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Characterization of Immunoglobulin (Ig) isotypes, IgG subclasses and cytokines in the blood and genital tracts of HIV infected and healthy women from an observational cohort study (CAPRISA 082)

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PLAGIARISM DECLARATION

I, Mandisa Nokukhanya Zuma, hereby declare that this dissertation has not been published or submitted before, to this or any other tertiary institution. Work that has been previously published and was referred to in this dissertation has been adequately referenced, and that the data presented here is original. The Turnitin originality report is in Appendix I (page 119).

Mandisa Nokukhanya Zuma

This dissertation was compiled under the supervision of Dr D. Archary and Dr P. Sobia, at the Centre for the Aids Programme of Research in South Africa.

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DECLARATION

I, Mandisa Nokukhanya Zuma, declare as follows:

1. That the work described in this dissertation has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:

I wrote a research proposal, applied for BREC, and then ran the assays post-approval. After obtaining the data, I did the graphical presentation and analyses using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, California, USA). I then compiled a dissertation based on my findings and the literature.

3. That the contributions of others to the project were as follows:

Mesuli Mhlongo (Statistician) further analysed my data using R version 4.0.2 (R Foundation for Statistical Computing Software, Vienna, Austria).

Signed  Date**:** 2 September 2020

DEDICATION

Firstly, I would like to thank my parents and siblings for supporting me throughout the academic period. Secondly, I would like to thank my late grandparents for being my guardian angels through this journey. Most importantly, I would like to thank God Almighty for giving me the strength, knowledge, ability and opportunity to undertake this research study.

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Literature Review

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ABBREVIATIONS

ADCC:	Antibody Dependent Cellular Cytotoxicity
ADCP:	Antibody Dependent Cellular Phagocytosis
ADCVI:	Antibody Dependent Cell-mediated Viral Inhibition
AIDS:	Acquired Immunodeficiency Syndrome
APC:	Antigen Presenting Cell
ARV:	Antiretroviral
BCR:	B Cell Receptor
bNAbs:	Broadly Neutralizing Antibodies
BV:	Bacterial Vaginosis
CAPRISA:	Centre for the AIDS Programme of Research in South Africa
CD40L:	CD40 Ligand
CVL:	Cervico-vaginal Lavage
CLP	Common Lymphoid Progenitor
COC:	Combined Oral Contraceptives
DMPA:	Depot Medroxy Progesterone Acetate
DNA:	Deoxyribonucleic Acid
EBF:	Early B-cell factor
ECHO:	Evidence for Contraceptive options and HIV Outcomes
Fab:	Antigen Binding Fragment
Fc:	Fragment Crystallisable
FcRn:	Neonatal Fc Receptor
FEM-PrEP:	Pre-exposure prophylaxis study for HIV prevention among African women
FGF:	Fibroblast Growth Factor
FGT:	Female Genital Tract
GBV:	Gender Based Violence
GCSF	Granulocyte Colony Stimulating Factor
GEE:	Generalized estimating equation
GI:	Genital Inflammation
G-CSF:	Granulocyte Colony Stimulating Factor
GM-CSF:	Granulocyte Macrophage Colony-Stimulating Factor
Gp:	Glycoprotein
HESN:	Highly Exposed Seronegative

HLA:	Human Leukocyte Antigen
HIV:	Human Immunodeficiency Virus
HSCs	Hematopoietic Stem Cells
HSV:	Herpes Simplex Virus
IFN-α:	Interferon Alpha
IFN-β:	Interferon Beta
IFN-γ:	Interferon Gamma
Ig:	Immunoglobulin
IL	Interleukin
IL-1α	Interleukin 1 alpha
IL-1β:	Interleukin 1 Beta
IP-10:	Interferon Inducible Protein 10
iPREX:	Pre-Exposure Prophylaxis initiative
IUD	Intrauterine Device
LNG:	Levonorgestrel
MCP:	Monocyte Chemoattractant Protein
MFI:	Median Fluorescence Intensity
MHC:	Major Histocompatibility Complex
MIP-1α:	Macrophage Inflammatory Protein 1 Alpha
MIP-1β:	Macrophage Inflammatory Protein 1 Beta
NET-EN:	Norethisterone Enanthate
NK:	Natural Killer
PAMPS:	Pathogen-Associated Molecular Patterns Molecules
PBS:	Phosphate Buffered Saline
PBMC:	Peripheral Blood Mononuclear Cells
PWM:	PokeWeed Mitogen
PrEP:	Pre-Exposure Prophylaxis
PRRs:	Pattern Recognition Receptors
QIC	Quasi-likelihood information criterion
RNA:	Ribonucleic Acid
SANAC:	South African National AIDS Council
SHIV:	Simian/Human Immunodeficiency Virus
SIV:	Simian Immunodeficiency Virus
sCD27:	Soluble CD27 Ligand

sCD40L:	Soluble CD40 Ligand
STIs:	Sexually Transmitted Infections
TCR:	T Cell Receptor
TDF:	Tenofovir Disoproxil Fumarate
TGF-B:	Transforming Growth Factor Beta
Th:	T Helper
TNF-α:	Tumor Necrosis Factor Alpha
VEGF:	Vascular Endothelial Growth Factor
VOICE:	Vaginal and Oral Interventions to Control the Epidemic
WHO:	World Health Organisation

ABSTRACT

Background: Heterosexual transmission remains the dominant route of HIV infections in women. Immune responses that predict HIV acquisition during pre-exposure prophylaxis (PrEP) remain undefined. We hypothesized that increased genital tract antibodies and cytokines pre-HIV infection predict HIV acquisition in seroconverters compared to non-seroconverters irrespective of PrEP use.

Methods: Plasma and Softcup specimens were collected from n=12 seroconverters (cases) and n=48 non-seroconverters (controls) in the CAPRISA 082 study at five time points. Of 12 cases-, nine took PrEP, while 29 of 48 controls took PrEP. IgG1, IgG2, IgG3, IgG4, IgM and IgA, and nine cytokines: MIP-1α, MIP-1β, IP-10, MCP-1, and IL-8, TNF-α, IL-1α, IL-1β, and IL-6 pre- and post-HIV infection, were measured using multiplexed technology.

Results: Baseline levels of IgG subclasses, Ig isotypes, and mucosal cytokines were similar between cases and controls. Over time within the cases, plasma IgA significantly declined, in controls, plasma IgG2, IgG3, and IgM significantly declined over time ($p<0.05$). In cases and controls on PrEP, plasma IgG3 trended higher compared to no PrEP ($p<0.1$). Relative to baseline, only within the controls, mucosal IgG1, IgG2, IgG3, IgG4, IgM, and IgA declined significantly. Mucosal IgM significantly predicted four-fold increased HIV risk ($p=0.01$). Eight of nine cytokines in the genital tract were significantly elevated in the cases compared to controls (all $p<0.05$). In cases and controls who used PrEP relative to no PrEP, IP-10 was significantly lower ($p=0.04$ and $p=0.009$). Baseline mucosal IL-8 significantly correlated with mucosal IgG1, IgG2, total IgG, and IgM ($p<0.001$ for all).

Conclusions: Although no significant elevated genital antibodies or cytokines pre-HIV infection were found, significantly different patterns of antibodies and cytokines were observed in this cohort. Plasma IgG3, one of the most effective of the IgG's eliciting diverse antibody functions, was increased in PrEP users. Mucosal IgM was associated with increased HIV-acquisition risk, while pleiotropic IP-10, a reported risk factor was modulated in PrEP users among cases. Collectively, these data suggest that PrEP use may modulate or preserve specific immune responses that can modify HIV risk. As PrEP uptake increases, its effect on mucosal and systemic immunity is important for informing on prevention strategies where PrEP may be given alone or in combination with HIV vaccine for added efficacy.

CHAPTER ONE

INTRODUCTION
&
LITERATURE REVIEW

INTRODUCTION

The human immunodeficiency virus (HIV) is the main cause of death among women of reproductive age (Klaas et al., 2018). In developing countries worldwide, the HIV prevalence is two-fold higher in young women between the ages of 15-24 years when compared to men of their age (UNAIDS, 2020a). In addition, young women become HIV infected 5-7 years earlier compared to men of their age [as reviewed by (Kharsany and Karim, 2016)]. The South African National AIDS Council reported the HIV prevalence to be approximately four-fold higher among South African women compared to men (South African National AIDS Council, 2017). Understanding various behavioural and biological factors is crucial to informing why women remain vulnerable to HIV.

A combination of behavioural and biological factors account for the gender-biased HIV infection rate in women. These include; inter-generational sexual partnering (de Oliveira et al., 2017, Kharsany et al., 2018), gender based violence [as reviewed by (Wathuta, 2016)], vaginal douching (Scholes et al., 1993), semen exposure (Sharkey et al., 2012), lubricants (Fichorova et al., 2001). In addition, age of sexual debut (Peltzer, 2010, Pettifor et al., 2009, Zuma et al., 2010), multiple concurrent sexual partners (Adimora et al., 2014), women disempowerment (Langen, 2005), the inability to negotiate safe sex practices leading to low or no condom use (Pettifor et al., 2005), low marriage rates (Abdool Karim et al., 1991, Anglewicz and Reniers, 2014) fuel this disparity. Hormonal changes during different phases of the menstrual cycle (Kyongo et al., 2012), the use of hormonal contraceptives (Ahmed et al., 2019, Heffron et al., 2012, Morrison et al., 2015) and genital schistosomiasis (Kleppa et al., 2014) are other factors that also influence the vulnerability of women to HIV infection.

Genital inflammation is an established biological factor for increased risk for HIV. Genital inflammation is defined by the elevated levels of at least five of nine pro-inflammatory or chemotactic cytokines in the female genital tract (FGT). These include [interleukin (IL): IL-6, IL-8, IL-1α, IL-1β, tumor necrosis factor-alpha (TNF-α), interferon-gamma inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-alpha (MIP-1α), and macrophage inflammatory protein 1-beta (MIP-1β)]. The above-mentioned cytokines and chemokines were found to be associated with increased risk of HIV infection among African women (Masson et al., 2015). Furthermore, Mlisana et al., (2012) also reported increased genital cytokines: IL-1β, IL-6, and IL-8 to be associated with increased HIV infection risk among women with asymptomatic and symptomatic sexually transmitted infections (STIs) (Mlisana et al., 2012).

Besides the associations of genital cytokines and increased HIV risk, very little is understood about the profile of pre-existing antibodies in the genital tract that may confer protection, reduce risk or increase the risk for HIV-acquisition in women. Antibodies can elicit diverse functions such as neutralizing and non-neutralizing activities. Neutralizing antibodies directly kill through binding to pathogens [as reviewed by (Klasse, 2014)]. Previous *in vitro* studies demonstrated broadly neutralizing properties of

systemic and mucosal IgA and based on these data, IgA was hypothesized to confer protection to HIV *in vivo* (Jia et al., 2020). In a humanized mice study, anti-HIV polymeric IgA effectively inhibited the mucosal HIV transmission when compared to the monomeric IgA or IgG1 (Hur et al., 2012). In addition, neutralizing anti-HIV IgM was shown to effectively protect the Rhesus macaques against chimeric simian-human immunodeficiency virus (SHIV) transmission (Gong et al., 2018). Therefore, the understanding of the immunoglobulin and isotypes in the genital tract prior to HIV infection provides important information about baseline correlates of protection or risk in women who remain vulnerable to HIV.

In addition to neutralizing, antibodies also have non-neutralizing functions which are coordinated through their Fc region and include; antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC) [as reviewed by (Forthal, 2014)], activation of the complement system, and antibody-dependent cell-mediated viral inhibition(ADCVI) (Excler et al., 2014). IgG3 binds to the FcγRIIc and FcγRIIIa of the natural killer (NK) cells and activate the killing of antibody-coated target cells expressing peptides of pathogens on their surfaces [as reviewed by (Bruhns and Jönsson, 2015)]. Vaccine-induced VIV2 specific IgG inversely correlated with HIV-1 infection risk while Env-specific IgA correlated with increased HIV risk in the RV144 HIV-1 vaccine trial (Haynes et al., 2012, Zolla-Pazner et al., 2014). However, these findings have not yet been confirmed from immune correlates studies in HVTN702. Furthermore, the lack of efficacy from HVTN702 posed a major setback to the HIV vaccine field and highlights that PrEP as antiretroviral (ARVs) remains the main tool in HIV prevention.

To reduce HIV infections among women, PrEP in a form of Truvada® [as reviewed by (Sheth et al., 2016)] are now being offered at public health facilities. Previous studies using either topical PrEP in the form of a microbicide gel for e.g. the 1% tenofovir gel (Abdool Karim et al., 2010, van der Straten et al., 2014) and the dapivirine ring (Baeten et al., 2016) have produced mixed results due to low to no adherence, and therefore conferred very low to no protection in women (Marrazzo et al., 2015, Van Damme et al., 2012). Based on the discrepant efficacies from these clinical trials, these products were not further tested and are no longer available as HIV prevention technologies.

In addition to making PrEP available to at risk populations, it is important to identify the biomarkers that may predict HIV acquisition as breakthrough HIV infections are anticipated despite PrEP. Therefore, to address this research gap, this sub-study aimed to assess biological markers such as immunoglobulins (Ig) subclasses and isotypes and cytokines pre-and post-HIV infection among South African women at the site of vulnerability, the FGT. Immunoglobulins were also assessed in the blood to understand their relationships between the systemic and mucosal compartments in the presence or absence of oral PrEP, and to understand the effect of cytokines on antibodies in the mucosal environment.

1. LITERATURE REVIEW

Since the start of the pandemic, HIV has infected more than 75.7 million [55.9 million–100 million] people and approximately 32.7 million [24.8 million–42.2 million] people have died from AIDS-related illnesses (UNAIDS, 2020a). Globally, 38.0 million [31.6 million–44.5 million] people were living with HIV at the end of 2019 (UNAIDS, 2020a). HIV remains a public health challenge worldwide with sub-Saharan Africa accounting for more than 70% of global HIV infection [as reviewed in (Kharsany and Karim, 2016)]. In 2019, the global adult HIV prevalence was estimated to be 0.7% (Figure 1). Approximately 15% of young women aged between 15-24 years are living with HIV worldwide and 80% of them are living in sub-Saharan Africa [as reviewed in (Kharsany and Karim, 2016)] with adolescent girls aged between 15-19 years accounting for 83% of new HIV infections (UNAIDS, 2020a). South Africa has approximately 1% of the world's population but 20% of the global HIV infection cases at the end of 2019 (UNAIDS, 2020b). The data from the national survey conducted in 2015 reported 11.0% and 34.1% HIV prevalence among young women aged between 15-19 and 20-24 years respectively, in KwaZulu-Natal (Chimbindi et al., 2018) which is almost four-fold higher when compared to the national estimate of 8.7% (Abdool Karim et al., 2012), underscoring the vulnerability of young women to HIV in this province. Several biological and behavioural factors have been reported to be associated with high risk of HIV infection among women.

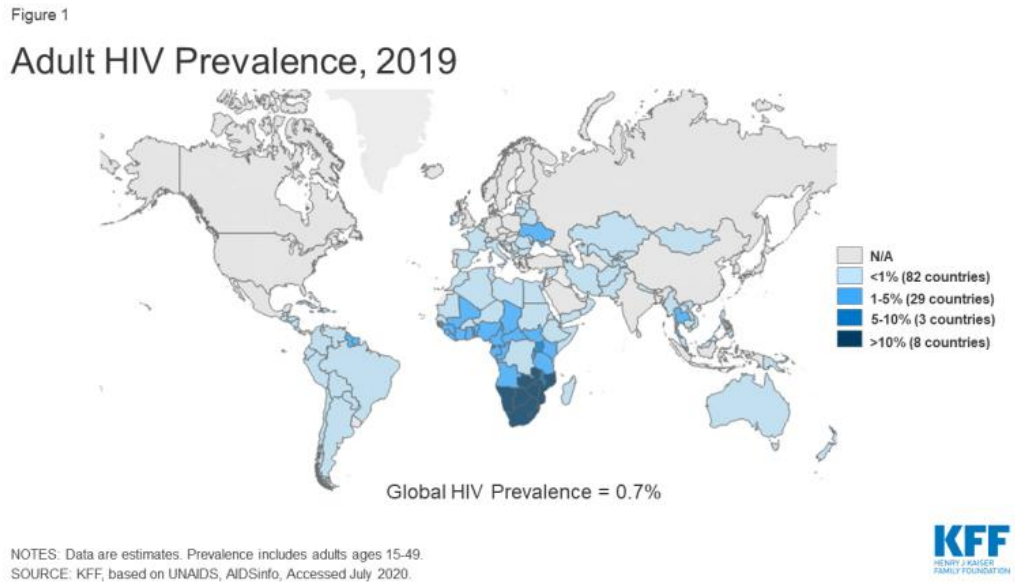

Figure 1: The global adult HIV prevalence. The countries located within sub-Saharan Africa have the highest percentage of people living with HIV. Southern and South Africa have the highest HIV prevalence rates in the world indicated by the dark blue shading [Adapted from (Kaiser Family Foundation, 2020)].

1.1 Social and behavioural factors in HIV acquisition

Several social and behavioural factors increase the vulnerability to HIV acquisition among women. These include: age-disparate sexual coupling (de Oliveira et al., 2017, Maughan-Brown et al., 2018), age at sexual debut (Peltzer, 2010, Pettifor et al., 2009, Zuma et al., 2010), multiple concurrent sexual partners (Adimora et al., 2014), low marriage rates (Abdool Karim et al., 1991, Anglewicz and Reniers, 2014), disempowerment of women (Langen, 2005), the inability to negotiate safe sex practices leading to low or no condom use (Pettifor et al., 2005), gender-based violence (Andersson et al., 2008) and the use of vaginal insertion products (Low et al., 2011) that further increases HIV risk especially in women.

1.2 Biological factors for HIV acquisition

In addition to social and behavioural factors, there are several biological factors that increase the vulnerability of HIV acquisition among women. These include: the large surface area of the FGT (Hladik and McElrath, 2008, Yeaman et al., 2004), pre-existing genital inflammation that can impact barrier function (Arnold et al., 2016, Masson et al., 2015), semen exposure (Sharkey et al., 2012), hormonal contraceptives, particularly depot medroxy progesterone acetate (DMPA) (Morrison et al., 2015), the phase of menstrual cycle (Kyongo et al., 2012), vaginal insertive practices (Low et al., 2011, McClelland et al., 2006, van de Wijgert et al., 2009) for e.g. the use of lubricants (Fichorova et al., 2001) vaginal microbial dysbiosis (Klatt et al., 2017), and STIs (Chen et al., 2007).

Because the FGT has a large surface area, there are more opportunities for HIV entry into HIV target cells [as reviewed in (Hladik and McElrath, 2008)]. Furthermore, micro abrasions to the vaginal epithelium as a result of friction during sexual intercourse increase the vulnerability of women to HIV infection [as reviewed in (Hladik and McElrath, 2008)]. Inflammation of the FGT, irrespective of the cause, also creates a favourable environment for HIV replication and opportunities for infection [as reviewed by (Passmore et al., 2016)]. Inflammation in the genital tract is defined as the upregulation of ≥ 5 of 9 inflammatory or chemotactic cytokines (IL-1α, IL-1β, IL-6, TNF-α, IL-8, IP-10, MCP-1, MIP-1α, and MIP-1β) (Masson et al., 2015). Elevated levels of inflammatory cytokine concentrations were reported to be associated with increased HIV infection as they signal to recruit and activate the HIV target cells and also lead to epithelial barrier disruption [as reviewed by (Passmore et al., 2016)]. Together, inflammation and immune activation can facilitate the translocation and transmission of viruses and bacteria (Nazli et al., 2010) and this mechanism of viral transmission can be further impacted by the use of hormonal contraceptives such as DMPA or through hormonal fluctuation occurring throughout the menstrual cycle.

DMPA is the most commonly used contraception in the sexual reproductive health services in South Africa [as reviewed by (Darroch and Singh, 2013)]. Thinning of vaginal epithelium have been shown in animal studies with DMPA use (Abel et al., 2004, Trunova et al., 2006). Data from studies of women

using injectable or oral contraceptives show a higher frequency of vaginal or cervical CCR5+ CD4+ T cells in comparison to women who did not use these forms of contraception respectively (Chandra et al., 2013, Prakash et al., 2002). Together, these data highlight the biological impact of exogenous hormones that compromises epithelial barrier integrity and target cell availability in the vaginal mucosa leading to increased risk for HIV infection.

However, human studies have produced conflicting data, with some reporting thinning of the vaginal epithelium (Heffron et al., 2012) and other studies reporting no difference between DMPA and non-DMPA users (Chandra et al., 2013, Miller et al., 2000). The study by Morrison et al., (2015) that was done in sub-Saharan African women reported a high risk of HIV acquisition amongst DMPA-users compared to combined contraceptives (COC), and norethisterone enanthate (NET-EN) users (Morrison et al., 2015). Recently, Molatlhegi et al., (2020) reported elevated DMPA levels to be associated with low genital tract cytokines [granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (MCSF), IL-16, cutaneous T-cell-attracting chemokine (CTACK), leukemia inhibitory factor (LIF), IL-1α, and stem cell growth factor-beta (SCGF-β)] (Molatlhegi et al., 2020). In contrast, Deese et al., (2015) reported elevated levels of MIP-1α, MIP-1β, IL-6, IL-8, IP-10, and RANTES concentrations among the DMPA users (Deese et al., 2015). Furthermore, Francis et al., (2016) reported high genital tract pro-inflammatory cytokines [IL-1α, IL-1β, IL-2, MIP-1β, IP-10, IL-8, and transforming growth factor-beta (TGF-β)], beta-defensins, IgA, IgG1, and IgG2 among DMPA users (Francis et al., 2016). Conversely, the Evidence for Contraceptive Options and HIV Outcomes (ECHO) Trial Consortium reported no difference in HIV risk between women on DMPA and women who were given either copper intrauterine devices (IUD) or levonorgestrel (LNG) implants (Ahmed et al., 2019).

Besides hormonal contraceptives, the use of vaginal insertive products may also influence HIV risk. A study by McClelland et al., (2006) reported high risk of HIV acquisition among women who cleansed their vagina using water or soap when compared to women who did not follow this practice (McClelland et al., 2006). However, some studies found no association between intravaginal practices and HIV risk (Myer et al., 2006, van de Wijgert et al., 2008) suggesting that there may be heterogeneity of risk based on the types of products used, frequency of use, and frequency of exposure to HIV through sexual intercourse etc.

Microbial dysbiosis in the cervico-vaginal compartment also increases risk for HIV acquisition (Klatt et al., 2017). For example, bacterial vaginosis (BV) is a condition caused by an overgrowth of anaerobic bacteria such as *Gardnerella vaginalis*, *Mobiluncu*s, *Prevotella* species, *Mycoplasma hominis* and the recently identified *Atopobium vaginae* (Verhelst et al., 2004, Verstraelen et al., 2004) which outcompete the normal vaginal flora (*Lactobacilli*) leading to microbial dysbiosis. BV was also reported to increase the susceptibility to HIV infection by approximately 60% [as reviewed by (Atashili et al., 2008)]. In

the presence of BV, there is depletion of the *Lactobacilli* species (McLean and Rosenstein, 2000). *Lactobacilli* are essential in maintaining a lower pH as they produce lactic acid and hydrogen peroxide, and in addition to maintaining low pH they also have antimicrobial action [as reviewed by (Amabebe and Anumba, 2018)]. Hydrogen peroxide also creates an unfavourable environment for survival of anaerobes and harsh environment for HIV [as reviewed by (Klebanoff and Coombs, 1991)], however if BV is present, then less hydrogen peroxide is produced. The ability of HIV to survive are favoured within a high pH environment, which may be likely present if there is BV (Taha et al., 1998). Vaginal dysbiosis is associated with increased vaginal pH and activation of CD4+ T-cells expressing increased levels of CCR5, which increases susceptibility to HIV [as reviewed by (Velloza and Heffron, 2017)]. Therefore, in the absence of a *Lactobacillus* dominant environment, the protection of the cervico-vaginal compartment is compromised, and remains vulnerable to opportunistic microbial infections like HIV (Hillier, 1998, Schmid et al., 2000).

Other STIs also increase risk for HIV infection [as reviewed by (Kalichman et al., 2011)]. The mechanism of HIV transmission by STIs is through the disruption of mucosal barriers and recruitment of CD4+ T-helper cells and macrophages to the site of infection, providing more HIV target cells [as reviewed by (Ward and Ronn, 2010)]. Several bacteria such as *Neisseria gonorrhoea*, *Chlamydia trachomatis*, and *Mycobacterium genitalium,* which cause various STIs, also increase susceptibility to HIV acquisition (Mlisana et al., 2012). Additionally, having one STI showed a three-fold increased HIV risk while the presence of two or more STIs increased the HIV risk to more than six-fold (Mlisana et al., 2012). Furthermore, asymptomatic STIs that are difficult to diagnose, also predispose women to HIV [as reviewed by (Kalichman et al., 2011)]. Besides bacterial infections, other viruses like human papillomavirus (HPV) (Liebenberg et al., 2019) and herpes simplex virus (HSV) (Looker et al., 2017) also increase HIV susceptibility during sexual intercourse (Auvert et al., 2010, van de Wijgert et al., 2009).

1.3 HIV genomic structure

HIV, an enveloped virus composed of RNA instead of DNA, is classified as a retrovirus, and belongs to the genus Lentivirus [as reviewed by (Rajarapu, 2014)]. The HIV genome consists of two indistinguishable single-stranded RNA molecules that are enclosed within the core of the virus particle [as reviewed by (Rajarapu, 2014)]. The HIV-1 RNA genome is composed of nine open reading frames which produce 15 proteins [as reviewed by (Frankel and Young, 1998)]. Three of these encode the Gag, Pol and Env polyproteins. Two Env proteins (surface gp120 and transmembrane gp41) are structural proteins forming the outer membrane envelope [as reviewed by (Frankel and Young, 1998)]. The four Gag proteins (which include the matrix, capsid, nucleocapsid and p6) forms core of the virion (Nagata et al., 2017). Three Pol proteins (protease, reverse transcriptase, and integrase) provide enzymatic functions [as reviewed by (Frankel and Young, 1998)]. HIV-1 encodes six accessory proteins:- Vif, Vpr

and Nef (found in the viral particle), Tat and Rev (provides essential gene regulatory functions) and Vpu (assists in assembly of the virion) (Nagata et al., 2017) (Table 1).

1.4 HIV structure

The virus is spherical in shape and has the outer layer known as envelope (Env). The viral Env is composed of a glycoprotein (gp)160, which is formed by three monomer gp120 subunits and three-gp41 units [as reviewed by (Garg and Blumenthal, 2008)]. Each gp120 spike is anchored by a gp41 stalk and together constitutes a heterodimer and three of these heterodimers come together to form the spike protein (Figure 2). Gp120 is used by the virus for attachment to the target CD4 cell receptor and a co-receptor (either CCR5 or CXCR4) [as reviewed by (Garg and Blumenthal, 2008)]. The binding of gp120 and CD4 results in conformational changes in gp120 thereby exposing the gp120 co-receptor binding sites and gp41 N and C terminal heptad repeat region [as reviewed by (Garg and Blumenthal, 2008)]. The interaction of gp120 with the co-receptor triggers the gp41 membrane domain to mediate virus entry [as reviewed by (Garg and Blumenthal, 2008)]. The gp41 N terminal is comprised of hydrophobic fusion peptide or protein (Freed et al., 1992). The hydrophobic fusion protein mediates the fusion of the viral and the target cell membrane (Gallo et al., 2003). The Gag proteins of HIV-1 play a vital role in virus particle assembly, release, and maturation, and also responsible for initiating productive infection [as reviewed by (Waheed and Freed, 2012)]. The core protein of HIV is p24 antigen (Sabin et al., 2001). Surrounding the core is the p17 matrix protein which is side by side to the lipid envelope containing the gp120 and gp41 Env glycoproteins (Ohagen and Gabuzda, 2000).

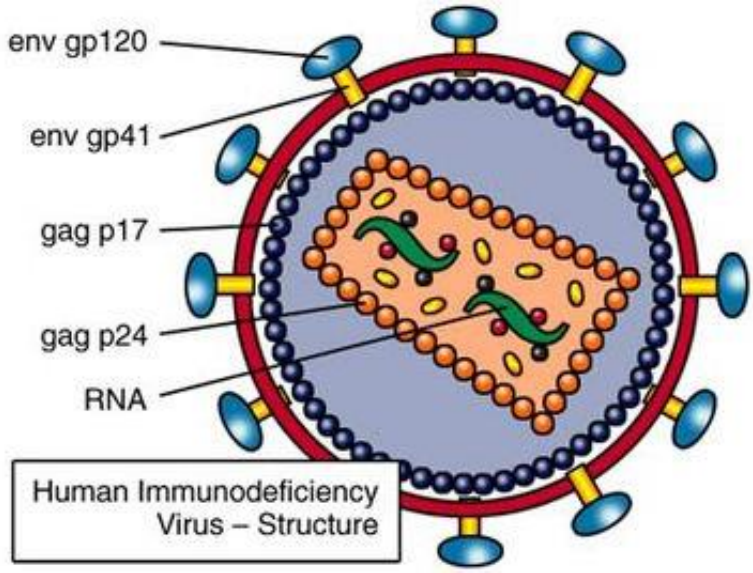

Figure 2: The structure of HIV. The viral envelope contains proteins from the host cell and relatively few copies of the HIV Envelope protein, which consists of a cap made of three molecules known as glycoprotein (gp) 120, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope [Adapted from (Perrone, 2015)].

Table 1: Summary of the HIV proteins and their roles

Gene	Protein	Role
Gag	p24	A capsid protein, which forms conical capsid. It is detectable prior to seroconversion (Sabin et al., 2001)
	p17	A matrix protein that forms the inner membrane layer, It plays vital role in the life cycle of HIV (Caccuri et al., 2016)
	p7	Nucleoprotein responsible for the formation of RNA complex (Ohagen and Gabuzda, 2000)
	p6	Binding site for several cellular and viral binding partners and is important for the formation of infectious viruses (Solbak et al., 2013)
Pol	P10	A protease responsible for the cleavage of Gag (Pr55) and Gag-pol (Pr160 Gagpol) precursor proteins thereby releasing the structural proteins and the viral enzymes (Speck et al., 2000)
	Reverse transcriptase	Reverse transcribe its RNA into the host's DNA [as reviewed by (Nisole and Saib, 2004)]
	Integrase	facilitates the integration of viral DNA into the host cell's DNA [as reviewed by (Nisole and Saib, 2004)]
Env	gp120	The binding site of HIV, that facilitates the binding and entry of HIV into the host cell (Raja et al., 2003)
	gp41	A transmembrane protein which mediates the fusion of the viral and the cell membrane (Gallo et al., 2003)
Accessory proteins	Vif, Vpr, and Nef	Modulate the infection and replication processes including Vif, Vpr, and Nef. [as reviewed by (Cullen, 1998)
	Tat, Rev	Provide essential regulatory functions and important for HIV replication (Nagata et al., 2017)
	Vpu	Assist in assemble of virion (Nagata et al., 2017)

1.5 Entry of HIV into the cells

The entry of HIV into host cell is through the binding of the viral envelope (Env) gp120 with the CD4 receptor on the target T cell [as reviewed by (Chan and Kim, 1998, Pierson and Doms, 2003)]. The CD4 binding results in a gp120 conformational change [as reviewed by (Sattentau and Moore, 1995)], which facilitates the exposure of the V3 crown motif on gp120 which can then more efficiently bind to the CCR5 co-receptor on the T cell (Feng et al., 1996, Moore, 1997, Trkola et al., 1996). In addition, the binding of the co-receptor (either CCR5 or CXCR4) with the gp120 results in structural changes in the Env glycoprotein, which then leads to the fusion of the host cell membrane and the virus envelope (Chan and Kim, 1998). The CCR5 co-receptor is used by HIV strains that have tropism to both T cells and macrophages (Quitadamo et al., 2018). Whereas, CXCR4 co-receptor –is used by lymphocyte-tropic HIV variants (Feng et al., 1996). The viruses that use the CCR5 (R5-tropic viruses) are mostly present during the asymptomatic stage of infection, while the viruses that use CXCR4 may be present during the later stages of infection (Berger et al., 1999, Miedema et al., 1994). Once the virus gets inside the CD4 T-cell, the HIV viral capsid is released (RNA and HIV enzymes – reverse transcriptase and integrase) [as reviewed by (Fanales-Belasio et al., 2010)]. It then uses the

reverse transcriptase to reverse transcribe its RNA into the host's DNA [as reviewed by (Nisole and Saib, 2004)] because HIV cannot enter the host cell's nucleus in the RNA form. Once the HIV is inside the host CD4 cell nucleus, HIV releases integrase enzymes which then facilitates the integration of viral DNA into the host cell's DNA [as reviewed by (Nisole and Saib, 2004)] (Figure 3). The RNA reverse transcription and the integration of DNA in the human genome generates the proviral DNA [as reviewed by (Craigie and Bushman, 2012)]. After the integration is successful, the virus then uses the CD4 T-cell's cellular machinery to replicate, making more copies of HIV proteins [as reviewed by (Kirchhoff, 2014, Nisole and Saib, 2004)]. The new HIV RNA and proteins that are generated by the CD4 T-cells, assemble into non-infectious virions [as reviewed by (Gelderblom, 1991)]. The immature HIV virions then bud off the host CD4 T-cell. Once outside the CD4 T-cell, HIV enzyme called protease is released to break down the proteins in non-infectious virions into smaller peptides [as reviewed by (Nisole and Saib, 2004)]. The proteases cleave the non-infectious virions thereby creating the infectious virus [as reviewed by (Nisole and Saib, 2004)]. In a nutshell, the HIV life cycle has seven stages; (i) Binding (the viral envelope firstly binds with CD4 cell receptor, then a co-receptor (either CCR5 or CXCR4), (ii) Fusion (the viral envelope fuses with the CD4 cell membrane), (iii) Reverse transcription (the reverse transcriptase enzyme reverse transcribes the HIV RNA into the HIV DNA), (iv) Integration (the integrase enzyme facilitates the integration of the HIV DNA into the host cell's DNA), (v) Replication (HIV RNA uses the CD4 cell's machinery to make viral proteins which are the building blocks for more copies of HIV), (vi) Assembly (the new HIV RNA and the HIV proteins are assembled at the cell's surface to form the immature HIV), (vii) Budding (the immature HIV bud-off the cell, and the enzyme protease cleaves the long chains of protein in this immature HIV creating infectious virus) as depicted in Figure 3 below.

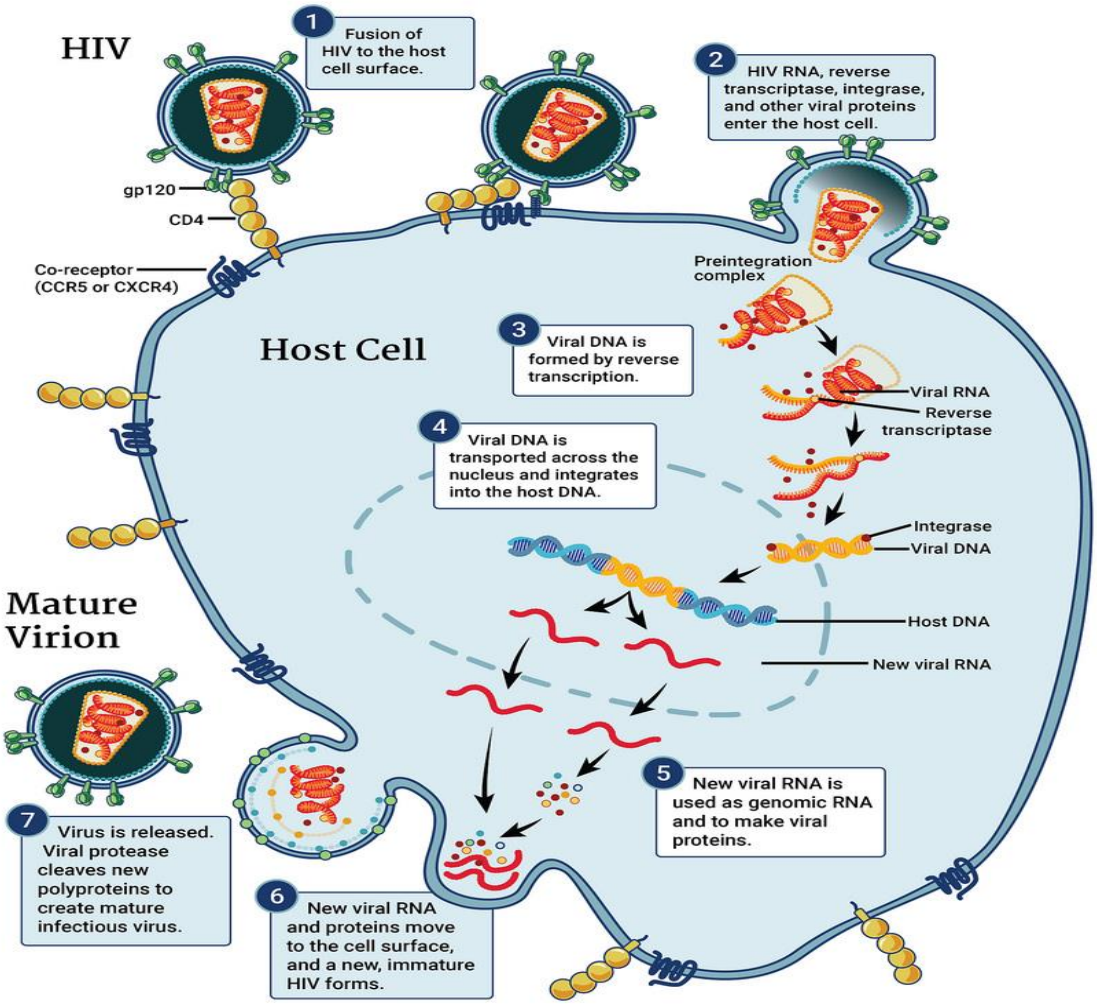

Figure 3: The diagram of HIV life cycle. There are seven stages of HIV life cycle summarized in the figure above. 1) Binding, 2) Fusion, 3) Reverse transcription, 4) Integration, 5) Replication, 6) Assembly 7) Budding. Reverse transcription and integration are facilitated by enzymes reverse transcriptase and integrase, respectively [Adapted from (National Institute of Allergy and Infectious Diseases, 2018)].

1.6 Immune response to HIV infection

The immune system is divided into the innate and adaptive immune response. The innate immune system provides the first line of defence against invading pathogen [as reviewed by (Espinosa and Rivera, 2016)]. The mucous layer forms the first form of innate immune defence (Figure 4). Cells associated with innate immune system include: natural killer cells (NK) (Vivier et al., 2011), mast cells [as reviewed by (St John and Abraham, 2013)], macrophages, dendritic, Langerhans cells [as reviewed by (Hirayama et al., 2017)], and neutrophils [as reviewed by (Selders et al., 2017)]. These cells assist in responding against invading HIV or any pathogen. The adaptive immune response, a second line of defence, and is classified into humoral and cell-mediated immunity arms. Humoral immunity is the antibody-mediated immune response, which arises from B cells and protects against extracellular pathogens (Casadevall, 2003). Cell-mediated immunity, is mediated by T cells and unlike humoral immunity, protects against intracellular pathogens (Casadevall, 2003).

1.6.1 The strategies of HIV to evade innate and adaptive immune responses

In order to evade the immune response, HIV modifies its pathogen-associated molecular pattern molecules (PAMPS) thereby preventing the binding of pattern recognition receptors (PRRs) in order to prevent the activation of innate immune response (Goto et al., 1994). The ability of HIV to randomly change its structure is the main strategy used by the virus to evade the immune response (Serra-Moreno et al., 2011). When the HIV replicates, the DNA-dependent RNA polymerase II (RNA pol II) which lacks the proofreading activity, produces synonymous and non-synonymous mutations which then results in mutations creating varied viral sequences particularly in the viral envelope [as reviewed by (Guha and Ayyavoo, 2013)].

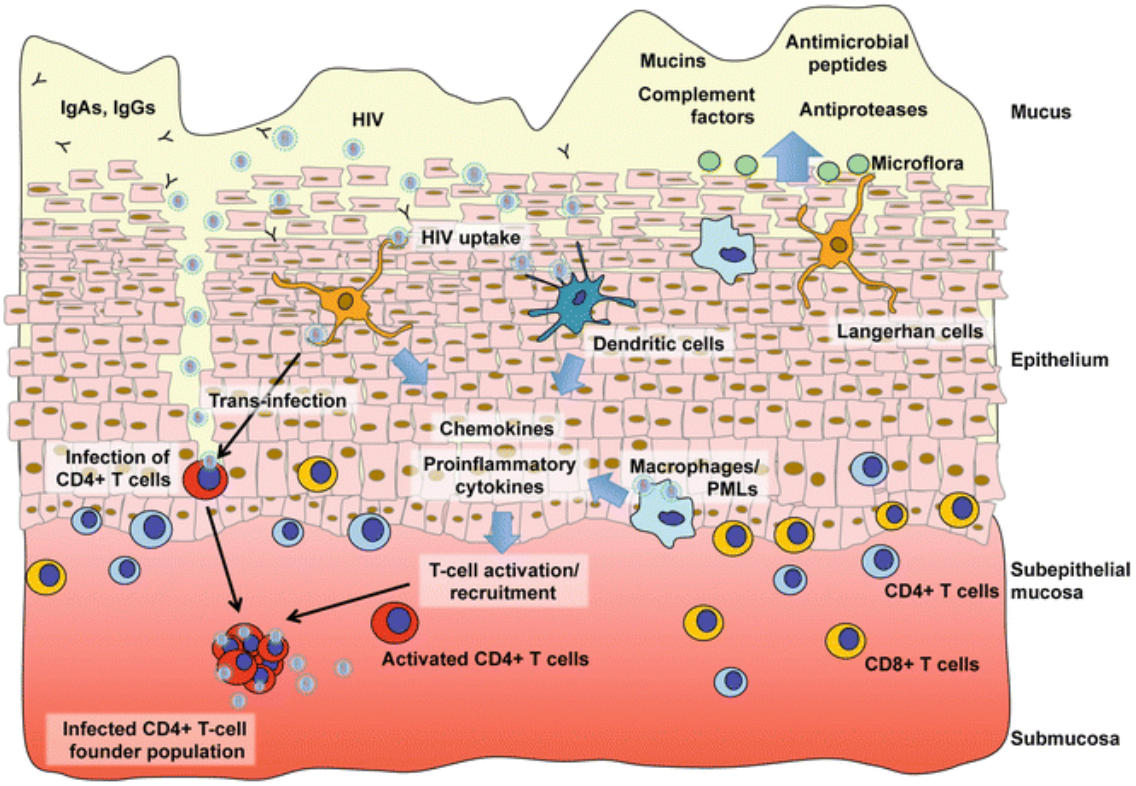

Figure 4: The innate and adaptive immunity of the vagina in the female genital tract. Innate immunity comprising of the mucous layer containing anti-microbial peptides and proteases, together with complement and microflora that create ideal conditions for killing of pathogens and prevention of subsequent infection. IgG and IgA are part of the adaptive immune response, with great interplay with the innate immune response to further enhance protection. Below the epithelium of the vagina, innate cells such as dendritic and Langerhans cells may be able to transfer invading pathogens such as HIV to target CD4 T cells to establish foci of infections in the submucosa binding sites [Adapted from (Broliden et al., 2014)].

