE PROTEINS IN MATERNAL BREAST MILK AND INFANT SALIVA

The concentration of SLPI and E/tr-2 in mucosal secretions is associated with protection against HIV infection *in vitro* (McNeely *et al.* 1995), (Drannik *et al.* 2012). In this study, the concentration of SLPI in maternal breast milk was not affected by maternal HIV status, since results showed similar concentrations in both HIV infected and HIV uninfected mothers' breast milk. These findings are contradicting

published data by Lin *et al.* 2004 and Bacqui *et al.* 1999 who found that SLPI concentrations were significantly elevated in HIV infected individuals compared to those that were HIV uninfected. However, both authors measured SLPI concentrations in saliva samples and not breast milk. Therefore variations may be due to different SLPI concentrations measurable in different mucosal secretions.

These findings suggests that the mammary gland is capable of producing SLPI regardless of maternal HIV status. SLPI is an anti-inflammatory protein (Moreau *et al.* 2008), therefore it is produced in response to inflammatory stimulation. It was speculated that HIV infected individuals may have high SLPI concentrations due to inflammation resulting from infection (Vogelmeier *et al.* 1997). In this dissertation, all HIV-infected women were taking ARV treatment and had reduced viral load and likely lower immune activation. Therefore it is possible that this would result in normalization of SLPI protein concentrations such that are equal to those observed in HIV-uninfected women.

In addition, the data presented in this study, shows that the concentration of SLPI is significantly lower in HIV exposed uninfected (HEU) infants compared to those that are HIV unexposed (HU) at birth. These concentrations were below the threshold (1-10µg/ml) associated with HIV inhibitory ability *in vitro* (McNeely *et al.* 1995). High SLPI concentrations are correlated with reduced risk of HIV transmission through oral and vaginal mucosae (Farquhar *et al.* 2002), (Shugars 2002), (Hocini *et al.* 1997). Interestingly, the concentration of the analyte peaked at 15 weeks and was significantly higher than in the HIV unexposed group, suggesting that infants produce sufficient concentrations of SLPI capable of blocking HIV infection at 15 and 36 weeks. It is possible that due to ongoing exposure through breast feeding, HIV-exposed infants up-regulate SLPI production.

The impact of HIV exposure status on the concentration of E/tr-2 was also investigated. The data suggests that the concentration of E/tr-2 in breast milk and infant saliva is not affected by HIV infection nor by exposure to the virus. However this is contradictory to findings by Iqbal et al. 2009 and Ghosh et al. 2010, who demonstrated that cervicovaginal secretions (CVL) from HIV infected women contained higher E/tr-2 concentrations than HIV uninfected women. It can be

speculated that the contradicting results are possibly due to differences in levels of E/tr-2 measurable at different mucosal secretions as well as variations in sample preparation.

THE EFFECT OF MATERNAL FEEDING MODE ON THE CONCENTRATION OF INNATE PROTEINS IN INFANT SALIVA

The data from this study shows that, at birth, the concentration of SLPI is significantly lower in breast-fed infant saliva compared to formula fed infants. These differences however, dissipated with advancing age, with both groups of infants having the same concentration of SLPI at 15 and 36 week of age. This observation did not support our hypothesis that infants who were exclusively breast fed would have high SLPI concentrations compared to those that are exclusively formula fed. It is possible that the concentration of SLPI in EBF-HEU infants increases over time due to ongoing HIV exposure during breast feeding. Furthermore, it can also be speculated that the significantly higher SLPI concentration in exclusively formula fed infants compared to exclusively breast fed infant saliva at birth was only by chance, since differences at later time points were not observed.

We did not find any significant differences when we compared E/tr-2 protein levels between EBF and EFF infant saliva. This may suggest that E/tr-2 production in infants occurs naturally. The infant immune system is dependent on maternal breast milk as a source of proteins and antibodies essential for immune protection and development (Newburg & Walker 2007). When it was examined whether there was an association between innate proteins present in maternal breast milk and the levels measured in infant saliva, none was found. This suggests that the production of SLPI and E/tr-2 in infant's saliva is independent of maternal breast milk. Previous studies have shown that SLPI and E/tr-2 are present in mucosal secretions at physiological concentrations (Mcneely *et al.* 1995, Wahl *et al.* 1997, Iqbal *et al.* 2009). SLPI is up-regulated by anti-inflammatory cytokines such as IL-10 and TGF-β (Py *et al.* 2009), and the up-regulation of E/tr-2 is dependent on pro inflammatory cytokines such as IL-1β and TNF-α (Ghosh, Fahey, *et al.* 2010). In human breast epithelial cells, the production of SLPI and E/tr-2 is induced by hormonal changes during the menstrual cycle (Dey et al. 2018). It is clear that the production of SLPI and E/tr-2 is dependent on a variety of factors independent of breast feeding. This may be the reason

that no association between innate proteins present in breast milk and infant saliva was observed in this dissertation.

STUDY LIMITATIONS AND CONCLUSIONS

Although the study was designed to measure and compare the concentration of SLPI and E/tr-2 in infant saliva (HIV Exposed uninfected (HEU), HIV Unexposed (HU) and Formula Feeding (FF) and breast milk (HIV infected (HIV+ve) and HIV uninfected (HIV-ve), it could have also measured maternal HIV viral load to see if there is an association/relationship with the concentration of SLPI and E/tr-2 that was measured in breast milk. Further studies would have to determine the association between ART regime and the concentration of SLPI and E/tr-2 in infant saliva and breast milk, which was a limitation in our study. Another limitation worth pursuing in future studies would be to look at the presence of foreign molecules in formula that could have resulted in the activation of infant immune cells, that may in turn affect the concentration innate proteins in infant saliva and maternal breast milk.

In conclusion, the data from this study suggests that the production of SLPI and E/tr-2 in infant saliva occurs naturally, and the upregulation of these analaytes is dependent on various factors but maternal feeding mode. In addition, HEU infants produce sufficient SLPI concentrations capable of blocking HIV infection at 15 and 36 weeks of life, as previously shown (Mcneely *et al.* 1995, Bacqui *et al.* 2002). Further studies will be required to investigate the ability of SLPI to block HIV infection *in vitro* using human saliva.

5. APPENDICES

APPENDIX I

List of materials and reagents

Micro-plate reader with wavelength set at 450nm and correction at either 540nm 0r 570nm.
A multichannel pipette, normal pipette and tips, 500ml sterile bottle, RO water Or dH20.
Squirt bottle/ automated plate washer.
Tubes either 2ml or facs tubes for solution, standard and control preparations
Vortex
Plate shaker

Human SLPI Quantikine ELISA (cat # DP100)

Human SLPI 96 well microplate (part #890147)

Assay diluent RD1Q (part #895079)

Calibrator diluent RD5T (part # 895175)

Human SLPI standard (part #890149)

Wash buffer concentrate (part # 895003)

Human SLPI conjugate (part # 890148)

Color reagent A (part #895000)

Color reagent B (part # 895001)

Stop solution (part # 895032)

Plate sealers

Human E/tr-2 Duoset ELISA (cat # DY1747)

Human Trappin-2 Capture antibody (part#842342)

Human Trappin-2 Detection antibody 9part # 842343)

Human Trappin-2 standard (part# 842344)

Streptavidin-HRP (part # 890803)

DuoSet ancillary reagents (catalogue #DY008)

96 well micro-plates (cat #DY990)

Plate sealers (cat #DY992)

PBS: 137mM NaCl, 2.7mM KCL, 8.1Mm Na_2HPO_4, pH 7.2-7.4, 0.2 filtered (cat #DY006)

Wash buffer: 0.05% tween® 20 in PBS, pH 7.2-7.4 (cat #WA126)

Reagent diluent: 1%BSA in PBS, pH 7.2-7.4, 0.2um filtered (cat# DY995)

Substrate solution: 1:1 mixture of color reagent A (H_2O_2) and color reagent B (tetramethylbenzidine) (cat#DY999)

Stop solution: 2 N H_2SO_4 (cat # DY994)

dH2O.