These mutations and variation in the sequences do not allow for efficient binding into the major histocompatibility complex (MHC) molecules. In order for HIV to evade antibody response, glycosylation, a form of co-translational and post-translational modification also occurs. Glycosylation is known to alter and hide proteins that would otherwise be antigenic. The glycan N301 protects the surface of CD4 binding site and the V3 loop epitopes from neutralizing antibodies (Ferreira et al., 2018). Therefore glycosylation and shifting Env glycan shield acts as a barrier to the recognition of viral

antigens, making it difficult for the natural immune system to recognize and eliminate the virus (Arey, 2012, Wei et al., 2003, Wyatt and Sodroski, 1998). Another strategy that the virus uses to escape the antibody response is by evolving the low-density envelope spikes. This hinders the bivalent binding of immunoglobulin G (IgG) to the virus, thereby minimizing the antibody avidity and neutralizing activity [as reviewed by (Klein and Bjorkman, 2010)].

1.6.2 B cell development

B cell development begins in foetal liver and then proceeds to further develop in the bone marrow [as reviewed by (Kondo, 2010)]. When inside the bone marrow, the stroma cells secrete cytokines such as IL-7 and -X-C motif chemokine 12 (CXCL12) that signals the developing B cells to differentiate, allowing the differentiation of hematopoietic stem cells (HSCs) into common lymphoid progenitor (CLPs) [as reviewed by (Kondo, 2010)]. When the transcription factors like E2A and early B-cell factor (EBF) are expressed, the CLPs develops to pro-B cells. Beginning from pro-B cells, the B cells in bone marrow undergo the heavy and light chain immunoglobulin (Ig) gene re-arrangement, and the variable, diversity, and joining [V(D)J] recombination, produces the immature B cells that express surface IgM [as reviewed by (Pelanda and Torres, 2012)]. These immature B cells then migrate to the spleen to differentiate into transitional B cells type 1 (T1) and transitional B cells type 2 (T2) [as reviewed by (Pelanda and Torres, 2012)]. The T1 B cells are the cells that recently migrated from bone marrow to spleen that further develop into T2 B cells, which are solely found in the primary follicles of the spleen (Loder et al., 1999). The B cells then become the immature B cells expressing the IgD and IgM on their surface [as reviewed by (Pelanda and Torres, 2012)].

1.6.3 B cell activation

B cell activation occurs in either T-cell dependent and/or T-cell independent pathway.

T cell dependent B cell activation

T cell dependent B cells' response require activation of both B cells and T cells upon antigen recognition [as reviewed by (Chaplin, 2003, Doherty et al., 2018)]. B cells that serve as antigen presenting cells (APCs) capture the antigen via B cell receptor (BCR) which is then internalized, processed and presented as peptide on the cell-surface via MHC class II molecules to the T cell receptor (TCR) on the surface of T cells [as reviewed by (Chaplin, 2003, Doherty et al., 2018)]. The interaction between the T and B cell surfaces is further enhanced by binding of costimulatory molecules CD28 on T cells with CD80 and CD86 on B cells [as reviewed by (Chaplin, 2003, Doherty et al., 2018)]. In addition, the second signal due to interaction between CD40 ligand (CD40L) on T cell, and CD40 receptor on the B cell, promotes B cell proliferation and differentiation [as reviewed by (Chaplin, 2003, Doherty et al.,

2018)]. The T cells also secrete cytokines such as interferon-gamma (IFN-γ), IL-4, IL-10 and IL-21 essential for B cell activation and Ig isotyping switching [as reviewed by (Doherty et al., 2018)].

The T cell independent B cell activation

For T cell independent B cell activation, involved antigens are able to induce and activate B cells without being in contact with the T helper cell (Maddaly et al., 2010). These antigens are divided into T-independent antigen type 1 (TI-1) and T-independent antigen type 2 (TI-2). TI-1 antigens are able to non-specifically activate B cells and stimulate both immature and mature B cells. An example of such an antigen is bacterial polysaccharides (Maddaly et al., 2010). TI-2 antigens require cytokines secreted by T helper cells for their activation, but not direct contact with cytokines in order to activate the B cells. TI-2 antigens includes; bacterial polysaccharides and flagellin (Maddaly et al., 2010). The TI antigen dependent B cell activation is weak and only produces IgM (Maddaly et al., 2010).

1.6.4 Antibodies

Antibodies (also known as immunoglobulins) are proteins that are part of the adaptive arm of immune response and are produced in response to infection. There are five isotypes of immunoglobulin namely; IgM, IgD, IgG, IgA, and IgE, each with their own site-specific function [as reviewed in (Mashoof and Criscitiello, 2016)]. These immunoglobulins are Y-shaped and have the antigen binding fragment (Fab) region, which is the antigen-binding site [as reviewed by (van Erp et al., 2019)], and the fragment crystallisable (Fc) region that determines the isotype [as reviewed by (Forthal, 2014)] (Figure 5). Antibodies have a variety of functions such as neutralizing and non-neutralizing activities. The antibodies neutralize the pathogen by binding its Fab region to the cognate epitope/epitopes of an antigen [as reviewed by (Forthal, 2014)]. Antibodies also have non-neutralizing activities that do not depend on the Fab region but the Fc region and includes; ADCP, ADCC [as reviewed by (Forthal, 2014)], activation of complement system, and ADCVI (Excler et al., 2014).

1.6.5 Immunoglobulin isotypes and IgG subclasses (IgG1-IgG4)

IgM is the third most abundant immunoglobulin in human blood and expressed on the surface of naïve B cells [as reviewed by (Mashoof and Criscitiello, 2016)]. It has a pentameric structure with ten binding sites that allow for them to bind multivalent target antigens with high avidity [as reviewed by (Wang et al., 2016)].

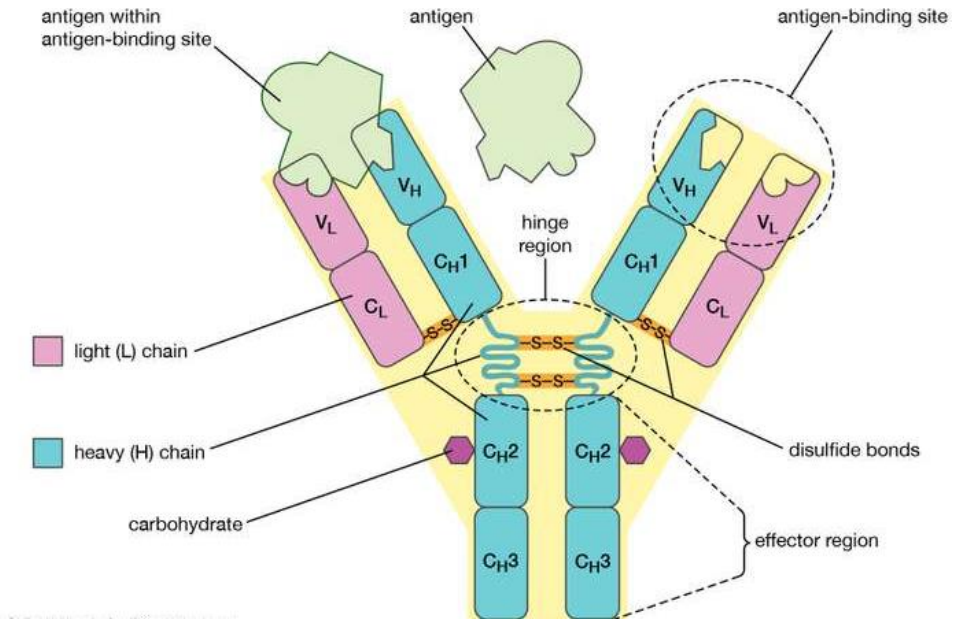

Figure 5: The structure of an antibody. Antibody has two antigen-binding sites and a constant region. The Fc region of the antibodies recognize different receptors, hence; each antibody has a unique Fc region. IgM and IgA have a different structural configuration from IgG, IgE, and IgD since IgM is a pentamer (with 10 binding sites), whereas IgA is a dimer (with 4 binding sites) [Adapted from (The Editors of Encyclopaedia Britannica, 2020)].

IgM is the first antibody to be produced as a response to infection and can bind to specific antigens without prior immunization (Jayasekera et al., 2007). IgM works by neutralizing pathogens through the activation of the complement pathway, recruiting phagocytic cells, and opsonisation (Jayasekera et al., 2007). Furthermore, it reduces inflammation by hindering T cell activation and pro-inflammatory cytokine production (Lobo et al., 2012). In relation to HIV, IgM was also reported to bind to CCR5 co-receptor therefore blocking binding of HIV-1 virion (Lobo et al., 2012, Lobo et al., 2008a, Lobo et al., 2008b). A study by Gong et al., (2018) generated HIV specific IgM from 33C6-IgG1 (gp120 specific IgG1) and administered the IgM intrarectally to the Rhesus macaques prior to intrarectal SHIV challenge (Gong et al., 2018). The generated 33C6-IgM was able to capture the virions and to neutralize the SHIV (Gong et al., 2018). The greater avidity to gp120 for 33C6-IgM compared to 33C6-IgG1 was observed, and four of the six Rhesus macaques were able to suppress the viremia (Gong et al., 2018). Monkeys given the gp120 specific IgM were also able to mount neutralizing antibodies in early breakthrough infected macaques at 6 weeks (Gong et al., 2018). In addition, the gp120 specific IgM form was able to clear 75% of the physical virus particles and 95% of infectious virus particles, indicating its important role in containing early infection (Gong et al., 2018). In conclusion, 33C6-IgM was found to prevent transmission of HIV and can be useful in future for vaccine development (Gong et al., 2018).

IgD is the least abundant immunoglobulin in blood [as reviewed by (Schroeder and Cavacini, 2010)]. IgD assist the short-lived anergic B cells to persist in the spleen for an extended period so that they can respond to the foreign antigens (Sabouri et al., 2016).

IgG is the most predominant isotype in the blood and extracellular fluid such as tears, cervico-vaginal mucus and saliva [as reviewed by (Mashoof and Criscitiello, 2016, Mestecky and Fultz, 1999)]. IgG has four subclasses (% in serum); IgG1 (60%), IgG2 (25%), IgG3 (10%) and IgG4 (5%) [as reviewed by (Jefferis, 2007). Immunoglobulin G (IgG) is the only antibody that is transferred from mother to infant *in utero* via neonatal Fc receptor (FcRn) (de Taeye et al., 2019). This receptor also maintains the high levels of serum IgG among adults (Vaughn and Bjorkman, 1998) as the FcRn has the ability to facilitate the bidirectional transfer of IgG between the luminal spaces and the subluminal spaces. IgG plays a role in complement activation and opsonisation of pathogens for phagocytosis [as reviewed (Mashoof and Criscitiello, 2016)].

IgG1 is the most predominant IgG subclass in serum (de Taeye et al., 2019). IgG1 Fc domains are able to bind all FcγRs (with different affinities) [as reviewed by (Bruhns and Jönsson, 2015)]. In addition, FcγRI is the high-affinity receptor for IgG1 whereas FcγRIIa and FcγRIIb are low-affinity receptors for IgG1 [as reviewed by (Bruhns and Jönsson, 2015)]. HIV-specific IgG1 binds to the antigen on the surface of the viral infected cell via the Fab region. Using the Fc portion of the antibody, this antibody-antigen-complex then binds to the Fc receptor (FcγRIII) of the effector cell for example on Natural killer cell (NK cell) (Pollara et al., 2015). This binding prompts the release of perforins and granzymes, which then disrupts the cell membrane and causes cell death respectively (Pollara et al., 2015). This mechanism is called ADCC (Smalls-Mantey et al., 2012). Studies have shown high ADCC activity among elite controllers compared to non or poor-controllers of HIV (Caskey et al., 2017, Lambotte et al., 2009). Another study found an association between higher plasma ADCC activity and a decrease in HIV mortality rate among infants (Pollara et al., 2015). Hypogammaglobinaemia, is a condition where there is a deficiency of IgG1 (Vidarsson et al., 2014), leading to increase the risk of infection. This lack of IgG subclass is associated with severe infection highlighting the importance of IgG1 in controlling infection and directing immunity [as reviewed by (Jefferis and Kumararatne, 1990)] towards non-neutralizing antibody functions such as ADCVI and ADCP.

IgG2 is the only IgG subclass which does not cross the placenta (de Taeye et al., 2019), it does not bind to high affinity receptors since it lacks a leucine at position 235 (de Taeye et al., 2019). IgG2 is divided into IgG2a and IgG2b. IgG2a binds to FcγRI, FcγRIII, and FcγRIV whereas IgG2b binds to FcγRIII and FcγRIV (Nimmerjahn et al., 2005). Ngo-Giang-Huong et al., (2001) previously reported reduced viral loads and elevated CD4+ T cells with gp41-specific IgG2 and p55- and p24-specific IgG2 (Ngo-Giang-Huong et al., 2001). IgG2 antibodies bind poorly to complement and are weak mediators of ADCC [as reviewed by (Tomaras and Haynes, 2009)].

IgG3 is the first subclass produced following infection (Sadanand et al., 2018). It has a long hinge region that strongly mediates ADCC, complement activation, and ADCP (Lu et al., 2007, Richardson et al., 2019). However, IgG3 is particularly susceptible to proteolysis because of the longer hinge region [as

reviewed by (Vidarsson et al., 2014)] and as a result has a shorter half-life (Stapleton et al., 2011). IgG3 plays a crucial role in rapidly clearing viruses [as reviewed by (Damelang et al., 2019)]. IgG3 like IgG1 Fc domains, are able to bind all FcγRs, resulting in phagocytosis of viral particles through FcγRI (the high-affinity FcγR) and FcγRIIa and FcγRIIb (low affinity FcγRs) that activate or inhibit phagocytic cell activity, respectively [as reviewed by (Bruhns and Jönsson, 2015)]. IgG3 has the ability to bind the FcγRIIc and FcγRIIIa on NK cells and activates the ADCC of the cells that are infected by the virus [as reviewed by (Bruhns and Jönsson, 2015)].

IgG4 is the least abundant subclass among healthy individuals (approximately 1-4% of total IgG) (Della-Torre et al., 2015), and results from repeated antigen exposure [as reviewed by (Lighaam and Rispens, 2016)]. HIV-1 specific IgG4 is dominant among chronically infected individuals [as reviewed by (Tomaras and Haynes, 2009)]. IgG4 does not activate complement and binds with low affinity to all the FcγRs compared to IgG1 and IgG3 [as reviewed by (Bruhns and Jönsson, 2015)].

IgA is the second most common immunoglobulin in serum [as reviewed by (Kulkarni and Ruprecht, 2017)]. IgA plays an important role in mucosal immunity and is the predominant isotype in the gastrointestinal, respiratory tracts [as reviewed by (Mashoof and Criscitiello, 2016)], breast milk, tears, and saliva (Cerutti et al., 2011, Cerutti and Rescigno, 2008, Fagarasan, 2008) but not in the cervico-vaginal secretions of the FGT. There are two types of IgA: IgA1, which is prominent in serum and lymphoid tissues, and IgA2, prominent in most mucosal secretions [as reviewed by (Kulkarni and Ruprecht, 2017)]. IgA is produced in both a monomeric and dimeric form. Dimeric IgA is most common in mucosal secretions [as reviewed by (Kulkarni and Ruprecht, 2017)]. Activated plasma cells produce polymeric IgA (pIgA), mainly the secretory dimer, which bind to the polymeric Ig receptor (pIgR) on epithelial cells (Kaetzel et al., 1991). This complex is taken in the cell through endocytosis and secreted on the luminal side of the cell. The pIgR is then cleaved through proteolysis and the dimeric IgA is released into the lumen as secretory IgA (sIgA) (Kaetzel et al., 1991). The function of pIgR is to transport the polymeric IgM and dimeric IgA from the lamina propia through the epithelial barrier to the mucosal surfaces [as reviewed by (Turula and Wobus, 2018)] and protect the immunoglobulins from proteolytic degradation (Stadtmueller et al., 2016). *In vitro,* systemic and mucosal IgA had properties of broadly neutralizing which were postulated to confer *in vivo* protection to HIV infection (Jia et al., 2020).

IgE is lower in blood and extracellular fluids than any of the other isotypes, however, it can easily disseminate out of blood and enter into other tissues [as reviewed (Mashoof and Criscitiello, 2016)]. IgE has a half-life of 2 days and cannot cross the placenta [as reviewed by (Burton and Oettgen, 2011)]. Blood IgE levels were found to be elevated in HIV infected patients compared to uninfected patients (Israël-Biet et al., 1992). In addition, IgE inversely correlated with the CD4 counts. These findings collectively suggest that IgE is a marker of HIV disease progression (Israël-Biet et al., 1992).

1.6.6 Diverse functions of antibodies

Antibodies are distinctive and can be categorized according to the function they perform. They can be either neutralizing or non-neutralizing. Neutralizing antibodies bind to the pathogen and block the pathogen entry into the cell and neutralize their infectivity [as reviewed by (Klasse, 2014)] (Figure 6A). Non-neutralizing antibodies clears the virus in diverse mechanisms including; opsonization, ADCC (Figure 6B), ADCP, ADCVI (Figure 6C), and complement activation [as reviewed by (Forthal, 2014)].

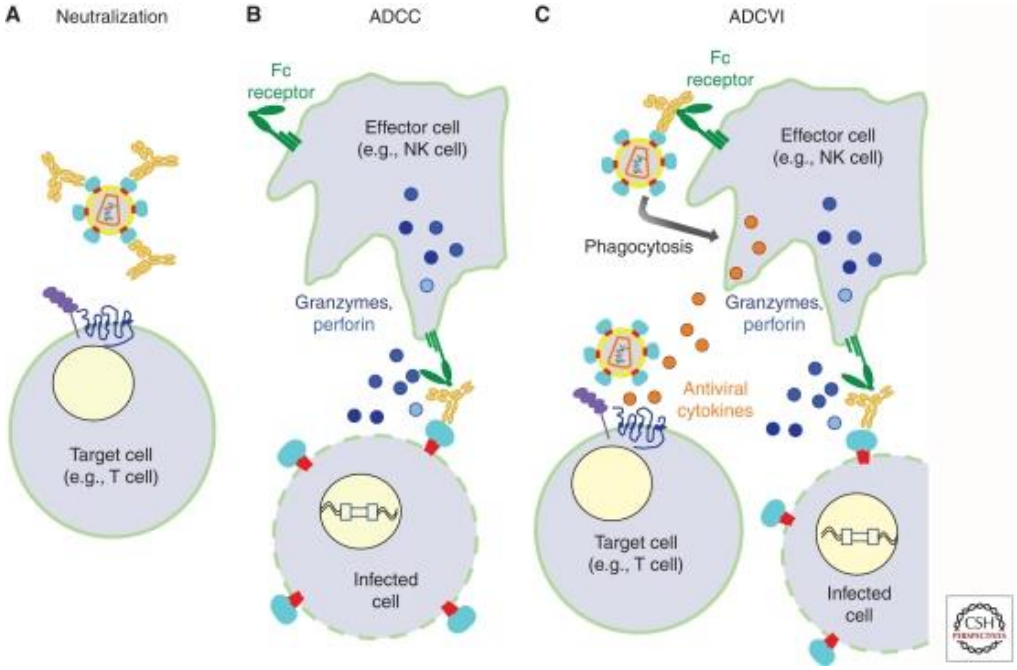

Figure 6: Illustration of the mechanism of virus elimination using antibodies to effect neutralization, ADCC, and ADCVI. (A) Antibodies neutralize the cell-free virus by binding to the HIV-1 glycoproteins thereby blocking the viral particles from interacting with CD4 and CCR5, which are the receptors on target cells that are required to initiate infection. (B) ADCC showing Fc receptor-bearing effector cells recognizing and killing antibody-coated target cells expressing peptides of pathogens on their surfaces. (C) ADCVI measures the effects of ADCC-mediated cell killing, which lead to reduced virus production, as well as virus inhibition by antiviral cytokines and other secondary effects of FcR-virus interactions such as phagocytosis [Adapted from (Overbaugh and Morris, 2012)].

Opsonization is a process that enhances phagocytosis; antibody coat the surface of the antigen by binding to specific antigenic determinants. These bound antibodies are then recognized by phagocytic cells. Phagocytic cells like macrophages have the specific Fc receptors on their surface. Once phagocytes bind the antibody via Fc receptors, these pathogens are eliminated by phagocytosis (Mevorach, 2000).

ADCC is the process of killing antibody coated target cells by certain leucocytes such as eosinophils and NK cells etc. having specific Fc receptors for these bound antibodies. These cells produce perforins and granzymes that penetrate and lyse the target cell (Sinclair et al., 1988) (Figure 6B).

The complement system is of importance in innate immune response against common pathogen. Complement activation results in proteolytic cascades, which cease in pathogen opsonization and lysis,

and also induce inflammatory responses by producing potent pro-inflammatory molecules (Dunkelberger and Song, 2010).

Non-neutralizing antibodies were also reported to inversely correlate with the risk of HIV infection. In the RV144 HIV-1 vaccine efficacy trial, the binding of vaccine-induced IgG to the V1V2 Env regions of multiple strains of HIV-1 correlated inversely with the risk of HIV infection (Haynes et al., 2012, Zolla-Pazner et al., 2014) suggesting that the elicitation of these antibodies through vaccination may be promising. However, more recently the HVTN702 vaccine trial (a slight variation of the vaccine used in the RV144 Thai vaccine trial) that was largely based on the RV144 Trial failed to show efficacy in preventing HIV in sexually active, young HIV-uninfected men and women across 14 sites in South Africa (UNAIDS, 2020c). The type/s of immune responses elicited by this vaccine have yet to be published (UNAIDS, 2020c).

Besides non-neutralizing antibodies, eliciting neutralizing antibodies through vaccination have become pivotal to the HIV vaccine development for prevention. With the high-throughput neutralization assays and technological advances to effectively screen memory B cells and isolate HIV antigen-specific B cells, there has been a large number of HIV neutralizing antibodies that have been discovered. These neutralizing antibodies can be classed as either entry inhibitors or fusion inhibitors. Entry inhibitors prevent the virus from entering the cell for e.g. a CD4 binding site antibody such as VRC01 and VRC07 (Rudicell et al., 2014, Walker et al., 2011). Fusion inhibitors sterically hinder and prevent the fusion of the viral membrane to the target cell membrane for e.g. 4E10, 2F5 and 10E8 (Huang et al., 2012, Muster et al., 1993, Zwick et al., 2001). These neutralizing antibodies can have two main properties: they can neutralize a wide variety of strains from different subtypes (this is breadth), and are required in very small amounts to effect neutralization (this is potency). These antibodies are referred to as potent broadly neutralizing antibodies (bNAbs). For example, the bNAb CAP256 was isolated from a female participant infected with subtype C HIV strain and was enrolled into the CAPRISA 002 acute infection study (Moore et al., 2011). The CAP256 bNAb targets the V1V2 region. This antibody was able to neutralize about 76% of heterologous viruses, predominately-neutralizing subtype C and A viruses over subtype B (Moore et al., 2011). CAP256 was also extremely potent and requires minute amounts to achieve neutralization of pseudovirus. Recently, the CAP256-VRC26.25 antibody that was isolated from the same donor was able to neutralize all of the subtype C viruses and at even lower concentrations, than its predecessor (CAP256) underscoring the effectiveness of this antibody (Doria-Rose et al., 2016) and moving into passive immunity studies. A few of these bNAbs are currently being tested in several Phase 1 passive immunity studies as proof of concept as HIV prevention strategies in at risk women. Other V2-specific bNAb such as PGDM1400 (Sok et al., 2014, van der Velden et al., 2018) was also reported to be efficacious in preventing SHIV infections following passive immunization prior to SHIV-challenge (Julg et al., 2017).

These and numerous other bNAbs have shown promise as proof of concept for passive immunity studies in humans to prevent HIV infection. Currently, CAPRISA has been spearheading phase I passive immunization studies in KwaZulu-Natal referred to as the CAPRISA012A (Mahomed et al., 2019) and CAPRISA012B trials testing VRC07, V3-specific-PGT121, and CAP256-VRC 26.25 as both subcutaneous and intravenous administrations. Although broadly neutralizing antibodies show great potential for the prevention of HIV, the practicality, cost and logistics to making such an intervention available through passive immunization at large scale may not be feasible. Other biomedical strategies using antiretroviral drugs show great promise for HIV prevention. Pre-exposure prophylaxis (PrEP) using antiretroviral drugs have been proven to be highly efficacious in people at high risk of HIV infection [as reviewed by (Eakle et al., 2018)].

1.7 Efficacy of PrEP

Pre-exposure prophylaxis (PrEP) in the form of antiretroviral drugs as topical or oral formulations for HIV prevention has been tested in human clinical trials. In 2010, the CAPRISA 004 1% tenofovir gel trial demonstrated a modest 39% efficacy (Abdool Karim et al., 2010). Since this trial, there has been an explosion of studies of oral PrEP and treatment as prevention (TasP) initiated during infection for the prevention of HIV in heterosexual serodiscordant couples (Baeten et al., 2012, Thigpen et al., 2012) and men who have sex with men (MSM) (Grant et al., 2010). Currently Truvada® (tenofovir disoproxil fumarate (TDF) with emtricitabine (FTC)- TDF-FTC®), has been approved as PrEP after demonstrating high efficacies in studies including MSM and heterosexual couples (Baeten et al., 2012, Thigpen et al., 2012). Clinical trials in African women however have produced inconsistent levels of protection ranging from -49% to 76% (Baeten et al., 2012, Marrazzo et al., 2015, Thigpen et al., 2012, Van Damme et al., 2012). Varying levels of adherence among women mainly accounted for the inconsistent efficacies seen in these studies. Pharmacokinetic modelling of oral TDF-FTC® indicated 85% adherence which translates to that at least six doses of drug/week and 28% adherence translating to 2 doses/week, is required to achieve protective drug exposure in the FGT and the lower gastrointestinal tract respectively (Cottrell et al., 2016). In the pre-exposure prophylaxis study for HIV prevention among African women referred to as the FEM-PrEP trial, only 24% of the women allocated to daily oral TDF/FTC had detectable drugs (Van Damme et al., 2012) while in the Vaginal and Oral Interventions to Control the Epidemic (VOICE) trial, detectable drug levels were found in 25%, 29%, and 30% of women in the daily tenofovir gel, daily oral TDF-FTC® and daily oral TDF groups (Marrazzo et al., 2015) respectively. Even in the monthly intra-vaginal dapivirine ring trial, significant rates of protection were observed in women older than 21 years (56%; 95% CI [31 to 71], $p<0.001$) in contrast to the women 21 years or younger (-27%; 95%CI [-133 to 31], $p=0.45$), with low adherence among young women undermining the protective effect of PrEP (Baeten et al., 2016, Nel et al., 2016).

1.7.1 Biological factors undermining PrEP efficacy

Besides low adherence, there are other factors that contribute to the lower protection among PrEP users. Vaginal microbial dysbiosis contributed mostly to the presence of anaerobes such as *Gardnerellla vaginalis* and non-*Lactobacillus* dominant species in women were shown to further reduce the protective efficacy of tenofovir gel in the CAP004 trial (Klatt et al., 2017). Klatt et al., (2017) reported a 61% reduction rate of HIV acquisition in *Lactobacillus*-dominant women on tenofovir but only an 18% reduction rate among non-lactobacillus-dominant women (Klatt et al., 2017). Furthermore, this *in vitro* study suggested that *G. vaginalis, P. bivia, P. amnii, and M. mulieris*, degrade the tenofovir gel, resulting in different efficacies observed, while *L. iners* or *L. crispatus* were not associated with tenofovir gel metabolism (Klatt et al., 2017).

In addition, genital inflammation was reported to also undermine the efficacy of tenofovir gel. Genital inflammation is defined by the presence of ≥5 of nine inflammatory cytokines (MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1α, IL-1β, IL-6, and TNF-α) (Masson et al., 2015). Tenofovir gel had a 57% protection against HIV infection among women who did not have genital inflammation while the protection was only 3% among women who had genital inflammation (McKinnon et al., 2018). Furthermore, from the participants who had high adherence to tenofovir gel (80% or more usage), the level protection was increased to 75% among women without genital inflammation in contrast to -10% among women who had genital inflammation (McKinnon et al., 2018), indicating no protective effect. The mechanisms by which inflammation modifies HIV risk is through the attraction of HIV target cells like CD4 T cell subsets through chemokines and cytokines, causing immune activation thereby proving ideal conditions to facilitate HIV infection [as reviewed by (Appay and Kelleher, 2016)].

Inflammation linked to immune activation in the FGT facilitates HIV infection, but also viral replication, which then promotes the establishment of infection at the mucosa and subsequent dissemination of virus (Miller et al., 2005). A study by Mlisana et al., (2012) reported elevated levels of genital cytokines such as IL-1β, IL-6, IL-8 and soluble CD40L (sCD40L) to be associated with increased risk of HIV acquisition among women (Mlisana et al., 2012). Masson et al., (2015) reported a three-fold increase of infection risk in women who had genital inflammation, defined as having 5 of 9 key pro-inflammatory cytokines elevated in the genital tract, compared to women who did not have genital inflammation (Masson et al., 2015). A study by Liebenberg et al., (2017) reported mucosa-biased gradients or elevated genital concentrations relative to blood of IP-10, MIP-1β, IL-8 and MCP-1 to be associated with high HIV risk among women (Liebenberg et al., 2017). In addition, many studies have confirmed that there is no correlation between the cytokines in the FGT to that in the peripheral circulation (Castle et al., 2002, Lajoie et al., 2012, Masson et al., 2015). These data indicate that genital inflammation is localized and therefore any assessment of inflammation has to be performed in samples taken from the FGT.

1.7.2 The effect of PrEP on Immunity

PrEP use can also modulate systemic immunity. Oral PrEP has been associated with decreased immune activation among HIV uninfected healthy individuals (Castillo-Mancilla et al., 2015). Among these participants, both the levels of soluble CD14 (sCD14) and soluble CD27 (sCD27), markers for T cell activation, and HLA-DR/CD38 co-expression for CD8 + T cells and CD4+ T cells in blood were reduced (Castillo-Mancilla et al., 2015). In a macaque model of breakthrough SHIV infections, animals on PrEP showed decreased immune activation, increased SHIV-specific central memory T cells, and no significant reductions in CD4+ T cell counts when compared to control macaques (Kersh et al., 2012). In contrast a study on HIV negative heterosexual men and women who were on PrEP (Truvada®) found no difference in the CD8+ T cell and CD4+ T cell responses, or NK cell activity when compared to individuals on placebo (Pattacini et al., 2015). These findings suggest that PrEP may have a heterogeneous response on immunity depending on their levels of exposure to HIV, and whether there is seroconversion that happens in the presence of PrEP or sub-optimal levels of PrEP. Another human study found no difference in immune activation and inflammation before and after ARV treatment (Gandhi et al., 2017).

A study by Naranbhai et al., (2015) assessed the effect of pre-existing immune activation for increased risk of HIV among women enrolled in CAPRISA 004 trial (Naranbhai et al., 2012). NK cell activation, high platelet counts, and high pro-inflammatory cytokine levels were observed in the blood of HIV infected women using the 1% tenofovir gel (Naranbhai et al., 2012). The elevated levels of systemic innate immune activation was associated with increased risk of HIV infection (Naranbhai et al., 2012).

1.7.3 Effect of PrEP on antibody responses

Besides the effects of PrEP on T cell activation, PrEP use has been shown to modulate HIV-specific antibody responses. A study by Laeyendecker et al., (2015) showed that women who seroconverted in the CAPRISA 004 tenofovir gel trial reported a delayed antibody avidity and maturation (Laeyendecker et al., 2015); however, time to seroconversion was not different to women who did not use the tenofovir gel. This data suggested that prior use of the topical tenofovir may have likely interfered with antibody binding avidity maturation among participants who became HIV infected (Laeyendecker et al., 2015), with possible implications for testing and confirming incident of HIV infections. Indeed, in macaques on oral PrEP (FTC or Truvada®) with breakthrough HIV infections, showed delayed antibody binding avidity for anti-p27, gp120, gp160, and gp41 with no impact on time to seroconversion (Curtis et al., 2011). Despite these findings, there were other studies from seroconverters in the CAPRISA 004 tenofovir gel trial that showed preservation of gag-specific CD4 T cell responses (Mureithi et al., 2012) and increased titres and HIV-specific antibody responses in women with prior tenofovir gel use compared to the placebo gel users (Archary et al., 2016). In women who used the tenofovir gel, antibodies specific to HIV envelope protein gp120, reverse transcriptase protein p66, and capsid protein

p24 were increased in the blood and genital tracts post seroconversion (Archary et al., 2016). Together, these data highlight that PrEP has an effect on immunity and can affect both innate and adaptive immunity before, during, and after HIV seroconversion. The humoral immune response can be skewed by the presence of certain cytokines and the impact of inflammation on antibody responses remains less well defined.

1.8 Cytokines and their effect on Ig isotype and IgG subclasses

The type of cytokine present in tissues or mucosal sites can influence the immunoglobulin types and subclasses thereby affecting the humoral response functioning (Kawano et al., 1994). IL-4 and IL-3 play a vital role in B cells class switching as well as inducing co-expression of IgG4 and IgE (Gascan et al., 1991). In mice models, TGF-β was reported to be associated with IgA class switching (van Ginkel et al., 1999); this underscores the contribution of cytokines in immunoglobulin class switching. In human models, IL-2, IL-5, and IL-10 were associated with B-cells activation and differentiation into Ig-producing cells [as reviewed by (Lacy and Stow, 2011)] implying the link between cytokines and B cell maturation.

A study by Kawano et al., (1994) investigated the contribution of IFN-γ and IL-6 in inducing IgG subclasses from peripheral blood mononuclear cells (PBMCs) stimulated with pokeweed mitogen (PWM). In the presence of IFN-γ, IgG1 production was suppressed while the secretion of IgG2 was enhanced (Kawano et al., 1994). Following addition of anti-IFN-γ antibody, IgG2 production was inhibited while the production of IgG1 was enhanced. This underscores the effect of IFN-γ in influencing IgG1 and IgG2 production (Kawano et al., 1994). In addition, IFN-γ was not able to influence the IgG2 production in the presence of PBMCs that were depleted of the surface IgG2-bearing cells indicating that, IFN-γ is not likely to impact switching (Kawano et al., 1994). IFN-γ and IL-6 do not affect class switching. IL-6 is able to differentially influence the production of all of the IgG (Kawano et al., 1994). Another study performed in a murine model showed that IL-4 mainly induced the production of IgG1 and this was less so with IL-13 and IL-10. However, if IL-10 was combined with IL-4 a higher percentage of cells underwent class switching; however, this was not the case with the IL-10 and IL-13 cytokine combination (Tangye et al., 2002). Besides cytokine impacting subclass switching, pro-inflammatory cytokines (IL-6, IL-8, and chemotactic MCP-1) were associated with high levels of total IgG and IgG1 whereas IgG2 and IgM had a positive correlation with MCP-1 and IL-6 concentrations respectively (Pillay et al., 2019). Together these data showed how cytokines alone or in combination could affect immunoglobulin subclass and isotype switching.

The information reviewed above underscores the need to assess the isotypes and cytokines present in women pre- and post-HIV infection to establish biological relationships between these biomarkers in

the genital tract. In so doing, we may be able to devise a way of assessing inflammation not only by using cytokines as a measure, but also immunoglobulins in the genital tract for HIV risk assessment.

This is the first study to assess the isotypes, IgG subclasses and cytokines in the blood and genital tracts of women using oral PrEP at the pre- and post-HIV infection stages. This study will give us an opportunity to identify the biomarkers in black South African women that may predict HIV acquisition; and study various changes in the immune responses over time at both the pre-HIV infection and the post-HIV infection stages relative to those of women who remained healthy throughout this study's period of observation.

1.9 Hypothesis:

Increased genital tract antibodies and cytokines pre-HIV infection predict HIV acquisition in seroconverters compared to non-seroconverters irrespective of PrEP use.

1.10 Aims:

1.10.1 To assess the isotypes and IgG subclasses in the blood and genital tracts of women pre- and post-HIV infection.

1.10.2 To assess cytokines in the genital tracts of women pre- and post-HIV infection.

1.11 Objectives:

1.11.1 To compare the isotypes and Ig subclasses in the blood and genital tracts of healthy and HIV infected women at baseline, 3-; 6-; 9- and 12-month intervals.

1.11.2 To compare cytokines in genital tracts of healthy and HIV infected women at baseline, 3-; 6-; 9- and 12-month intervals.

1.12 Research Question: Do pre-HIV infection profiles of isotypes, subclasses, and cytokines predict HIV acquisition in young women?

1.13 Significance of the study: This study will give us an opportunity to identify various biomarkers prior to HIV acquisition, which can be used as an immunological tool or diagnostic to assess for HIV risk especially in HIV endemic and high-risk populations.

CHAPTER TWO

MATERIALS & METHODS

2. MATERIALS AND METHODS

2.1 Study design and population

This study used the samples that were stored from the CAPRISA 082 study "Prospective Study of HIV Risk Factors and Prevention Choices in Young Women in KwaZulu-Natal, South Africa". This observational study was approved by the Biomedical Research Ethics Committee (BREC) for the University of KwaZulu-Natal (UKZN) BREC Reference BE458/15 (See Appendix II, page 120). In this sub-study BREC Reference BREC/00000091/2019 (See Appendix II, pages 121 and 122), 60 participants were included, comprising 12 seroconverters [cases (n=12 HIV positive)] and 48 non-seroconverters [controls (n=48 HIV negative women)]. In this sub-study, baseline; 3-; 6-; 9- and 12-month visits (5 time points) were included for the experimental analyses. The time points that were chosen were based on the sample availability for the majority of women in the sub-study. There were many missing samples for the women in the subsequent time points (>12-month visit). These five time points over 12 months of observation provided adequate sampling to assess longitudinally the changes in the antibodies and cytokines pre- and post-HIV infection.

2.2 Recruitment and Screening for eligibility into CAPRISA 082 study

The study staff recruited HIV uninfected women aged between 18 and 30 years from an urban clinic site (eThekwini), and a rural site (Vulindlela) in KwaZulu-Natal, South Africa. Potential study participants were screened for eligibility and those who qualified and satisfied the eligibility criteria were enrolled in the study within 30 days of screening. Participants had monthly visits for the first three months post-enrolment; thereafter visits were scheduled every three months.

Participants were asked to provide a consent form for screening. The participants were assigned a participant identification number. For eligibility into the study two rapid HIV tests were performed. Those who tested positive in one or both tests were referred to an HIV/AIDS treatment programme and were exited out of the study. If both tests were negative, the participants were allowed to proceed with screening process and were asked to provide details pertaining to demographic information, behavioural eligibility information, locator information, and a medical history. The participants also underwent a physical examination and a urine pregnancy testing. In addition, the participants were also evaluated for the presence of STIs based on symptoms and were offered syndromic management based on South African Department of Health guidelines during 2016.

Eligibility criteria

Eligibility criteria included females between 18-30 years old, being able and willing to provide informed consented form and locator information, sexually active for at least 3 times in the last three months, HIV uninfected, not pregnant and had to have agreed to adhere to study visits and procedures.

The exclusion criteria were known HIV positive status, any medical or mental health condition that in the opinion of the investigator would preclude comprehension of informed consent, or preclude study participation, and pregnancy.

2.3 Specimens collection and processing

2.3.1 Plasma collection and processing

The blood samples were collected from participants who agreed to be part of the CAPRISA 082 study by signing the consent form. Collection of blood by venipuncture was performed according to the CAPRISA SOP Nr CPBL006, version 005, and collected in vacutainer tubes. Plasma was separated from the blood by centrifugation and stored in cryovials at -80°C until required.

2.3.2 Softcup collection and processing

Mucosal specimens were collected from women using the Softcup collection device. When the women came in for their study visit, either a study nurse or a clinician inserted the Softcup into the vagina of the women for up to two hours or for the duration of the study visit. The Softcups were then removed and placed into a sterile 50ml conical tube and transported for further processing to the CAPRISA Mucosal Immunology Laboratory within four to six hours of collection. These Softcups were then centrifuged at 2,000 rpm for 10 minutes at room temperature (Archary et al., 2015). The Softcups were again centrifuged to separate the mucous pellet from fluid supernatant. An extra centrifugation was done if there was remaining mucous observed in the supernatant. The five-fold dilution of the Softcup supernatants was done with sterile phosphate-buffered saline (PBS). For example, a volume of 50 µL of the clear supernatant was diluted with 200 µL of PBS, prior to storage at -80°C. On thawing, PBS was added to dilute the stored Softcups supernatants to a further five-fold resulting in a 25-fold diluted Softcup supernatants that were used for the downstream isotyping and cytokine multiplex assays.

2.4. MILLIPLEX® MAP Human Isotyping Magnetic Bead Panel Multiplex Assay

Human Immunoglobulin Isotyping Magnetic Bead Panel kit from Merck (Millipore Corporation, Billerica, USA) was used to quantify antibody subclasses IgG1, IgG2, IgG3, and IgG4 and isotypes IgM and IgA on the Bio-Plex 200 multiplex system (Bio-Rad, Hercules, CA), according to manufactures instruction. The levels were determined by median fluorescent intensity (MFI) plate readout on the Bio-Plex 200 multiplex system (Bio-Rad, Hercules, CA). The serial dilution of plasma samples were optimized to be between 1:20,000 to 1:100,000. The serial dilution of Softcup samples were optimized to be 1:2,000; 1:4,000; 1:8,000; and 1:15,000.

2.4.1 Principle of the Isotyping Assay

In this assay, magnetic beads are coated with specific capture antibodies, which is added to the 96 well plate. Samples are then added to the respective wells of the plate. These samples contain the analytes that bind to the capture antibody bound to the beads. After the bead captures an analyte from a test sample, a biotinylated detection antibody is added, and this reaction mixture is then incubated with Streptavidin-Phycoerythrin conjugate, the reporter molecule, to complete the reaction on the surface of

the microsphere (Figure 1). The fluorescence captured by Luminex machine determines the immunoglobulin isotype and or/ subclass.

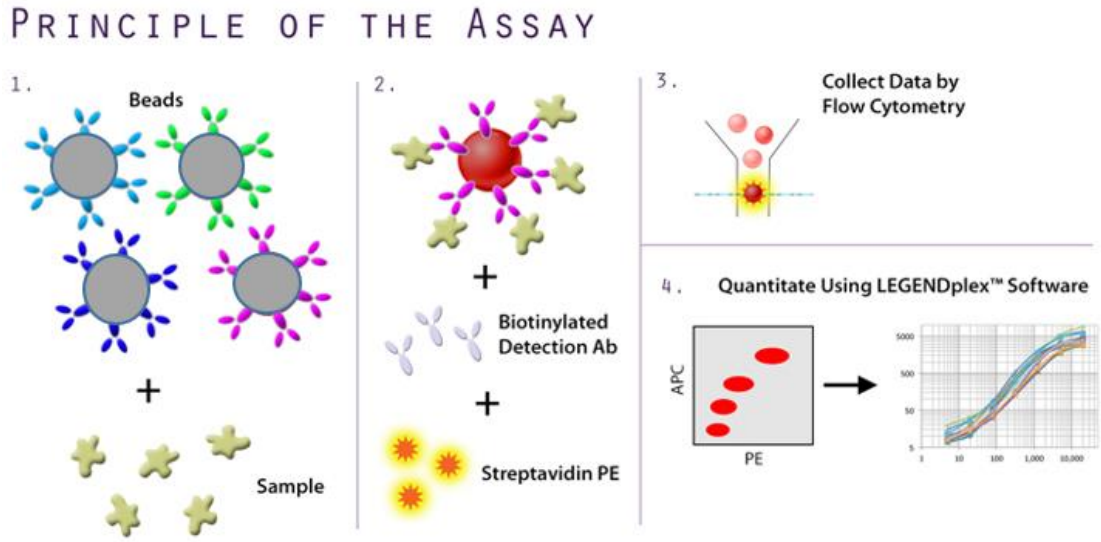

Figure 1: The principle of human immunoglobulin isotyping assay used for plasma and Softcup samples. Initially, the samples containing the analytes bind to the beads coated with a captured antibody. The beads then capture analytes from a test sample. Thereafter, the biotinylated detection antibody is introduced into the antibody-conjugated beads. The reaction mixture is then incubated with Streptavidin-Phycoerythrin conjugate and a signal is produced in proportion to the amount of immunoglobulin bound [Adapted from (Nordic BioSite, 2014)].

2.4.2 Assay preparation

The master plate was designed according to the protocol (Figure 2). The samples were thawed on ice overnight at 4°C.

1. The wash buffer and assay buffer were kept at room temperature until required.
2. The 10X wash buffer was mixed to bring all salts into solution. A volume of 60 ml of 10X wash buffer was diluted with 540 mL of deionized water, to make a 1X wash buffer. This 1X wash buffer was used to prime The Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA).
3. The positive control provided in the kit was reconstituted with 250 µL of deionized water. To mix the contents of the tube thoroughly, vortexing was done at high speed for 15 seconds, and then the vial was placed on ice for 15 minutes.
4. The samples were initially prepared in a 96 well-round-bottomed (U-shaped) plate by following the plated design in figure 2. The dilutions that were used for plasma isotyping were optimized to be between 1:20,000 to 1:100,000. For 1:20,000 dilution, a volume of 10 µL of each sample was diluted with 190 µL assay buffer (Figure 2). The serial dilution of Softcup samples were optimized to be 1:2,000; 1:4,000; 1:8,000; and 1:15,000. A final volume of 50 µL of diluted plasma and Softcup supernatant (as stated above) was added to the plates. Random samples were prepared in duplicate to account for intraplate variation.

Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	BLANK	200132 Base	200132 3m	200132 6m	200132 9m	120087 Base	120087 3m	120087 6m	120087 9m	120087 E11m	200135 Base
B	S1	S1	200135 3m	200135 6m	200135 9m	200135 12m	200173 Base	200173 6m	200173 9m	200173 12m	200173 15m	200174 Base
C	S2	S2	200174 3m	200174 12m	200174 E15m	200182 Base	200182 3m	200182 6m	200182 9m	120054 Base	120054 3m	120054 9m
D	S3	S3	120054 E17m	200184 Base	200184 3m	200184 6m	200184 9m	200195 Base	200195 3m	200195 9m	200195 12m	200199 Base
E	S4	S4	200199 3m	200199 6m	200199 9m	200199 12m	200217 Base	200217 3m	200217 6m	200217 9m	200119 3m	200119 6m
F	S5	S5	200119 E8m	200224 Base	200224 3m	200224 6m	200224 9m	200224 12m	200227 Base	200227 9m	200227 12m	200248 Base
G	S6	S6	200248 6m	200248 9m	200248 E12m	200257 Base	200257 3m	200257 6m	200257 9m	200257 E12m	200227 12m	200248 Base
H	S7	S7	200248 6m	200248 9m	200248 E12m	200257 Base	200257 3m	200257 6m	200257 9m	200257 E12m	CONTROL	CONTROL

Figure 2: The master plate layout for human isotyping assay. This plate was designed to accommodate the blanks, standards, samples, and controls. The same design was used for both plasma and Softcup isotyping.

5. Prior to use, the human immunoglobulin isotyping standard (provided) was reconstituted with 500 µL deionized water. The vial containing the reconstituted standards was vortexed at high speed for 15 seconds, and then the vial was placed on ice for 15 minutes. This was used as standard 7. A volume of 200 µL of assay buffer was transferred into tubes 6 to 1. Thereafter a three-fold serial dilution was prepared by transferring 100 µL of reconstituted standard (standard 7) to tube 6, this tube was vortexed and the 100 µL form tube 6 was transferred to tube 5. The same procedure was repeated until tube 1 (Figure 3).

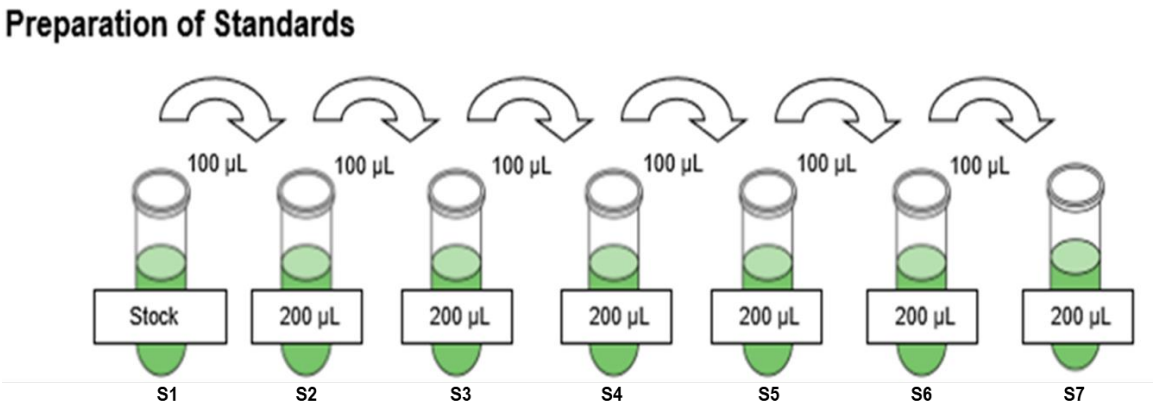

Figure 3: The standards preparation. This represents the serial dilutions of standards. Into the original stock, 500 µL of deionized water was added. The vial was then vortex and kept on ice for 15 minutes. To the 6 polypropylene tubes, 200 µL of assay buffer was added. From the original stock (S1), 100 µL was pipetted and transferred into tube 2 (S2), the solution was vortex and again 100 µL from tube 2 was transferred to tube 3 (S3) and the same procedure was done until tube 7 (S7) (Adapted from Milliplex® MAP instruction manual).

6. The master mix of beads was prepared by firstly sonicating each of the six antibody-bead vials for 30 seconds, followed by vortexing for 1 minute. A volume of 150 µL was added from each antibody-bead vial to the mixing bottle. The final volume of 900 µL was brought to 3.0 ml by

adding 2,100 µL of assay buffer to the mixing bottle, followed by thorough vortexing the mixing bottle**.**

2.4.3 Procedure

1. A volume of 50 µL of each Standard or Control was added into the appropriate wells. Assay buffer was used for 0 ng/ml standard (background). A volume of 50 µL of samples (diluted) was added into the appropriate wells.
2. The mixing bottle was vortex mixed at medium speed for 15 seconds and then sonication was done for 15 seconds using a sonication bath. A volume of 25µL of the mixed beads was vortexed and added to each well.
3. The plate was sealed with a plate sealer; it was then wrapped with foil (to prevent photobleaching) followed by incubation with agitation on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm for 1 hour at room temperature (20-25°C). The contents of the wells were gently removed and washed 2 times with 200 µL of wash buffer using the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). Before plate washing, the plate washer was primed with 30ml of the 1X wash buffer.
4. A volume of 25µL of anti-human κ and λ light chain detection antibody was added into the plate. Then the plate was sealed with a plate sealer and wrapped with the foil followed by incubation with agitation on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm for 30 minutes at room temperature (20- 25°C).
5. During the last 5 minutes of incubation, 75 µL of the Streptavidin-Phycoerythrin was diluted in polystyrene tubes (covered with foil) with 2.925 mL assay buffer, followed by vortexing to mix.
6. Once the 30 minutes incubation period was over, the diluted Streptavidin-Phycoerythrin was vortexed and a volume of 25 µL was added to each well containing the 25 µL of detection antibody; the plate was sealed and wrapped with foil, and then incubated with agitation on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm for 30 minutes at room temperature (20-25^{0}C).
7. The plate was then washed to remove the excess Streptavidin-Phycoerythrin and detection antibodies. To avoid aspiration related bead loss, the plate was allowed to soak for 60 seconds prior to aspiration. The wells were then resuspended with 150 µL of sheath fluid. The beads were agitated on a plate shaker for 5 minutes to ensure uniform mixing. The plates were read on the Bio-Plex 200 multiplex system (Bio-Rad, Hercules, CA) using Bioplex Manager 6.0 software (Bio-Rad, Hercules, CA).

2.5 MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel Cytokine/Chemokine Multiplex Assay

The concentrations of 28 cytokines were measured in Softcup specimens using Luminex multiplexing technology. The cytokines were measured in the 25-fold diluted Softcup samples as described above. The cytokine panel included the following: (IL-1β, IL-1Rα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-1α, IL-13, IL-15, IL-17α, eotaxin, G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF). Their concentrations were measured using the MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel from Merck (Millipore Corporation, Billerica, USA). The Bio-Plex Manager software version 6 was used to collect the data, and the sample concentrations were calculated from standard curves by using a 5PL regression formula. The sensitivities for the cytokines in this kit ranged between 0.7 and 47.9 pg/ml for each of the 28 cytokines measured.

2.5.1 Principle of the Cytokine Assay

The beads that are coated with the specific capture antibody are added to the 96 well plate. Samples containing the analytes bind to the captured antibody. After the bead captures an analyte from a test sample, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with the Streptavidin-Phycoerythrin conjugate, the reporter molecule to complete the reaction on the surface of the microsphere (Figure 4). The median fluorescence intensity captured by the Luminex® 200™ machine determines the cytokine concentrations in the samples.

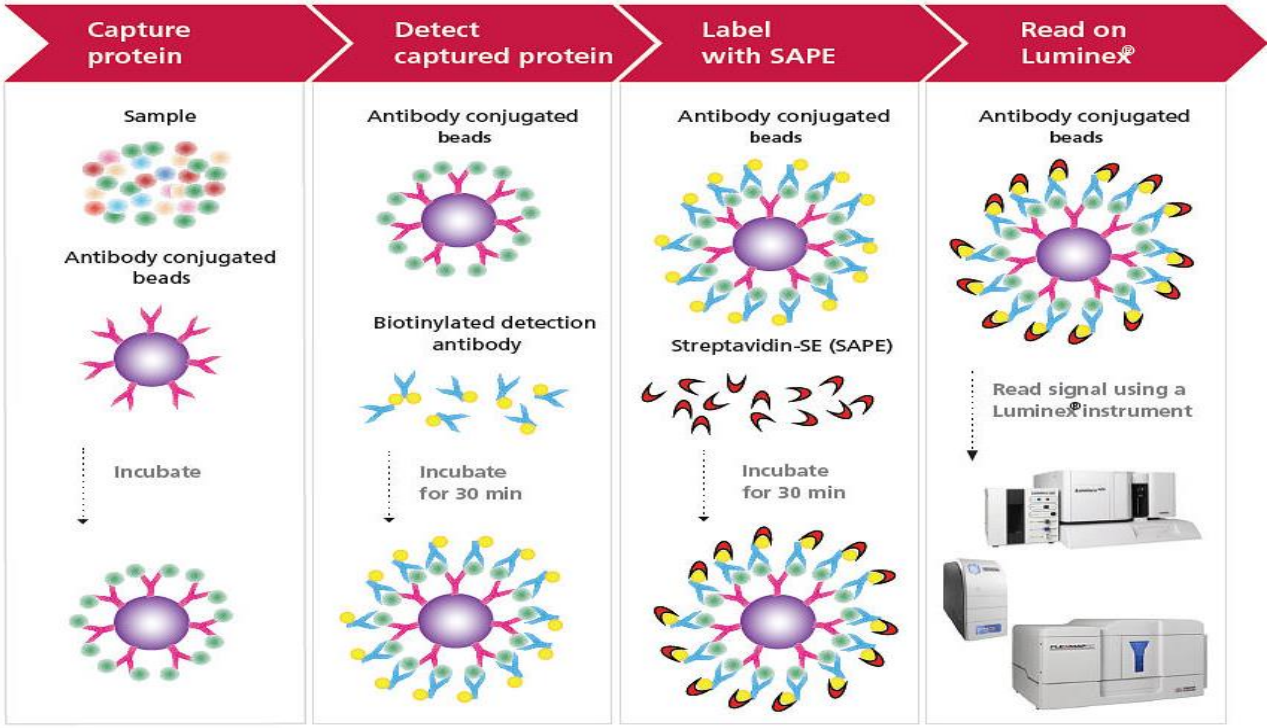

Figure 4: The principle of human cytokine/chemokine assay used for Softcup samples. Initially, the samples containing the analytes bind to the beads coated with the captured antibody. The beads then capture analytes from a test sample. Thereafter, the biotinylated detection antibody is introduced into the antibody-conjugated beads. The reaction mixture is then incubated with Streptavidin-Phycoerythrin conjugate and a signal is produced in proportion to the amount of cytokine bound [Adapted from (Shah, 2014)].

2.5.2 Assay preparation

The master plate was designed according to the protocol as shown in Figure 5. The samples were thawed on ice overnight at 4°C.

1. The wash buffer and assay buffer were kept at room temperature until required.
2. The 10X wash buffer was mixed thoroughly through constant agitation to bring all salts into solution. 60 mL of 10X wash buffer was diluted with 540 mL of deionized water, to make a 1X wash buffer. In addition, 30mL of 1X wash buffer was used to prime The Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA).
3. Two quality controls as provided in the kit were each reconstituted with 250 µL deionized water. The vials were inverted several times to allow for uniform, mixing, thereafter the vials were vortexed. The vials were placed at room temperature for 5-10 minutes.
4. The samples were initially prepared in the 96 well round bottom (U-shaped) plate by following the plated design in figure 5. The dilution that was used for plasma isotyping was 1:1 where a volume of 25 µL of each sample was diluted with 25 µL of assay buffer. Random samples were prepared in duplicate for intraplate variation (Figure5).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	BLANK	C2	C2	200246 Base	200246 Base	200246 3m	200246 3m	200246 6m	200246 6m	200246 9m	200246 E12m
B	S1	S1	120069 Base	120069 3m	120069 6m	120069 E9m	120073 Base	120073 Base	120073 3m	120073 6m	120073 E9m	120077 Base
C	S2	S2	120077 3m	120077 E9m	120115 Base	120115 3m	120033 Base	120033 3m	120033 6m	120033 9m	120033 E12m	120123 base
D	S3	S3	120123 6m	120123 9m	120123 12m	120152 Base	120152 3m	120152 6m	120152 9m	200016 Base	200016 3m	200016 6m
E	S4	S4	200016 9m	200028 Base	200028 3m	200028 6m	200028 9m	200028 12m	120053 Base	120053 3m	120053 E5	200032 Base
F	S5	S5	200032 3m	200032 6m	200032 9m	200032 12m	200036 Base	200036 3m	200036 6m	200037 Base	200037 3m	200037 6m
G	S6	S6	200037 9m	200037 12m	200041 Base	200041 3m	200041 6m	200041 9m	200041 12m	120074 Base	120074 3m	120074 6m
H	C1	C1	200043 Base	200043 3m	200043 6m	200043 9m	200043 E11	200056 Base	200056 6m	200056 E9m	120055 3m	120055 6m

Figure 5: The master plate layout of the Softcup cytokine assay. This 96-well plate was sketched to fit in the controls, standards, blanks, and samples. The samples were diluted 25 times using PBS.

5. Prior to use, the human cytokine standards provided in the kit were reconstituted with 250 µL deionized water to give a 10,000 pg/mL concentration. The vial was vortexed at high speed for 15 seconds, and then the vial was placed at room temperature for 15 minutes. This was used as standard one. A volume of 200 µL of assay buffer was added into each of the tubes 2 to 6. A five-fold serial dilution was prepared by transferring 50 µL of reconstituted standard (standard 1) to tube 2, the tube was then vortexed and then 50 µL from the second tube was transferred to tube 3. The same procedure was followed until tube 6 to achieve this serial five-fold dilution for all the standards (Figure 6).

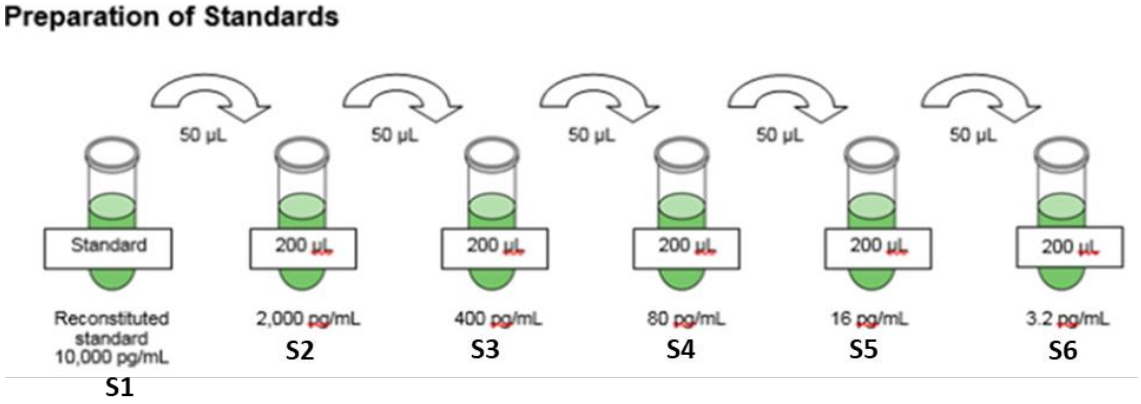

Figure 6: The serial dilutions of standard preparations. The same serial dilution procedure as a human isotyping assay was followed for the cytokine assay. Briefly, Tube 1 was vortexed and 50 µL of the reconstituted standard (tube 1) was transferred to a polystyrene tube 2 containing 200 µL of assay buffer to make a five-fold dilution. The same procedure was done until tube 6 (Adapted from Milliplex® MAP instruction manual). S1 (reconstituted standard 10,000 pg/ml), S2 (2,000 pg/ml), S3 (400 pg/ml), S4 (80 pg/ml), S5 (16 pg/ml), and S6 (3.2 pg/ml).

2.5.3 Procedure

1. A volume of 200 µL of wash buffer was added into each well of the magnetic plate followed by sealing the plate with a plate sealer and mixing on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm for 10 minutes at room temperature (20-25°C).
2. The wash buffer was decanted and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. A volume of 25 µL of each standard, and/or control were added into the appropriate wells. Assay buffer was used for 0 pg/mL standard (Background).
4. Precisely 25 µL of assay buffer was added to the sample wells. A volume of 25 µL of Softcup specimens was added into the appropriate wells.
5. The premixed bottle containing the beads was sonicated for 30 seconds and vortexed for 5 minutes to ensure uniform mixing. Thereafter, a volume of 25 µL of premixed beads was added to each well.
6. The plate was sealed with a plate sealer and foil to prevent photobleaching followed by incubation overnight at 4 ^{o}C while shaking.
7. The next day (morning), the well contents were gently removed and the plate was washed 2 times with 200 µL of wash buffer.
8. A volume of 25 µL of detection antibodies was added into each well.
9. The plate was sealed, covered with foil and incubated with agitation on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm for 1 hour at room temperature (20- 25°C).
10. During the last 5 minutes of incubation, 75 µL of Streptavidin-Phycoerythrin was diluted in polystyrene tubes (covered with foil) with 2.925 mL assay buffer, followed by vortexing to mix the contents uniformly.
11. Once the 30 minutes incubation period was over, 25 µL of diluted Streptavidin-Phycoerythrin was added to each well containing the 25 µL of detection antibodies. The plate was sealed, covered with foil, and incubated with agitation on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm for 30 minutes at room temperature (20- 25°C).
12. The well contents were gently removed and the plate was washed 2 times with 200 µL of 1X wash buffer using the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). The beads were resuspended in a volume of 150 µL of sheath fluid. The beads were mixed by agitating on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm for 5 minutes. The plate was read on the Bio-Plex 200 multiplex system (Bio-Rad, Hercules, CA) using Bioplex Manager 6.0 software (Bio-Rad, Hercules, CA). The MFI data were analysed using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples. (Note: to account for the concentrations in the diluted samples the calculated concentrations were multiplied by the dilution factor).

2.6 Statistical Analyses

Based on the distribution of the data and depending on whether the data were skewed, non-parametric Mann-Whitney and Unpaired t-tests were used to determine the differences in isotypes, IgG subclasses, and the cytokines between the cases and the controls at different time points. The Wilcoxon and the Paired t-tests were used to determine the isotypes, IgG subclasses, and the cytokines within the cases or controls. The One-way ANOVA followed by Dunn's multiple comparisons test was performed to compare different isotypes and IgG subclasses within plasma and the genital tract at baseline. A p-value of <0.05 was considered statistically significant. Pearson's correlation was used to compare the relationship of Ig isotypes and IgG subclasses between the plasma and the genital tract. The Quasi-likelihood information criterion (QIC) was conducted to determine whether the isotypes and IgG subclasses were associated with HIV outcome after adjusting for the number of study visits irrespective of PrEP use. Linear mixed model analyses were conducted to determine the impact of the genital tract cytokines on mucosal antibody responses irrespective of HIV infection status and PrEP use. GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, California, USA) was used for graphical presentation and analyses. R version 4.0.2 (R Foundation for Statistical Computing Software, Vienna, Austria) was used for statistical data analyses.

CHAPTER THREE

RESULTS

3. RESULTS

3.1 Baseline Demographics

Plasma and cervico-vaginal secretions collected through Softcup obtained from 12 HIV infected (cases) and 48 HIV uninfected (controls) women were used to characterize the immunoglobulins and cytokines in women pre- and post-HIV infection. Of the 12 cases, 9 (75%) were on PrEP, and three (25%) were not on PrEP. Of the 48 controls, 29 (60%) were on PrEP while 19 (40%) were not on PrEP. One of the 12 seroconverters (8%) reported to have an HIV positive male partner. There was no data reported as to whether the HIV positive male partner was on ARVs or not. Five of 12 seroconverters (42%) did not know the status of their male partners; the remaining six (50%) had male partners that were HIV negative. Among seroconverters, 6 of 12 (50%) were aged between 21-24 years with a median of 23.5 years (IQR 21.75-25.5), completed their matric, were on Depo-Provera, had three or more sexual partners, and had bacterial vaginosis (BV) (Table 1). Among controls (HIV uninfected), 24 of 48 (50%) were aged between 21-24 years with a median of 22 years (IQR 20-23.25), and all of them were on Depo-Provera (Table 1).

Table 1: The demographic characteristics of the participants

Demographic Characteristics	Category	Controls (HIV-) n=48 (%)	Cases (HIV+) n=12 (%)	p-value
Age group	18 to 20	13 (27.1)	2 (16.7)	0.70
	21 to 24	24 (50)	6 (50)	
	25 and above	11 (22.9)	4 (33.3)	
Education level	No matric	15 (31.3)	4 (33.3)	0.90
	Matric	27 (56.3)	6 (50)	
	Tertiary education	6 (12.5)	2 (16.7)	
Site	eThekwini	18 (37.5)	5 (41.7)	0.79
	Vulindlela	30 (62.5)	7 (58.3)	
Number of sexual partners	One partner	15 (31.3)	4 (33.3)	0.97
	Two partners	11 (22.9)	2 (16.7)	
	Three partners	11 (22.9)	3 (25)	
	Four or more partners	11 (22.9)	3 (25)	
Partner's age difference	Age difference not reported	2 (4.2)	0 (0)	0.19
	Partner younger or same age	7 (14.6)	4 (33.3)	
	Age difference <=4	30 (62.5)	4 (33.3)	
	Age difference > 4	9 (18.8)	4 (33.3)	
Relationship status	Stable partner	42 (87.5)	11 (91.7)	0.86
	Casual	5 (10.4)	1 (8.3)	
	Single	1 (2.1)	0 (0)	
Contraceptive use				
	Not reported	13 (2.7)	4 (33.3)	*
	Depo-Provera	24 (50)	6 (50)	
	Implant	6 (12.5)	2 (16.7)	
	Nur-isterate	4 (8.3)	-	
	Oral contraceptive	1 (2.1)	-	
Sexually Transmitted Infections				
Neisseria gonorrhoea infections	Unknown	1 (2.1)	1 (8.3)	0.15
	No	45 (93.8)	9 (75)	
	Yes	2 (4.2)	2 (16.7)	
Chlamydia Trachomatis infections	Unknown	1 (2.1)	1 (8.3)	0.37
	No	37 (77.1)	10 (83.3)	
	Yes	10 (20.8)	1 (8.3)	
Yeast infections	Unknown	1 (2.1)	1 (8.3)	0.50
	No	40 (83.3)	10 (83.3)	
	Yes	7 (14.6)	1 (8.3)	
Nugent scores	Negative (0-3)	-	-	0.10
	Intermediate (4-6)	18	1	
	BV (7-10)	12	6	
	Not reported	18	5	

The Chi-square test was used for statistical analyses. A p-value <0.05 was considered statistically significant.* denotes the two types of contraceptives that were not used by the women enrolled in this study, therefore, this was precluded and the Chi-square analyses for contraceptive use could not be performed.

3.2 Plasma immunoglobulins and isotypes in cases and controls over time

3.2.1 The intergroup analyses of cases and controls

The immunoglobulin titres between the cases and the controls were the same

To assess the immunoglobulin titres, immunoglobulin isotypes (IgA and IgM), four IgG subclasses (IgG1, IgG2, IgG3, IgG4) and the total IgG (sum of IgG1+IgG2+IgG3+IgG4) were measured (Log_{10} ng/ml) from the plasma of the HIV infected (cases) and HIV negative (controls) participants at baseline, 3-; 6-; 9-, and 12-month visits. Women who seroconverted (cases) while on PrEP (9) and not on PrEP (3) were followed up in this sub-study, they were compared to women who remained negative (controls) while on PrEP (29) and not on PrEP (19). When comparing the immunoglobulins titres among the cases and the controls irrespective of PrEP use, there were no significant differences found at baseline, 3-; 6-; 9-; and the 12-month visits (Figures 1.1-1.5).

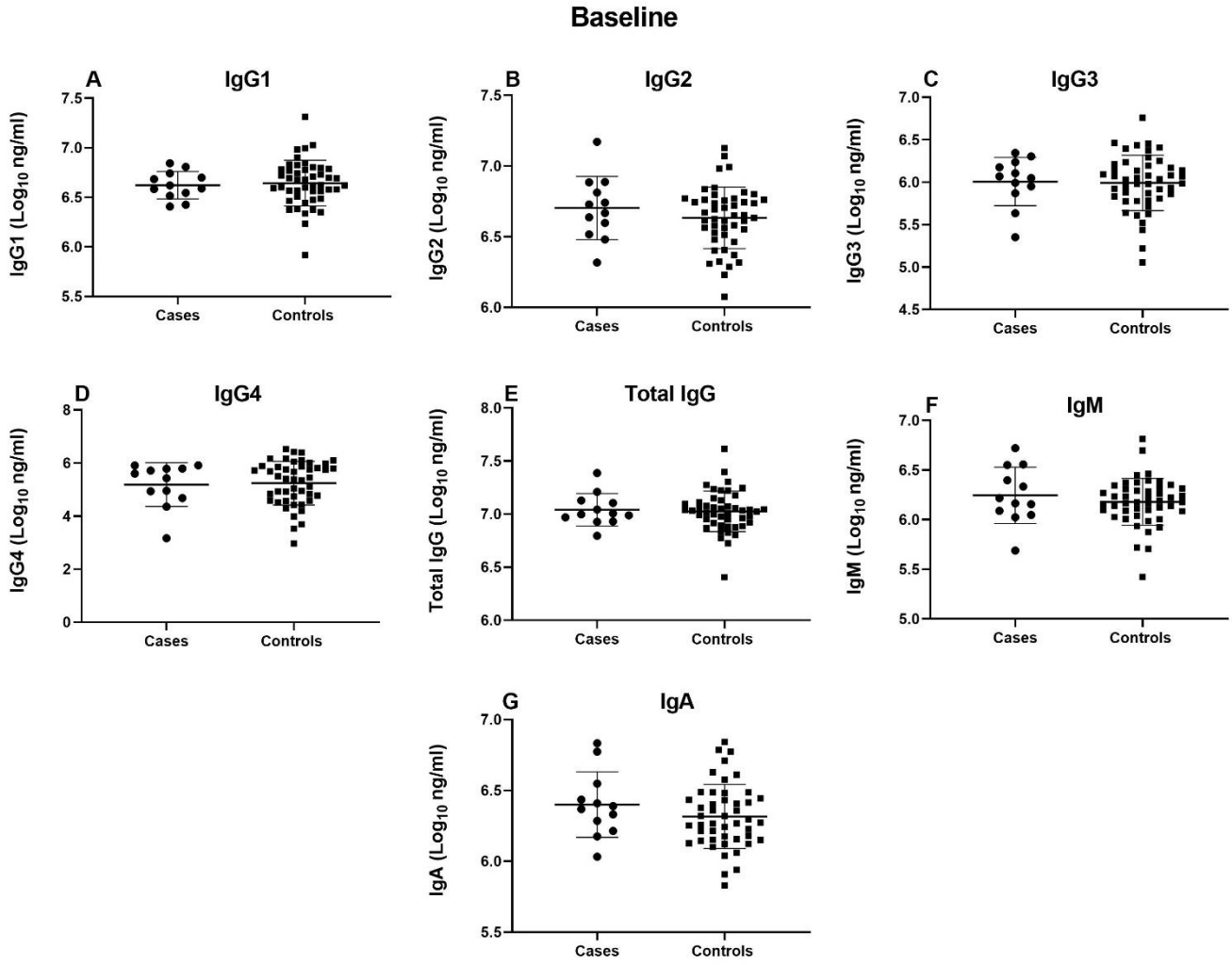

Figure 1.1: Cross-sectional analyses of immunoglobulin titres (Log_{10} ng/ml) in the plasma between cases and controls at baseline [cases (n=12); controls (n=47)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, cases represent women who subsequently became HIV infected. The Unpaired t-tests and Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One participant from the control group did not have the baseline sample.

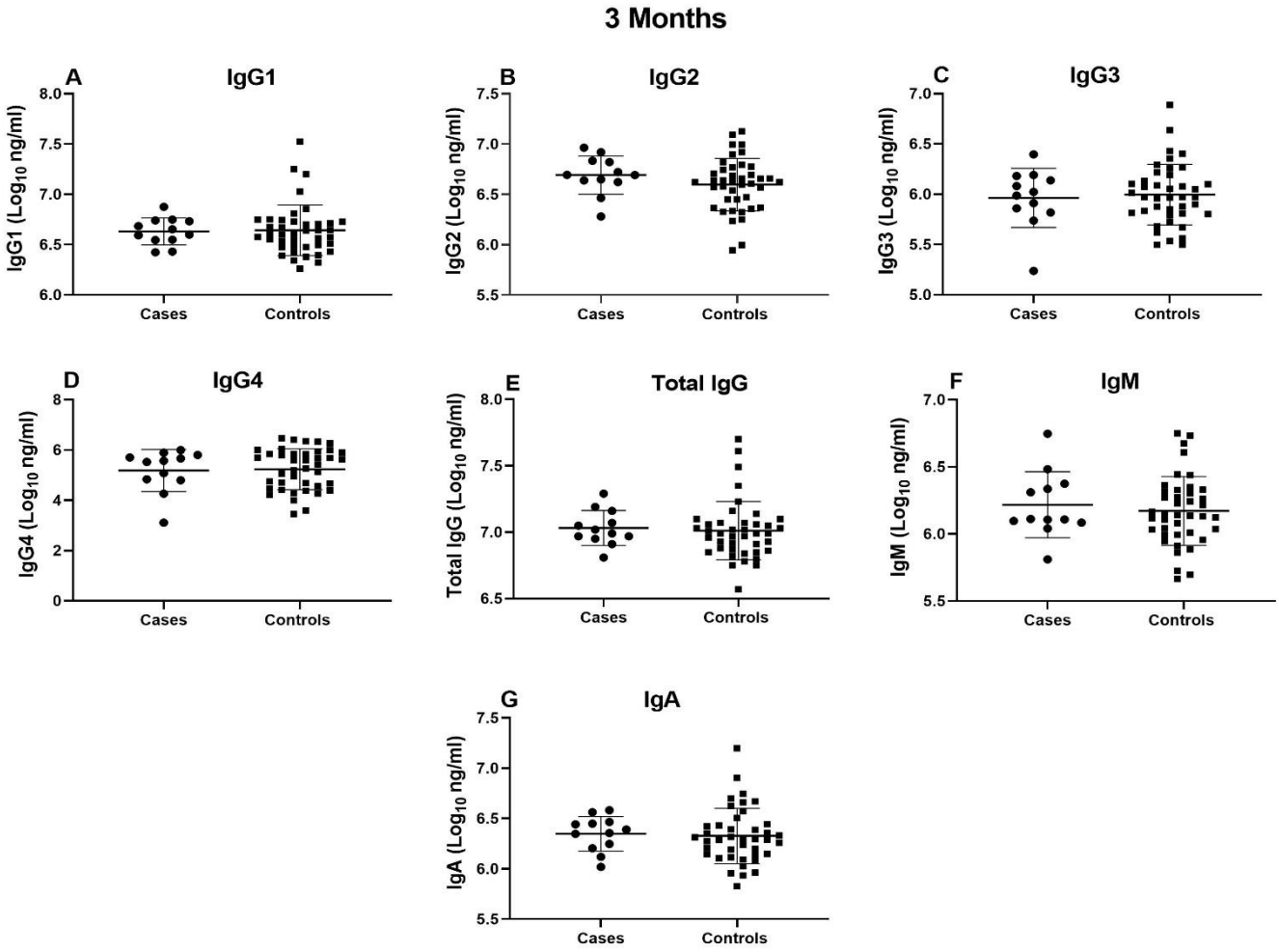

Figure 1.2: Cross-sectional analyses of immunoglobulin titres (Log_{10} ng/ml) in the plasma between cases and controls at the 3-month visit [cases (n=12); controls (n=42)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Unpaired t-tests and Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eight participants from the control group did not have the 3-month samples.

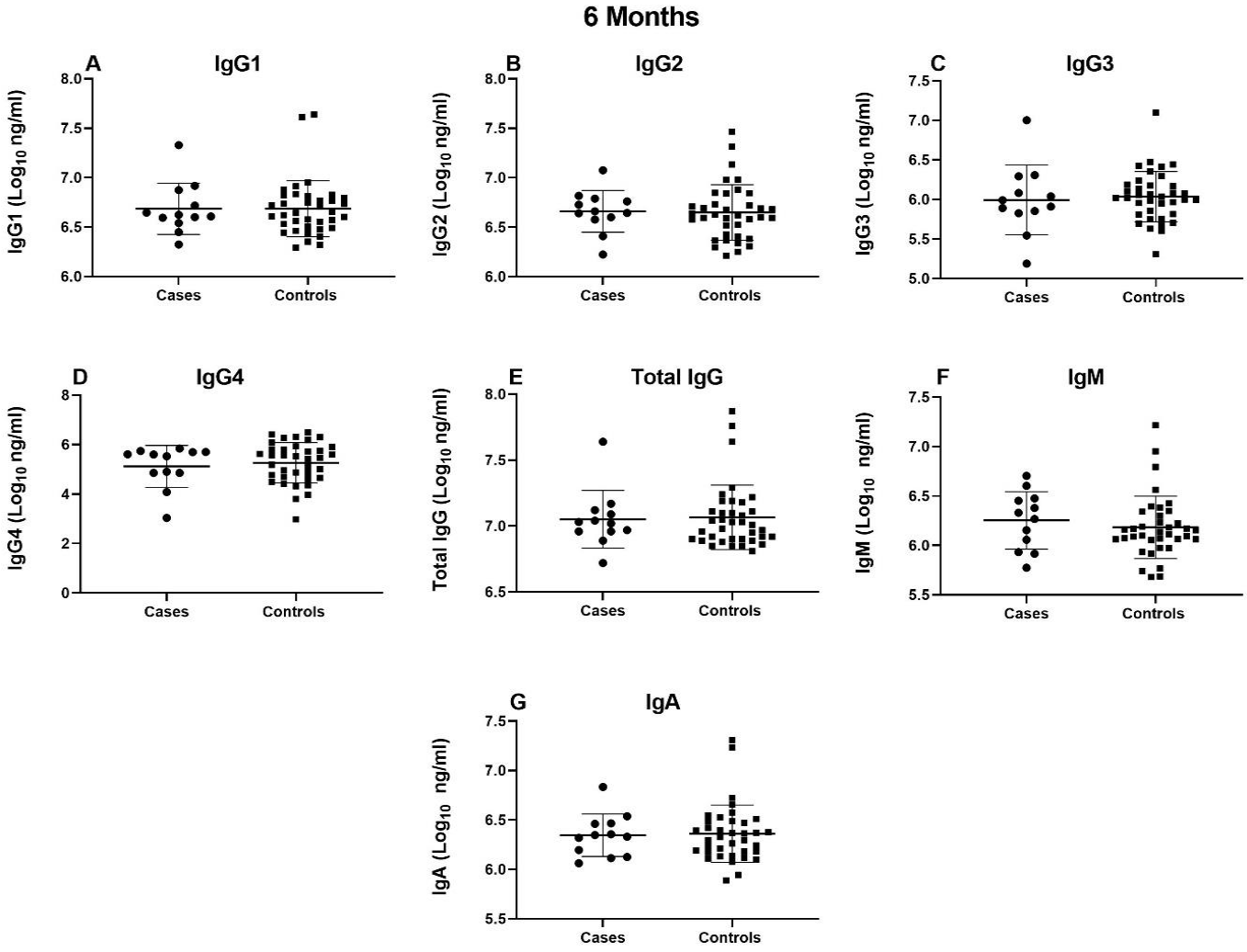

Figure 1.3: Cross-sectional analyses of immunoglobulin titres (Log_{10} ng/ml) in the plasma between cases and controls at the 6-month visit [cases (n=12); controls (n=37)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Unpaired t-tests and Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eleven participants from the control group did not have the 6-month samples.

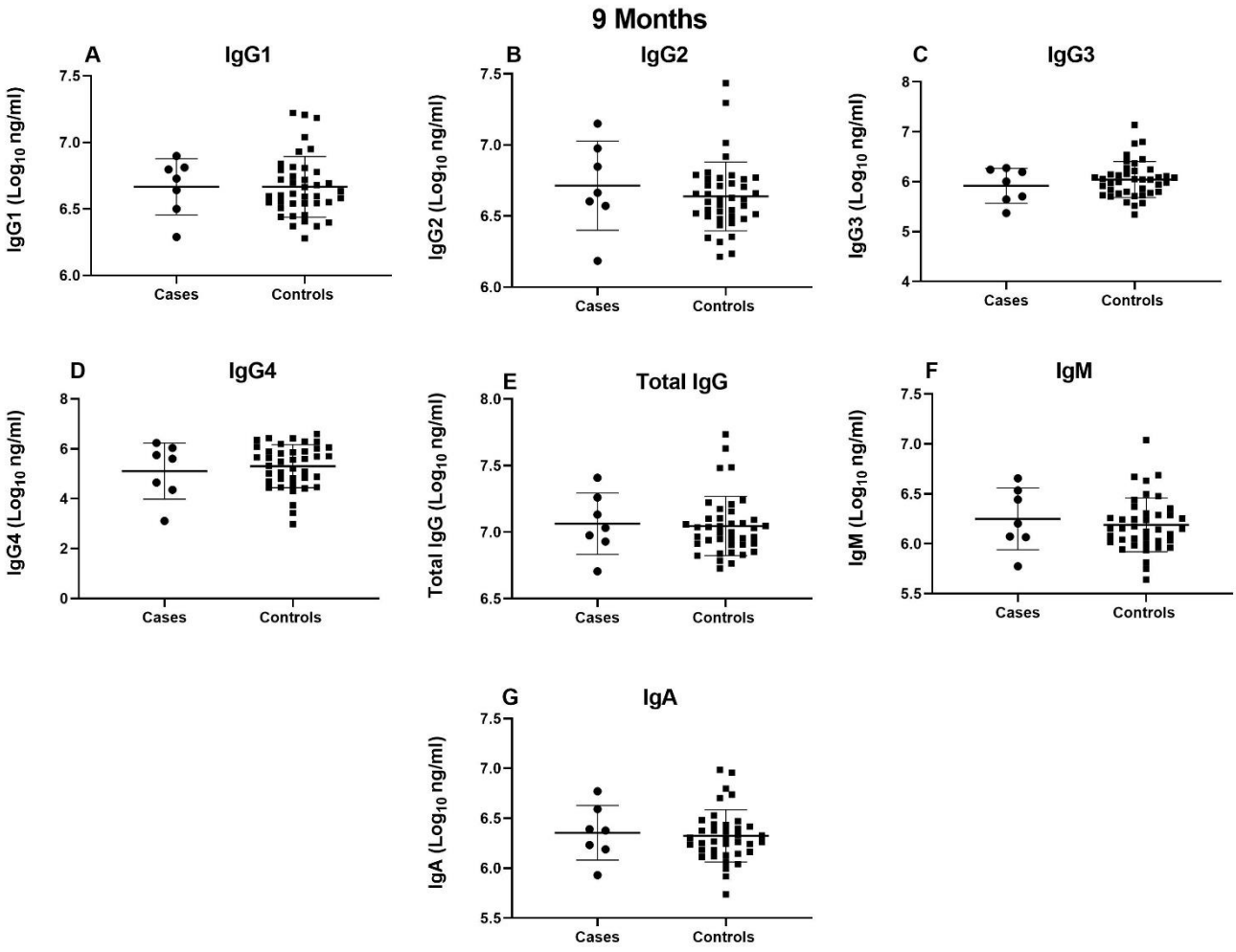

Figure 1.4: Cross-sectional analyses of immunoglobulin titres (Log_{10} ng/ml) in the plasma between cases and controls at the 9-month visit [cases (n=7); controls (n=40)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Unpaired t-tests and Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Four participants from the cases and eight participants from the control group did not have the 9-month samples.