APPENDIX II

Reagents preparation

Secretory leukocyte protease inhibitiors (SLPI)

Human SLPI standard was prepared for both saliva and breast milk, by reconstituting lyophilised SLPI standard 1ml of RD5Q, then serial dilutions were made as depicted below

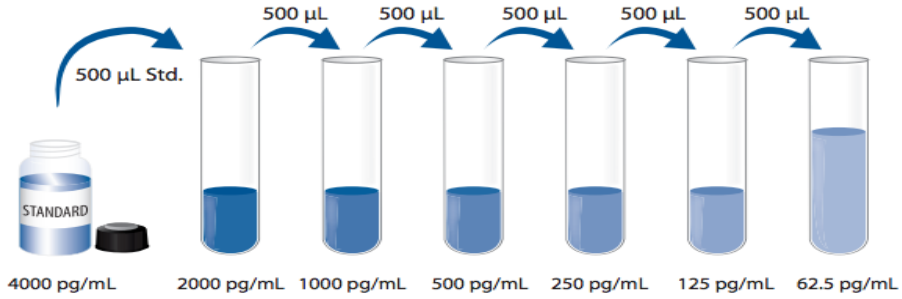


Figure 2.1: SLPI standard preparation and standard curve serial dilutions.

Elafin/trappin-2

E/tr-2 Standard preparation was prepared by reconstituting rTr-2 standard with 0,5ml of reagent diluent (i.e. STD stock). The standard was allowed to sit for a minimum of 15 min, gentle agitation was made prior to making dilutions. The highest standard was prepared by adding 18,2ul of the stock to 981,8ul of reagent diluent. As depicted by the picture below

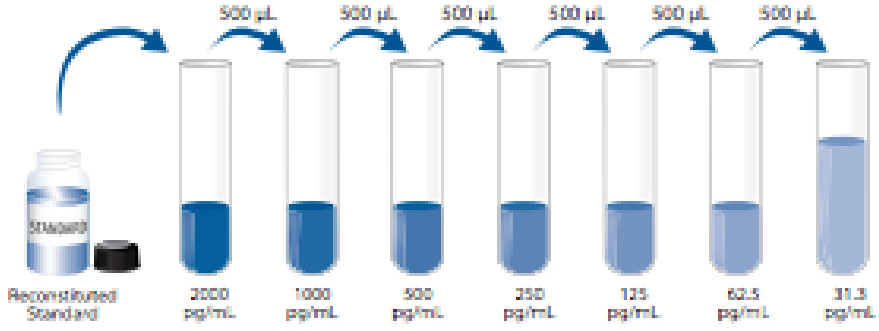


Figure 2.1: E/tr-2 standard preparation and standard curve serial dilutions

Capture antibody (for coating the plate overnight)

1ml of PBS was added to lyophilized capture antibody (i.e. to create capture Antibody stock). From the stock 55,6ul of the stock was added to 99445ul of PBS, to make a working solution with a concentration of 0,4ug/ml.
100ul of the capture antibody per well was used to coat the plate into each Well. The plate was sealed using provided sealers and incubated the plate at room temperature overnight.

Reagent diluent preparation

2,5ml of reagent diluent concentrate was added to 22,5ml of dH_2O to make 1% of reagent diluent.

Wash buffer preparation

To prepare 600ml of wash buffer, 24ml of the wash buffer concentrate was added to 576ml of dH_2O. Adjust the volume of WB with the number of wells to wash. i.e. for 64 wells use 20ml of the WB concentrate into 480ml of dH_2O.

Detection antibody prep

1ml PBS was added to lyophilised detection antibody (i.e. detection antibody stock)
27,8ul was pipetted out from the stock and added to 9972,2ul of reagent diluent.

Streptavidin-HRP

A working solution of streptavidin-HRP was prepared by making a dilution of 1:200 for HRP in reagent diluent as indicated on the vial.

APPENDIX III

After optimisation 80 fold dilution was selected as a suitable dilution to measure SLPI analyte table 3.1.1.

Table 3.1.1. Dilution factor optimisation in infant saliva for measuring SLPI.

SLPI Standard			HU saliva					HEU saliva				FF saliva			
STD. conc pg/m	optical density @450 nm		dilution	Optical density @450 nm		SLPI conc pg/ml	Neat SLPI conc.(averaged)	Optical density @450 nm		SLPI conc pg/ml	Neat SLPI conc (average)	Optical density @450 nm		SLPI conc pg/ml	Neat SLPI conc pg/ml
4000	1,60	1,72	1:40	1,81	1,91	5034,79	201380	1,85	1,78	5027,75	192960	1,07	1,06	2005,53	80200
2000	1,01	1,02	1:80	1,43	1,24	2823,74	225880	1,30	1,33	2707,61	219960	0,65	0,71	1111,86	88920
1000	0,63	070	1:160	0,83	0,77	1380,78	552200	0,30	0,30	432,936	171200	0,14	0,14	186,16	74400
500	0,34	0,37	1:320	0,12	0,11	154,17	245600	0,09	0,08	106,875	17200	0,06	0,06	70.23	111200
250	0,18	0,20	1:640	0,02	0,03	0	0	0,02	0,024	0	0	0,001	0,02	0	0
125	0,1	0,08	-	0,03	0,03	0	0	0,03	0,012	0	0	0,005	0,047	0	0
62.5	0,05	0,05													

After optimisation 32 fold dilution was selected as a suitable dilution to measure SLPI analyte table 3.1.2.

Table 3.1.2 Dilution factor optimisation results in maternal breast milk for measuring the SLPI protein

SLPI Standard			HIV+ve breast milk						HIV –ve breast milk				
conc pgml	optical density @450 nm		dilution	Optical density @450 nm		SLPI conc pg/ml		Neat SLPI conc.(averaged)	Optical density @450 nm		SLPI conc pg/ml		Neat SLPI conc (average)
4000	1,89	2,06	1:32	0,67	0,72	765,11	822,03	25392	0,98	1,05	1245,08	1355,55	41600
2000	1,32	1,41	1:64	0,36	0,33	368,21	326,04	22208	0,56	0,62	608,29	679,82	41184
1000	0,79	0,86	1:128	0,19	0,20	178,67	184,94	23168	0,31	0,32	321,53	303,62	39936
500	0,47	0,47	1:256	0,09	0,09	75,47	72,52	18816	0,17	0,17	150,78	154,88	38912
250	0,26	0,27	1:512	0,05	0,05	32,73	34,65	16895	0,08	0,08	68,60	69,58	35072
125	0,15	0,15	1:1024	0,02	0,03	9,85	9,86	9881.5	0,04	0,04	30,82	28,90	29696
62.5	0,07	0,08											

After optimisation 128 fold dilution was selected as a suitable dilution to measure SLPI analyte in infant saliva table 3.1.3.