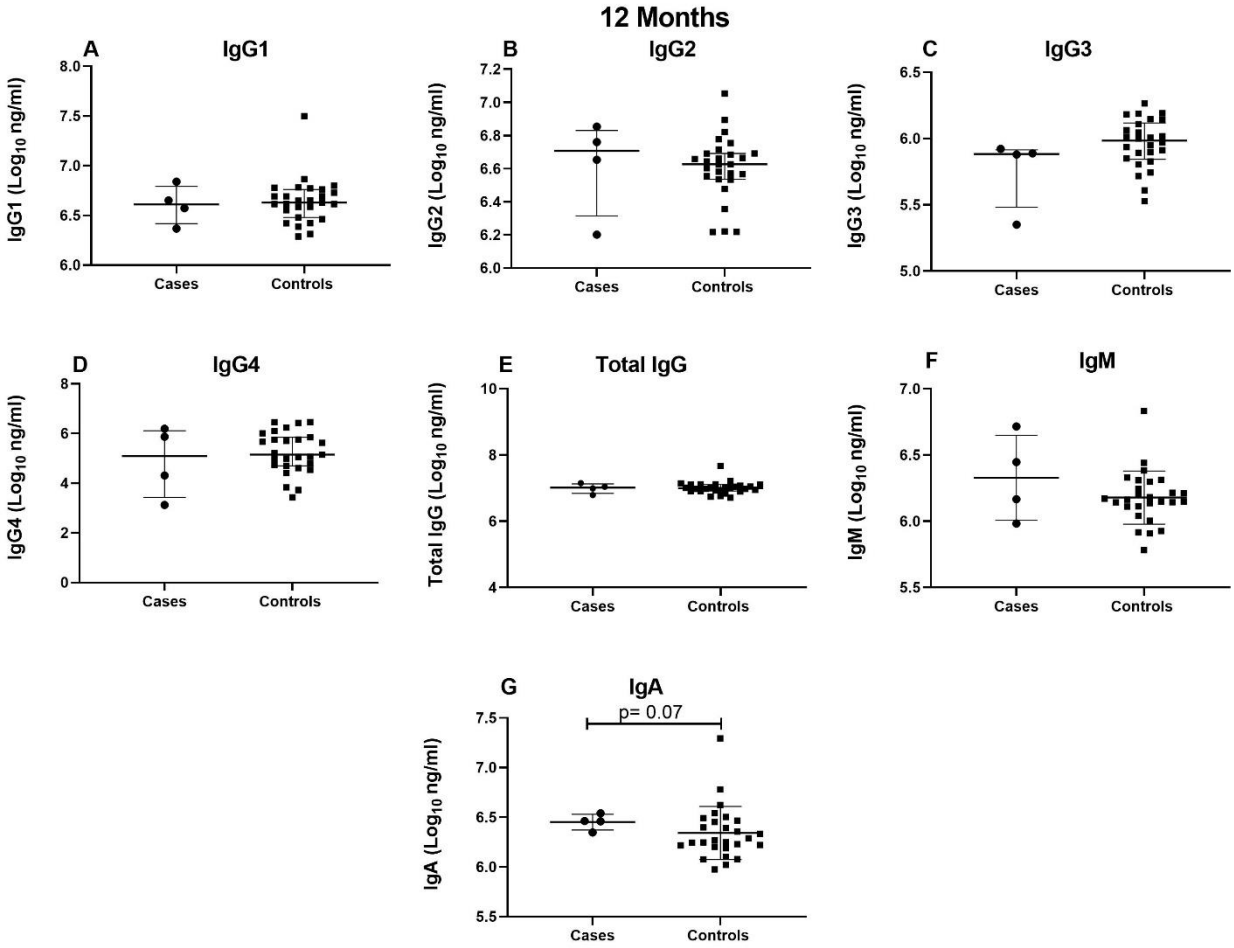

Figure 1.5: Cross-sectional analyses of immunoglobulin titres (Log_{10} ng/ml) in the plasma between cases and controls at the 12-month visit [cases (n=4); controls (n=27)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Unpaired t-tests and Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eight participants from the cases and one participant from the control group did not have the 12-month samples.

3.2.2 The intragroup analyses of immunoglobulins and isotypes in the cases

Plasma IgA was significantly lower at the 6-month visit compared to the baseline

Seeing that there were no differences between the cases and controls, we further analysed the data by comparing the baseline to the various time-points within the cases (intragroup analyses). There were no significant differences in IgG1 (Figure 2A), IgG2 (Figure 2B), IgG3 (Figure 2C), IgG4 (Figure 2D), total IgG (Figure 2E), and IgM (Figure 2F) titres within the cases over time. However, IgA was significantly lower at the 6-month visit [median 6.35 log_{10} ng/ml (IQR 6.22-6.46 log_{10} ng/ml)] compared to baseline [median 6.39 log_{10} ng/ml (IQR 6.27-6.492 log_{10} ng/ml)] (p= 0.02) (Figure 2G).

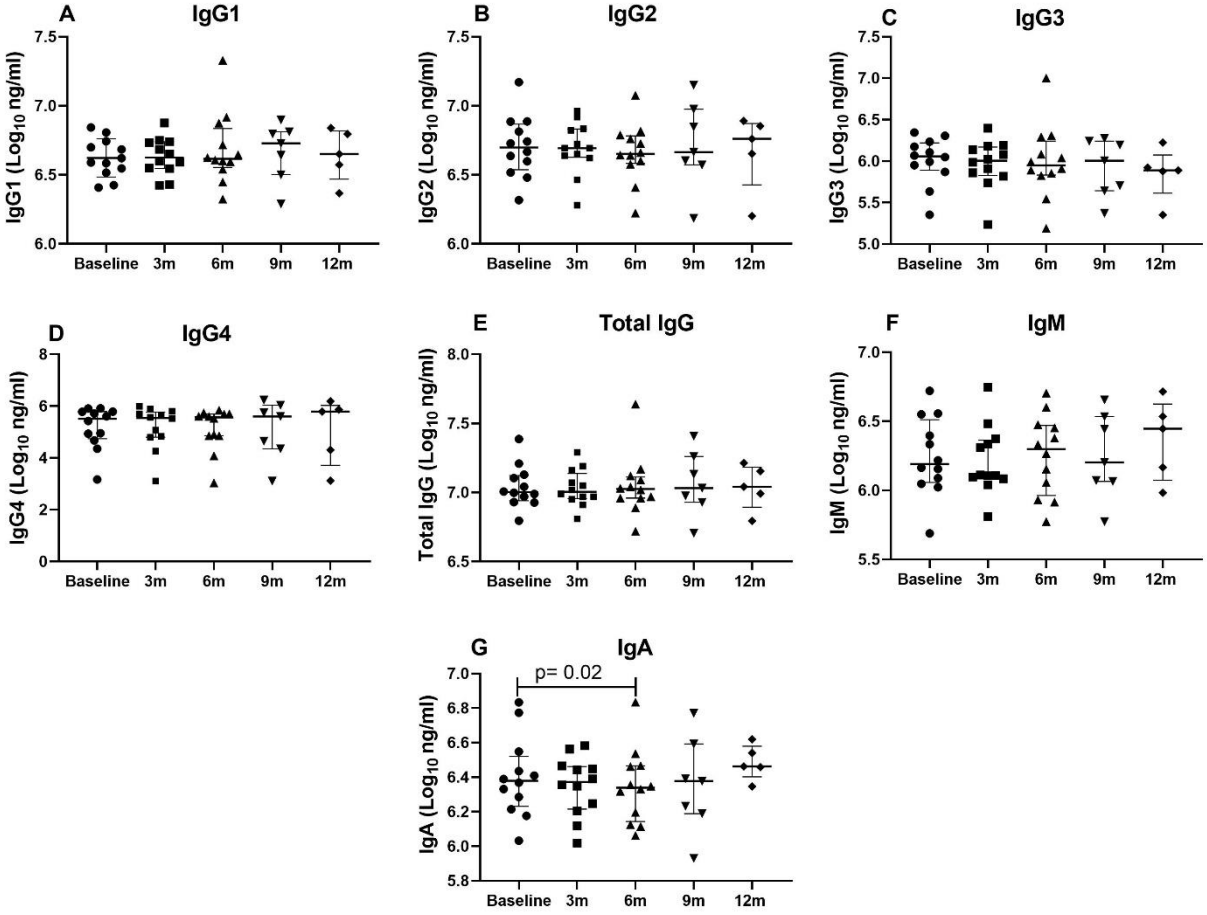

Figure 2: The longitudinal follow up of cases at baseline compared to various time points for plasma immunoglobulins (Log_{10} ng/ml) IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). At baseline (n=12), at 3-month (n=12), at 6-month (n=12), at 9-month (n=7), and at 12-month visits (n=5). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, cases represent women who subsequently became HIV infected. The Paired t-tests were used to compare the IgG1, IgG2, IgG3, Total IgG, IgM, and IgA titres, and the Wilcoxon tests were used to compare the IgG4 titres within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Five participants and seven participants from the cases did not have the 9- and 12-month samples, respectively.

3.2.3 The intragroup analyses of immunoglobulins and isotypes in the controls

Plasma IgG2, IgG3, total IgG, and IgM were significantly lower at the 12-month visit compared to the baseline

The similar intragroup analyses comparing the baseline immunoglobulins to subsequent time points were done within the controls. At the 12-month visit, IgG2 [6.63 (6.54-6.69) (p= 0.04)], IgG3 [6.00 (5.85-6.11) (p= 0.001)], total IgG [7.00 (6.91-7.11) (p= 0.03)], and IgM [6.16 (6.11-6.30) (p= 0.04)] were significantly lower when compared to the baseline IgG2 [6.65 (6.51-6.76)], IgG3[6.03 (5.81-

6.20)], total IgG [7.03 (6.92-7.11)], and IgM [6.18 (6.09-6.31)] , respectively, (Figure 3B, 3C, 3E, and F).

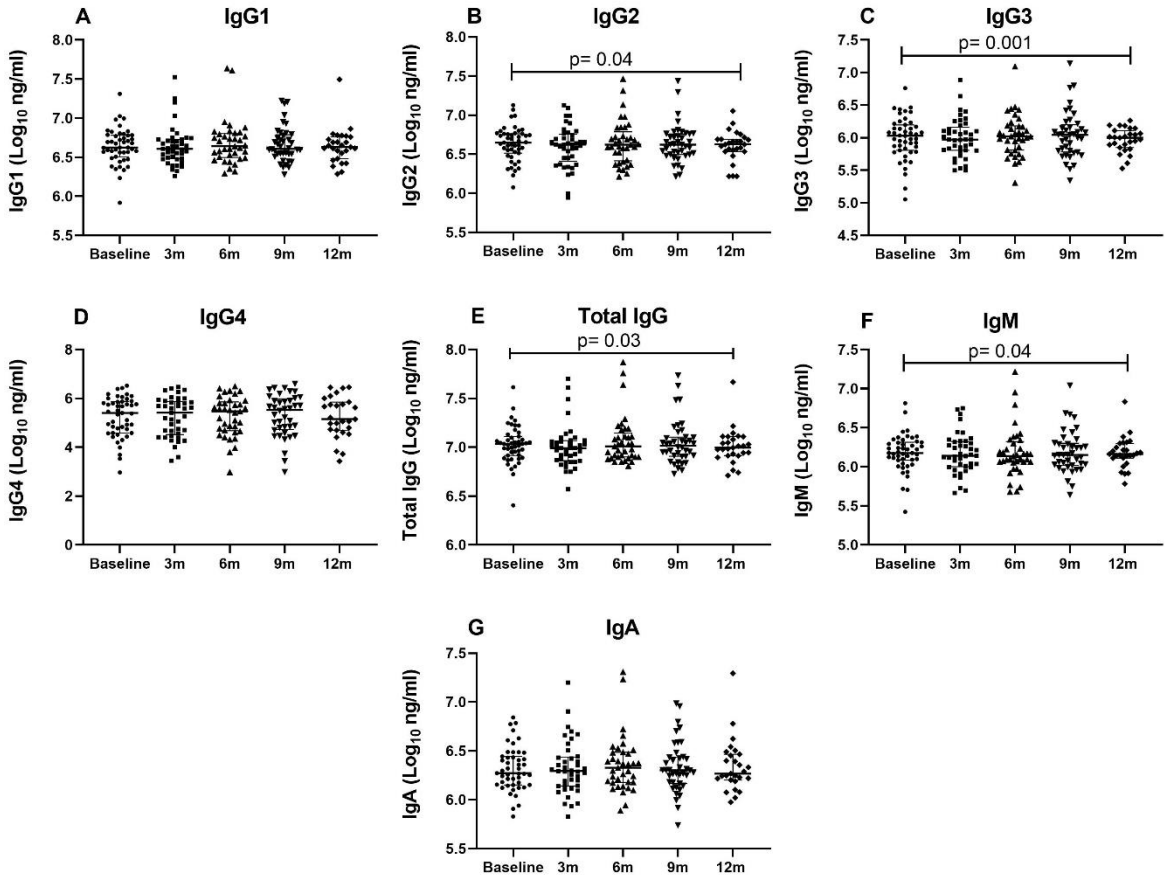

Figure 3: The longitudinal follow up of controls at baseline compared to various time points for plasma immunoglobulins (Log_{10} ng/ml) IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). At baseline (n=47), at 3-month (n=41), at 6-month (n=37), at 9-month (n=40), and at 12-month visits (n=27). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Paired t-tests were used to compare the IgG1, IgG2, IgG3, IgG4, and IgM titres, and the Wilcoxon tests were used to compare the Total IgG and IgA titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One, four, eleven, eight, and twenty-one seven participants from the cases did not have the baseline, 3-; 6-; 9- and 12-month samples, respectively.

3.2.4 The comparison of plasma immunoglobulin titres in the cases between women on PrEP and not on PrEP

Plasma immunoglobulin titres (IgG1, IgG3, IgM, and IgA) trended to significance within the participants using PrEP compared to the participants not using PrEP

After further stratifying for PrEP use among the cases and controls, the results illustrated in Figure 4 and 5 were obtained for cases and controls, respectively. The comparison was done at baseline, 3-; 6-; 9-; and the 12-month visits. However, we did not analyse the 12-month visit amongst cases due to missing data. No significant differences were among cases on PrEP compared to the cases not on PrEP

at the baseline, 3-, and the 6-month visits (Supplementary Figure 1; 2; and 3, respectively). Only at the 9-month visit within the cases, the immunoglobulin titres (IgG1, IgG3, IgM and IgA) trended to significance where the participants on PrEP had higher medians compared participants not on PrEP, IgG1 [PreP: 6.44 (6.143-6.60); no PrEP: 5.92 (5.77-6.07)] (p= 0.07) (Figure 4A), IgG3 [PrEP: 6.85 (6.43-7.06); no PrEP: 6.10 (6.01-6.19)] (p= 0.06) (Figure 4C), IgM [PrEP: 6.44 (6.13-6.60); no PrEP 5.92 (5.77-6.07)] (p= 0.07) (Figure 4F), and IgA [PrEP; 6.39 (6.305-6.77); no PrEP: 6.06 (5.93-6.19)] (p= 0.07) (Figure 4G) respectively.

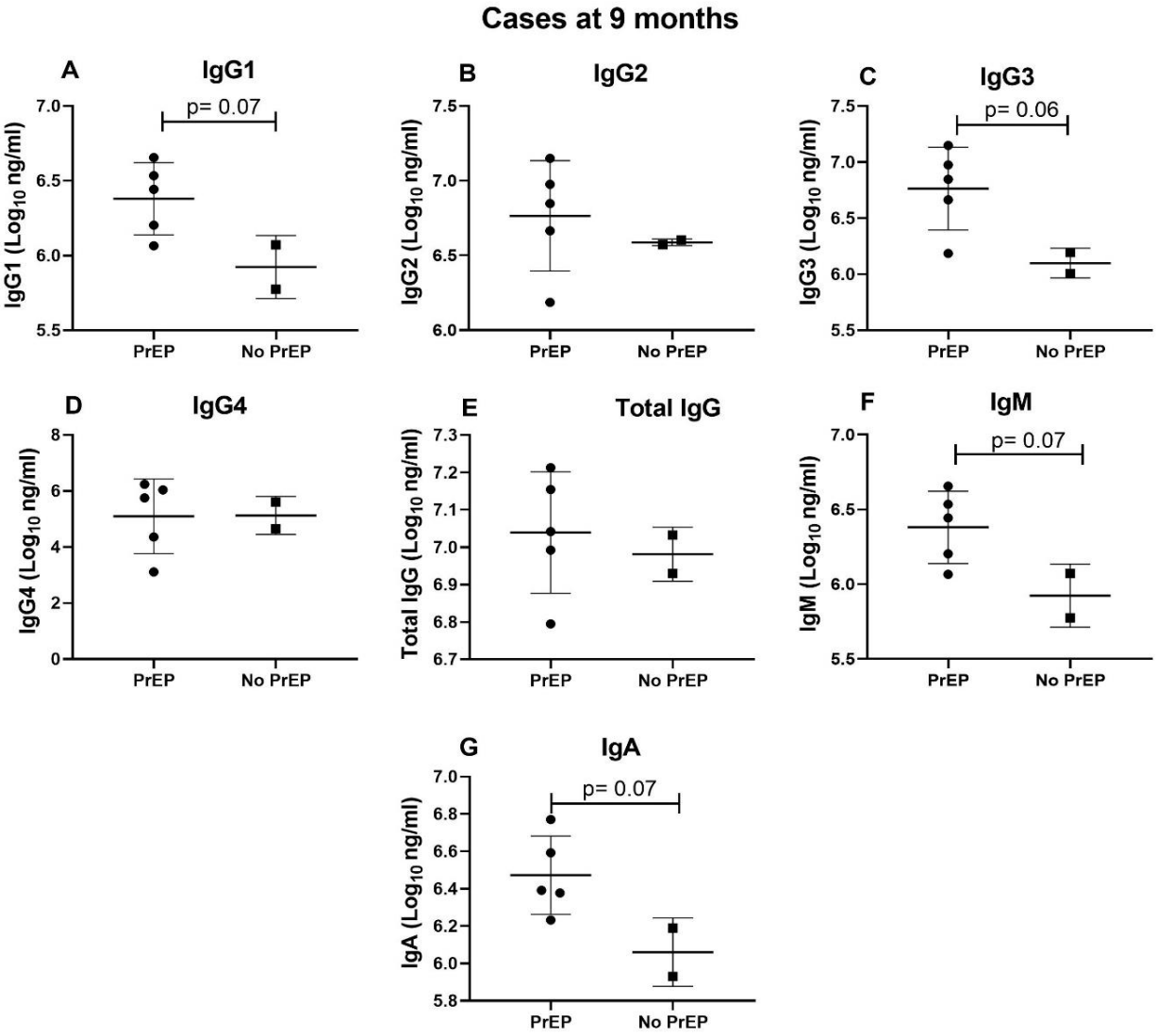

Figure 4: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the cases using PrEP (n=5) and not using PrEP (n=2) at the 9-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Unpaired t-tests were used to compare the IgG1, IgG2, IgG3, IgG4, Total IgG, IgM, and IgA titres within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Four participants from the PrEP group and 1 participant from the no PrEP group did not have the 9-month samples.

3.2.5 The comparison of plasma immunoglobulin titres among the controls on PrEP and not on PrEP

Plasma IgG3 and IgG4 within the PrEP users trended to significance compared to non-PrEP

Similar analyses were done within the controls with no significant differences observed between PrEP users and non-PrEP users at baseline, 3-; 9-; and 12-month visits (Supplementary Figure 4, 5, 6, and 7, respectively). At the 6-month visit, IgG3 within the PrEP users were higher compared to non-PrEP users [PrEP: 6.08 (5.95-6.42); no PrEP: 6.00 (5.81-6.09)] (p= 0.07) (Figure 5C) and IgG4 [PrEP: 5.67 (4.79-6.05); no PrEP: 5.01 (4.51-5.58)] (p= 0.06) (Figure 5D). In addition to isotyping in the plasma, we also performed the isotyping experiments in the genital tract using Softcup specimens.

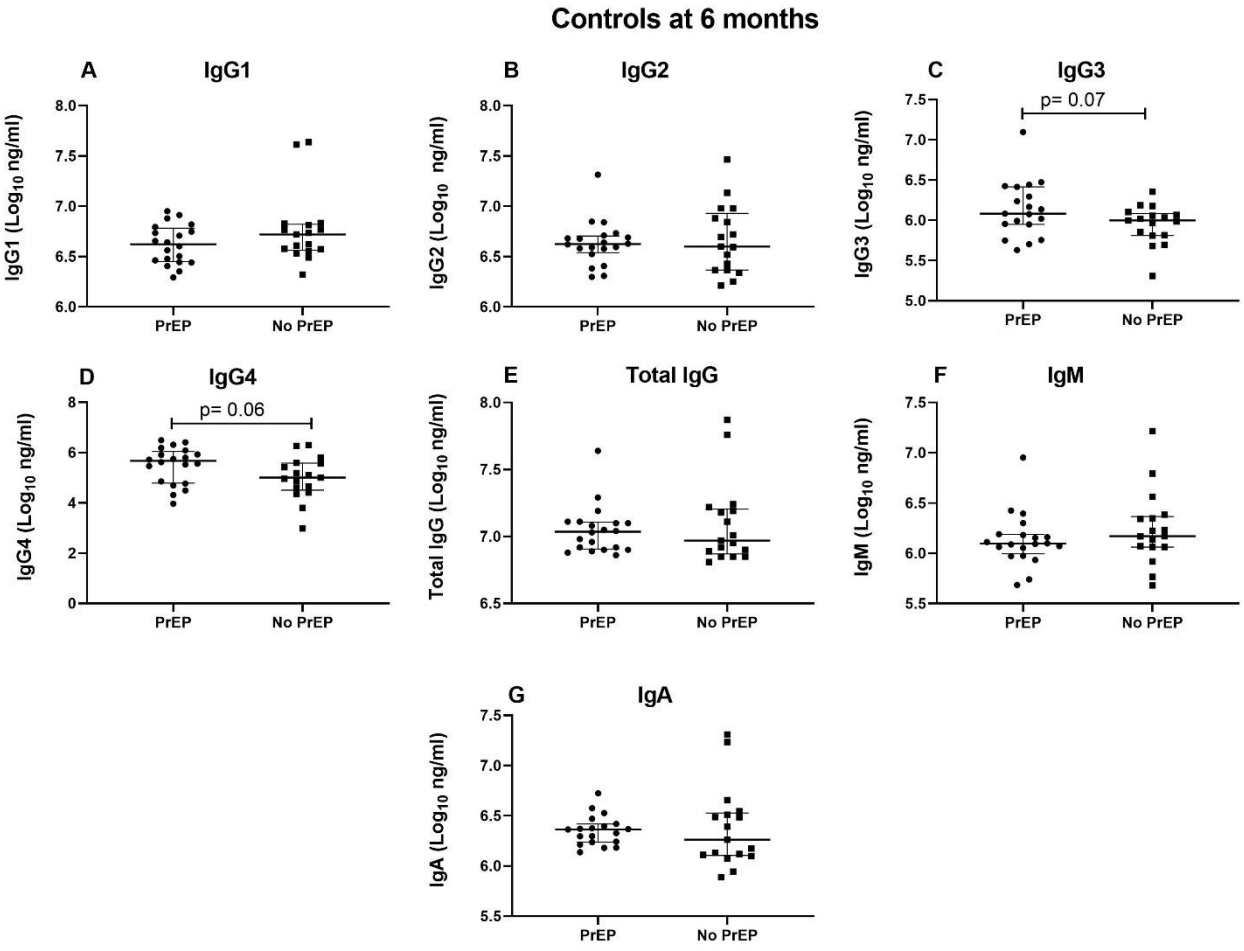

Figure 5: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the controls using PrEP (n=20) and not using PrEP (n=17) at the 6-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Mann-Whitney tests were used to compare the IgG1, IgG2, Total IgG, IgM, and IgA titres and the Unpaired t-tests were used to compare the IgG3 and IgG4 titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Nine participants from the PrEP group and two participants from the no PrEP group did not have the 9-month samples.

3.3 Mucosal immunoglobulins and isotypes in cases and controls over time

The genital tract specimens that were collected using the Softcups from the 60 women (n=12 cases; n=48 controls) at different visits (Baseline, 3-; 6-; 9-, and 12-month visit) as previously described, were used for mucosal isotyping.

3.3.1 The intergroup analyses of cases and controls

Genital tract IgG2 and Total IgG were significantly higher in cases compared to controls at the 12-month visit

There were no significant differences observed between cases and controls at baseline, 3-, and the 6-month visits (Supplementary Figures 8.1; 8.2; and 8.3, respectively). However, at the 9-month visit, IgG2 trended to significance among the cases [6.39 (6.03-7.03)] compared to the controls [5.87 (5.64-6.17)] (p= 0.06). Similarly total IgG trended to significance in the cases [6.39 (5.66-8.39)] compared to the controls [5.87 (3.84-8.41)] (p= 0.06) (Supplementary Figures 8.4). At the 12-month visit, cases had significantly high titres of IgG2 [6.55 (6.22-6.72)] and total IgG [7.34 (6.52-8.06)] compared to controls; [5.34 (4.88-5.85)] (p= 0.0004) (Figure 6B) and [6.01 (5.60-6.43)] (p= 0.002) (Figure 6E), respectively. In addition, IgM trended higher in the cases [5.69 (3.76-6.85)] compared to the controls [4.11 (3.45-4.76)] (p= 0.07) (Figure 6F).

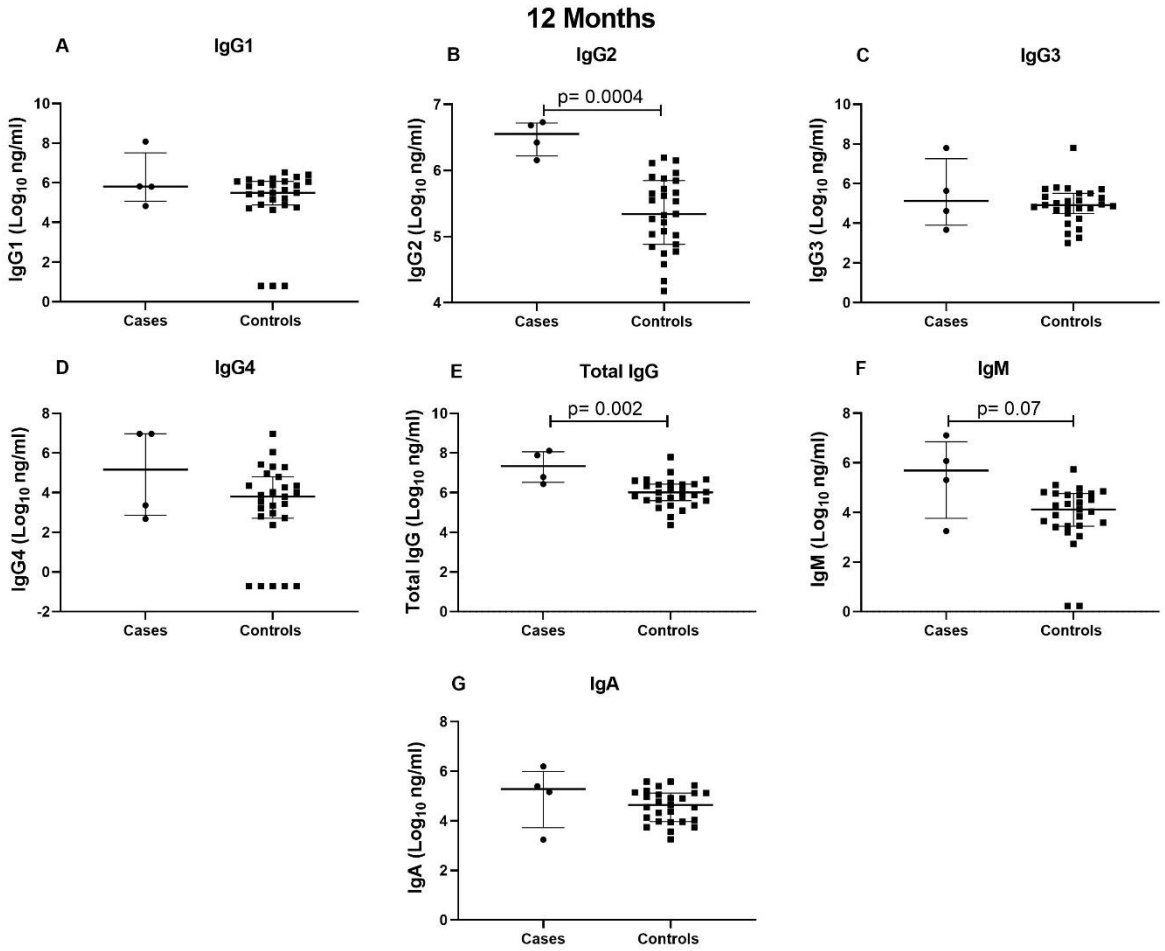

Figure 6: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens between the cases (n=4) and the controls (n=27) at the 12-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Mann-Whitney tests were used to compare the IgG4, and the IgM titres between the cases and the controls, and Unpaired t-tests were used to compare the IgG1, IgG2, IgG3, Total IgG, and IgA titres between the cases and the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eight cases and one control did not have the 12-month samples.

3.3.2 Intragroup analyses within the cases in the genital tract

Mucosal IgG2 and total IgG were significantly lower at the 3-month visits compared to baseline in cases

The longitudinal follow up was done within the cases at baseline versus 3-; 6-; 9-, and 12-month visits (Figure 6). There was no significant difference in the titres of IgG4 (Figure 7D), IgM (Figure 7F), and IgA (Figure 7G) between the baseline and different visits. Significantly lower titres of IgG2 were observed at the 3-month visit [5.56 (5.14-6.04)] when compared to the baseline [5.90 (5.59-6.16)] (p= 0.02) (Figure 7B). In addition, total IgG was significantly lower at the 3-month visit [6.09 (5.82-6.57)] when compared to the baseline [6.34 (6.15-7.80)] (p= 0.01) (Figure 7E). Moreover, IgG1 trended to significance when comparing between the baseline [5.86 (5.61-6.11)] and the 3-month visit [5.53 (5.11-6.18)] (p= 0.07) (Figure 7A), and IgG3 trended to significance when comparing between the baseline

[5.52 (5.02-7.80)] and the 3-month [5.45 (4.92-5.70)] (p= 0.06), and the 6-month visits [4.91 (4.37-5.45)] (p= 0.08) (Figure 7C).

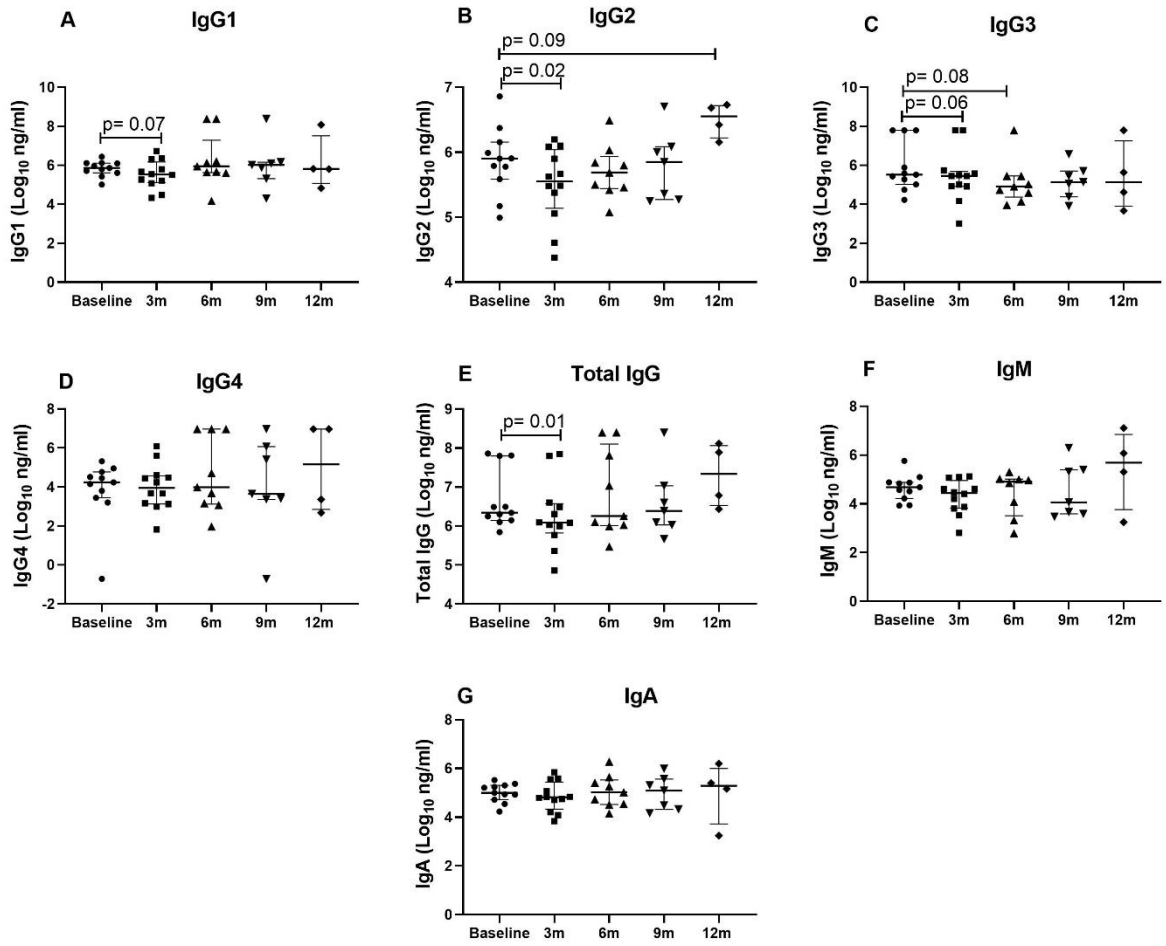

Figure 7: The longitudinal follow up of cases at baseline compared to various time points in the mucosal immunoglobulins (Log_{10} ng/ml) for IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). At baseline (n=11), at 3-month (n=12), at 6-month (n=8), at 9-month (n=7), and at 12-month visits (n=4). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, cases represent women who subsequently became HIV infected. Wilcoxon tests were used to compare the IgG1, IgG2, IgG3, IgG4, and Total IgG titres within the cases, and Paired t-tests were used to compare IgM, and IgA within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One, four, five, and eight cases did not have for the baseline, 6-;9-, and the12-month samples, respectively.

3.3.3 Intragroup analyses of the controls in the genital tract

Mucosal immunoglobulins titres were higher at baseline compared to 3-; 6-; 9-, and 12-month visits in controls

The same analysis was done within the control group (Figure 8). Significantly higher titres of IgG1 were observed at the baseline [5.95 (5.54-6.45)] when compared to the 9-month [5.53 (5.24-5.91)] (p= 0.004) and the 12-month visits [5.49 (4.88-5.91)] (p= 0.03), while a trend to significance was observed at the 3-month visit [5.74 (5.21-6.10)] compared to the baseline [5.95 (5.54-6.45)] (p= 0.07) (Figure

8A). IgG2 was significantly lower at the 9-month visit [5.29 (5.05-5.62)] when compared to the baseline [5.71 (5.25-6.08)] (p= 0.04) (Figure 8B). In addition, IgG3 [4.82 (4.43-5.28)] and IgG4 [3.64 (2.93-4.50)] were significantly lower at the 9-month visit compared to baseline [IgG3: 5.19 (4.75-5.59); p= 0.02; IgG4: 4.04 (3.44-5.64); p= 0.005, respectively] (Figure 8C and 8D). At the 6-month visit, lower titres of IgG4 [3.70 (3.06-4.64)] (p= 0.05) were observed while the titres were higher at the 12-month visit [4.64 (3.97-5.13)] (p= 0.08) both compared to the baseline [4.04 (3.44-5.64)] (Figure 8D). The total IgG titres were higher at baseline [4.40 (3.44-5.64)] when compared to the 9-month visit [3.64 (2.93-4.50)] (p= 0.01) (Figure 8E). The IgM titres were significantly higher at baseline [4.51 (4.08-5.21)] when compared to the 6-month [4.38 (3.78-4.73)] (p= 0.04) and the 9-month visits [4.15 (3.66-4.57)] (p= 0.02) (Figure 8F). Moreover, the IgA titres were higher at baseline [4.95 (4.60-5.58)] when compared to the 9-month visit [4.67 (4.29-5.06)] (p= 0.01) and trended to significance when compared to the 12-month visit [4.64 (3.97-5.13)] (p= 0.09) (Figure 8G).

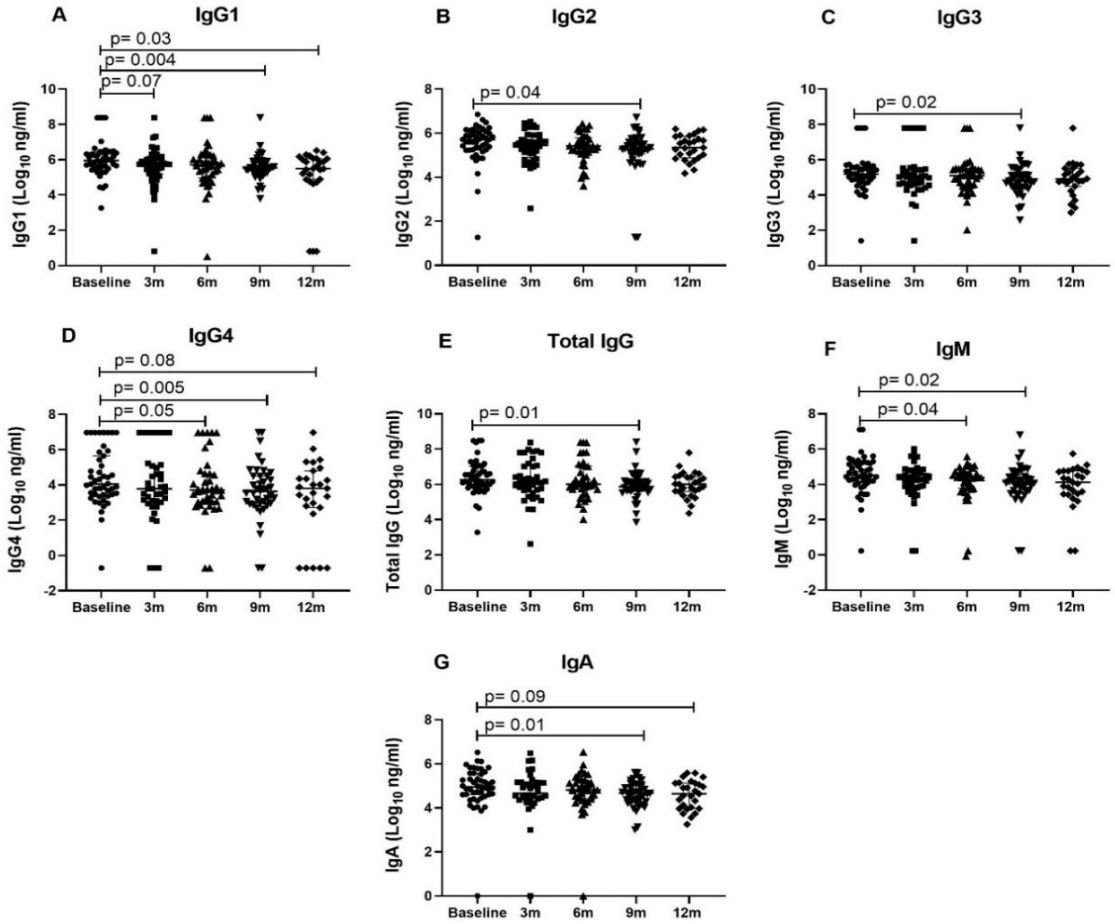

Figure 8: The longitudinal follow up of controls at baseline compared to various time points in the mucosal immunoglobulins (Log_{10} ng/ml) for IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). At baseline (n=47), at 3-month (n=40), at 6-month (n=41), at 9-month (n=41), and at 12-month visits (n=27). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, cases represent women who subsequently became HIV infected. Wilcoxon tests were used to compare the IgG1, IgG2, IgG3, IgG4, Total IgG, IgM, and IgA titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One, eight, seven, seven, and twenty-one controls did not have the baseline, 3-; 6-; 9-, and the 12-month samples, respectively.

3.3.4 Mucosal immunoglobulins and isotypes within cases and the controls (intragroup analyses) on PrEP and not on PrEP

The immunoglobulin titres in the genital tract were investigated between PrEP users and non-PrEP users within the cases at baseline, 3-, and 6-month visits and there were no significant differences observed (Supplementary Figures 9; 10; and 11, respectively). Furthermore, similar analyses for the 9- and the 12-month visits were not possible, as missing data precluded these analyses. The similar analyses were done within the controls at baseline, 3-; 6-; 9-, and 12-month visit, similarly, there were no significant differences observed (Supplementary Figures 12; 13; 14; 15; and 16, respectively).

3.4 Correlations of the immunoglobulin titres between plasma and genital tract

The IgG2 at baseline, 3-month, and 12-month visits correlated strongly within the cases

To determine whether the concentrations (Log_{10} ng/ml) of immunoglobulins correlated between the plasma and the genital tract, we conducted these analyses within the cases and controls. Within the cases, IgG2 titres correlated directly and significantly between the compartments at baseline (r= 0.67, p= 0.02) (Figure 9A), 3-month (r= 0.64, p= 0.03) (Figure 9B), and the 12-month visits (r= 0.98, p= 0.02) (Figure 9C).

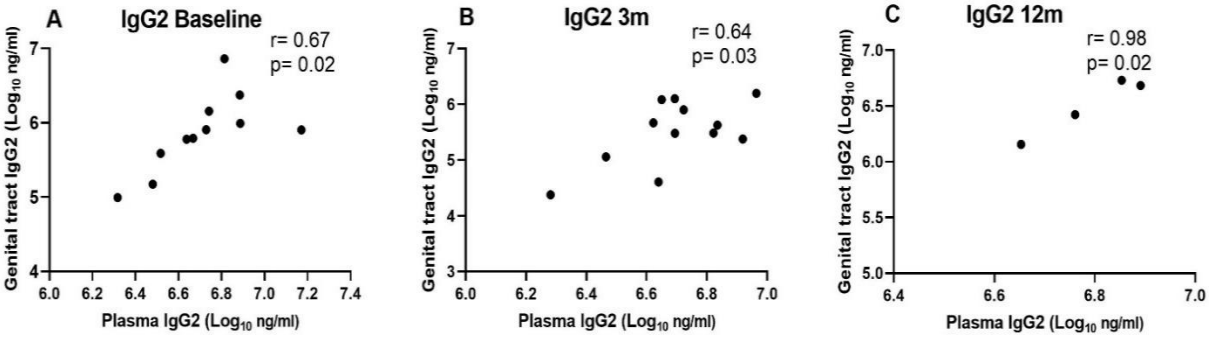

Figure 9: Correlations of IgG2 (Log_{10} ng/ml) between the plasma and the mucosal specimens in the cases. Figure 9A [Plasma (n=12); Mucosal specimens (n=11)], 9B [Plasma (n=12); Mucosal specimens (n=12)], and 9C [Plasma (n=4); Mucosal specimens (n=4)] are the correlation plots between the samples from the two compartments. At baseline, cases represent women who subsequently became HIV infected. The Pearson's correlation coefficient test was used to compare IgG2 subclass between the plasma and the genital tract, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One case did not have mucosal specimens at the baseline, and eight cases did not have both the plasma and the mucosal specimens at the 12-month visit.

The IgG4 at baseline and the 9-month visit correlated strongly within the cases

In the cases, IgG4 titres also correlated directly and significantly between the compartments at baseline (r= 0.78, p= 0.004) (Figure 10A) and at the 9-month visit (r= 0.81, p= 0.03) (Figure 10B)

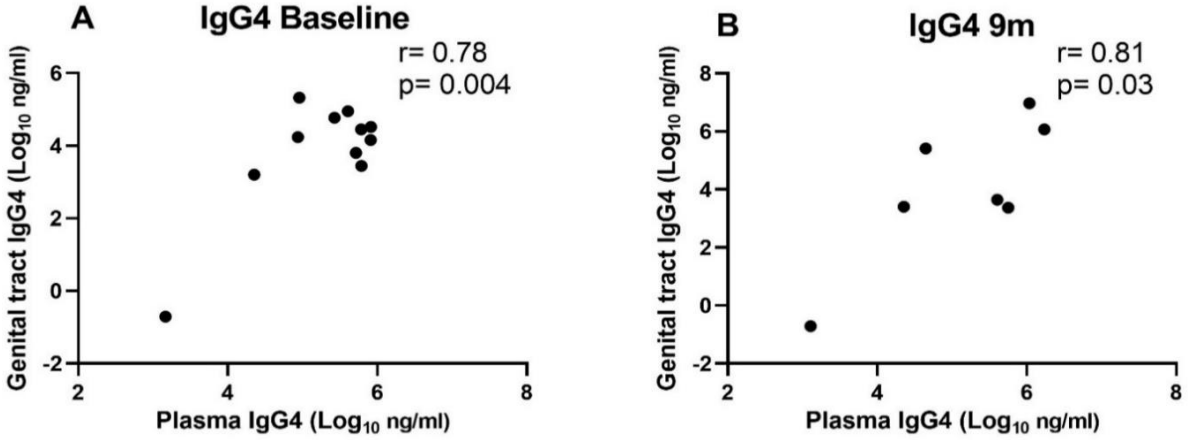

Figure 10: Correlations of IgG4 (Log_{10} ng/ml) between the plasma and the mucosal specimens in the cases. Figure 10A [Plasma (n=12); Mucosal specimens (n=11)] and 10B [Plasma (n=7); Mucosal specimens (n=7)] are the correlation plots between the samples from the two compartments. At baseline, cases represent women who subsequently became HIV infected. The Pearson's correlation coefficient test was used to compare IgG4 subclass between the plasma and the genital tract, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One case did not have the mucosal specimens at the baseline and five samples did not have both the plasma and mucosal specimens at the 9-month visit.

IgG4 correlated strongly at baseline and 12-month visit within the controls

Within the controls, IgG4 titres also correlated relatively strongly between the compartments at baseline (r= 0.57, p< 0.0001) (Figure 11A) and the 12-month visit (r= 0.69, p< 0.0001) (Figure 11C), and weakly correlated between the two compartments with the p-value trending to significance at the 3-month visit (r= 0.31, p= 0.06) (Figure 11B).

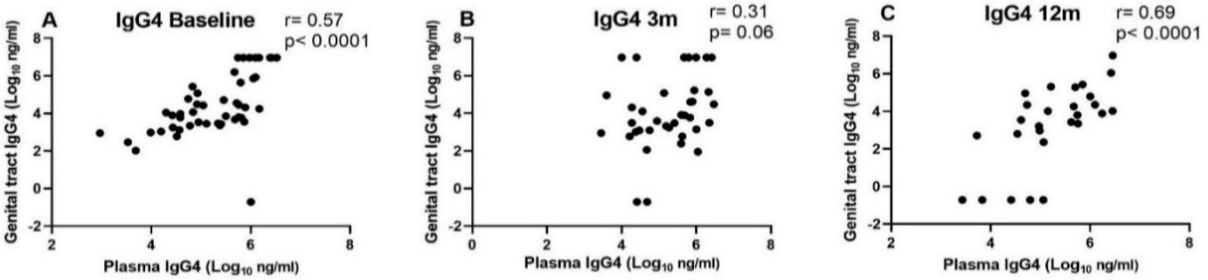

Figure 11: Correlations of IgG4 titres (Log_{10} ng/ml) between the plasma and the mucosal specimens in the controls. Figure 11A [Plasma (n=48); Mucosal specimens (n=47)]; 11B [Plasma (n=41)]; Mucosal specimens (n=40)], 11C [Plasma (n=27)]; Mucosal specimens (n=28)] are the correlation plots between the samples from the two compartments. At baseline, cases represent women who subsequently became HIV infected. The Pearson's correlation coefficient test was used to compare IgG4 subclass between the plasma and the genital tract, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One control did not have the baseline mucosal specimen, seven controls did not have 3-month plasma sample and eight controls did not have 3-month mucosal specimens. In addition, and twenty one controls did not have the 12-month plasma sample and twenty controls did not have the 12-month mucosal specimens.

3.5 The comparison of the immunoglobulins at baseline within the plasma and the mucosal samples irrespective of HIV status

Irrespective of the compartments, at baseline, IgG1 titres were consistently significantly higher compared to IgG3, IgG4, IgM, and IgA (Tables 2 and 3). Similarly, IgG2 titres were significantly higher than IgG4, IgM, and IgA titres (Tables 2 and 3). IgG2 was significantly higher than IgG3 in the plasma (Table 2) but not in the genital tract (Table 3). In addition, we found significantly lower IgG3 titres compared to IgA in the plasma (Table 2). In contrast, IgG3 was significantly greater than IgG4 and IgM in the mucosal compartment (Table 3). IgG4 titres remained the lowest in both plasma and the genital tract when compared to all IgG subclasses or IgM/IgA isotypes (Tables 2 and 3). On further analyses of each of the IgG subclasses and isotypes between the plasma and the genital tract revealed consistently and significantly higher levels in the plasma (Table 4).

Table 2: Comparisons of IgG subclasses and isotype titres at baseline in the plasma

Immunoglobulin isotypes and subclasses	Mean rank 1 (ng/ml)	Mean rank 2 (ng/ml)	Mean rank difference (ng/ml)	Adjusted *p*-value
IgG1 vs IgG2	281.8	283.9	-2.08	ns
IgG1 vs IgG3	281.8	110.9	170.8	<0.0001
IgG1 vs IgG4	281.8	51.21	230.6	<0.0001
IgG1 vs IgM	281.8	153.5	128.3	<0.0001
IgG1 vs IgA	281.8	193.2	88.6	0.001
IgG2 vs IgG3	283.9	110.9	172.9	<0.0001
IgG2 vs IgG4	283.9	51.2	232.7	<0.0001
IgG2 vs IgM	283.9	153.5	130.3	<0.0001
IgG2 vs IgA	283.9	193.2	90.7	0.001
IgG3 vs IgG4	110.9	51.2	59.7	ns
IgG3 vs IgM	110.9	153.5	-42.6	ns
IgG3 vs IgA	110.9	193.2	-82.3	0.001
IgG4 vs IgM	51.21	153.5	-102.3	<0.0001
IgG4 vs IgA	51.21	193.2	-142	<0.0001
IgA vs IgM	193.2	153.5	39.7	ns
IgA vs Total IgG	193.2	374.4	-181.2	<0.0001
IgM vs Total IgG	153.5	374.4	-220.9	<0.0001

One-Way ANOVA was used for statistical analysis. A p-value of <0.05 was statistically significant. The Dunn's multiple comparison test was used to provide the adjusted p-value, ns refers to not significant.

Table 3: Comparisons of IgG subclasses and isotype titres at baseline in the genital tract

Immunoglobulin isotypes and subclasses	Mean rank 1 (ng/ml)	Mean rank 2 (ng/ml)	Mean rank difference (ng/ml)	Adjusted *p*-value
IgG1 vs IgG2	277.7	244.7	33.1	ns
IgG1 vs IgG3	277.7	194.2	83.5	0.003
IgG1 vs IgG4	277.7	118.0	159.7	<0.0001
IgG1 vs IgM	277.7	120.2	157.7	<0.0001
IgG1 vs IgA	277.7	159.2	118.5	<0.0001
IgG2 vs IgG3	244.7	194.2	50.8	ns
IgG2 vs IgG4	244.7	118.0	126.6	<0.0001
IgG2 vs IgM	244.7	120.2	124.5	<0.0001
IgG2 vs IgA	244.7	159.2	85.4	0.002
IgG3 vs IgG4	194.2	118.0	76.1	0.01
IgG3 vs IgM	194.2	120.2	74.0	0.02
IgG3 vs IgA	194.2	159.2	34.9	ns
IgG4 vs IgM	118.0	120.2	-2.1	ns
IgG4 vs IgA	118.0	159.2	-41.2	ns
IgA vs IgM	159.2	120.2	39.1	ns
IgA vs Total IgG	159.2	313.8	-154.5	<0.0001
IgM vs Total IgG	120.2	313.8	-193.6	<0.0001

One-Way ANOVA was used for statistical analysis. A p-value of <0.05 was statistically significant. The Dunn's multiple comparison test was used to provide the adjusted p-value, ns refers to not significant.

Table 4: Comparisons of IgG subclasses and isotype titres between the plasma and the genital tract

Immunoglobulin isotype and subclasses	Mean rank 1 (ng/ml)	Mean rank 2 (ng/ml)	Mean rank difference (ng/ml)	Adjusted *p*-value
Plasma IgG1 vs Mucosal IgG1	391.2	285.1	106.1	0.001
Plasma IgG2 vs Mucosal IgG2	391.7	247.5	144.2	<0.0001
Plasma IgG3 vs Mucosal IgG3	325.8	203.8	122.1	<0.0001
Plasma IgG4 vs Mucosal IgG4	226.5	134.5	92.0	0.01
Plasma total IgG vs Mucosal total IgG	408.5	326.9	81.6	0.03
Plasma IgM vs Mucosal IgM	352.3	122.4	229.9	<0.0001
Plasma IgA vs Mucosal IgA	367.5	160.5	207.0	<0.0001

One-Way ANOVA was used for statistical analysis. A p-value of <0.05 was statistically significant. The Dunn's multiple comparison test was used to provide the adjusted the p-value.

3.6 Mucosal cytokine analyses in the cases and the controls over time

The concentrations (pg/ml) of 28 cytokines were assessed in this sub-study. Of the 28, five chemotactic cytokines (MIP-1α, MIP-1β, IP-10, MCP-1, and IL-8), and four inflammatory cytokines (TNF-α, IL-1α, IL-1β, and IL-6) were analysed in the FGT using the cervico-vaginal secretions collected through Softcup at baseline; 3-; 6-; 9-; and 12-month visits. These cytokines were compared between the 12 HIV infected (cases) and 48 HIV uninfected (controls) women. A cross-sectional analysis was done between the cases and the controls, and longitudinal analyses were also done within the cases and controls.

3.6.1 Intergroup analyses of mucosal cytokines between the cases and the controls at baseline; 3-; 6-; 9-, and 12-month visits

Elevated concentrations of five mucosal chemotactic cytokines and three inflammatory cytokines were observed in cases at the 12-month visit

There were no significant differences observed in cytokine concentrations (Log_{10} pg/ml) among the cases and the controls at baseline (Figure 12.1) and the 3-month visit (Figure 12.2). At the 6-month visit, IL-1β was significantly higher among the cases [median 3.73 Log_{10} pg/ml (IQR 3.02-3.84 Log_{10} pg/ml)] compared to the controls [2.52 (2.06-3.43)] (p= 0.01) (Figure 12.3E), while TNF-α [cases 2.08

(1.72-2.53); controls 1.26 (0.30-2.16)] (p= 0.07) (Figure 12.3C), IL-1α [cases 4.05 (3.97-4.40); controls 3.79 (3.50-4.09)] (p= 0.06) (Figure 12.3D), and IL-8 [cases 5.02 (4.54-5.31); controls 4.58 (4.17-5.06)] (p= 0.06) (Figure 12.3I) trended to significance with the higher medians observed among the cases. At the 9-month visit, MCP-1 was significantly higher among the cases [3.49 (2.74-4.58)] when compared to the controls [3.12 (2.68-3.85)] (p= 0.04) (Figure 12.4G). At the 12-month visit, the cases had elevated genital concentrations of five chemotactic cytokines (MIP-1α, MIP-1β, IP-10, MCP-1, and IL-8) and three inflammatory cytokines (TNF-α, IL-1α, and IL-1β) when compared to the controls (Figure 12.5).

The subsequent analyses highlight the significant differences of the 12-month levels of the cytokines in between cases and controls. Pro-inflammatory IL-6 trended to significance with a higher median observed in the cases [3.37 (2.40-5.03)] compared to the controls [2.49 (1.91-3.09)] (p= 0.09) (Figure 12.5H). MIP-1α was significantly higher in the cases [2.96 (2.40-3.66)] compared to the controls [2.19 (0.19-2.37)] (p= 0.01) (Figure 12.5A). Elevated concentrations of MIP-1β were observed among the cases [2.97 (2.58-3.50)] compared to the controls [2.08 (1.90-2.53)] (p= 0.02) (Figure 12.5B). TNF-α was also significantly higher in the cases [3.17 (2.64-3.56)] compared to the controls [0.96 (0.30-2.08)] (p= 0.001) (Figure 12.5C). Furthermore, IL-1α was significantly higher in the cases [4.97 (4.74-5.34)] compared to the controls [3.81 (3.15-4.21)] (p= 0.0002) (Figure 12.5D). Moreover, elevated IL-1β concentrations were observed in the cases [4.83 (4.39-5.03)] compared to the controls [2.62 (1.90-3.31)] (p= 0.0002) (Figure 12.5E). Significantly higher genital concentrations of IP-10 were observed among the cases [4.91 (3.68-5.44)] compared to the controls [3.74 (3.31-4.19)] (p= 0.02) (Figure 12.5F). In addition, MCP-1 was significantly higher among the cases [4.31 (3.29-5.84)] compared to the controls [3.16 (2.58-3.57)] (p= 0.003) (Figure 12.5G). IL-8 was significantly higher in the cases [5.31 (4.73-5.82)] compared to the controls [4.60 (3.98-5.18)] (p= 0.02) (Figure 12.5I).

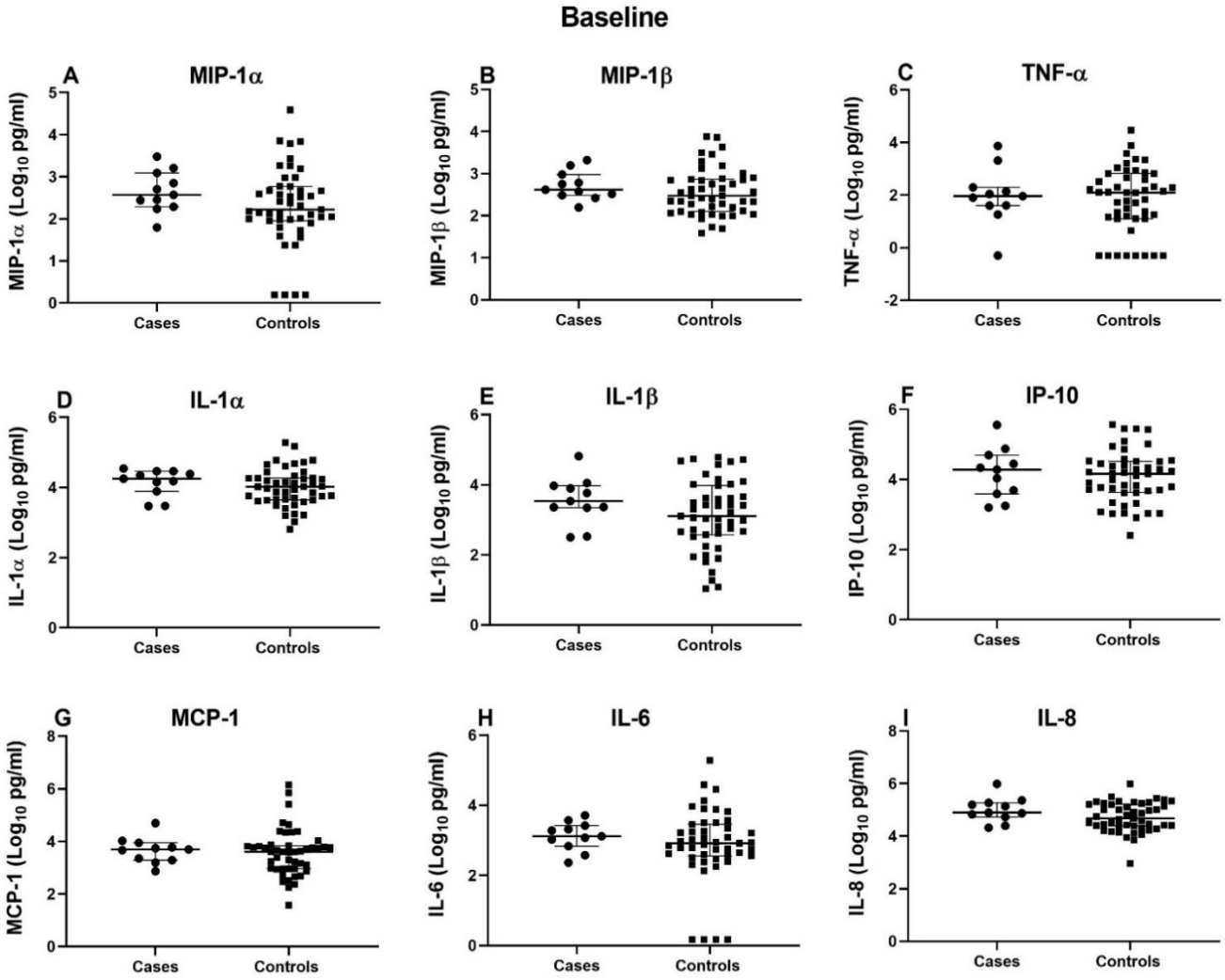

Figure 12.1: Cross-sectional analyses of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens between cases and controls at baseline [cases (n=11); controls (n=47)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, cases represent women who subsequently became HIV infected. Unpaired t-tests and the Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One case and one control did not have the baseline samples.

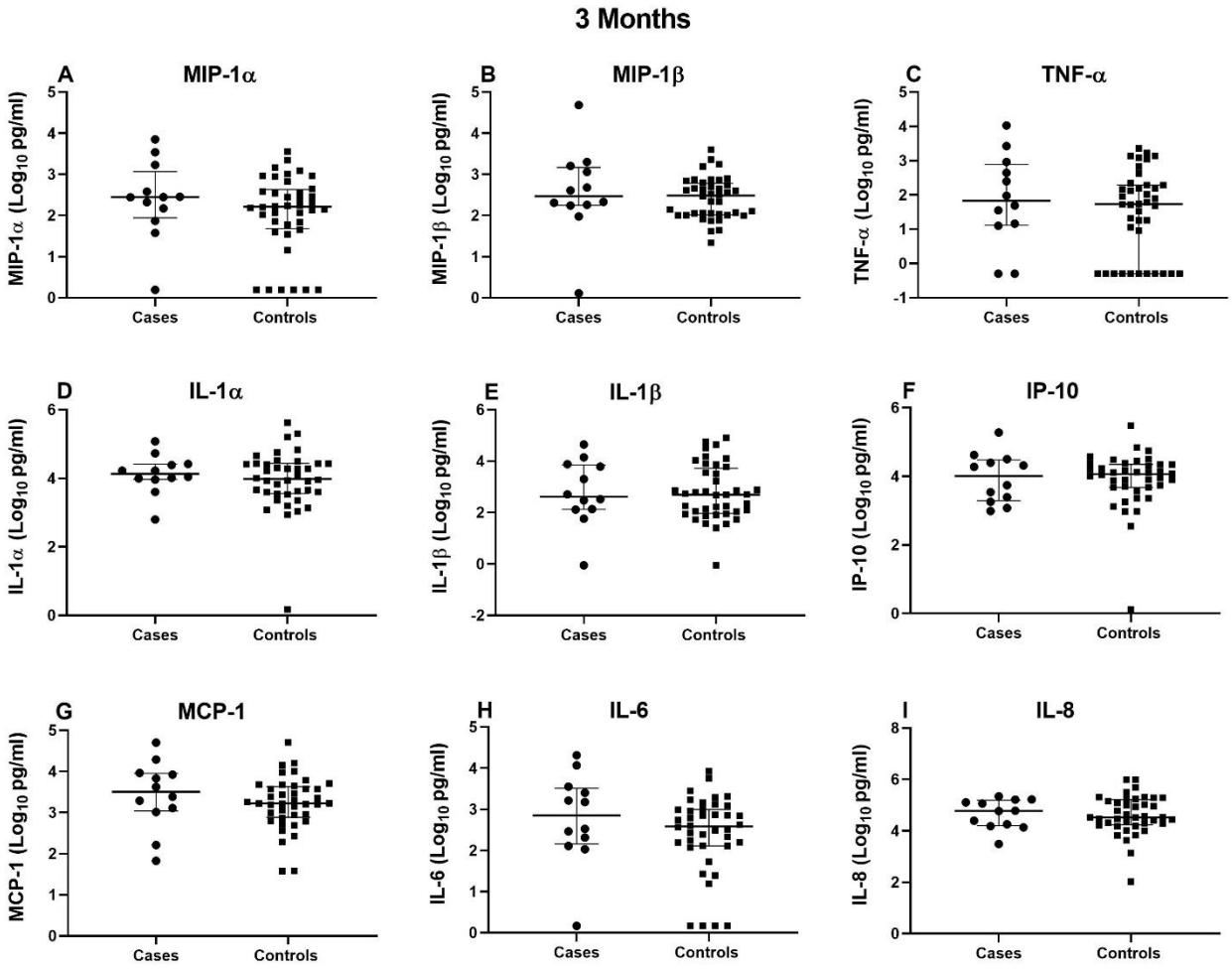

Figure 12.2: Cross-sectional analyses of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens between cases and controls at the 3-month visit [cases (n=12); controls (n=40)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Unpaired t-tests and the Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eight controls did not have the baseline samples.

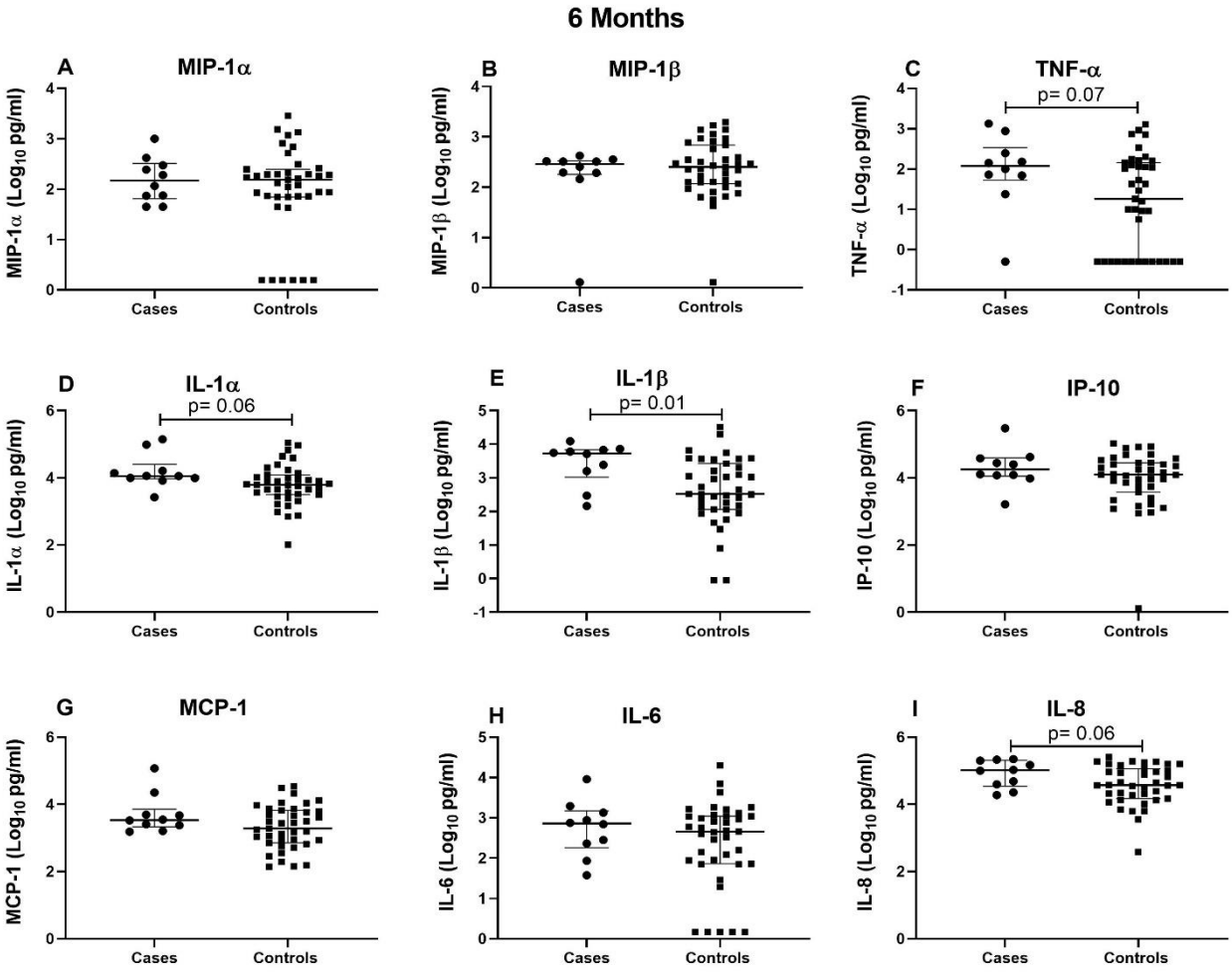

Figure 12.3: Cross-sectional analyses of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens between cases and controls at the 6-month visit [cases (n=10); controls (n=39)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Unpaired t-tests and the Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Two cases and nine controls did not have the 6-month samples.

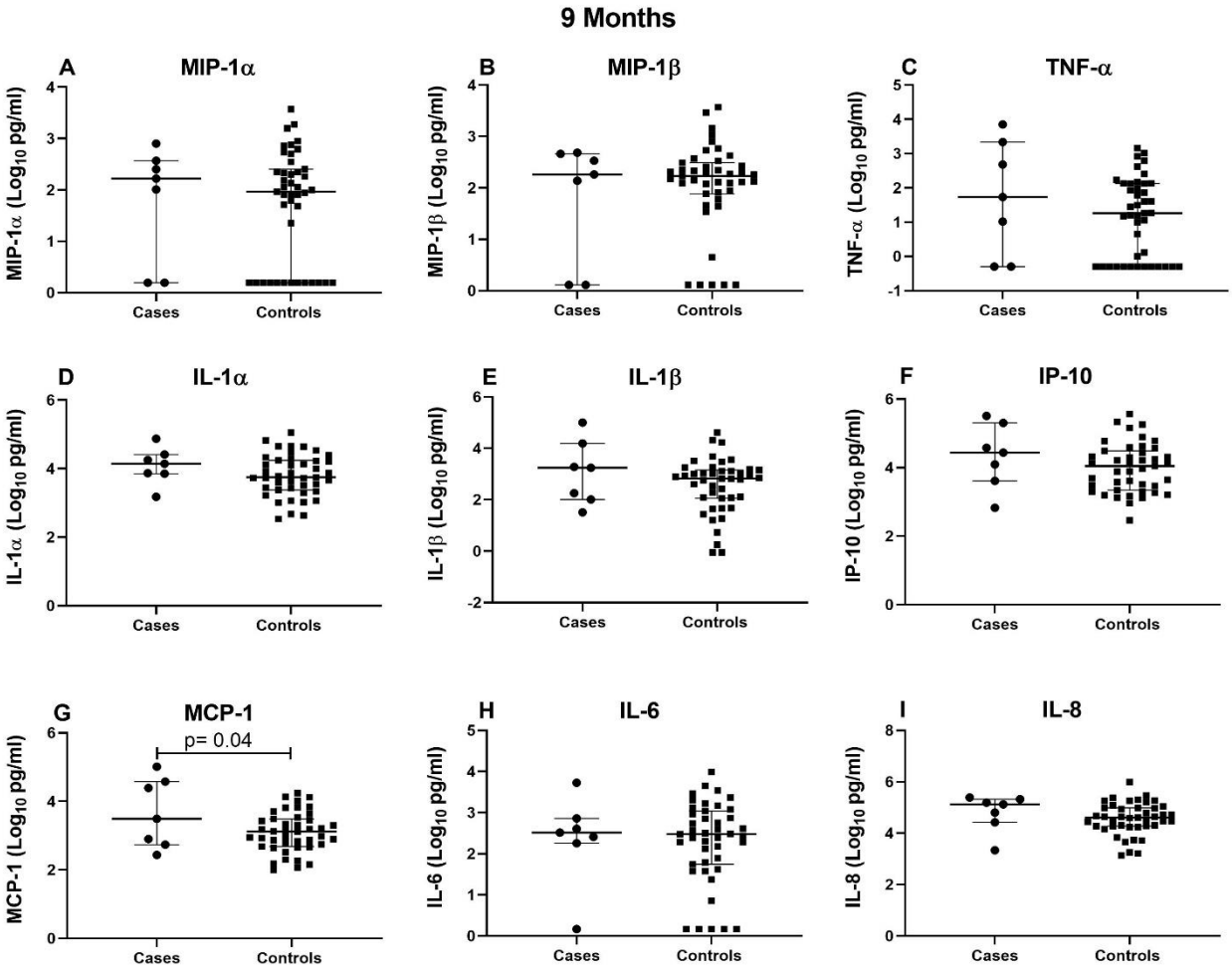

Figure 12.4: Cross-sectional analyses of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens between cases and controls at the 9-month visit [cases (n=7); controls (n=43)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Unpaired t-tests and the Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Five cases and five controls did not have the 9-month samples.

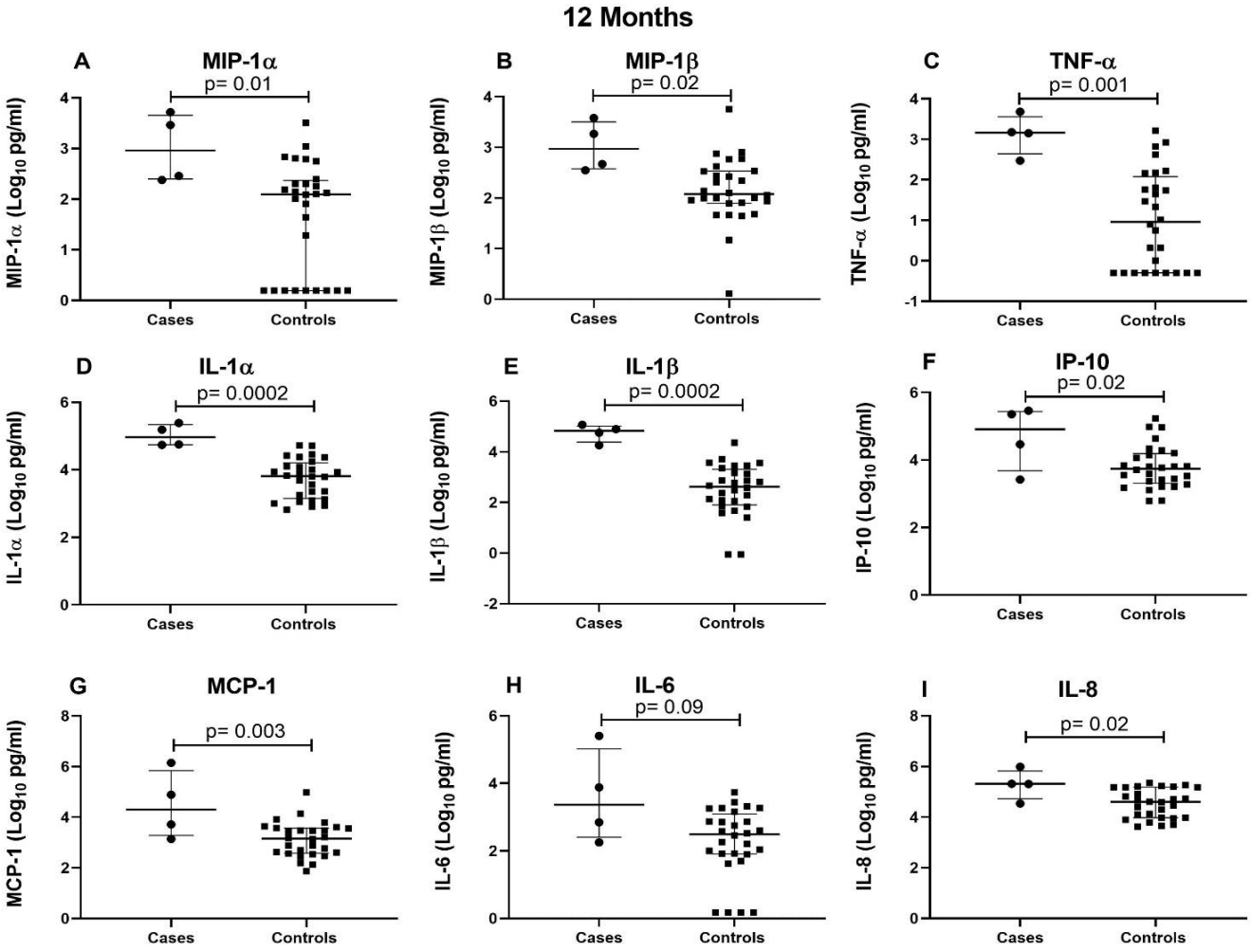

Figure 12.5: Cross-sectional analyses of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens between cases and controls at the 12-month visit [cases (n=4); controls (n=28)]. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Unpaired t-tests and the Mann-Whitney tests were used to compare the two groups, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eight cases and one control did not have the 12-month samples.

3.6.2 Intragroup analyses of cytokines in the genital tract of cases

Mucosal MIP-1α decreased while IL-1β was significantly increased over time

The cases were followed up longitudinally from the baseline up to the 12-month visit. The concentrations of genital cytokines (MIP-1β, TNF-α, IL-1α, IP-10, MCP-1, IL-6, and IL-8) at baseline were similar to the other time points (Figure 13B, C, D, F, G, H, and I). Only, MIP-1α was significantly lower at the 6-month visit [2.17 (1.81-2.57)] compared to the baseline time point [2.57 (2.29-3.09)] (p= 0.02) (Figure 13A). In contrast, IL-1β was significantly increased at the 12-month visit [4.83 (4.34-5.03)] compared to baseline [3.58 (3.35-3.97)], (p= 0.02) (Figure 13E).

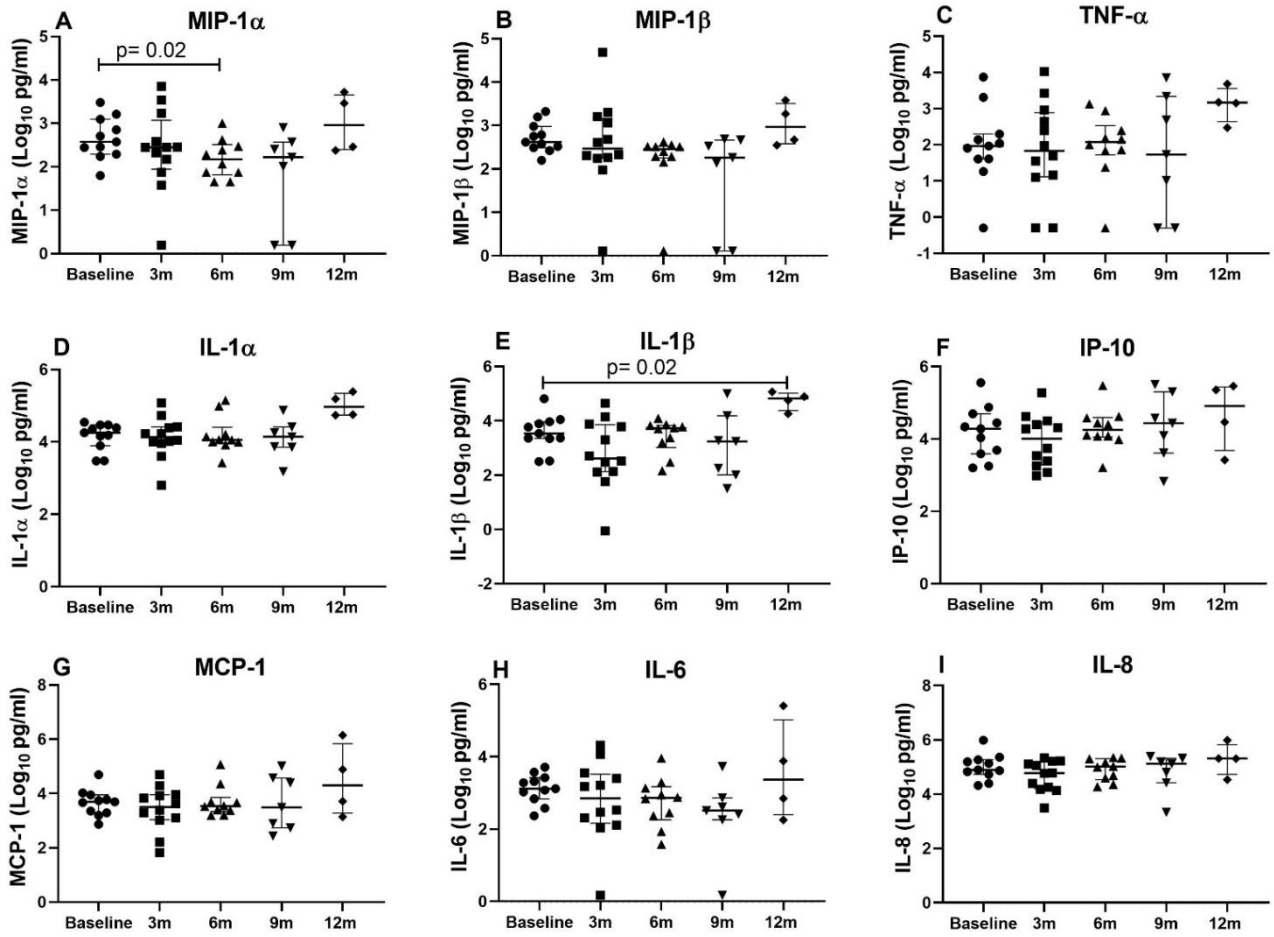

Figure 13: The longitudinal follow up of cases at baseline compared to various time points in the mucosal specimens for MIP-1α (Log_{10} pg/ml) (A), MIP-1β (B), TNF-α (C), IL-1α (D), IL-1β (E), IP-10 (F), MCP-1 (G), IL-6 (H), and IL-8 (I). At baseline (n=11), at 3-month (n=12), at 6-month (n=10), at 9-month (n=7), and at 12-month visits (n=4). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, cases represent women who subsequently became HIV infected. Paired t-tests were used to compare MIP-1α, MIP-1β, TNF-α, IL-1β, IP-10, MCP-1, IL-6, and IL-8 concentrations (Log_{10} pg/ml), and the Wilcoxon tests were used to compare IL-1α concentrations (Log_{10} pg/ml) within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One, two, five, and eight cases did not have the baseline, 6-; 9-, and 12-month samples, respectively.

3.6.3 Intragroup analyses of the controls in the genital tract

Majority of mucosal cytokines were significantly higher at the baseline compared to other time points in the controls

The similar longitudinal analyses were conducted in the controls and in the cases (Figure 14). MIP-1α was significantly higher at baseline [2.22 (1.96-2.77)] compared to the 6-month [2.19 (1.85-2.39)] (p = 0.04), 9-month [1.97 (0.19-2.41)] (p= 0.002), and the 12-month visits [2.10 (0.19-2.37)] (p= 0.001) (Figure 14A). Additionally, MIP-1β was significantly higher at baseline [2.48 (2.10-2.87)] compared to the 9-month [2.23 (1.89-2.49)] (p= 0.002) and the 12-month visits [2.08 (1.90-2.53)] (p= 0.002) (Figure 14B). Similarly, TNF-α was significantly higher at baseline [2.10 (1.11-2.82)] compared to the 6-month [1.26 (-0.30-2.16)] (p= 0.02), the 9-month [1.26 (-0.30-2.13)] (p= 0.002), and the 12-month

visits [0.96 (-0.30-2.08)] (p= 0.01) (Figure 14C). Furthermore, IL-1α was significantly higher at baseline [(4.02 (3.65-4.27)] compared to the 9-month [3.75 (3.38-4.24)] (p= 0.04), and trended to significance compared to 12-month visits [3.81 (3.15-4.21)] (p= 0.09) (Figure 14D). Significantly higher concentrations of IL-1β were observed at baseline [3.11 (2.58-3.98)] compared to the 6-month [2.52 (2.06-3.43)] (p= 0.02), the 9-month [2.82 (2.06-3.16)] (p= 0.01), and the 12-month visits [2.62 (1.90-3.31)] (p= 0.01) (Figure 14E). Elevated IP-10 concentrations were observed at baseline [4.16 (3.63-4.52)] compared to the 12-month visit [3.74 (3.31-4.19)] (p= 0.02) (Figure 14F). MCP-1 was significantly higher at baseline [3.61 (2.97-3.84)] in comparison to the 9-month [3.12 (2.68-3.49)] (p= 0.002), and the 12-month visits [3.16 (2.58-3.57)] (p= 0.01) (Figure 14G). In addition, the elevated IL-6 concentrations were observed at baseline [2.91 (2.56-3.46)] compared to the 3-month [2.59 (2.11-3.00)] (p= 0.03), the 6-month [2.66 (1.86-3.04)] (p= 0.01), the 9-month [2.48 (1.75-3.04)] (p= 0.002), and the 12-month visits [2.49 (1.91-3.09)] (p= 0.04) (Figure 14H). IL-8 trended to significance at the 9-month [4.61 (4.27-4.99)] (p= 0.06) and the 12-month visits [4.60 (3.98-5.18)] (p= 0.05) all compared to baseline [4.68 (4.39-5.21)], with the higher medians observed at baseline (Figure 14I).