Table 3.1.3 Dilution factor optimisation results in maternal breast milk for measuring the SLPI protein

E/tr-2 Standard			HU saliva					HEU saliva				FF saliva			
conc pg/ml	optical density @450 nm		dilution	Optical density @450 nm		SLPI conc pg/ml	Neat SLPI conc.(averaged)	Optical density @450 nm		SLPI conc pg/ml	Neat SLPI conc (average)	Optical density @450 nm		SLPI conc pg/ml	Neat SLPI conc pg/ml
2000	2,77	2,83	1:32	2,47	2,43	621,74	19895	1,95	1,97	305,01	9760	2,20	2,30	457,77	14648
1000	2,76	2,72	1:64	2,20	2,10	394,71	25261	1,27	1,48	148,96	9533	2,68	2,67	983,45	62940
500	2,48	2,38	1:128	1,49	1,44	165,58	21193	0,77	0,87	70,72	9052	1,52	2,22	549,60	70348
250	2,02	1,80	1:256	0,99	0,89	84,84	21719	0,47	0,47	37,86	9694	2,20	2,05	384,71	98485
125	1,14	1,11	1:512	0,25	0,57	32,87	16829	0,15	0,24	17,50	8960	1,01	0,92	87,926	45018
62.5	0,69	0,66	1:1024	0,29	0,33	25,26	25861	0,03	0,12	9,927	10165	0,51	0,70	49,793	50988
31.25	0,32	0,34													

After optimisation 64 fold dilution was selected as a suitable dilution to measure SLPI analyte in maternal breast milk table 3.1.4.

Table 3.1.4 Dilution factor optimisation results in maternal breast milk for measuring the SLPI protein

E/tr-2 Standard			HIV+ve breast milk					HIV –ve breast milk			
conc pgml	optical density @450 nm		dilution	Optical density @450 nm		SLPI conc pg/ml	Neat SLPI conc.(averaged)	Optical density @450 nm		SLPI conc pg/ml	Neat SLPI conc (average)
2000	2,77	2,83	1:32	1,97	1,80	279,15	8932	1,59	1,44	176,59	5650
1000	2,76	2,72	1:64	1,36	1,19	131,54	8418	1,04	1,04	96,51	6176
500	2,48	2,38	1:128	0,86	0,75	69,23	8861	0,70	0,67	52,18	6720
250	2,02	1,80	1:256	0,52	0,47	40,14	10275	0,38	0,35	29,64	7587
125	1,14	1,11	1:512	0,28	0,23	21,78	11152	0,20	0,17	16,76	8587
62.5	0,69	0,66	1:1024	0,14	0,13	13,53	13855	0,11	0,12	13,43	13755
31.25	0,32	0,34									

APPENDIX IV

Spike and Recovery (sample preparation and results)

SLPI spiking stock preparation (saliva):

SLPI standard was reconstituted with 0.5ml of reagent diluent, therefore working spiking stock concentration was 400pg/ml i.e. 10X concentrated than the highest standard (R&D systems). Spiking stock and sample preparations was executed as depicted in the layout below.

SLPI concentration that was used to spike infant saliva was calculated as following:

$C_1V_1 = C_2V_2$

40000 x9.6 = C_2x480

=800pg/ml

Therefore 800pg/ml was added to the first well of the ELISA plate

C_1= concentration of the spiking stock

V_1= spiking stock volume used to spike breast milk

V_2= total volume of sample after 1:80 dilution

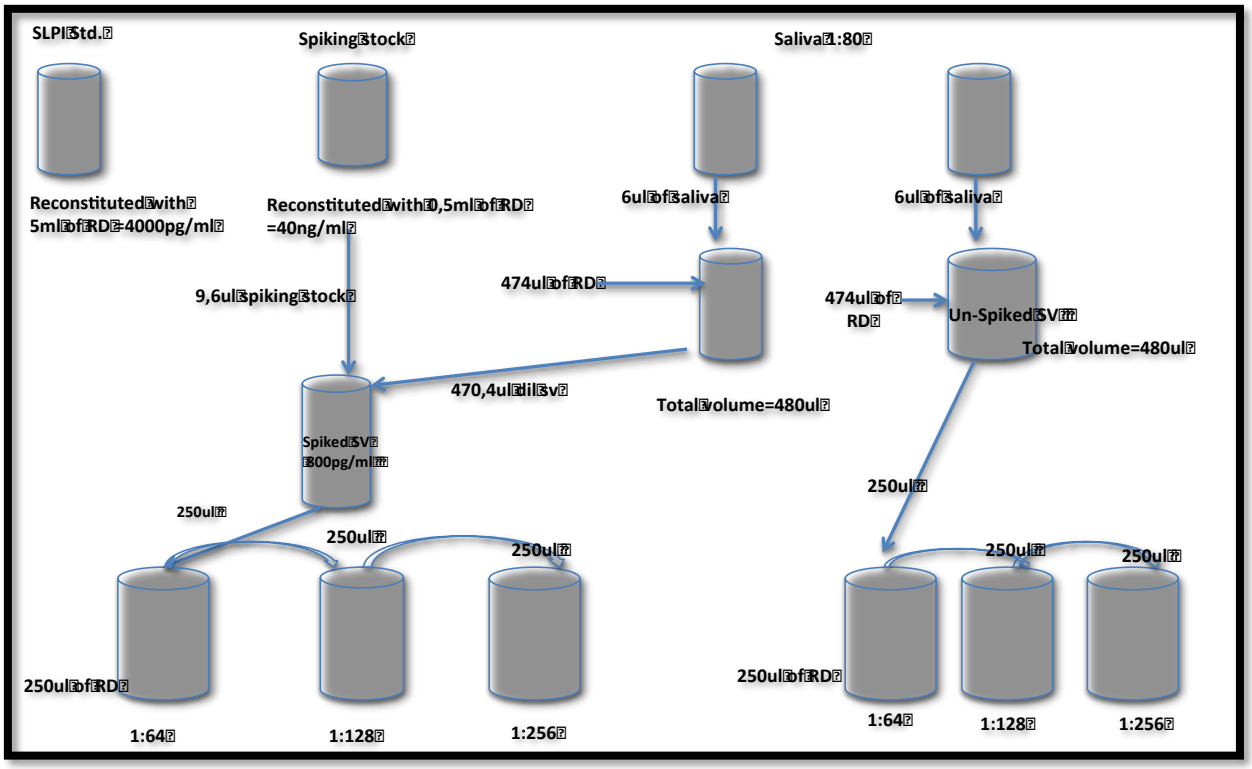


Figure 4.1: Sample preparation and serial dilution layout for spike and recovery and linearity testing for SLPI analyte in infant saliva.

SLPI spiking stock preparation (Breast milk):

SLPI standard was reconstituted with 0.5ml of reagent diluent, therefore working spiking stock concentration was 40000pg/ml i.e. 10X concentrated than the highest standard (R&D systems). Spiking stock and sample preparations was executed as depicted in the layout below.