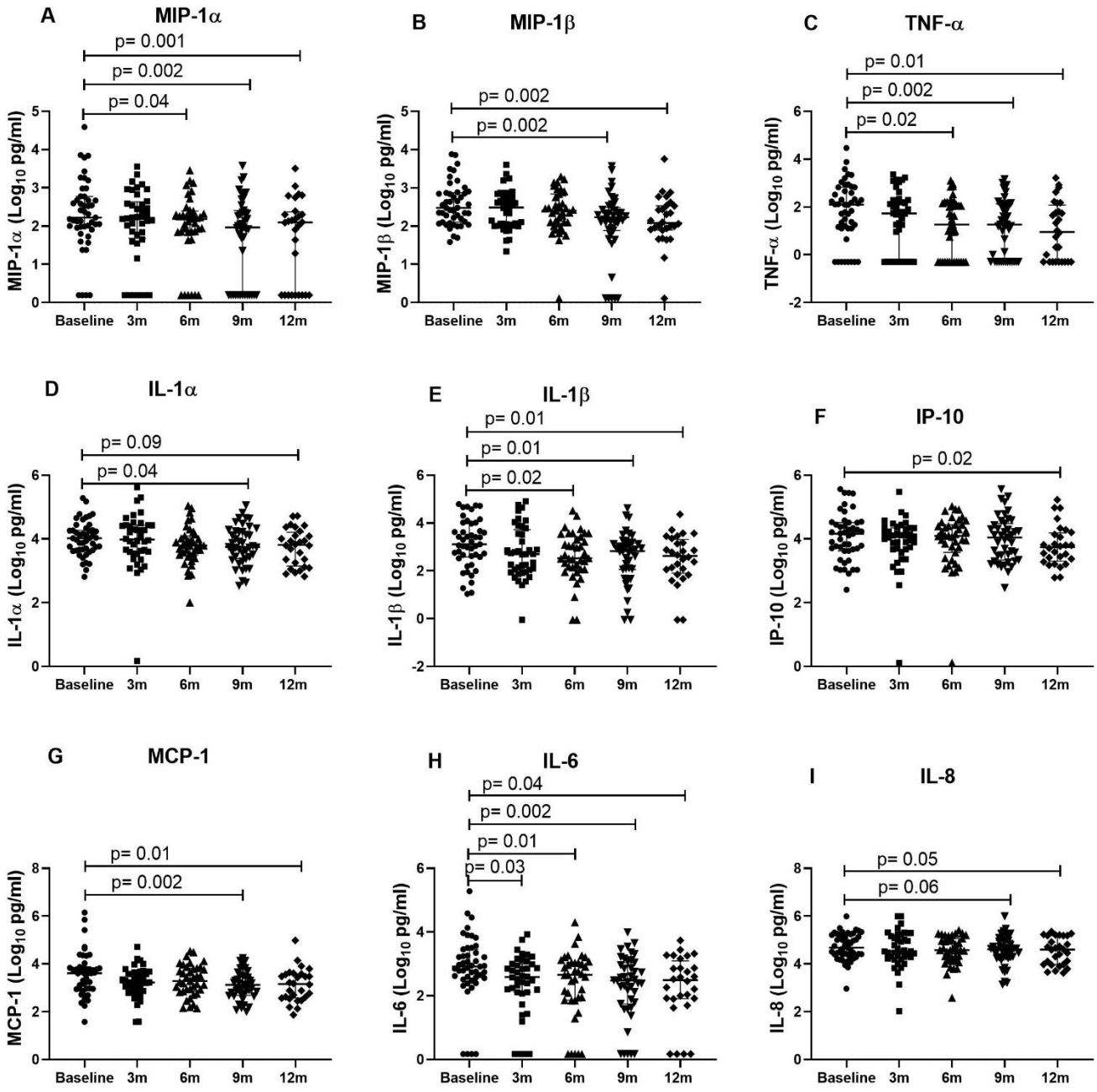

Figure 14: The longitudinal follow up of controls at baseline compared to various time points in the mucosal specimens for MIP-1α (Log_{10} pg/ml) (A), MIP-1β (B), TNF-α (C), IL-1α (D), IL-1β (E), IP-10 (F), MCP-1 (G), IL-6 (H), and IL-8 (I). At baseline (n=47), at 3-month (n=40), at 6-month (n=39), at 9-month (n=43), and at 12-month visits (n=28). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, cases represent women who subsequently became HIV infected. Paired t-tests were used to compare the MIP-1β, IL-1α, IL-1β, and IP-10 concentrations (Log_{10} pg/ml), and the Wilcoxon tests were used to compare MIP-1α,TNF-α, MCP-1, IL-6, and IL-8 concentrations (Log_{10} pg/ml) within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One, eight, nine, five, and twenty controls did not have the baseline, 3-; 6-; 9-, and 12-month samples, respectively.

3.6.4 Mucosal cytokine concentrations within cases (intragroup analyses) on PrEP and not on PrEP

PrEP use significantly altered genital tract IP-10 compared to non-PrEP users in the cases

Out of twelve cases, nine were on PrEP while three were not on PrEP. When comparing the mucosal cytokines among these cases at baseline, IP-10 was the only cytokine that was significantly lower among the cases on PrEP [4.04 (3.42-4.39)] compared to cases not on PrEP [5.22 (4.88-5.56)] (p= 0.04) (Supplementary Figure 17F). No further significant differences were found among cases on PrEP compared to the cases not on PrEP at the 3-month visit (Supplementary Figure 18). Furthermore, similar analyses for 6-; 9- and the 12-month visits were not possible, as missing data precluded these analyses.

3.6.5 Mucosal cytokine concentrations within controls (intragroup analyses) on PrEP and not on PrEP

Chemokine- MCP-1 was significantly higher among non-PrEP users when compared to participants on PrEP in the controls

Of the 48 controls, 19 did not take PrEP while 29 elected to take PrEP. The mucosal cytokine concentrations were analysed between these participants at different visits. At baseline, MCP-1 was significantly higher among non-PrEP users [3.81 (3.24-4.34)] compared to PrEP users [3.49 (2.75-3.77)] (p= 0.02) (Figure 15G). In addition, at baseline MIP-1α [2.53 (2.15-2.78)], IL-1α [4.13 (3.77-4.45)], IP-10 [4.21 (3.83-4.86)], and IL-6 [3.03 (2.77-3.57)] trended to significance with the higher medians in non-PrEP users compared to the PrEP users [MIP-1α: 2.03 (1.75-2.74) (p= 0.08); IL-1α: 3.87 (3.62-4.25) p= 0.10; IP-10: 3.81 (3.12-4.40) p= 0.06, and IL-6: 2.82 (2.39-3.24) p= 0.07] (Figure 15 A, D, F, and H). Among controls on PrEP compared to the controls not on PrEP, MIP-1β (Figure 15B), TNF-α (Figure 15C), IL-1β (Figure 15E), and IL-8 (Figure 15I) were similar.

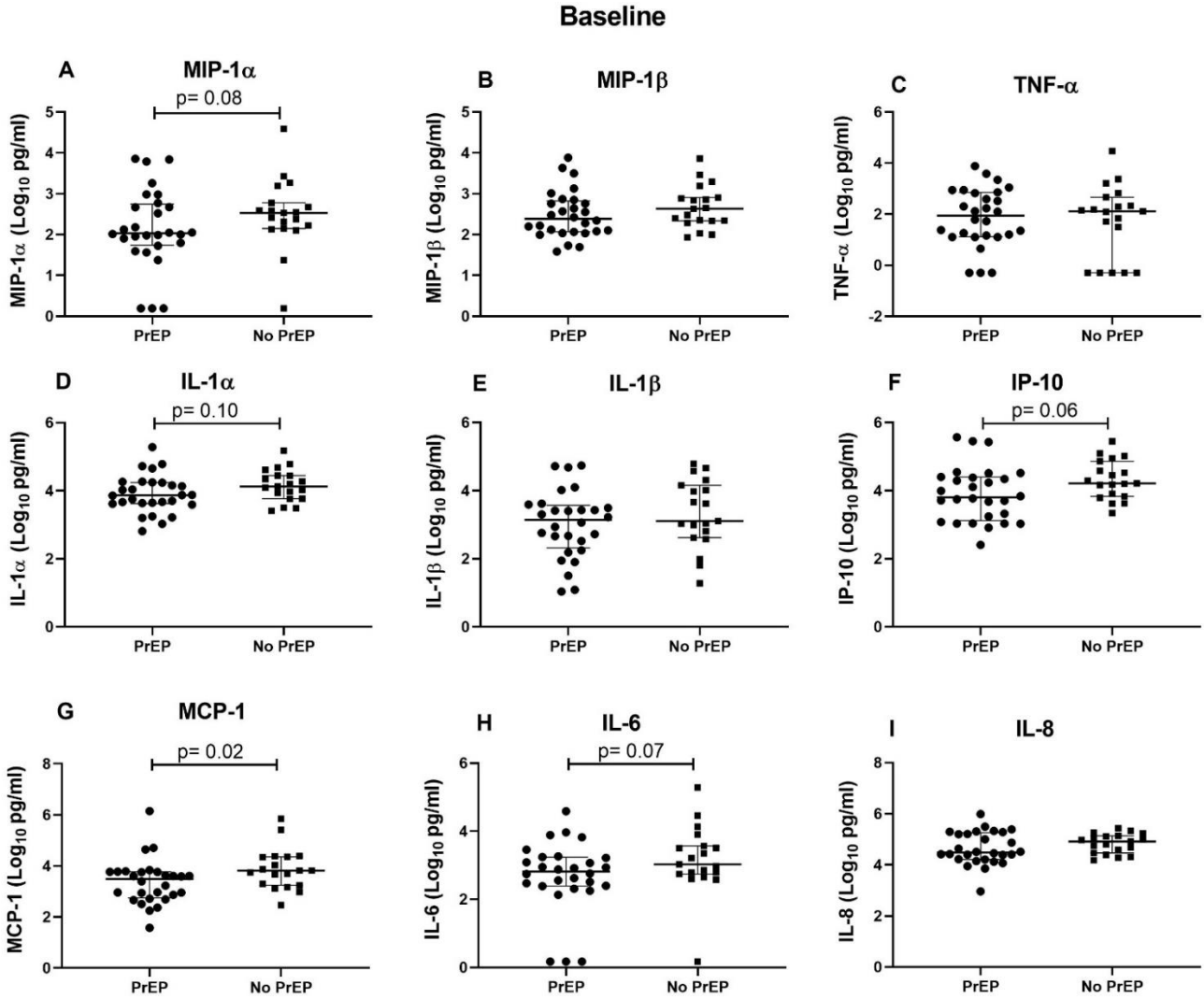

Figure 15: The comparison of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens within the controls using PrEP (n=29) and not using PrEP (n=19) at baseline. MIP-1α (A), MIP-1β (B), TNF-α (C), IL-1α (D), IL-1β (E), IP-10 (F), MCP-1 (G), IL-6 (H), and IL-8 (I). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, cases represent women who subsequently became HIV infected. Mann-Whitney tests were used to compare MIP-1α, TNFα, MCP-1, and IL-6 concentrations (Log_{10} pg/ml), and the Unpaired t-tests were used to compare MIP-1β, IL-1α, IL-1β, IP-10, and IL-8 concentrations (Log_{10} pg/ml) within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done.

Mucosal MCP-1 and IP-10 were significantly affected by PrEP use when compared in the controls

At the 3-month visit, IL-1α trended to significance with the higher median observed among participants not using PrEP [4.29 (3.73-4.48)] compared to participants who were using PrEP [3.87 (3.24-4.37)] (p= 0.07) (Figure 16A). Similarly, at the 6-month visit, IP-10 was significantly higher among participants not using PrEP [4.42 (4.00-4.70)] compared to participants using PrEP [3.92 (3.22-4.25)] (p= 0.009) (Figure 16B). In addition, MCP-1 was significantly higher among women who were not using PrEP [3.70 (3.07-4.03)] compared to participants using PrEP [3.12 (2.71-3.66)] (p= 0.04) (Figure 16C). There were no significant differences observed at the 9-month visit (Supplementary Figure 19).

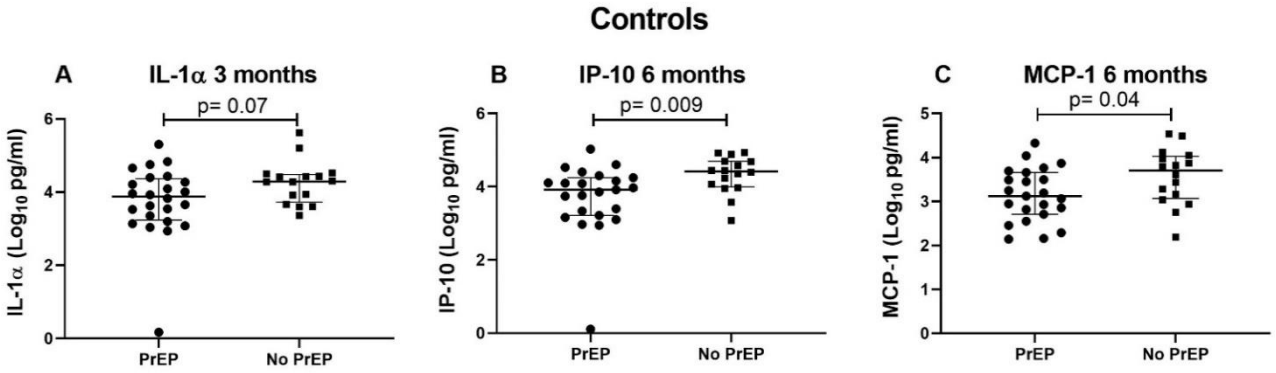

Figure 16: The comparison of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens within the controls using PrEP (n=24) and not using PrEP (n=16) at the 3-month (A) and the controls using PrEP (n=23) and not using PrEP (n=16) at the 6–month visits (B and C). IL-1α (A), IP-10 (B), and MCP-1 (C). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Mann-Whitney tests were used to compare IL-1α, and IP-10 concentrations (Log_{10} pg/ml) within the controls and the Unpaired t-tests were used to compare MCP-1 concentrations (Log_{10} pg/ml) within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Five controls on PrEP and three controls not on PrEP did not have the 3-month samples. In addition, six controls on PrEP and three controls not on PrEP did not have the 6-month samples.

In the controls, PrEP use significantly affected mucosal IP-10, MCP-1, and IL-8 compared to participants not on PrEP

At the 12-month visit, significantly elevated IP-10 concentrations were observed among participants not using PrEP [4.43 (3.71-4.98)] compared to participants using PrEP [3.55 (3.21-3.83)] (p= 0.004) (Figure 17B). Similarly, MCP-1 was significantly higher among participants not on PrEP [3.55 (3.21-3.80)] compared to PrEP users [2.88 (2.51-3.48)] (p= 0.03) (Figure 17C). IL-8 was also significantly higher among participants not on PrEP [5.18 (4.61-5.23)] compared to PrEP users [4.42 (3.40-4.77)] (p= 0.04) (Figure 17D). In addition, MIP-1β trended to significance with the higher median observed from no PrEP group [5.18 (4.61-5.27)] compared to participants using PrEP [4.41 (3.97-4.77] (p= 0.07) (Figure 17A).

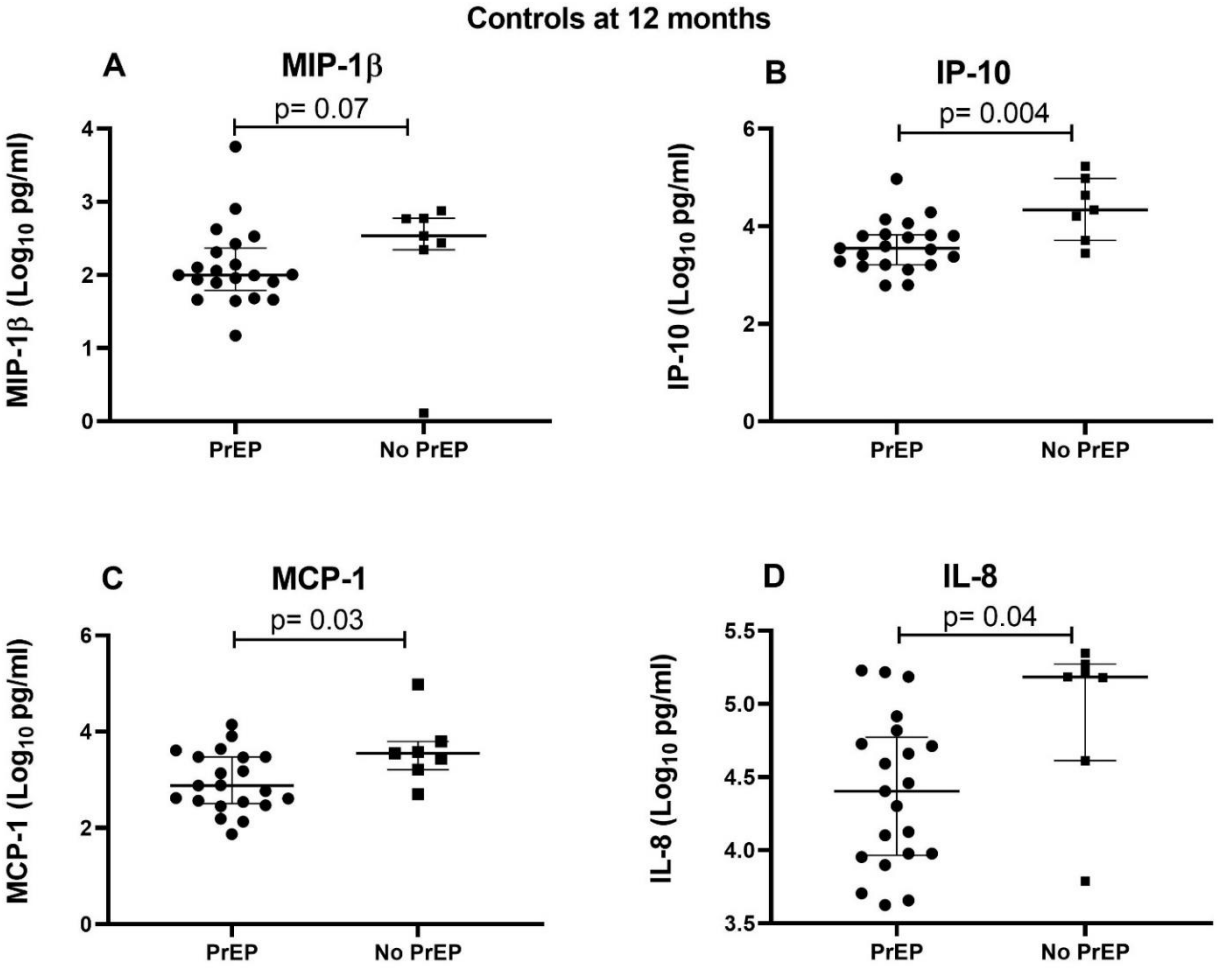

Figure 17: The comparison of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens within the controls using PrEP (n=21) and not using PrEP (n=7) at the 12-month visit. MIP-1β (A), IP-10 (B), MCP-1 (C), and IL-8 (D). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Mann-Whitney tests were used to compare MIP-1β, and IL-8 concentrations (Log_{10} pg/ml) within the controls and the Unpaired t-tests were used to compare IP-10 and MCP-1 concentrations (Log_{10} pg/ml) within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eight controls on PrEP and twelve controls not on PrEP did not have the 12-month samples.

3.7 The association between the plasma and the mucosal IgG subclasses/isotypes and HIV outcome

Mucosal IgM was significantly associated with increased risk of HIV acquisition

To determine if immunoglobulin titres could predict HIV infection, a generalized estimating equation (GEE) using the Quasi-likelihood information criterion (QIC) was conducted. In the plasma, no associations were observed between the various IgG subclasses/isotypes and HIV outcomes. However, in the genital tract, a 1log (ng/ml) increase in IgM was significantly associated with a 43% increased chance of HIV seroconversion after adjusting for the number of study visits (p= 0.01) (data not shown). In addition, linear mixed model analyses were conducted to determine the impact of the genital tract cytokines on mucosal antibody response (Table 5) irrespective of HIV infection status and PrEP use.

3.8 The association between the mucosal cytokines and IgG subclasses and isotypes at the baseline irrespective of HIV status and PrEP use

Mucosal pro-inflammatory IL-8 significantly associated with IgG1, IgG2, total IgG, and IgM

IL-8 levels were significantly associated with IgG1, IgG2, total IgG, and IgM levels. For every 1 pg/ml increase in IL-8, IgG1, IgG2, total IgG, and IgM increased by 0.80 ng/ml ($p< 0.001$), 0.55 ng/ml ($p< 0.001$), 0.65 ng/ml ($p< 0.001$), and 0.69 ng/ml ($p< 0.001$), respectively (Table 5). Similarly, IP-10 levels were significantly positively associated with IgG4, total IgG, and IgA ($p< 0.001$ for all) (Table 5). In addition, chemotactic MIP-1β showed a significant positive association with IgA ($p< 0.001$) (Table 5). Also, IL-6 levels were significantly positively associated with IgG2 and IgM levels ($p< 0.001$ for both). Moreover, EGF levels, which is a growth factor in the epithelium, were significantly positively associated with IgG3, IgG4, and IgA levels ($p\leq 0.002$) (Table 5). G-CSF and IL-9 showed a significant positive association with IgG1 ($p< 0.001$) and IgG3 ($p= 0.02$), respectively. However, eotaxin was negatively associated with IgG1 ($p= 0.003$) but positively associated with IgG4 ($p< 0.001$) (Table 5).

Table 5: Linear mixed model reflecting the association between the mucosal cytokines and IgG subclasses and isotypes at the baseline

Cytokine	Beta Estimates ng/ml	Confidence interval	*p*-value
		IgG1	
IL-8	0.80	0.54 to 1.05	<0.001
Eotaxin	-0.45	-0.75 to -0.15	0.003
G-CSF	0.47	0.24 to 0.70	<0.001
		IgG2	
IL-8	0.55	0.42 to 0.67	<0.001
IL-6	0.21	0.14 to 0.29	<0.001
		IgG3	
EGF	0.60	0.38 to 0.81	<0.001
IL-9	0.19	0.04 to 0.35	0.02
		IgG4	
EGF	0.49	0.18 to 0.81	0.002
Eotaxin	0.63	0.29 to 0.98	<0.001
IP-10	0.69	0.45 to 0.93	<0.001
		Total IgG	
EGF	0.10	-0.08 to -0.29	ns
IL-8	0.65	0.45 to 0.85	<0.001
IP-10	0.29	0.12 to 0.46	<0.001
		IgM	
IL-6	0.46	0.33 to 0.58	<0.001
IL-8	0.69	0.49 to 0.88	<0.001
		IgA	
MIP-1β	0.22	0.10 to 0.34	<0.001
EGF	0.25	0.09 to 0.40	0.002
IP-10	0.50	0.38 to 0.62	<0.001

Abbreviations: IL, interleukin; TNF, tumor necrosis factor; IP-10, interferon-γ inducible protein-10; MCP1, monocyte chemoattractant protein-1; MIP-1, macrophage inflammatory protein-1; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor. One-Way ANOVA was used for statistical analyses and $p<0.05$ were considered statistically significant. The p-values were not adjusted. ns refers to not significant.

CHAPTER FOUR

DISCUSSION

CONCLUSIONS &

FUTURE DIRECTIONS

4. DISCUSSION

Since most HIV infections in women occur through heterosexual transmission, it is important to investigate both the mucosal and systemic immune responses in parallel. In addition, with the widespread use of PrEP, understanding its effect on mucosal and systemic immunity is important for informing on prevention strategies where PrEP and HIV vaccines may be given alone or in combination for added efficacy. We tested the hypothesis that increased genital tract antibodies and cytokines pre-HIV infection predict HIV acquisition in seroconverters compared to non-seroconverters irrespective of PrEP. Analyses of the immune responses in these two compartments namely, blood and FGT provides important insights into their interplay and how they are regulated. In addition, such studies provide clues towards identifying the biomarkers that may predict HIV acquisition. Therefore, in this sub-study, we aimed to assess biological markers such as immunoglobulins and cytokines pre-and post-HIV infection among at risk South African women, in the blood and the FGT, the primary site for heterosexual HIV transmission.

Understanding the distribution and kinetics of circulating immunoglobulins in the presence or absence of HIV infection over time is important. We found higher plasma IgA amongst the cases compared to the controls at the 12-month visit post-infection suggesting that HIV itself may have driven higher responses for this isotype. In contrast, in the study by Mestecky et al., (2011), plasma IgA levels were similar among the HIV infected individuals compared to highly exposed but persistently seronegative (HESN) women, and the total IgG and IgM were significantly higher in HIV-1-infected women when compared to HESN women (Mestecky et al., 2011). We conducted a similar analysis in the genital tract because previous data showed that IgG rather than IgA predominates in the genital tract (Bélec et al., 1995, Wei et al., 2012). These analyses in the genital tract revealed significantly higher mucosal IgG2 and the total IgG in the cases compared to the controls at the 12-month visit. The strong and significantly elevated mucosal IgG2 levels likely also drove the significant difference between the cases and the controls for the total IgG. These data corroborate Mestecky et al., (2011) who reported the significantly high levels of mucosal total IgG among the HIV positive individuals compared to HESN women (Mestecky et al., 2011). Previous studies have reported that IgA plays an important role in mucosal immunity and is the predominant isotype in the gastrointestinal, respiratory tracts [as reviewed by (Mashoof and Criscitiello, 2016)], breast milk, tears, and saliva (Cerutti et al., 2011, Cerutti and Rescigno, 2008, Fagarasan, 2008). However, IgA is not predominant in the cervico-vaginal secretions of the FGT. These findings are in keeping with our data because the IgA was elevated in plasma and not in the genital tract.

In addition to the cross-sectional analysis, we then did the longitudinal follow up of the immunoglobulins over time. Within the cases, HIV infection affected IgA with significantly decreased circulating levels during the primary phase of infection. When similar analyses were done within the controls, plasma IgG2, IgG3, total IgG, and IgM significantly decreased at the 12-month visit into the

study relative to the baseline. Within the cases, mucosal IgG2 and the total IgG significantly declined over time and is consistent with the general decrease of this subclass (McGowan et al., 2006, Raux et al., 2000). However, at the 12-month visit into the study, IgG2 significantly increased relative to the baseline time point. Ngo-Giang-Huong et al., (2001) previously reported reduced viral loads and elevated CD4+ T cells with gp41-specific IgG2 and p55- and p24-specific IgG2 (Ngo-Giang-Huong et al., 2001). However, in our study, we did not conduct any HIV-specific subclass antibody assays and therefore we do not know whether the elevated IgG2 is related to a decrease in viral load or an increase in CD4+ T cell count. All the mucosal IgG subclasses significantly declined at the 9-month visit into the study relative to the baseline levels, in the controls. Collectively, the significant decreases were observed in both the plasma and the mucosal levels of the IgG subclasses and isotypes and may reflect how the compartments mirror each other, likely indicating the systemic-mucosal axis for humoral responses.

IgG2 in the cases and IgG4 in both the cases and the controls showed strong and significant direct correlations between the plasma and the mucosal compartment whereas suggesting that these immunoglobulins are reflected between the compartments. These data corroborate findings from our group as we previously found highly significant direct correlations for HIV-specific IgG between the blood and the genital tract (Archary et al., 2015, Archary et al., 2016, Mkhize et al., 2016); this might suggest that IgG4 is the strong predictor of correlation between the two compartments irrespective of HIV status. However, whether PrEP use may have altered/impacted these immunoglobulins in the plasma and the genital tract remains less defined.

In this study, some participants elected to take PrEP while others did not. Amongst the cases, nine of 12 elected to take PrEP while three of 12 did not. In the control group, 29 of 48 were on PrEP while 19 of 48 were not on PrEP. We hypothesized that increased genital tract antibodies and cytokines pre-HIV infection predicted HIV acquisition in seroconverters compared to non-seroconverters irrespective of PrEP. In our study, elevated plasma IgG1, IgG3, IgM, and IgA were found amongst the cases on PrEP when compared to the cases not on PrEP at the 9-month visit into the study. Previous studies have reported that PrEP modulates immunity in healthy individuals (Castillo-Mancilla et al., 2015) and can impact the avidity and binding affinities of antibodies in the post seroconversion stage (Laeyendecker et al., 2015). However, in the present study, the immunoglobulins were higher among PrEP users post seroconversion suggesting that HIV itself drove these responses. IgG1 and IgG3 have specific functions that are highly effective including complement activation, direct neutralization, and Fc receptor-mediated non-neutralizing antibody functions (Lu et al., 2007, Richardson et al., 2018, Richardson et al., 2019). IgG3 is highly effective through its characteristic long hinge region that strongly mediates ADCC, complement activation, and ADCP (Lu et al., 2007, Richardson et al., 2019). During HIV infection, the IgM levels increase as a compensatory mechanism to reduce inflammation by inhibiting T cell activation and pro-inflammatory cytokine production (Lobo et al., 2012). IgM can also perform

diverse functions by blocking the CCR5 co-receptor on target CD4 T cells thereby inhibiting HIV infection (Lobo et al., 2012, Lobo et al., 2008a, Lobo et al., 2008b) or effectively neutralizing SHIV with HIV-specific IgM and conferring protection against mucosal SHIV transmission (Gong et al., 2018). *In vitro,* systemic and mucosal IgA had broadly neutralizing properties, which were postulated to confer *in vivo* protection to HIV infection (Jia et al., 2020). However, whether the elevated IgAs in the cases on PrEP were able to neutralize the virus remains undefined. The higher systemic IgA in cases on PrEP even post-seroconversion is also a point of interest. Lund et al., (2016) reported elevated genital HIV-1-neutralizing IgAs responses in HIV exposed seronegative women in serodiscordant relationships on oral PrEP compared to the women not on oral PrEP (Lund et al., 2016). Collectively, these data may indicate that oral PrEP impacts the magnitude of circulating IgG subclasses or isotypes (IgM and IgA), in the post-seroconversion stage.

In the original observational study, PrEP use was inconsistent amongst the women that seroconverted and the elevated or increased plasma IgG subclasses and isotypes were in response to HIV infection. However, these findings need to be verified with larger sample size. In contrast, among the controls, high levels of plasma IgG3 and IgG4 were observed amongst the PrEP users compared to non-PrEP users, however, the role of PrEP in increasing circulating levels of these subclasses remain undefined. For the mucosal levels of immunoglobulins, no differences were found between women on PrEP or not on PrEP irrespective of their HIV infection status suggesting that oral PrEP may not affect the mucosal humoral immune response.

Genital inflammation is an established risk factor for HIV acquisition in women (Masson et al., 2015). In the present study, of the 28 cytokines and to satisfy the definition of inflammation, five mucosal chemotactic cytokines: MIP-1α, MIP-1β, IP-10, MCP-1, IL-8, and four inflammatory cytokines: TNF-α, IL-1α, IL-1β, and IL-6 were investigated in the cases and the controls. The mucosal chemotactic cytokines MIP-1α, MIP-1β, IP-10, MCP-1, IL-8, and inflammatory cytokines TNF-α, IL-1α, and IL-1β significantly increased at the 12-month visit into the study among the cases when compared to the controls. Furthermore, mucosal inflammatory IL-6 was also increased at the 12-month into the study among the cases when compared to the controls. The increase in IL-6 is not surprising. IL-6 expression is activated by IL-1β and TNF-α [as reviewed by (Hunter and Jones, 2015)]. IL-6 also regulates T cells, can prompt Th17 cell development and function, inhibit regulatory T cells, and promote the development of pro-inflammatory CD4 T cell responses [as reviewed by (Jones and Jenkins, 2018)]. TNF-α enhances the permeability of the endothelial cells and promotes the recruitment of monocytes, lymphocytes, and neutrophils to the site of inflammation (Mackay et al., 1993, Pasparakis et al., 1996). IP-10 in the plasma is upregulated with HIV infection and is associated with HIV disease progression [as reviewed by (Lei et al., 2019)]. We had similar findings of elevated mucosal IP-10 only at 12 months into the study and this coincided with most women having seroconverted already and likely experiencing disease pathogenesis. IP-10, MIP-1α, and MIP-1β are chemotactic for the dendritic cells,

monocytes, T cells, and the macrophages (Garlet et al., 2003). MIP-1α and MIP-1β specifically bind to the HIV co-receptor CCR5 and recruit the CCR5+ target cells that enhance the HIV infection [as reviewed by (Mueller and Strange, 2004)]. IP-10 is pivotal for the trafficking of CXCR3+ leukocytes and homing to inflamed tissues, and perpetuates the inflammation resulting in tissue damage [as reviewed by (Liu et al., 2011)]. Liebenberg et al., (2017) confirmed that mucosa-biased gradients or elevated genital concentrations relative to the blood of IP-10, MIP-1β, IL-8, and MCP-1 are associated with increased HIV risk among women (Liebenberg et al., 2017). IL-8 is a chemotactic factor for HIV target cells including basophils, NK cells, T cells, and neutrophils [as reviewed by (Harada et al., 1994)]. In addition, elevated inflammatory cytokines in the FGT are associated with altered mucosal barrier proteins, protease expression, and an influx of HIV-specific target cells (Arnold et al., 2016). The global expression of elevated cytokines fitting the definition of genital inflammation is linked to increased immune cell activation subsequently increasing the risk for HIV acquisition. Immune activation facilitates HIV infection and viral replication to promote the establishment of infection at the mucosa and subsequent dissemination of the virus (Miller et al., 2005).

In addition to the comparison between the cases and the controls, the mucosal cytokines were also followed up longitudinally within the cases and the controls. MIP-1α and IL-1β were significantly increased in cases whereas in the controls; MIP-1α, MIP-1β, TNF-α, IL-1β, IP-10, MCP-1, and IL-6 significantly decreased at 12 months relative to the baseline levels. When the data were further stratified for PrEP use in the controls, IP-10, MCP-1, and IL-8 significantly decreased over time with PrEP use. Collectively, these data suggest that PrEP can significantly alter the profile of cytokines in the genital compartment.

Other factors can also affect the genital cytokine concentrations. These include STIs and BV (Mlisana et al., 2012), the number of sexual partners and hormonal contraceptive use particularly DMPA (Deese et al., 2015, Molatlhegi et al., 2020). However, in our study, we did not account for hormonal contraception use. Recently, Molatlhegi et al., (2020) reported elevated DMPA levels to be associated with low genital tract cytokines (GCSF, MCSF, IL-16, CTACK, LIF, IL-1α, and SCGF-β) (Molatlhegi et al., 2020). In contrast, Deese et al., (2015) reported elevated levels of MIP-1α, MIP-1β, IL-6, IL-8, IP-10, and RANTES concentrations among the DMPA users (Deese et al., 2015). Furthermore, Francis et al., (2016) reported high genital tract pro-inflammatory cytokines (IL-1α, IL-1β, IL-2, MIP-1β, IP-10, IL-8, and TGF-β), beta-defensins, IgA, IgG1, and IgG2 among DMPA users (Francis et al., 2016). Collectively, these data suggest that DMPA may be linked with increased HIV risk through an elevated inflammatory cytokine profile in the genital mucosal environment. In addition to DMPA, endogenous hormones might influence the cervico-vaginal cytokine profile during the different stages of the menstrual cycle (Boily-Larouche et al., 2019).

Cytokines play important roles in B cell function such as antibody production [as reviewed by (Vazquez et al., 2015)]. Certain inflammatory cytokines and chemokines play an essential role in Ig isotype and IgG subclass switching (Avery et al., 2008). In semen, pro-inflammatory cytokines and chemokines (IL-6, IL-8, and MCP-1) were associated with high levels of IgG1 and total IgG whereas IgG2 and IgM had a positive correlation with MCP-1 and IL-6 concentrations, respectively (Pillay et al., 2019). In the present study, IL-6 levels were significantly associated with IgG2 and IgM levels. IL-6 may not be pivotal for IgG class switching but it does enhance the differential production of IgG subclasses (Kawano et al., 1994). IL-6 also leads to B cell differentiation, proliferation, and activates B cell to produce IgG, IgM, and IgA antibodies (Urashima et al., 1996, Yasukawa et al., 1987). Our data also shows a significant association between IgM and IL-6. As previously mentioned the increase in IgM appears to be a compensatory drive during HIV infection to reduce inflammation and perhaps inhibit T cell activation and further pro-inflammatory cytokine production (Lobo et al., 2012). Besides the effect of IL-6 on B cells, IL-6 is also involved in the development of cytotoxic T cells and induces their activity (Jones et al., 2018). In addition, IL-6 can simultaneously induce the production of Th17 cells, a subset of CD4 T cells that are prime targets for HIV infection in the genital tract and inhibit regulatory T cells (Jones et al., 2018). In a study on mice, chemotactic MIP-1β enhanced both humoral and cellular mucosal immunity responses (Lillard et al., 2003). The significant association of IgA with MIP-1β in our study may similarly reflect the elevated antigen-specific mucosal secretory IgA, which is tracked with MIP-1β (Lillard et al., 2003). In the present study, the magnitude of IgG1, IgG2, total IgG, and IgM were directly associated with IL-8. IL-8 can elicit other functions, IL-8 was shown to activate and recruit neutrophils (Swensson et al., 1991) which could then lead to phagocytosis through IgG opsonizing HIV. IL-9 regulates T cell dependent B cell differentiation (Fawaz et al., 2007). Our study found the magnitude of IgG3 levels to be significantly positively associated with IL-9. IgG3 plays a crucial role in triggering the effector functions such as ADCC, ADCP, and complement activation [as reviewed by (Damelang et al., 2019)], which are crucial for virus clearance. Previously, elevated mucosal IP-10 was associated with an increased risk of HIV acquisition (Liebenberg et al., 2017). The significant association between IP-10 and IgG4 and IgA levels suggest that IP-10 can locally affect subclass as well as isotype. Although IgA can directly neutralize HIV virions (Jia et al., 2020), the protective effect of mucosal IgA in this study remains undefined.

IgG1 showed significant direct associations with G-CSF. G-CSF has been shown to directly affect B cell development and likely antibody production (Winkler et al., 2013). The lack of IgG1 has been linked with susceptibility to HIV infection (Vidarsson et al., 2014), underscoring the importance of this subclass to be present in sufficient levels to confer protection from HIV. The findings of a negative association between eotaxin and IgG1 and the positive association with IgG4 is challenging, the function of IgG4 and the significance of this subclass is not fully defined. Together, these data suggest that cytokines may exert or affect IgG subclasses differentially and whether we can use such subclasses

and isotypes as a proxy for the profiles of cytokines in the genital tract milieu to understand the local mucosal inflammation status remains less well defined.

The present study has several limitations. Firstly, the small sample size precluded certain statistical analyses. In addition, there were missing data points, further affecting the analyses. Some of the women on the PrEP arm did not take PrEP consistently; as a result, the majority of the women on PrEP seroconverted compared to women not on PrEP. These findings in itself may suggest that women in the PrEP group behave differently to women who did not take PrEP. In addition, measurements of PrEP in the blood were not done on all women, and PrEP adherence data relied on pill count and self-reporting. Therefore, there was no accurate assessment for PrEP adherence. Even though higher levels of plasma IgG subclasses and isotype were found in the post-seroconversion stage of the PrEP users, we did not perform mechanistic studies to define the functions of these elevated antibodies. The data we obtained from the mucosal antibodies and cytokines did not account for the variation of these immune responses with the different stages of the menstrual cycle and the effect of exogenous hormones. Despite these limitations, we have matching blood and mucosal specimens with longitudinal follow-up in a cohort of at risk South African women from KwaZulu-Natal. In addition, we have collected mucosal specimens through the Softcup collection method, which allowed for the robust detection of mucosal antibodies and cytokines.

4.1 Conclusions

The plasma immunoglobulins were similar between the cases and the controls in all the time points, however, IgA trended to a significance with a higher median observed among the cases post-seroconversion. The increase in IgA among the cases may be due to its neutralizing activity against the virus. Mucosal IgG2 was higher among the cases post-infection when compared to the controls, increase in IgG2 might be driven by HIV since the IgG2 subclass has been previously reported to control the viral load and is associated with elevated CD4+ T cell count. However, in this study, we did not conduct the HIV specific Ig isotypes and/or IgG subclass assay; therefore, it is not known whether the increase in IgG2 is related to a reduced viral load and/or increased CD4+ T cell count. The increasing trend of plasma IgG3 in women who used PrEP suggests that oral PrEP affects the expression of this highly versatile and functional IgG subclass. IgG3 is particularly important since it is the first subclass produced following infection and strongly mediates effector functions. We found mucosal IgM to be associated with four-fold increase risk of HIV infection. These data suggest that IgM may be used as a biomarker to predict HIV infection. Higher chemotactic and inflammatory cytokines observed in the cases when compared to the controls suggest that HIV infection itself skewed these responses. In addition, the baseline pro-inflammatory IL-8 significantly correlated with IgG1, IgG2, total IgG, and IgM, underscoring its association between the mucosal profile of these Ig isotypes and IgG subclasses with this cytokine irrespective of HIV status or PrEP use. Reduced genital cytokine concentrations were

found with PrEP use among the cases and the healthy controls. In the cases, IP-10, the only cytokine significantly modulated in PrEP users compared to no-PrEP users, suggests that PrEP use modulates this pleiotropic, chemotactic cytokine. Collectively, these data suggest that PrEP can significantly modify the profile of immunoglobulins and cytokines in the genital and systemic compartments. As PrEP uptake increases, its effect on mucosal and systemic immunity is important for informing on prevention strategies where PrEP and HIV vaccines may be given in combination for added efficacy.

4.2 Future Directions

In future studies, our data provide evidence that the IgM isotype needs to be interrogated longitudinally in the genital tract and in a larger cohort offered PrEP to verify whether this isotype can be used as a proxy for HIV infection risk. Based on these findings, IgM could be used as a biomarker to predict the HIV infection risk in women. To establish robust biomarkers for inflammation, both cytokines and immunoglobulins need to be measured in parallel, and in large cohorts, prior to and post-seroconversion to determine the interplay of the cytokine milieu and the local expression of immunoglobulins. In addition, future studies should look at the association between the activation status of CD4+ target T cells, the Th17 cell subset and the quantity of IgM in the genital tract to understand whether IgM is a compensatory mechanism for immune activation and inflammation. In so doing, it may be established if mucosal IgM alone can be used as an immune correlate of risk. Furthermore, future studies should assess the cervical mucus for mucins such as MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6 to understand their interactions with mucosal antibodies. Future studies should also try defining the role and dynamics of FGT microbiome and epithelial barrier function in relation to the mucosal system. In addition, the social-behavioural characteristics of the cohorts should be interrogated in order to better understand the drivers of PrEP uptake. Together, these future investigations will provide a road map for prevention studies to better identify immune correlates in at risk African women who remain particularly vulnerable to HIV infection.

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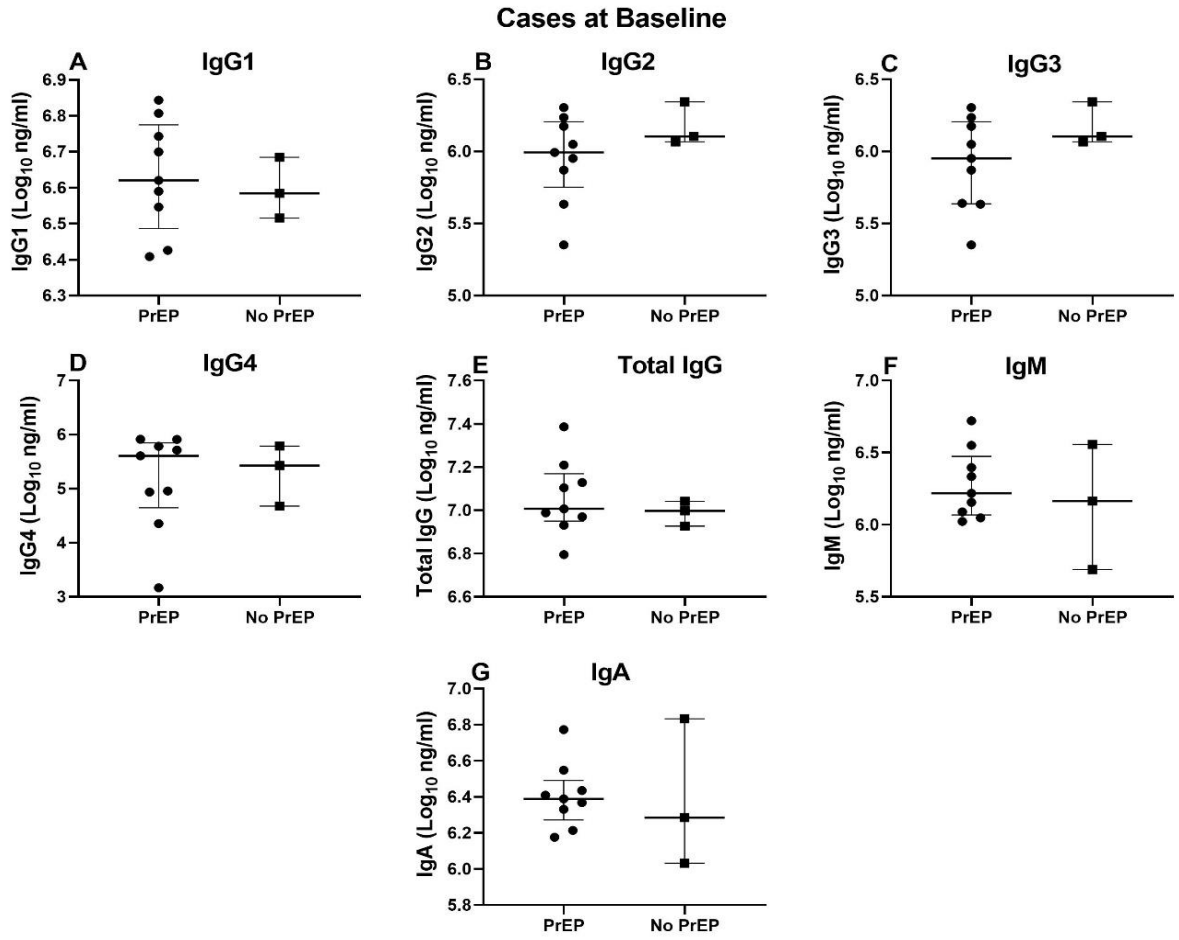
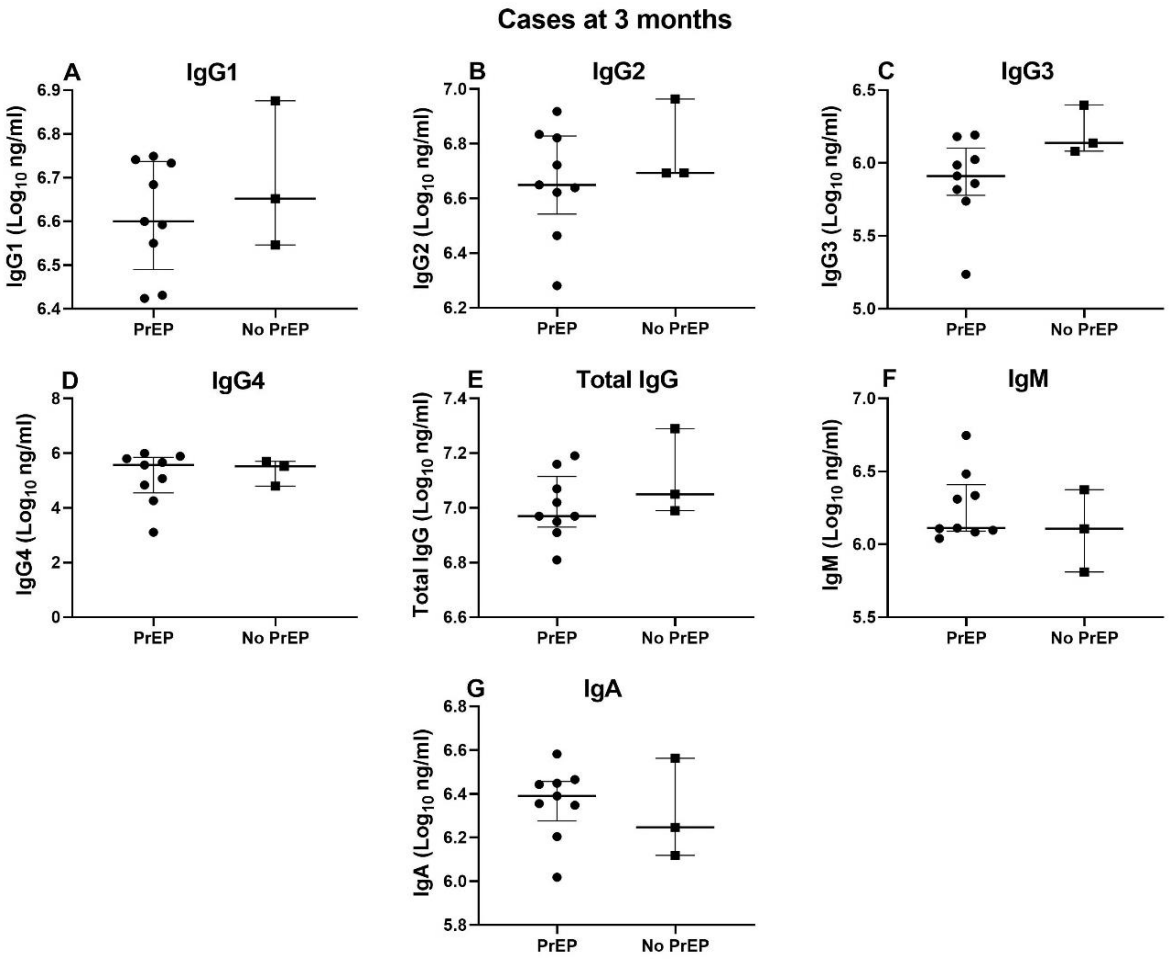
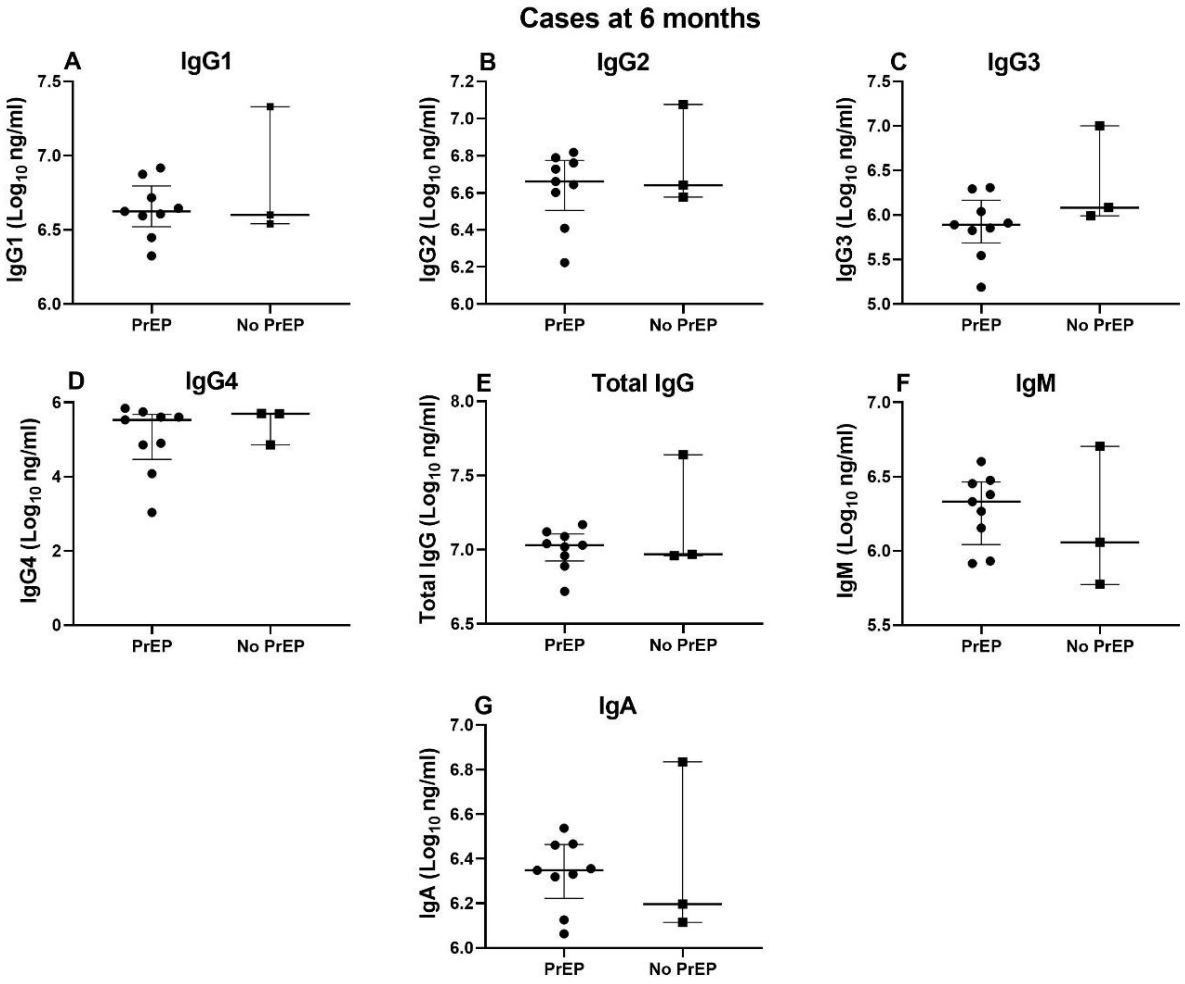
6. SUPPLEMENTARY FIGURES



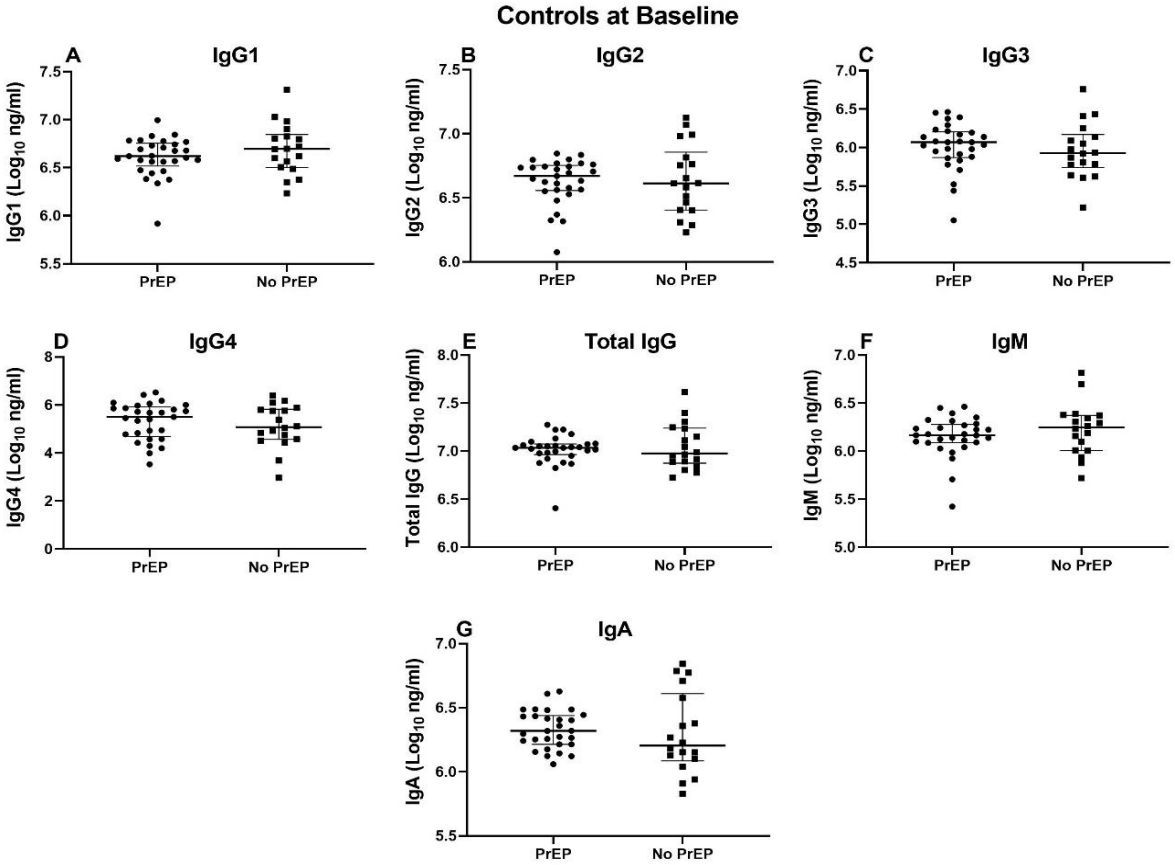
Supplementary Figure 1: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the cases using PrEP (n=9) and not using PrEP (n=3) at baseline. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare IgG4 titres and the Unpaired t-tests were used to compare the IgG1, IgG2, IgG3,Total IgG, IgM, and IgA titres within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done.



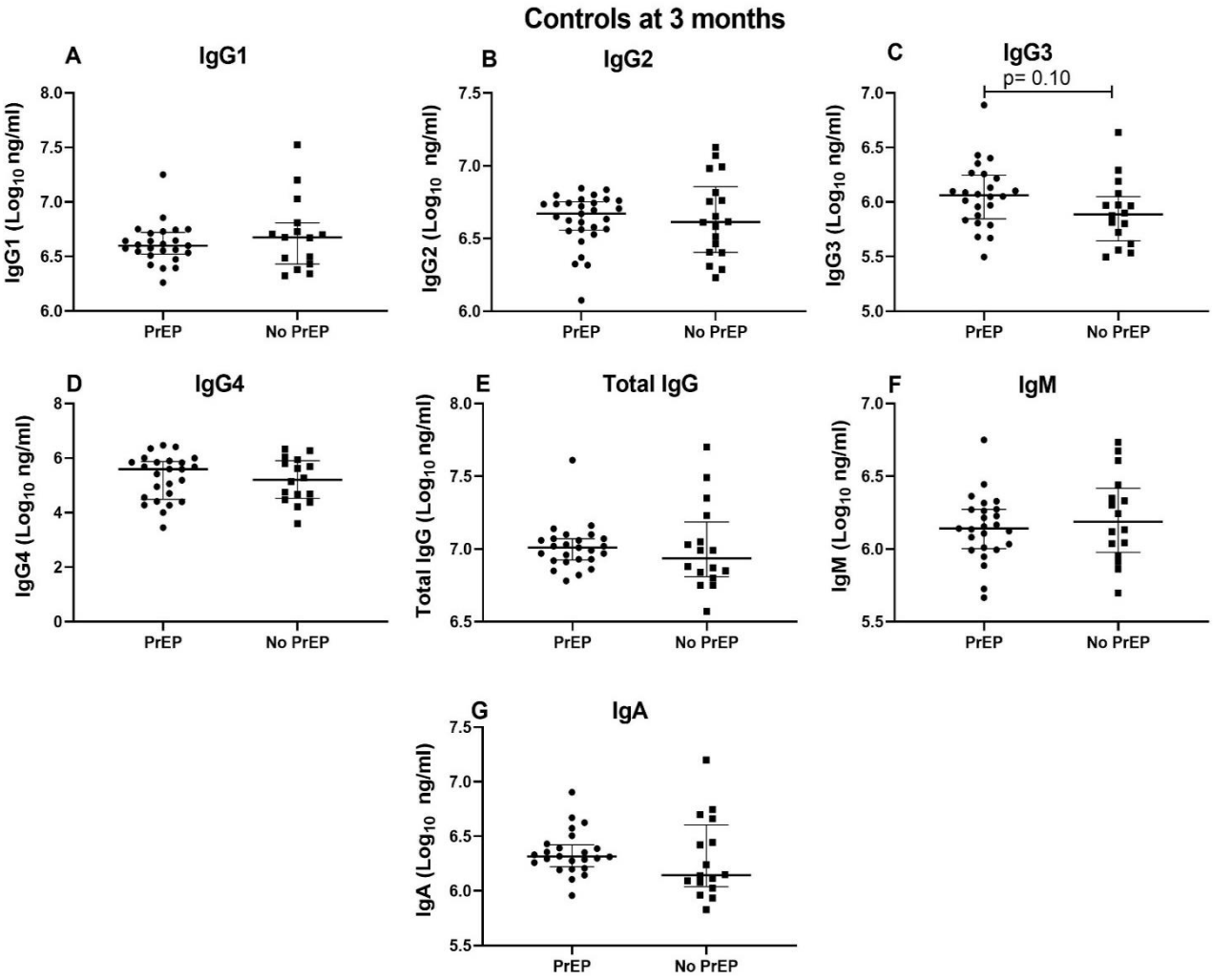
Supplementary Figure 2: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the cases using PrEP (n=9) and not using PrEP (n=3) at the 3-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare IgG2 titres and the Unpaired t-tests were used to compare the IgG1, IgG3, IgG4, Total IgG, IgM, and IgA titres within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done.



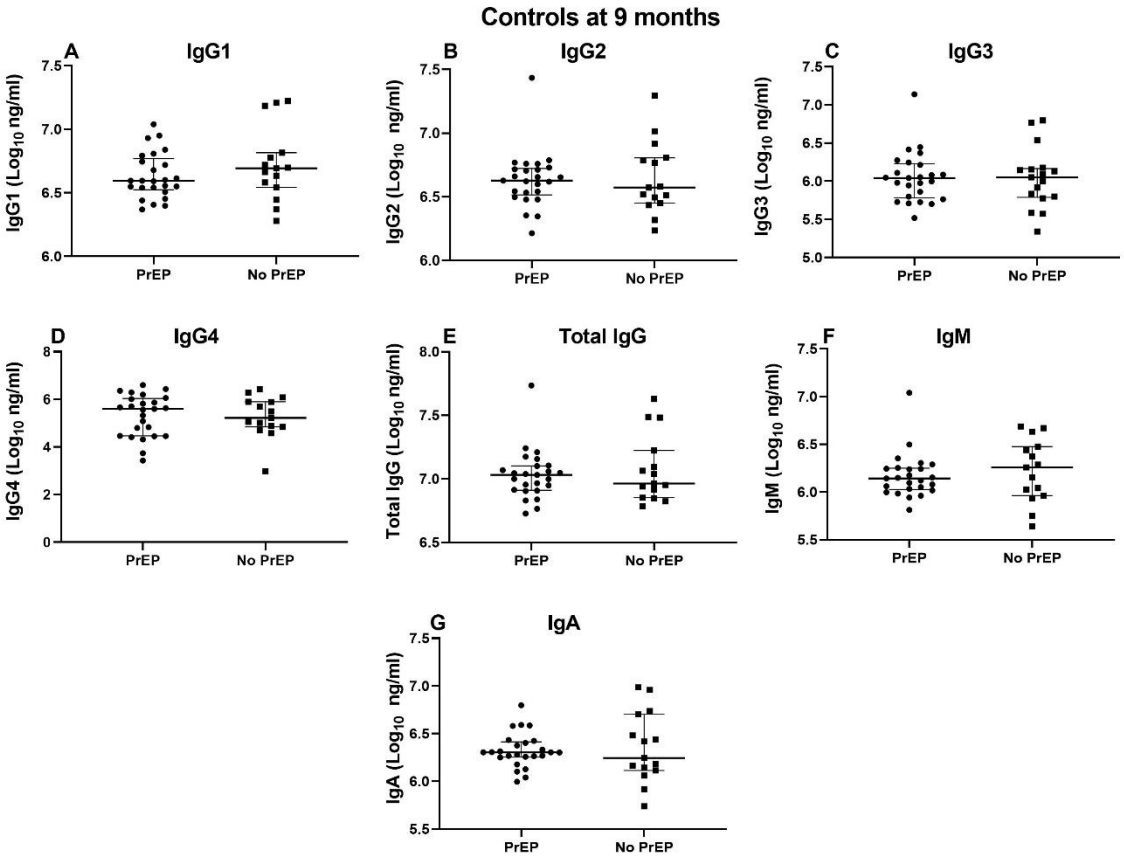
Supplementary Figure 3: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the cases using PrEP (n=9) and not using PrEP (n=3) at the 6-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Unpaired t-tests were used to compare the IgG1, IgG2, IgG3, IgG4, Total IgG, IgM, and IgA titres within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done.



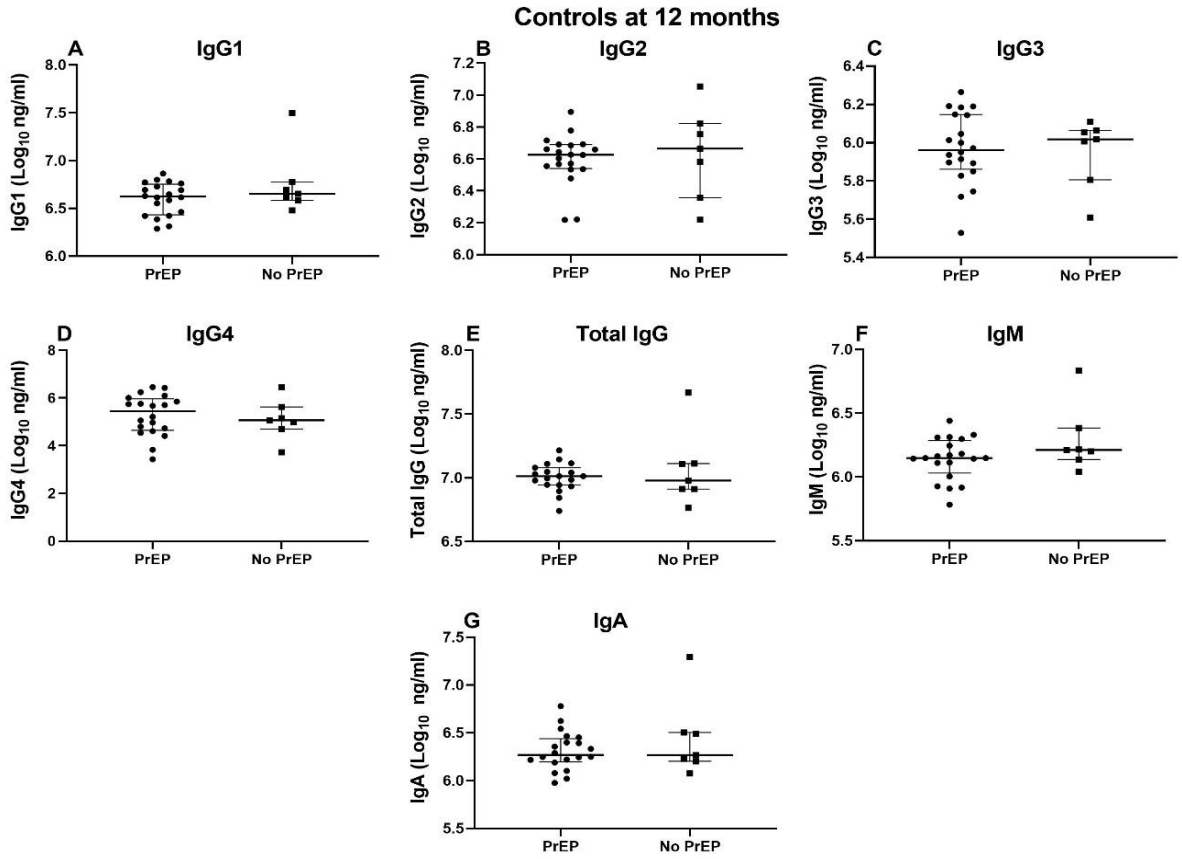
Supplementary Figure 4: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the controls using PrEP (n=29) and not using PrEP (n=18) at baseline. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, Total IgG, and IgM titres, and the Unpaired t-tests were used to compare the IgG4 and IgA titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One control not on PrEP did not have the baseline sample.



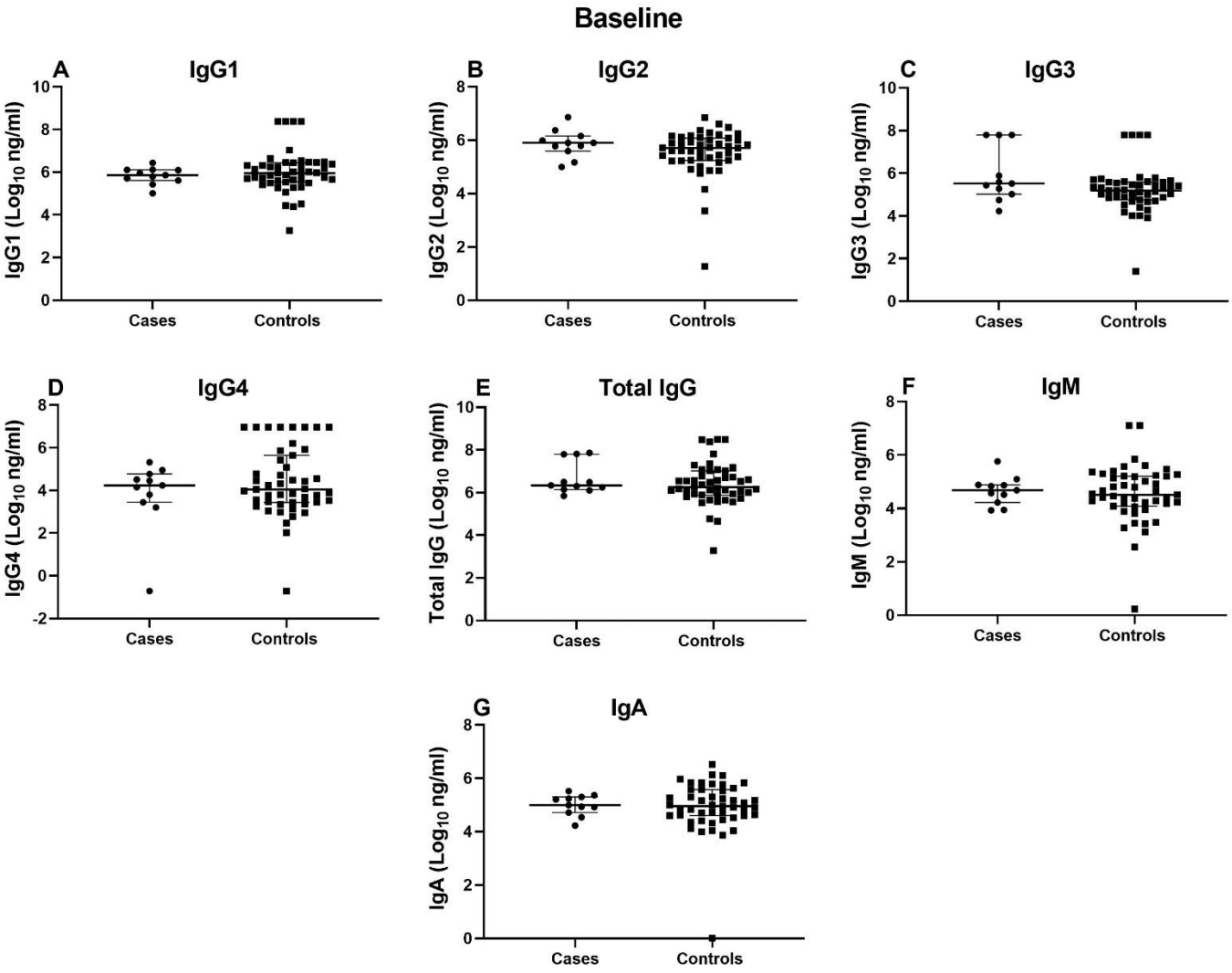
Supplementary Figure 5: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the controls using PrEP (n=25) and not using PrEP (n=16) at the 3-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG4, Total IgG, and IgM titres, and the Unpaired t-tests were used to compare the IgG3 and IgA titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Four controls on PrEP and three controls not on PrEP did not have the 3-month samples.



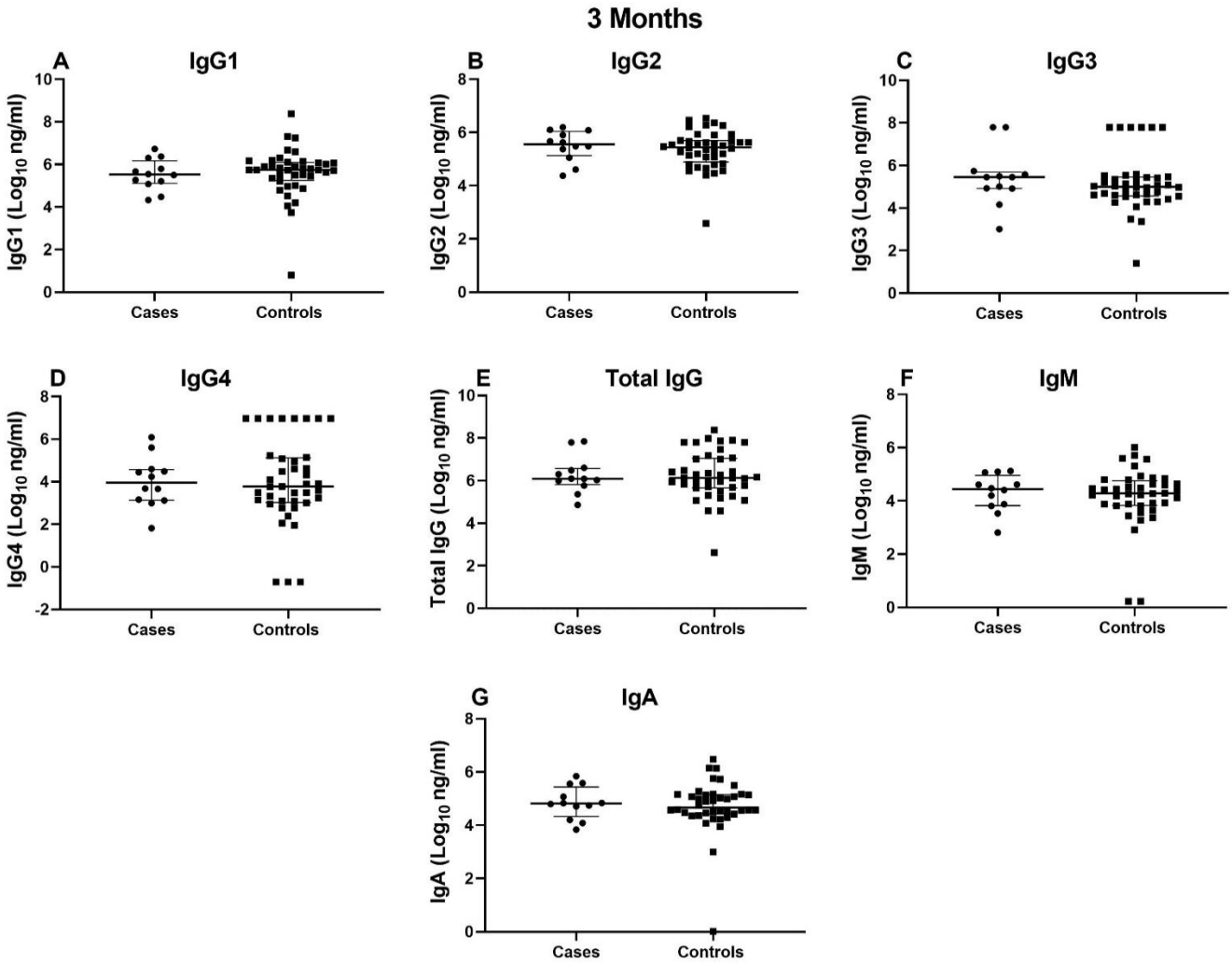
Supplementary Figure 6: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the controls using PrEP (n=25) and not using PrEP (n=15) at the 9-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, Total IgG, and IgM titres, and the Unpaired t-tests were used to compare the IgG4 and IgA titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Four controls on PrEP and not on PrEP did not have the 9-month samples.



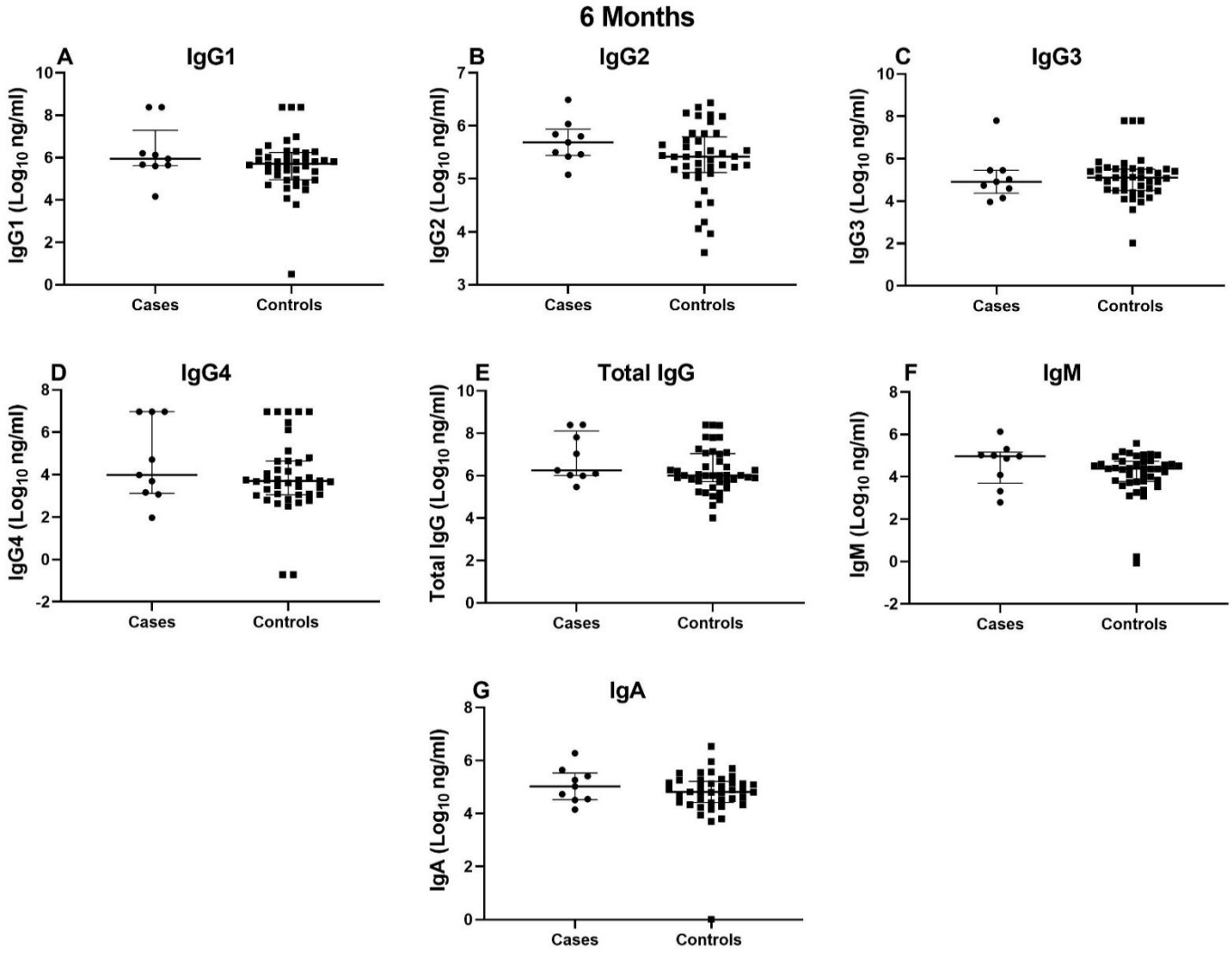
Supplementary Figure 7: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the plasma within the controls using PrEP (n=20) and not using PrEP (n=7) at the 12-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, Total IgG, and IgM titres, and the Unpaired t-tests were used to compare the IgG4 and IgA titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Nine controls on PrEP and twelve controls not on PrEP did not have the 3-month samples.



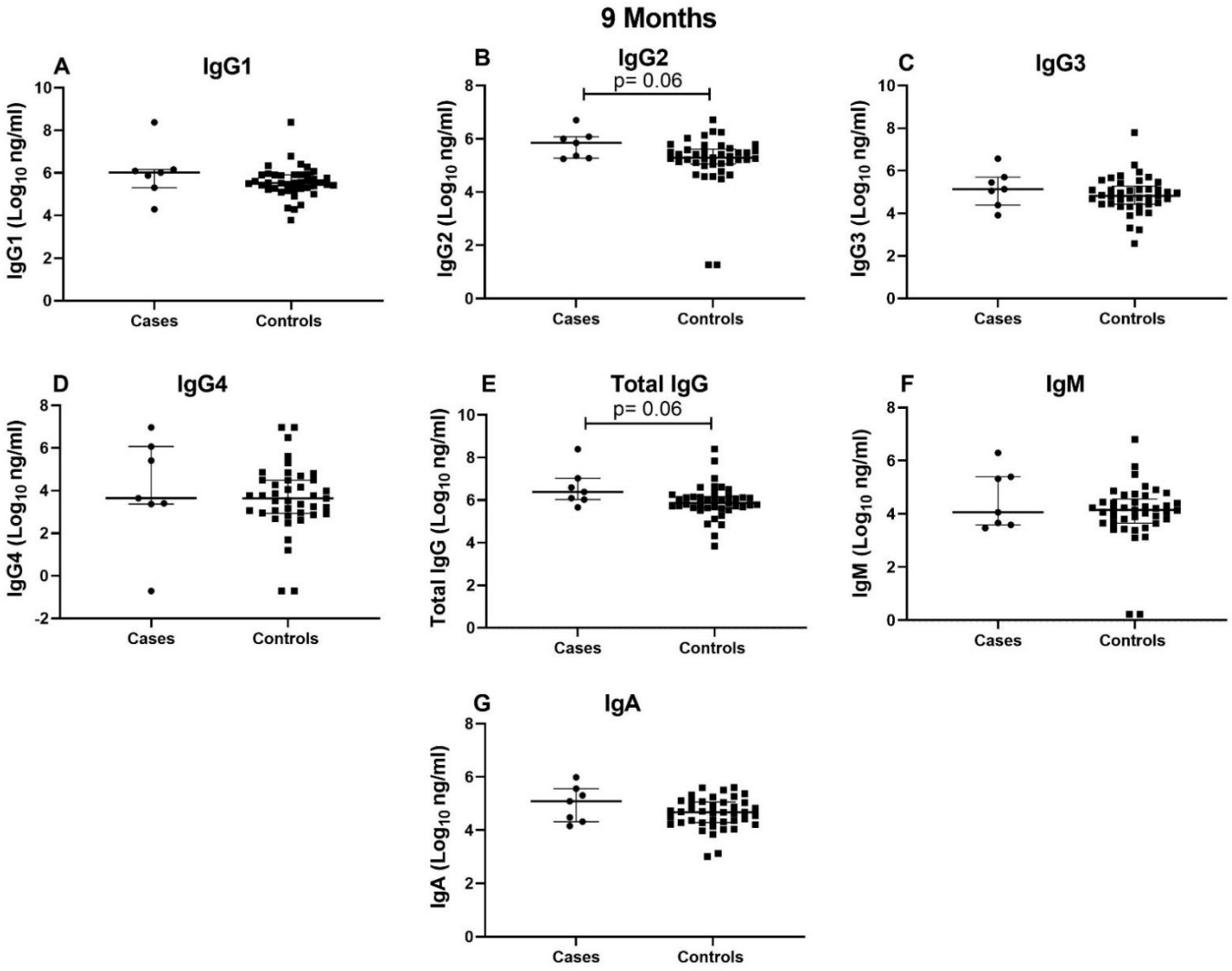
Supplementary Figure 8.1: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens between the cases (n=11) and the controls (n=47) at baseline. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, IgG4, Total IgG, IgM, and IgA titres between the cases and the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One case and one control did not have the baseline samples.



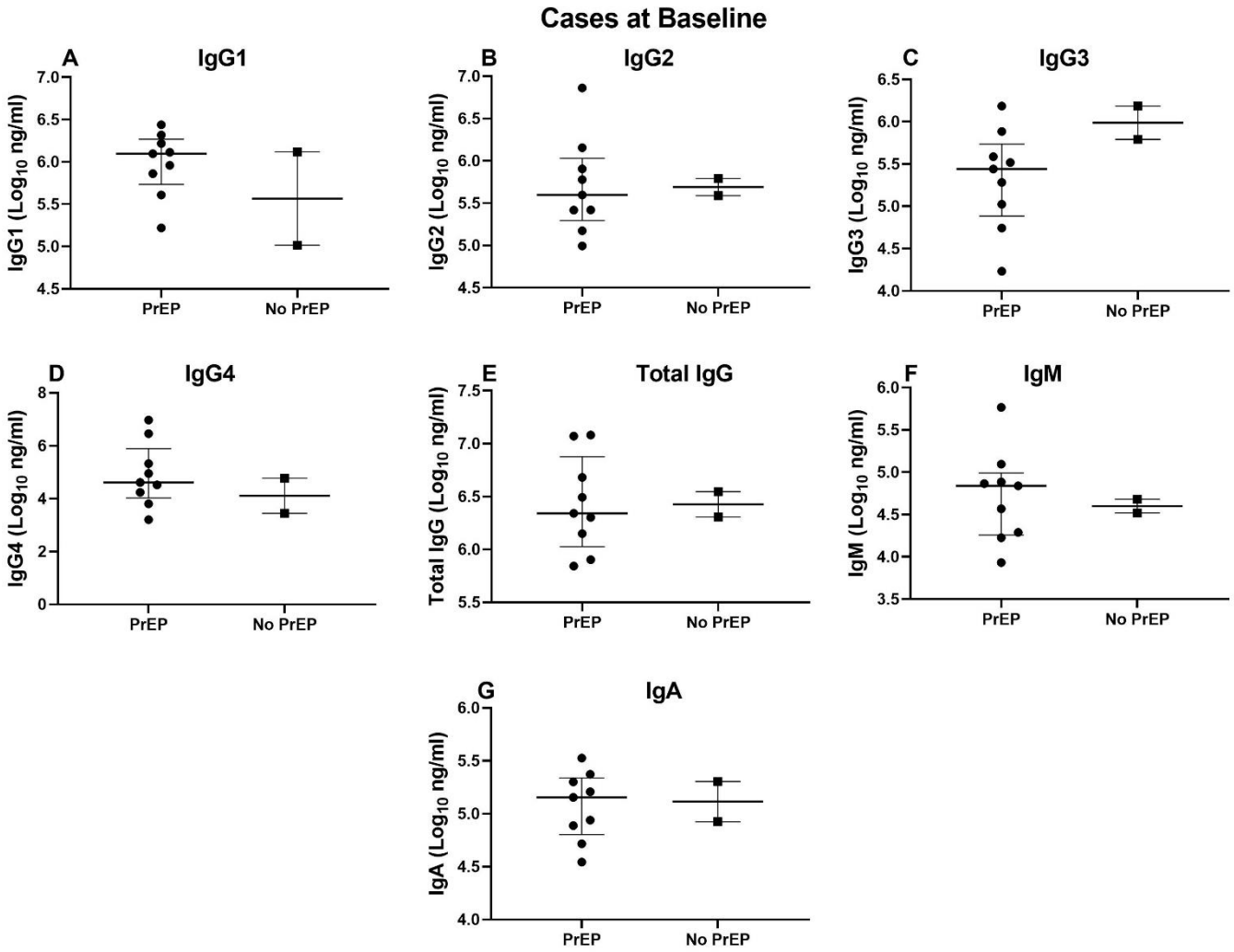
Supplementary Figure 8.2: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens between the cases (n=12) and the controls (n=40) at the 3-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, IgG4, IgM, and IgA titres, and the Unpaired t-tests were used to compare the Total IgG titres between the cases and the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eight controls did not have the 3-month samples.