SLPI concentration that was used to spike maternal breast milk was calculated as following:

$C_1V_1 = C_2V_2$

$40000 \times 13 = C_2 \times 640$

$=812.5pg/ml$

Therefore 8125pg/ml was added to the first well of the ELISA plate

C_1= concentration of the spiking stock

V_1= spiking stock volume used to spike breast milk

V_2= total volume of sample after 1:32 dilution

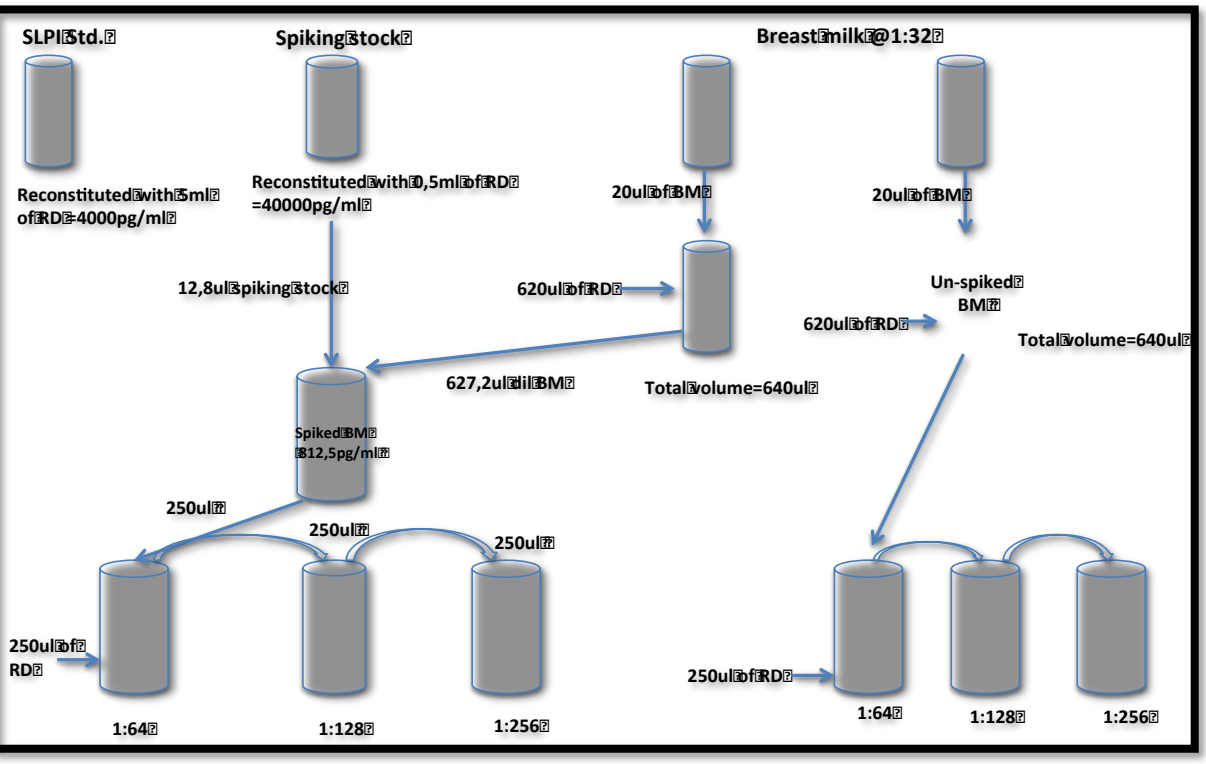


Figure 4.2: Sample preparation and serial dilution layout for spike and recovery and linearity testing for SLPI analyte in maternal breast milk.

E/tr-2 spiking stock preparation (Breast milk and saliva):

A volume of 7.28ul of E/tr-2 standard was added to 32.72ul of reagent diluent to prepare the spiking stock, therefore working concentration was 200pg/ml i.e. 10X concentrated than the highest standard (R&D systems). The same spiking stock concentration was applied in both breast milk and saliva.

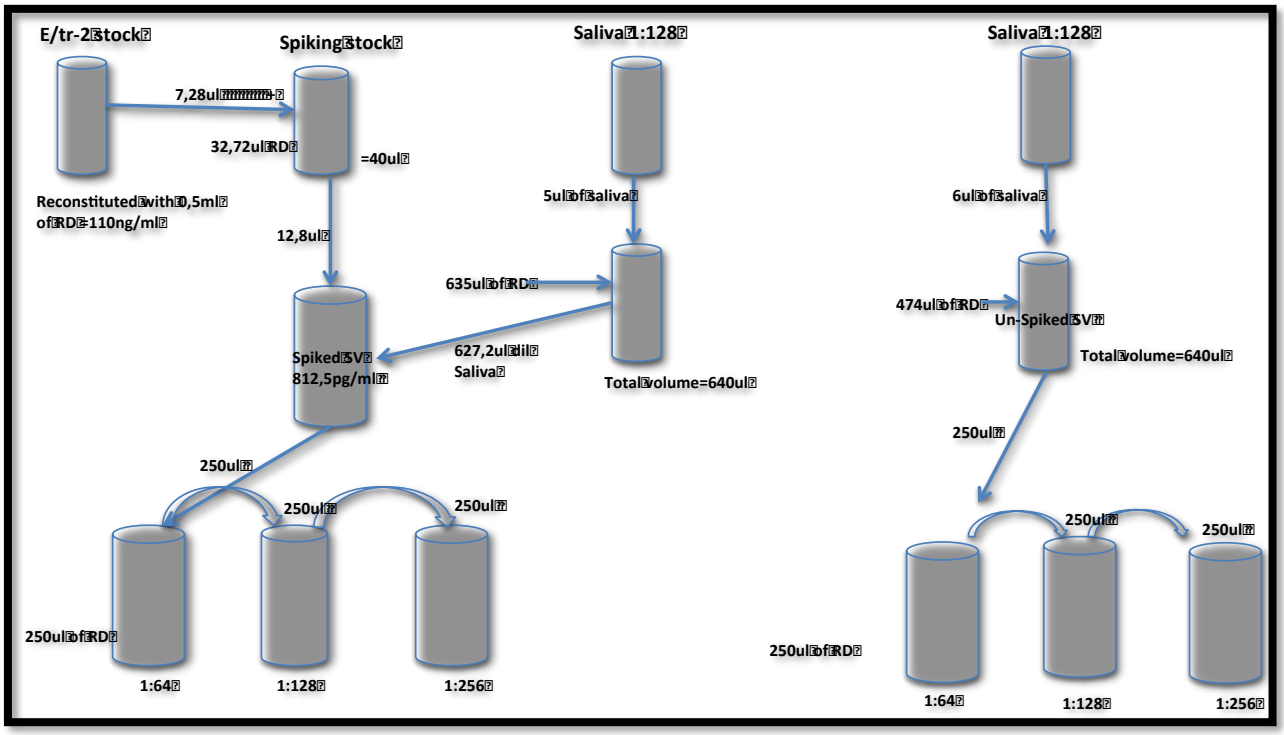


Figure 4.3: Sample preparation and serial dilution layout for spike and recovery and linearity testing for E/tr-2 analyte in infant saliva.

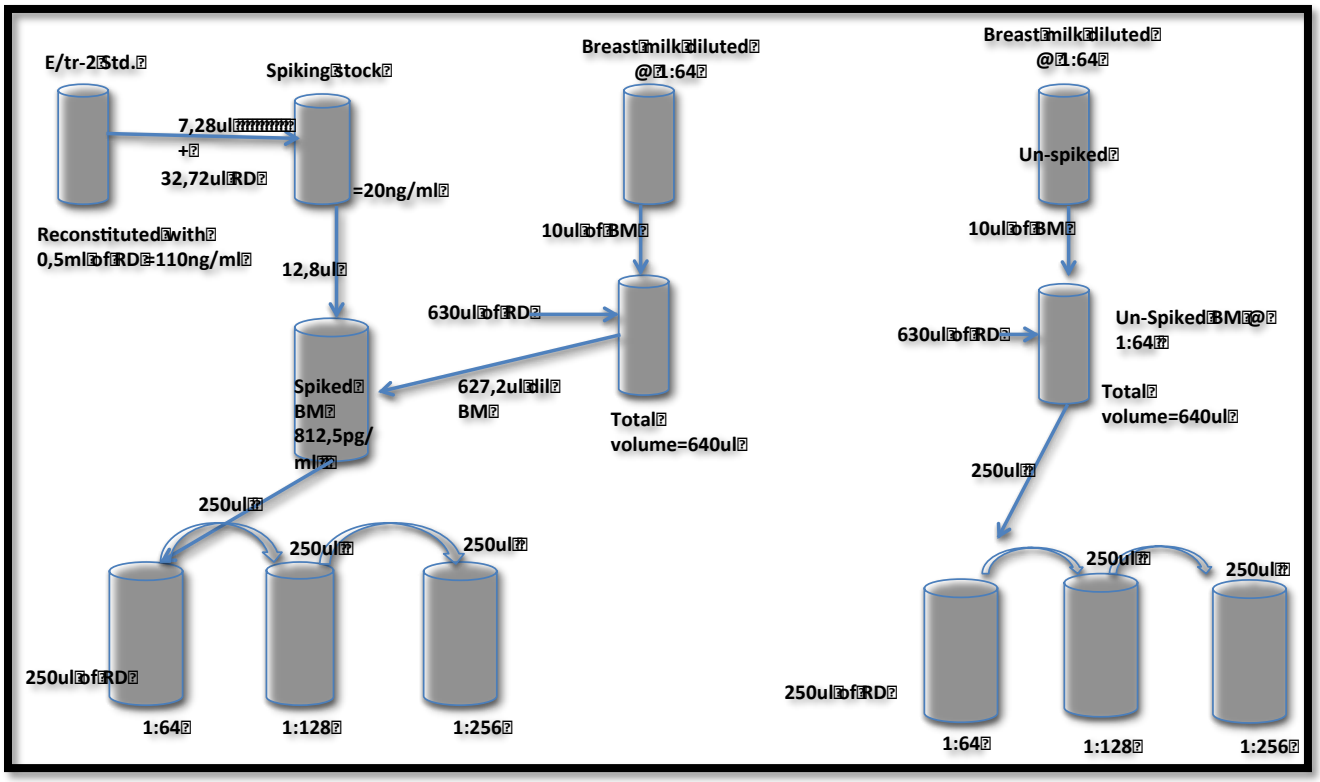


Figure 4.4: Sample preparation and serial dilution layout for spike and recovery and linearity testing for E/tr-2 analyte in maternal breast milk.

SLPI % recovery calculations for spiked saliva

% Recovery (1:80) = Observed – spiked x 100
Expected
= 1683.28-738.94 x 100
800
=118%

% Recovery (1:160) = 838.79-381.52 x 100
400
=114%

% Recovery (1:320) = 423.67-197.50 x 100
200
=113%

% Recovery (1:640) = 201.07-95.10 x 100
100
= 105.97%

SLPI linearity calculations for spiked saliva

% Linearity (1:160) = Observed value (ng/mL) of 1:2 dilution x 100
Expected value (ng/mL) divided by 2
= 838.79 x 100
841.64
=99%

% Linearity (1:320) = 423.67 x 100
420.8
=100%

% Linearity (1:640) = 201.07 x 100
210.41
=95.6%

SLPI % recovery calculations spiked breast milk

% Recovery (1:32) = Observed – spiked x 100
Expected
=1656.32-749.24 X100
812.5
= 111%

% Recovery (1:64) = Observed – spiked x 100
Expected
=796.50-364.80 X100
406
= 107%

% Recovery (1:128) = Observed – spiked x 100
Expected
=398.25-180.83 X100
203.13
= 107%

% Recovery (1:256) = Observed – spiked x 100
Expected
=189.31-85.52 x 100
101.57
= 102%

SLPI %linearity calculations for spiked breast milk

% Linearity (1:64) = Observed value (ng/mL) of 1:2 dilution x 100
Expected value (ng/mL) divided by 2
=796.50 x 100
828
=96%

% Linearity (1:128) = 398.25 x 100
414.08
=96%

% Linearity (1:256) = 189.31 x 100
207.04
=91%

E/tr-2 concentration that was used to spike infant saliva and maternal breast milk was calculated as following:

$C_1V_1 = C_2V_2$

$20000.13 = C_2 .640$

=406.25pg/ml

Therefore 406.25pg/ml was added to the first will of the ELISA plate

C_1= concentration of the spiking stock

V_1= spiking stock volume used to spike breast milk

V_2= total volume of breast milk and saliva (1:32 and/ 1:64)

E/tr-2 % recovery calculations for spiked saliva

% Recovery (1:128) = Observed – spiked x 100
Expected
= 750-370 x 100
406.25
=93%

% Recovery (1:264) = 420.57-229.08 x 100
203.125
=94%

% Recovery (1:512) = 162.015-65.781 x 100
103.125
=94%

% Recovery (1:1024) = 84.63-44.25 x 100
51.65
= 78%

E/tr-2 linearity calculations for spiked saliva

% Linearity (1:128) = Observed value (ng/mL) of 1:2 dilution x 100
Expected value (ng/mL) divided by 2
= 183 x 100
203.12
=91%

% Linearity (1:264) = 98 x 100
101.5
=97%

% Linearity (1:512) = 49 x 100
50.75
=96%

E/tr-2 % recovery calculations spiked breast milk

% Recovery (1:64) = Observed – spiked x 100
Expected
= 682-270.84 x 100
406.25
=101%

% Recovery (1:128) = 350.82-174.35 x 100

203.125
=86%

% Recovery (1:256) = 195.44-90.77 x 100
103.13
=102%

% Recovery (1:512) = 90.47-47.65 x 100
51.65
= 84%

E/tr-2 linearity calculations spiked breast milk

% Linearity (1:128) = Observed value (ng/mL) of 1:2 dilution x 100
Expected value (ng/mL) divided by 2
= 198.82 x 100
203.125
=98%

% Linearity (1:256) = 95 x 100
101.56
=94%

% Linearity (1:512) = 50.7 x 100
50.78
=100%

REFERENCES

Adkins, B., 1999. T-cell function in newborn mice and humans. *Immunology Today*, 20(7), pp.330–335. Available at: https://www.sciencedirect.com/science/article/pii/S0167569999014735 [Accessed April 2, 2019].

Ahmed, A.H. et al., 2016. The Effect of Interactive Web-Based Monitoring on Breastfeeding Exclusivity, Intensity, and Duration in Healthy, Term Infants After Hospital Discharge. *Journal of Obstetric, Gynecologic & Neonatal Nursing*, 45(2), pp.143–154. Available at: https://www.sciencedirect.com/science/article/abs/pii/S0884217515000477 [Accessed April 2, 2019].

De Almeida, P.D.V. et al., 2008. Saliva composition and functions: A comprehensive review. *Journal of Contemporary Dental Practice*.

Andreas, N.J., Kampmann, B. & Mehring Le-Doare, K., 2015. Human breast milk: A review on its composition and bioactivity. *Early Human Development*, 91(11).

Antoniades, C.G. et al., 2014. Secretory leukocyte protease inhibitor: A pivotal mediator of anti-inflammatory responses in acetaminophen-induced acute liver failure. *Hepatology*.

Baier, M. et al., 1995. HIV suppression by interleukin-16. *Nature*, 378(6557), pp.563–563. Available at: http://www.nature.com/articles/378563a0 [Accessed April 4, 2019].

Baqui, A.A.M.A., Meiller, T.F. & Falkler, W.A., 1999. Enhanced Secretory Leukocyte Protease Inhibitor in Human Immunodeficiency Virus Type 1-Infected Patients. , 6(6), pp.808–811.

Basha, S., Surendran, N. & Pichichero, M., 2014. Immune responses in neonates. *Expert Review of Clinical Immunology*, 10(9), pp.1171–1184. Available at:

http://www.tandfonline.com/doi/full/10.1586/1744666X.2014.942288 [Accessed April 2, 2019].

Becquart, P. et al., 1999. Secretory leukocyte protease inhibitor in colostrum and breast milk is not a major determinant of the protection of early postnatal transmission of HIV. *AIDS (London, England)*, 13(18), pp.2599–602. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10630534 [Accessed April 4, 2019].

Belyakov, I.M. & Ahlers, J.D., 2012. Mucosal immunity and HIV-1 infection: Applications for mucosal AIDS vaccine development. *Current Topics in Microbiology and Immunology*.

Berger, E.A. et al., 1998. A new classification for HIV-1. *Nature*, 391(6664), pp.240–240. Available at: http://www.nature.com/articles/34571 [Accessed April 2, 2019].

Beyrer, C. et al., 2012. Global epidemiology of HIV infection in men who have sex with men. *The Lancet*, 380(9839), pp.367–377. Available at: https://www.sciencedirect.com/science/article/pii/S0140673612608216 [Accessed April 2, 2019].

Bingle, C.D., 2011. Towards defining the complement of mammalian WFDC-domain-containing proteins. *Biochemical Society transactions*, 39(5), pp.1393–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21936821 [Accessed April 4, 2019].

Bland, R.M. et al., Breast Health Problems Are Rare in Both HIV-Infected and HIV-Uninfected Women Who Receive Counseling and Support for Breast-Feeding in South Africa. Available at: https://academic.oup.com/cid/article-abstract/45/11/1502/334848 [Accessed April 2, 2019].

Bleul, C.C. et al., 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 94(5), pp.1925–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9050881

[Accessed April 2, 2019].

Boćko, D. & Frydecka, I., 2003. [Structure and function of lymphocyte TCR/CD3 complex]. *Postepy higieny i medycyny doswiadczalnej*, 57(5), pp.519–529. Available at: https://europepmc.org/abstract/med/14737968 [Accessed April 2, 2019].

Briz, V., Poveda, E. & Soriano, V., 2006. HIV entry inhibitors: mechanisms of action and resistance pathways. *Journal of Antimicrobial Chemotherapy*, 57(4), pp.619–627. Available at: http://academic.oup.com/jac/article/57/4/619/669426/HIV-entry-inhibitors-mechanisms-of-action-and [Accessed April 2, 2019].

Castellote, C. et al., 2011. Premature Delivery Influences the Immunological Composition of Colostrum and Transitional and Mature Human Milk. *The Journal of Nutrition*, 141(6), pp.1181–1187.

Coutsoudis, A. et al., 1999. Influence of infant-feeding patterns on early mother-to-child transmission of HIV-1 in Durban, South Africa: a prospective cohort study. *The Lancet*, 354(9177), pp.471–476. Available at: https://www.sciencedirect.com/science/article/pii/S0140673699011010 [Accessed April 2, 2019].

Daher, K.A., Selsted, M.E. & Lehrer, R.I., 1986. Direct inactivation of viruses by human granulocyte defensins. *Journal of virology*, 60(3), pp.1068–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3023659.

Dey, T. et al., 2018. Proteases and Their Inhibitors in Chronic Obstructive Pulmonary Disease. *Journal of Clinical Medicine*, 7(9), p.244.

Dieterich, C.M. et al., 2013. Breastfeeding and health outcomes for the mother-infant dyad. *Pediatric clinics of North America*, 60(1), pp.31–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23178059 [Accessed April 3, 2019].

Doumas, S., Kolokotronis, A. & Stefanopoulos, P., 2005. MINIREVIEW Anti-Inflammatory and Antimicrobial Roles of Secretory Leukocyte Protease Inhibitor. *INFECTION AND IMMUNITY*, 73(3), pp.1271–1274.

Drannik, A.G. et al., 2012. Trappin-2/Elafin Modulate Innate Immune Responses of Human Endometrial Epithelial Cells to PolyI:C. *PLoS ONE*, 7(4).

Duncan, B. et al., 1993. Exclusive Breast-Feeding for at Least 4 Months Protects Against Otitis Media. *Pediatrics*, 91(5). Available at: https://pediatrics.aappublications.org/content/91/5/867.short [Accessed April 2, 2019].

Et, J., 1981. James O Wesgard et al, A Multi rule Shewart Chart for Quality Control in Clinical Chemistry. Clin. Chem. 1981; 27/3. 493-501. , pp.493–501.

Farquhar, C. et al., Salivary Secretory Leukocyte Protease Inhibitor Is Associated with Reduced Transmission of Human Immunodeficiency Virus Type 1 through Breast Milk.

Farquhar, C. et al., 2002. Salivary Secretory Leukocyte Protease Inhibitor Is Associated with Reduced Transmission of Human Immunodeficiency Virus Type 1 through Breast Milk. *The Journal of Infectious Diseases*, 186(8), pp.1173–1176. Available at: https://academic.oup.com/jid/article-lookup/doi/10.1086/343805 [Accessed April 4, 2019].

Farquhar, C., Farquhar, C. & John-Stewart, G., 2003. The role of infant immune responses and genetic factors in preventing HIV-1 acquisition and disease progression. *Clin Exp Immunol*, 134, pp.367–377.

Field, C.J., 2005. The Immunological Components of Human Milk and Their Effect on Immune Development in Infants. *The Journal of Nutrition*, 135(1), pp.1–4. Available at: https://academic.oup.com/jn/article/135/1/1/4663581 [Accessed April 3, 2019].

Freed, E.O., 2001. HIV-1 Replication. *Somatic Cell and Molecular Genetics*, 26(1/6), pp.13–33. Available at: http://link.springer.com/10.1023/A:1021070512287 [Accessed April 2, 2019].

Frenkel, E.S. & Ribbeck, K., 2015. Salivary mucins in host defense and disease prevention. *Journal of Oral Microbiology*, 7(1), p.29759. Available at:

https://www.tandfonline.com/doi/full/10.3402/jom.v7.29759.

Garvy, B.A. & Harmsen, A.G., 1996. The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice. *Inflammation*, 20(5), pp.499–512. Available at: http://link.springer.com/10.1007/BF01487042 [Accessed April 2, 2019].

Gervassi, A.L. & Horton, H., Is Infant Immunity Actively Suppressed or Immature? Available at: http://www.la-press.com. [Accessed April 2, 2019].

Ghosh, M., Fahey, J. V., et al., 2010. Anti-HIV Activity in Cervical-Vaginal Secretions from HIV-Positive and -Negative Women Correlate with Innate Antimicrobial Levels and IgG Antibodies K. Broliden, ed. *PLoS ONE*, 5(6), p.e11366. Available at: http://dx.plos.org/10.1371/journal.pone.0011366 [Accessed April 4, 2019].

Ghosh, M., Shen, Z., et al., 2010. Trappin-2/Elafin: A novel innate anti-human immunodeficiency virus-1 molecule of the human female reproductive tract. *Immunology*.

Gil, A. & Rueda, R., 2002. Interaction of early diet and the development of the immune system. *Nutrition Research Reviews*, 15(02), p.263. Available at: http://www.journals.cambridge.org/abstract_S095442240200015X [Accessed April 4, 2019].

Gipson, T.S. et al., 1999. Regulatory Effects of Endogenous Protease Inhibitors in Acute Lung Inflammatory Injury. *The Journal of Immunology*, 162(6), pp.3653–3662. Available at: http://www.jimmunol.org/content/162/6/3653.short [Accessed April 4, 2019].

Gomez, S.A. et al., 2009. Secretory Leukocyte Protease Inhibitor. *American Journal of Respiratory and Critical Care Medicine*, 179(3), pp.247–253. Available at: http://www.atsjournals.org/doi/abs/10.1164/rccm.200804-615OC [Accessed April 4, 2019].

Gregory, K.E. & Walker, W.A., 2013. Immunologic Factors in Human Milk and Disease Prevention in the Preterm Infant. *Current Pediatrics Reports*, 1(4),

pp.222–228.

Henrick, B.M. et al., 2017. Breastfeeding behaviors and the innate immune system of human milk: Working together to protect infants against inflammation, HIV-1, and other infections. *Frontiers in Immunology*.

Henry, R.J. & Segalove, M., 1952. the Running of Standards in Clinical. , pp.305–311.

Henson, P.M., Vandivier, R.W. & Douglas, I.S., 2006. Cell Death, Remodeling, and Repair in Chronic Obstructive Pulmonary Disease? *Proceedings of the American Thoracic Society*, 3(8), pp.713–717. Available at: http://pats.atsjournals.org/cgi/doi/10.1513/pats.200605-104SF [Accessed April 4, 2019].

Hiemstra, P.S. et al., 1996. Antibacterial activity of antileukoprotease. *Infection and Immunity*, 64(11), pp.4520–4524. Available at: https://iai.asm.org/content/64/11/4520.short [Accessed April 4, 2019].

Hocini, H. et al., 1997. High-level ability of secretory IgA to block HIV type 1 transcytosis: contrasting secretory IgA and IgG responses to glycoprotein 160. *AIDS research and human retroviruses*, 13(14), pp.1179–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9310284.

Idziorek, T. et al., 1998. Recombinant human IL-16 inhibits HIV-1 replication and protects against activation-induced cell death (AICD). *Clinical and experimental immunology*, 112(1), pp.84–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9566794 [Accessed April 4, 2019].

Iqbal, S.M. et al., 2009. Elevated elafin/trappin-2 in the female genital tract is associated with protection against HIV acquisition. *AIDS*, 23(13), pp.1669–1677. Available at: https://insights.ovid.com/crossref?an=00002030-200908240-00006 [Accessed April 4, 2019].

Janeway, C.A. & Medzhitov, R., 2002. I <scp>NNATE</scp> I <scp>MMUNE</scp> R <scp>ECOGNITION</scp>. *Annual Review of Immunology*, 20(1), pp.197–216. Available at:

http://www.annualreviews.org/doi/10.1146/annurev.immunol.20.083001.084359.

John-Stewart, G. et al., Breast-feeding and Transmission of HIV-1 for the Ghent IAS Working Group on HIV in Women and Children.

Kavanagh, K. & Dowd, S., 2004. Histatins: antimicrobial peptides with therapeutic potential. *Journal of Pharmacy and Pharmacology*, 56(3), pp.285–289.

Kazmi, S.H. et al., 2006. Comparison of human immunodeficiency virus type 1-specific inhibitory activities in saliva and other human mucosal fluids. *Clinical and Vaccine Immunology*.

Kuhn, L. et al., 2007a. High Uptake of Exclusive Breastfeeding and Reduced Early Post-Natal HIV Transmission K. P. Grietens, ed. *PLoS ONE*, 2(12), p.e1363. Available at: https://dx.plos.org/10.1371/journal.pone.0001363 [Accessed April 3, 2019].

Kuhn, L. et al., 2007b. High Uptake of Exclusive Breastfeeding and Reduced Early Post-Natal HIV Transmission K. P. Grietens, ed. *PLoS ONE*, 2(12), p.e1363. Available at: https://dx.plos.org/10.1371/journal.pone.0001363 [Accessed April 2, 2019].

Kuhn, L. et al., 2002. Human Immunodeficiency Virus (HIV)–Specific Cellular Immune Responses in Newborns Exposed to HIV In Utero. *Clinical Infectious Diseases*.

Lahey, T. et al., 2012. Selective impact of HIV disease progression on the innate immune system in the human female reproductive tract. *PLoS ONE*.

Ma, G. et al., 2004. Secretory leukocyte protease inhibitor binds to annexin II, a cofactor for macrophage HIV-1 infection. *The Journal of experimental medicine*, 200(10), pp.1337–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15545357 [Accessed April 4, 2019].

Maródi, L., 2006. Innate cellular immune responses in newborns. *Clinical Immunology*.

McClelland, M. et al., 2001. Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. *Nature*, 413(6858), pp.852–856. Available at: http://www.nature.com/articles/35101614 [Accessed April 2, 2019].

Mcneely, T.B. et al., *Inhibition of Human Immunodeficiency Virus Type 1 Infectivity by Secretory Leukocyte Protease Inhibitor Occurs Prior to Viral Reverse Transcription*, Available at: www.bloodjournal.org.

Mcneely, T.B. et al., Inhibition of Human Immunodeficiency Virus Type 1 Infectivity by Secretory Leukocyte Protease Inhibitor Occurs Prior to Viral Reverse Transcription.

Mcneely, T.B. et al., 1995. Secretory Leukocyte Protease Inhibitor: A Human Saliva Protein Exhibiting Anti-Human Immunodeficiency Virus 1 Activity In Vitro monocytes l lymphocytes * SLPI * retrovirus * saliva. *J. Clin. Invest*, 96, pp.456–464.

McNeely, T.B. et al., 1995. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *Journal of Clinical Investigation*.

Moreau, T. et al., 2008a. Multifaceted roles of human elafin and secretory leukocyte proteinase inhibitor (SLPI), two serine protease inhibitors of the chelonianin family. *Biochimie*.

Moreau, T. et al., 2008b. Multifaceted roles of human elafin and secretory leukocyte proteinase inhibitor (SLPI), two serine protease inhibitors of the chelonianin family. *Biochimie*, 90(2), pp.284–295.

Moriyama, A. et al., 1999. Secretory leukocyte protease inhibitor (SLPI) concentrations in cervical mucus of women with normal menstrual cycle. *Molecular Human Reproduction*, 5(7), pp.656–661. Available at: https://academic.oup.com/molehr/article-lookup/doi/10.1093/molehr/5.7.656 [Accessed April 4, 2019].

Muessig, K.E. et al., 2012. Does ART prevent HIV transmission among MSM? *Aids*, 26(18), pp.2267–2273.

Natchu, U.C.M. et al., 2012. Exclusive breastfeeding reduces risk of mortality in infants up to 6 mo of age born to HIV-positive Tanzanian women. *American Journal of Clinical Nutrition*, 96(5), pp.1071–1078.

Newburg, D.S. & Walker, W.A., 2007. Protection of the Neonate by the Innate Immune System of Developing Gut and of Human Milk. *Pediatric Research*, 61(1), pp.2–8. Available at: http://www.nature.com/doifinder/10.1203/01.pdr.0000250274.68571.18 [Accessed April 4, 2019].

Onyango-Makumbi, C. et al., 2010. Early weaning of HIV-exposed uninfected infants and risk of serious gastroenteritis: Findings from two perinatal HIV prevention trials in Kampala, Uganda. *Journal of acquired immune deficiency syndromes (1999)*, 53(1), pp.20–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19779355 [Accessed April 2, 2019].

Page-Shafer, K. et al., 2006. (C2) Saliva, breast milk, and mucosal fluids in HIV transmission. *Advances in dental research*.

Van De Perre, P. et al., 2012. HIV-1 reservoirs in breast milk and challenges to elimination of breast-feeding transmission of HIV-1. *Science Translational Medicine*.

Planer, J.D. et al., 2016. Development of the gut microbiota and mucosal IgA responses in twins and gnotobiotic mice. *Nature*.

Py, B. et al., 2009a. The phospholipid scramblases 1 and 4 are cellular receptors for the secretory leukocyte protease inhibitor and interact with CD4 at the plasma membrane. *PLoS ONE*.

Py, B. et al., 2009b. The Phospholipid Scramblases 1 and 4 Are Cellular Receptors for the Secretory Leukocyte Protease Inhibitor and Interact with CD4 at the Plasma Membrane L. Zhang, ed. *PLoS ONE*, 4(3), p.e5006. Available at: https://dx.plos.org/10.1371/journal.pone.0005006 [Accessed April 4, 2019].

Quinlivan, E.B. et al., 2012. Modeling the impact of Trichomonas vaginalis

infection on HIV transmission in HIV-infected individuals in medical care. *Sexually transmitted diseases*, 39(9), pp.671–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22902662 [Accessed April 2, 2019].

Raundhal, M. et al., 2015. High IFN-γ and low SLPI mark severe asthma in mice and humans. *Journal of Clinical Investigation*.

Reidpath, D.D. & Allotey, P., 2003. Infant mortality rate as an indicator of population health. *Journal of Epidemiology and Community Health*, 57(5), pp.344–346.

Rodger, A. et al., 2014. 153LB: HIV transmission risk through condomless sex if HIV+ partner on suppressive ART: PARTNER Study. *21st Conference on Retroviruses and Opportunistic Infections*, 0, pp.3–6. Available at: http://www.croiconference.org/sites/default/files/abstracts/153LB.pdf.

Sallenave, J.M. et al., 1997. Secretory leukocyte proteinase inhibitor is a major leukocyte elastase inhibitor in human neutrophils. *Journal of leukocyte biology*, 61(6), pp.695–702. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9201260 [Accessed April 4, 2019].

Saville, M. et al., 1994. Interleukin-10 suppresses human immunodeficiency virus-1 replication in vitro in cells of the monocyte/macrophage lineage. *Blood*, 83(12). Available at: http://www.bloodjournal.org/content/83/12/3591.short?sso-checked=true [Accessed April 4, 2019].

Scheib, H., Sperisen, P. & Hartley, O., Matters Arising HIV-1 Coreceptor Selectivity: Structural Analogy between HIV-1 V3 Regions and Chemokine b-Hairpins Is Not the Explanation. Available at: http://www.structure.org/cgi/content/full/14/4/645/DC1/. [Accessed April 2, 2019].

Shapiro, R.L. et al., 2007. Infant Morbidity, Mortality, and Breast Milk Immunologic Profiles among Breast-Feeding HIV-Infected and HIV-Uninfected Women in Botswana. *The Journal of Infectious Diseases*.

Shaw, L. & Wiedow, O., 2011. Therapeutic potential of human elafin. *Biochemical Society transactions*, 39(5), pp.1450–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21936832 [Accessed April 4, 2019].

Shugars, D.C., 2002. Endogenous Mucosal Antiviral Factors of the Oral Cavity. *The Journal of Infectious Diseases*, 179(s3), pp.S431–S435.

Skott, P. et al., 2002. Inhibitory function of secretory leukocyte proteinase inhibitor (SLPI) in human saliva is HIV-1 specific and varies with virus tropism. *Oral Diseases*.

Spadaro, M. et al., 2008. Lactoferrin, a major defense protein of innate immunity, is a novel maturation factor for human dendritic cells. *The FASEB Journal*, 22(8), pp.2747–2757. Available at: http://www.fasebj.org/doi/10.1096/fj.07-098038 [Accessed April 3, 2019].

Spector, S.A., 2001. Mother-to-infant transmission of HIV-1: the placenta fights back. *The Journal of clinical investigation*, 107(3), pp.267–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11160148 [Accessed April 2, 2019].

Van der Strate, B.W.A. et al., 2001. Antiviral activities of lactoferrin. *Antiviral Research*, 52(3), pp.225–239.

Supervie, V. et al., 2010. HIV, transmitted drug resistance, and the paradox of preexposure prophylaxis. *Proceedings of the National Academy of Sciences of the United States of America*, 107(27), pp.12381–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20616092 [Accessed August 3, 2018].

Uruakpa, F.O., Ismond, M.A.H. & Akobundu, E.N.T., 2002. Colostrum and its benefits: A review. *Nutrition Research*.

Velilla, P.A., Rugeles, M.T. & Chougnet, C.A., 2006. Defective antigen-presenting cell function in human neonates. *Clinical Immunology*.

Vogelmeier, C. et al., 1997. Comparative loss of activity of recombinant secretory leukoprotease inhibitor and alpha 1-protease inhibitor caused by different forms of oxidative stress. *The European respiratory journal*, 10(9), pp.2114–

9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9311513 [Accessed April 4, 2019].

Wahl, S. et al., 1997. Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-1. *Oral Diseases*.

Walker, A., 2010. Breast Milk as the Gold Standard for Protective Nutrients. *The Journal of Pediatrics*, 156(2), pp.S3–S7. Available at: https://www.sciencedirect.com/science/article/pii/S0022347609011287 [Accessed April 3, 2019].

White, M.R. et al., 2009. Multiple components contribute to ability of saliva to inhibit influenza viruses. *Oral Microbiology and Immunology*, 24(1), pp.18–24. Available at: http://doi.wiley.com/10.1111/j.1399-302X.2008.00468.x [Accessed April 3, 2019].

Wilen, C.B., Tilton, J.C. & Doms, R.W., 2012. HIV: Cell binding and entry. *Cold Spring Harbor Perspectives in Medicine*, 2(8).

Williams, S.E. et al., 2006. SLPI and elafin: one glove, many fingers. *Clinical Science*, 110(1), pp.21–35. Available at: http://www.clinsci.org/content/110/1/21.abstract [Accessed April 4, 2019].

Wood, L.F. et al., 2013. The oral mucosa immune environment and oral transmission of HIV/SIV. *Immunological Reviews*.

Wright, A.L. et al., 1998. Increasing Breastfeeding Rates to Reduce Infant Illness at the Community Level. *Pediatrics*, 101(5), pp.837–844.

Yolken, R.H. et al., 1992. Human milk mucin inhibits rotavirus replication and prevents experimental gastroenteritis. *The Journal of clinical investigation*, 90(5), pp.1984–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1331178 [Accessed April 3, 2019].

Yoshizawa, J.M. et al., 2013. Salivary biomarkers: Toward future clinical and diagnostic utilities. *Clinical Microbiology Reviews*.

Zaghouani, H., Hoeman, C.M. & Adkins, B., 2009. Neonatal immunity: faulty T-

helpers and the shortcomings of dendritic cells. *Trends in Immunology*.

Zeeuwen, P.L. et al., 1997. Identification and sequence analysis of two new members of the SKALP/elafin and SPAI-2 gene family. Biochemical properties of the transglutaminase substrate motif and suggestions for a new nomenclature. *The Journal of biological chemistry*, 272(33), pp.20471–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9252357 [Accessed April 4, 2019].

.