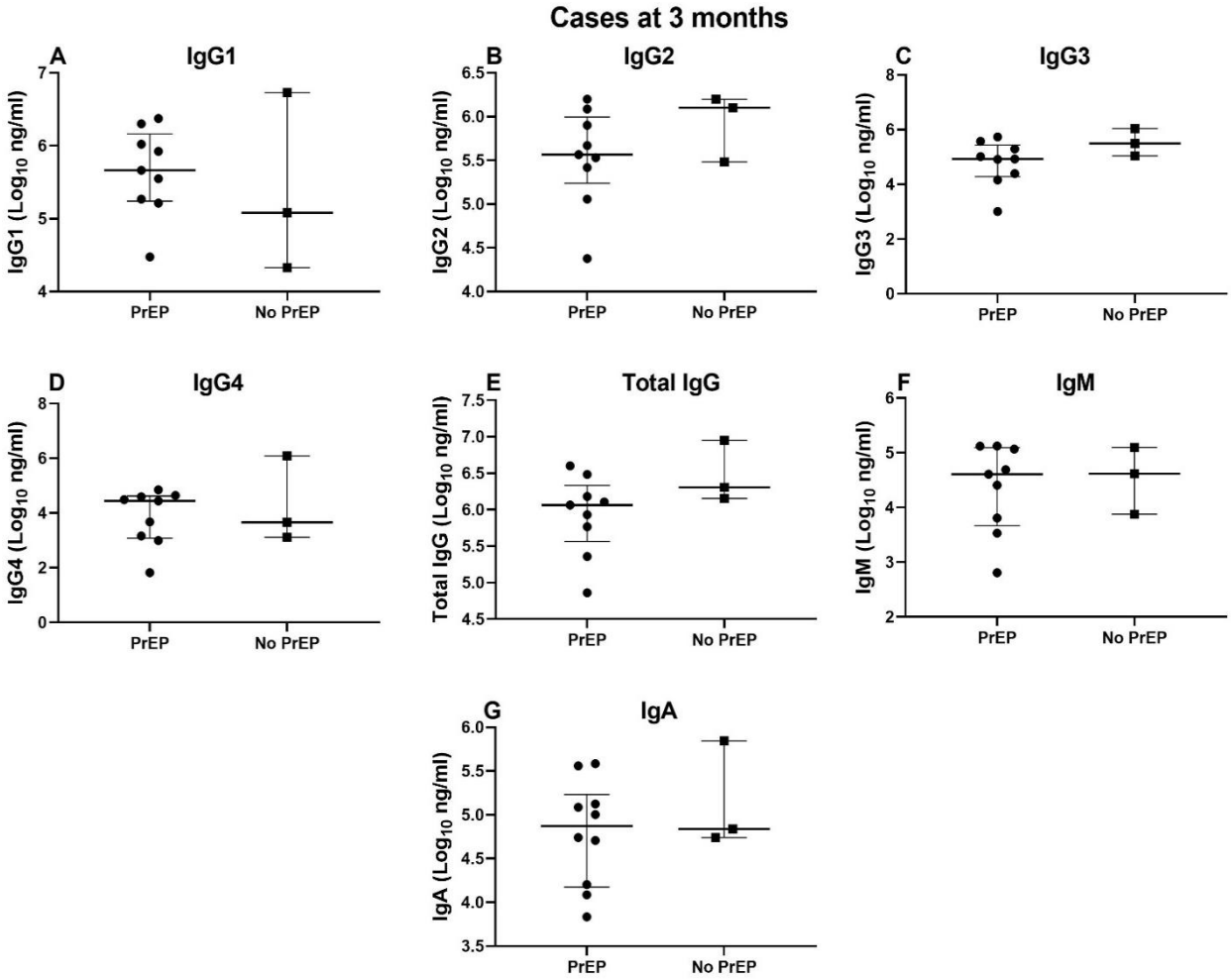
Supplementary Figure 8.3: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens between the cases (n=9) and the controls (n=41) at the 6-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, IgG4, IgM, and IgA titres, and the Unpaired t-tests were used to compare the Total IgG titres between the cases and the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Three cases and seven controls did not have the 6-month samples.



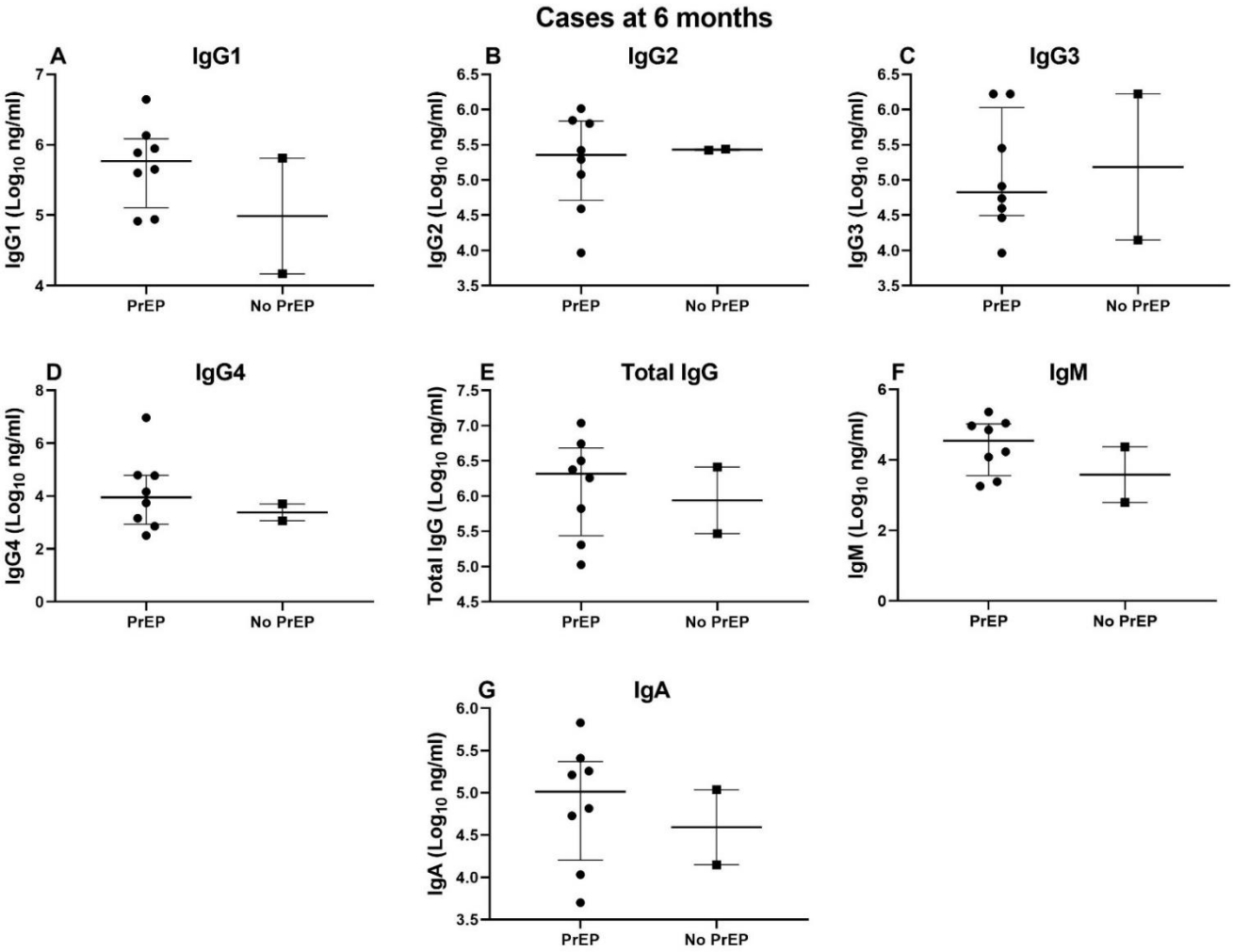
Supplementary Figure 8.4: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens between the cases (n=7) and the controls (n=41) at the 9-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, IgG4, and IgM titres, and the Unpaired t-tests were used to compare the Total IgG and IgA titres between the cases and the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Five cases and eight controls did not have the 9-month samples.



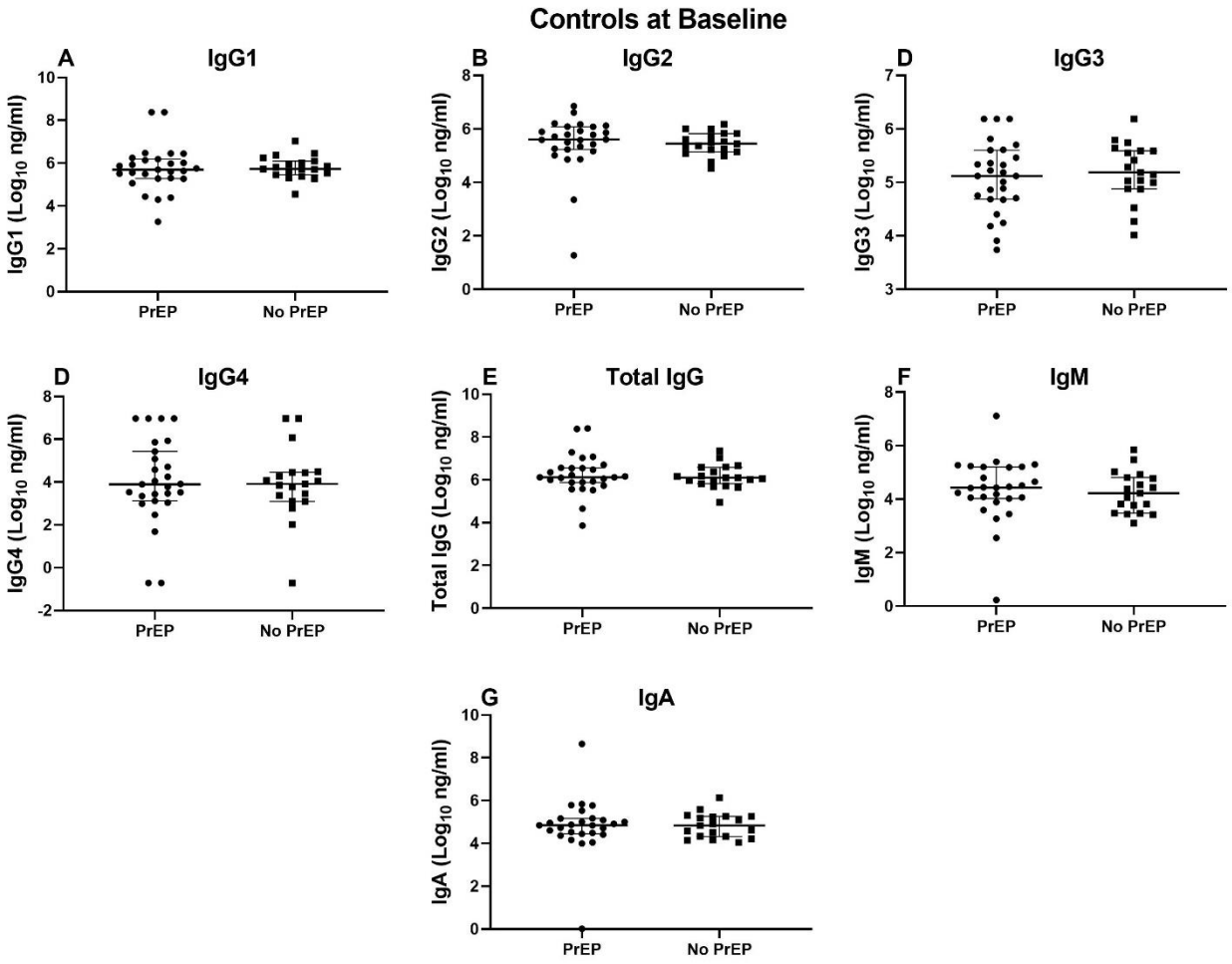
Supplementary Figure 9: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens within the cases using PrEP (n=9) and not using PrEP (n=2) at baseline. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, IgG4, Total IgG, IgM, and IgA titres within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One case not on PrEP did not have the baseline sample.



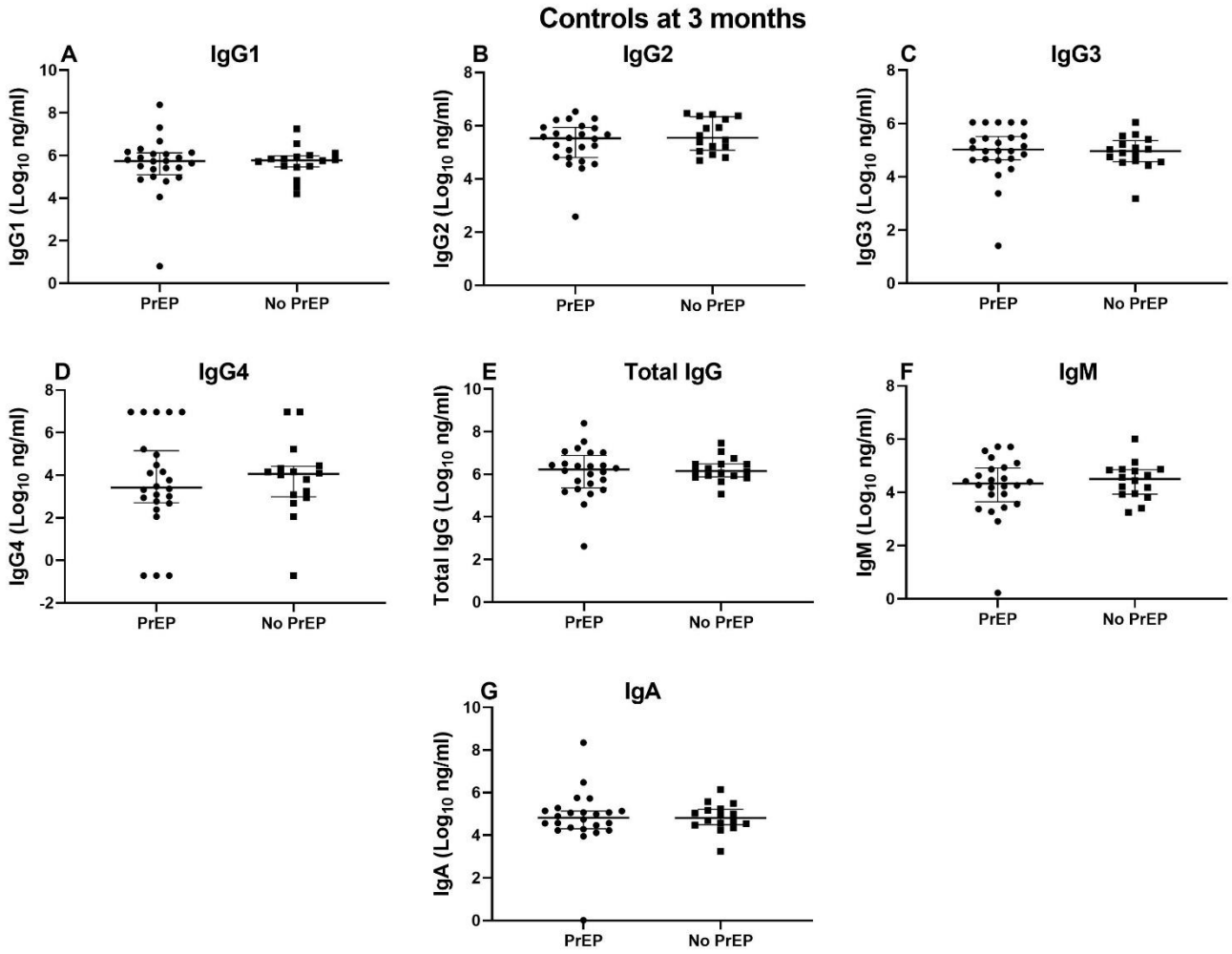
Supplementary Figure 10: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens within the cases using PrEP (n=9) and not using PrEP (n=3) at the 3-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Unpaired t-tests were used to compare the IgG1, IgG2, IgG3, IgG4, Total IgG, IgM, and IgA titres within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done.



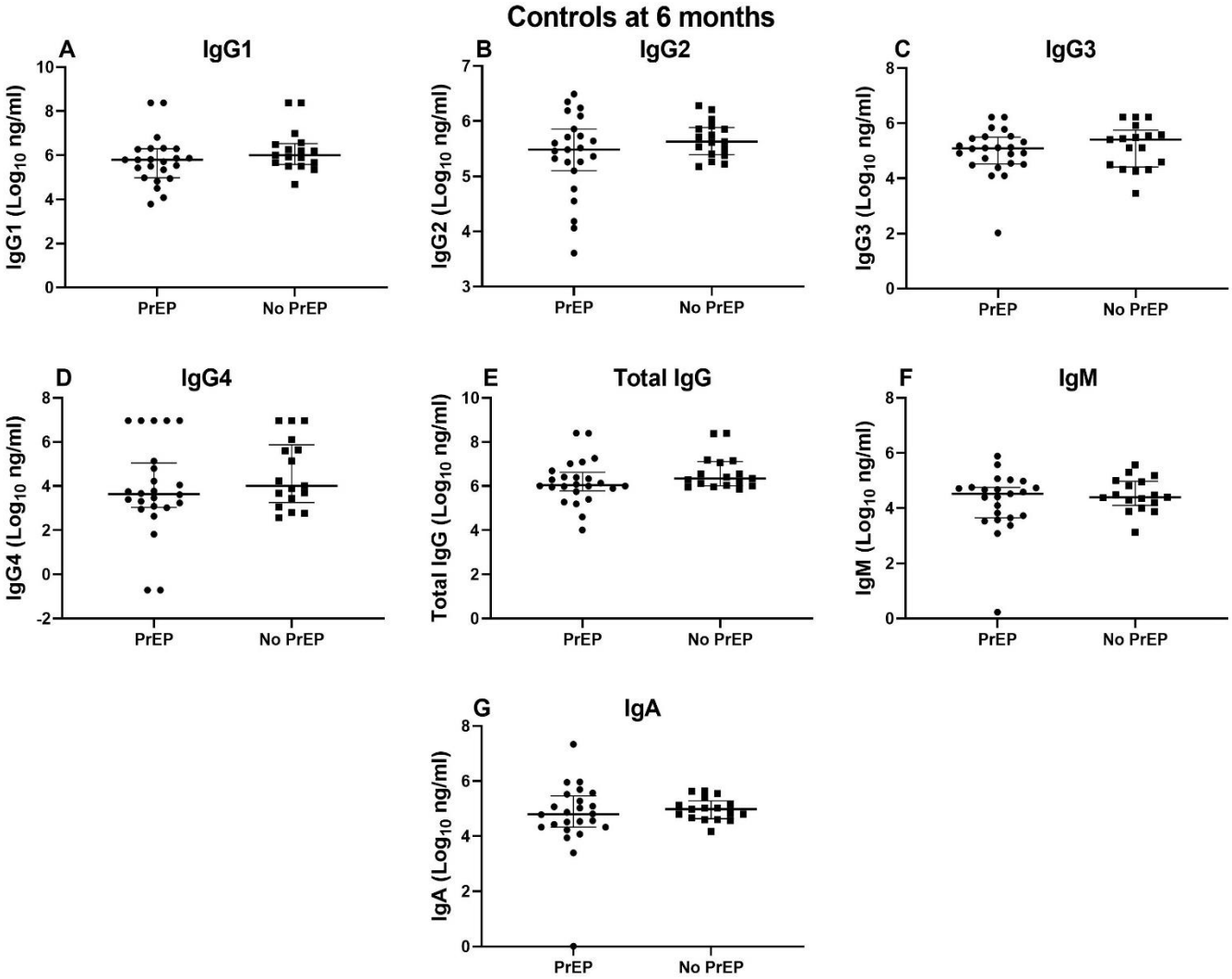
Supplementary Figure 11: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens within the cases using PrEP (n=8) and not using PrEP (n=2) at the 6-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, IgG4, Total IgG, IgM, and IgA titres within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One case on PrEP and one cases not on PrEP did not have the 6-month samples.



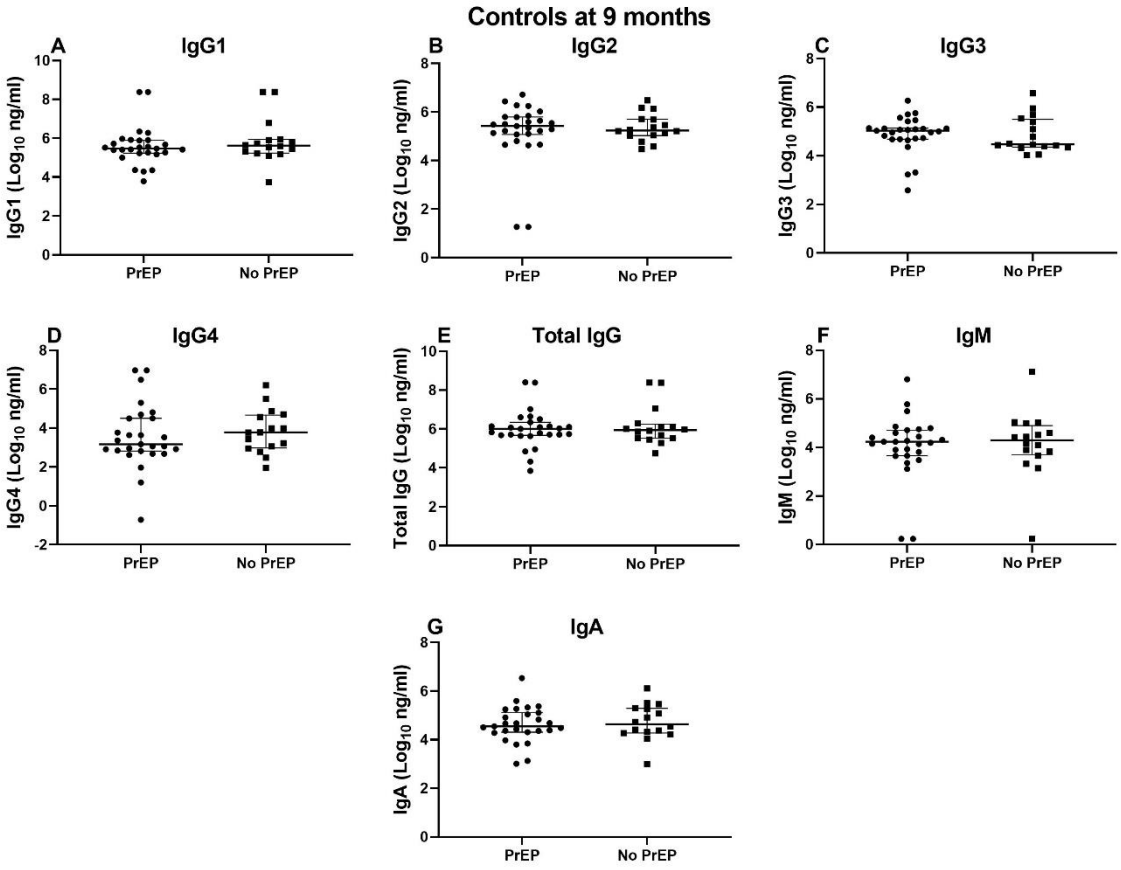
Supplementary Figure 12: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens within the controls using PrEP (n=27) and not using PrEP (n=19) at baseline. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG4, IgM, and IgA titres, and the Unpaired t-tests were used to compare IgG3 and Total IgG titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Two controls on PrEP did not have the baseline samples.



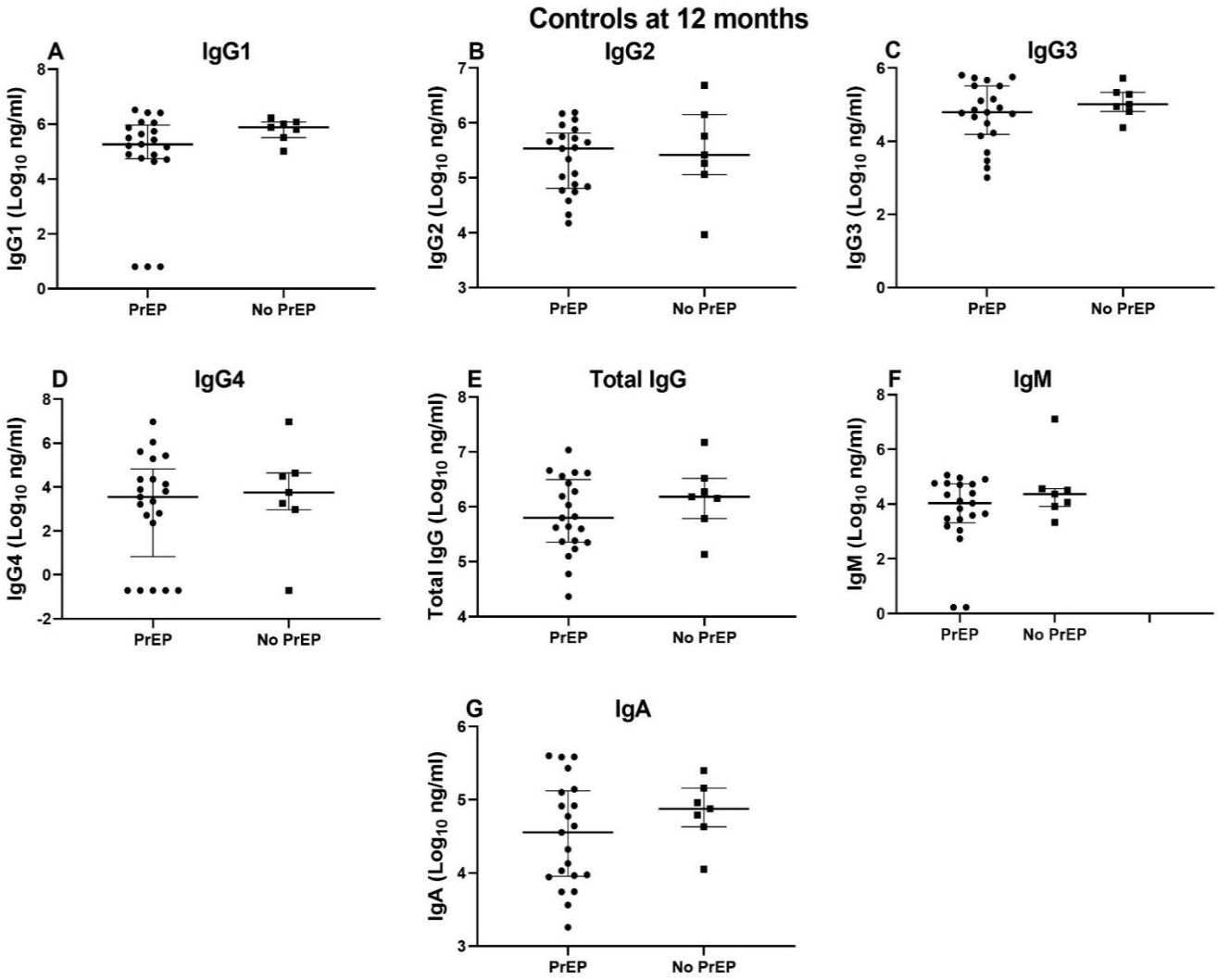
Supplementary Figure 13: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens within the controls using PrEP (n=24) and not using PrEP (n=16) at the 3-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3, IgG4, IgM, and IgA titres, and the Unpaired t-tests were used to compare the Total IgG titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Five controls on PrEP and three controls not on PrEP did not have the 3-month samples.



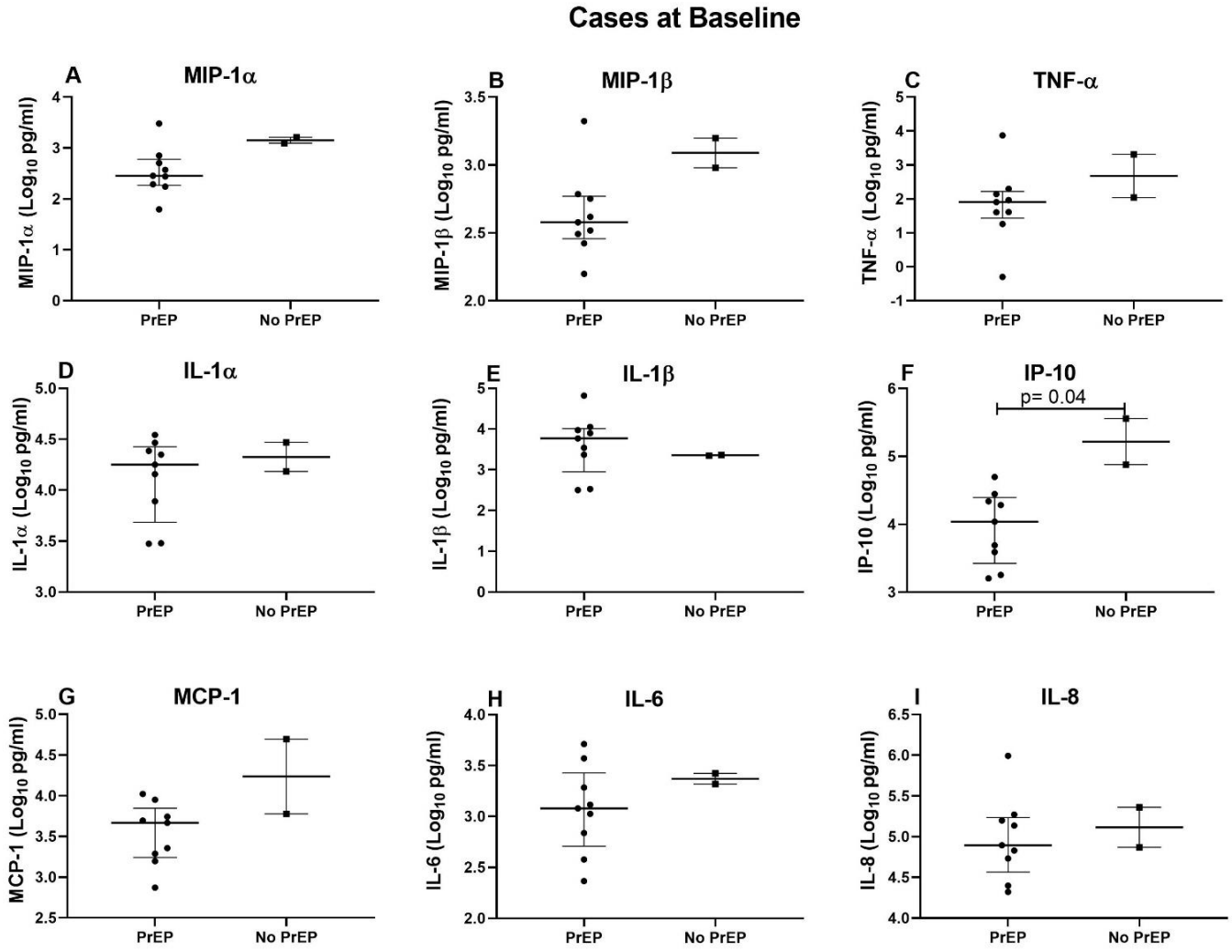
Supplementary Figure 14: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens within the controls using PrEP (n=23) and not using PrEP (n=17) at the 6-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG3, IgG4, Total IgG, IgM, and IgA titres, and the Unpaired t-tests were used to compare the IgG2 titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Six controls on PrEP and two controls not on PrEP did not have the 6-month samples.



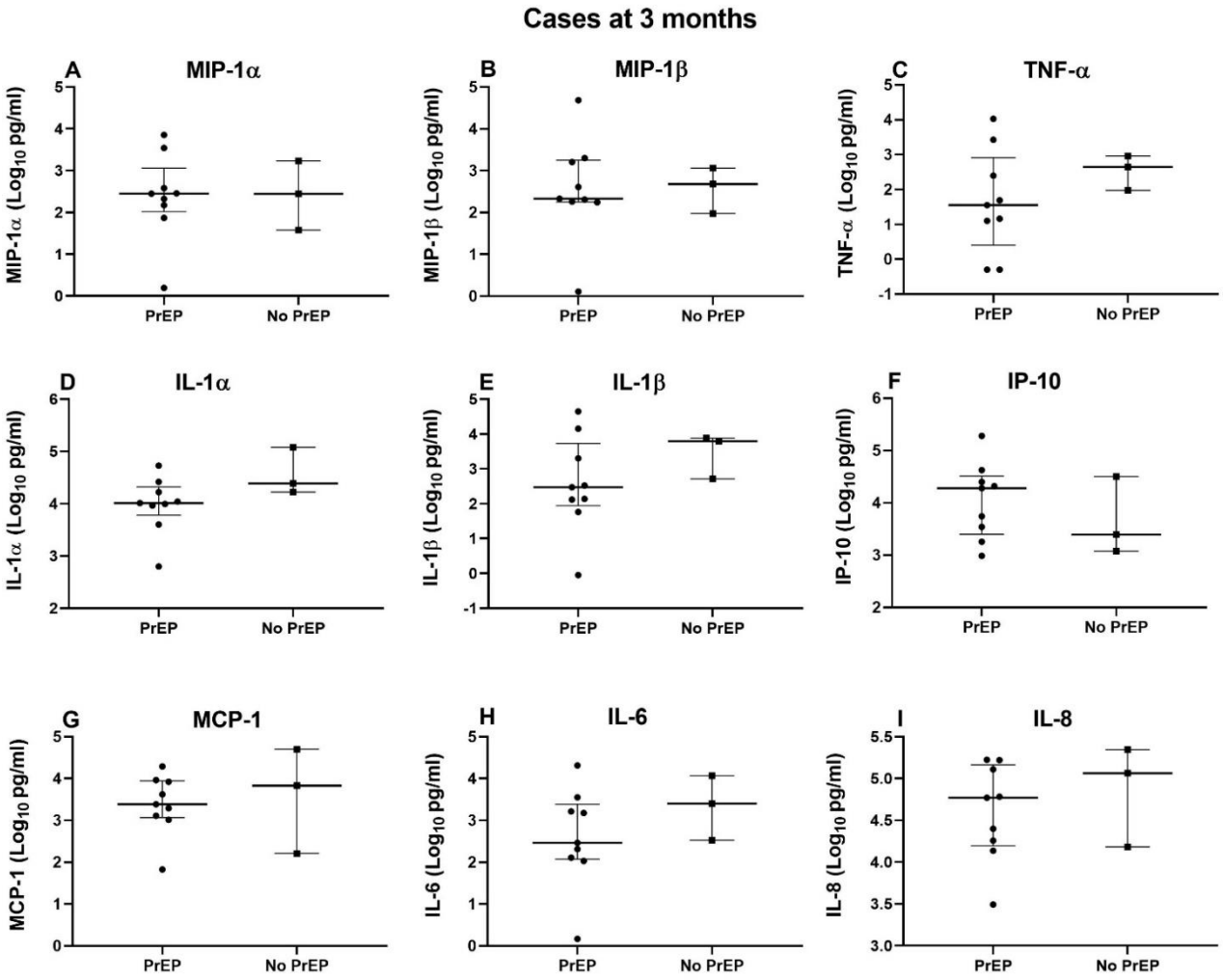
Supplementary Figure 15: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens within the controls using PrEP (n=27) and not using PrEP (n=16) at the 9-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG2, IgG3,Total IgG, and IgM titres, and the Unpaired t-tests were used to compare the IgG4 and IgA titres within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Two controls on PrEP and three controls not on PrEP did not have the 9-month samples.



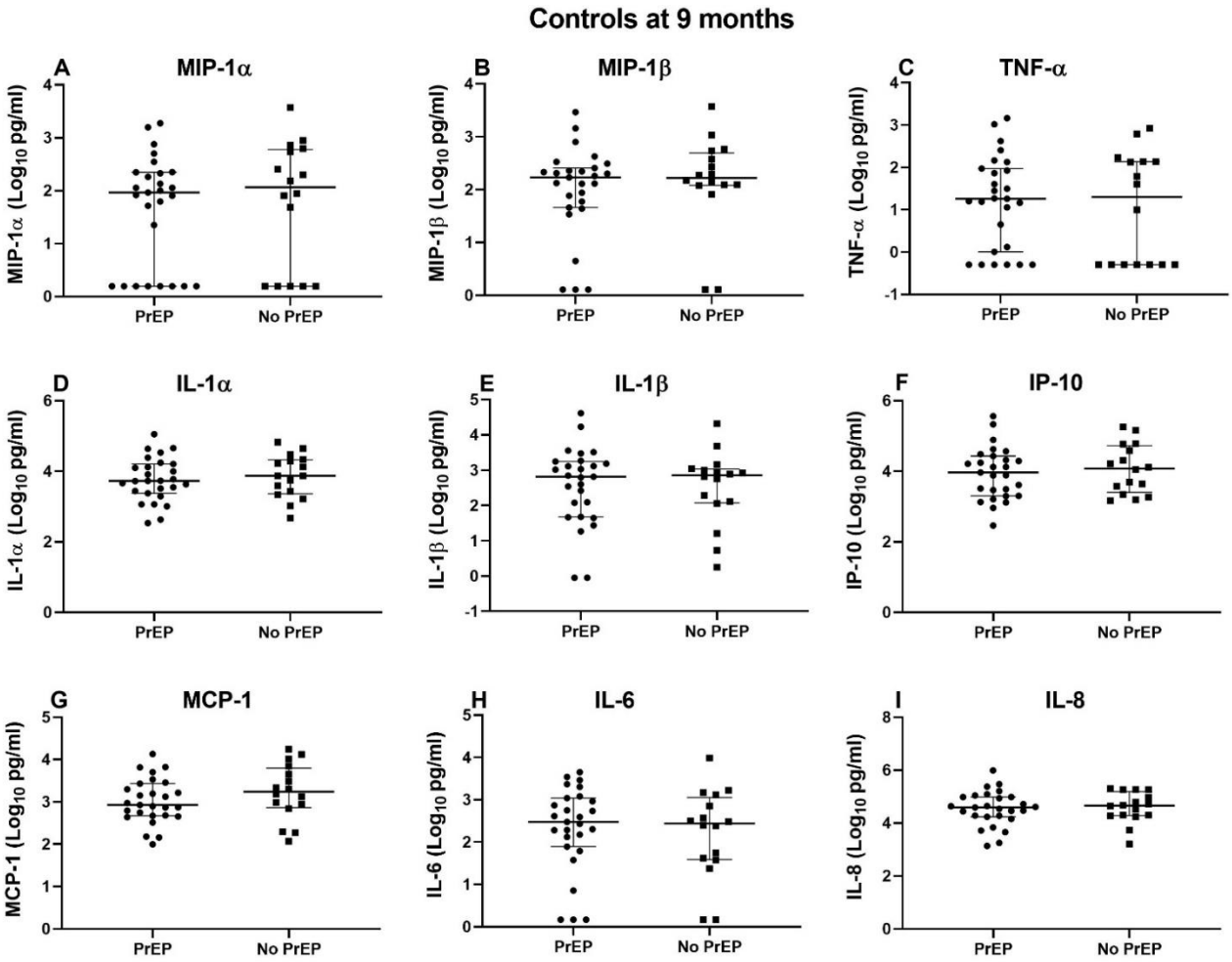
Supplementary Figure 16: The comparison of immunoglobulins and isotypes (Log_{10} ng/ml) in the mucosal specimens within the controls using PrEP (n=21) and not using PrEP (n=7) at the 12-month visit. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), Total IgG (E), IgM (F) and IgA (G). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare the IgG1, IgG3, IgG4, IgM, and IgA titres, and the Unpaired t-tests were used to compare the IgG2 and Total IgG titres, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Eight controls on PrEP and twelve controls not on PrEP did not have the 12-month samples.



Supplementary Figure 17: The comparison of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens within the cases using PrEP (n=9) and not using PrEP (n=2) at baseline. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare MIP-1α, MIP-1β, TNFα, IL-1α, IL-1β, IP-10, MCP-1, IL-6, and IL-8 concentrations (Log_{10} pg/ml) within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. One case not on PrEP did not have the baseline sample.



Supplementary Figure 18: The comparison of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens within the cases using PrEP (n=9) and not using PrEP (n=3) at the 3-month visit. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. Unpaired t-tests were used to compare MIP-1α, MIP-1β, TNFα, IL-1α, IL-1β, IP-10, MCP-1, IL-6, and IL-8 concentrations (Log_{10} pg/ml) within the cases, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done.



Supplementary Figure 19: The comparison of cytokine concentrations (Log_{10} pg/ml) in the mucosal specimens within the controls using PrEP (n=27) and not using PrEP (n=16) at the 9-month visit. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Mann-Whitney tests were used to compare MIP-1α, MIP-1β, TNFα, and IL-6 concentrations (Log_{10} pg/ml), and the Unpaired t-tests were used to compare IL-1α, IL-1β, IP-10, MCP-1, and IL-8 concentrations (Log_{10} pg/ml) within the controls, $p<0.05$ considered statistically significant and $p\leq0.1$ was considered to be trending to significance. Adjustment for multiple comparisons was not done. Two controls on PrEP and three controls not on PrEP did not have the 9-month samples.

7. APPENDIX I

7.1 Turnitin originality report

8/31/2020 Turnitin

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Turnitin Originality Report

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Ulrich F. Prodjinotho, Charlotte von Horn, Alex Y. Debrah, Linda Batsa Debrah et al. "Pathological manifestations in lymphatic filariasis correlate with lack of inhibitory properties of IgG4 antibodies on IgE-activated granulocytes", PLOS Neglected Tropical Diseases, 2017

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8. APPENDIX II

8.1 BREC approval letter for the original study

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

21 December 2017

Dr LE Mansoor
CAPRISA
NRMSM
leila.mansoor@caprisa.org

Dear Dr Mansoor

Protocol: CAPRISA 082: Prospective study of HIV risk factors and prevention choices in young women in KwaZulu-Natal, South Africa. Degree: Non-degree. BREC reference number: BE458/15

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	17 February 2018
Expiration of Ethical Approval:	16 February 2019

I wish to advise you that your application for Recertification dated 13 December 2017 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

This approval will be ratified by a full Committee at its meeting taking place on 13 February 2018.

Yours sincerely

[redacted]

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc: Kalendri.Naidoo@caprisa.org
cc: Jennifer.David@caprisa.org

8.2. BREC approval letter for the present study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 September 2019

Miss Mandisa Nokukhanya Zuma (214502628)
School of Lab Med & Medical Sc
Medical School

Dear Miss Mandisa Nokukhanya Zuma,

Protocol reference number: BREC/00000091/2019
Project title: Characterization of immunoglobulin (Ig) isotypes, IgG subclasses and cytokines in the blood and genital tracts of HIV infected and healthy women from an observational cohort (CAPRISA 082).
Degree: MMedSc

Full Approval (Expedited Application)

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 25 July 2019.

Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from **11 September 2019.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

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

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

The sub-committee's decision will be **noted** by a full Committee at its next meeting taking place on **08 October 2019.**

Yours sincerely

[redacted]

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

8.3 BREC recertification approval letter for the present study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

22 July 2020

Miss Mandisa Nokukhanya Zuma (214502628)
School of Laboratory Medicine and Medical Science
Medical School

Dear Miss Zuma,

Protocol reference number: BREC/00000091 /2019
Project title: Characterization of Immunoglobulin (Ig) isotypes, IgG subclasses and cytokines in the blood and genital tracts of HIV infected and healthy women from an observational cohort (CAPRISA 082).
Degree: MMedSc

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**11 September 2020**
Expiration of Ethical Approval:	**10 September 2021**

I wish to advise you that your application for recertification received on 17 July 2020 for the above study has been noted and approved by a subcommittee of the Biomedical Research Ethics Committee (BREC).The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 August 2020.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx