The effect of genital tract inflammation on HIV-specific binding antibodies, IgG subclass and Isotype transudation.

By

Thevani Pillay

Submitted in fulfilment of the requirements for the degree of Master of Medical Science in the Department of Medical Microbiology, Nelson R Mandela School of Medicine, College of Health Science, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal

Date: 6 March 2019

PLAGIARISM DECLARATION

I, Thevani Pillay, 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.

Thevani Pillay

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

Supervisor: Dr Derseree Archary

Co-supervisor: Dr Cheryl Baxter

DEDICATION

To my parents and sisters, thank you for all of your support, encouragement and motivation throughout my academic career- you have been my pillar of strength throughout my most difficult challenges.

To Ethan, thank you for all of your support and positive talks throughout my Masters, I appreciate your patience and willingness to always help me whenever I needed an extra pair or hands. Thank you for sharing this journey with me.

Most of all, I am thankful for having my late grandparents guide me through this journey- you are my guardian angels.

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Lastly, and most importantly, I would like to express my sincere thanks and gratitude to my supervisor, Dr D. Archary for her guidance, mentorship and support throughout my Masters research. I have been well-trained under her supervision, and this prepared me for the next chapter of my life.

POSTER PRESENTATIONS

1. HIV-specific binding antibody profiles in semen of HIV-infected heterosexual men from Cape Town, South Africa

Thevani Pillay, Abraham J. Olivier, Kapil Narain, Hoyam Gamieldien, Lenine Liebenberg, Sinaye Ngcapu, Jo-Ann Passmore, Derseree Archary.

8th SA AIDS Conference, Durban, South Africa, 13th June 2017.

HIV-specific binding antibody profiles in semen of HIV-infected heterosexual men from Cape Town, South Africa.

Thevani Pillay[1], Abraham J. Olivier[2], Kapil Narain[1], Hoyam Gamieldien[3], Lenine Liebenberg[1], Sinaye Ngcapu[1], Jo-Ann Passmore[3], Derseree Archary[1].

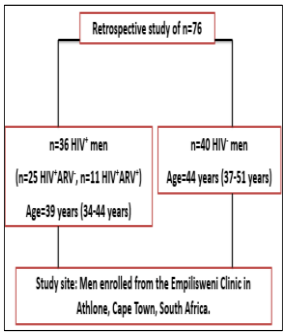
[1]Centre for the AIDS Programme of Research in South Africa, Durban; [2]TB/HIV Care Association; [3]University of Cape Town, South Africa.

Background

- Semen is a major vector for HIV-1 transmission across mucosal surfaces, during male to female or male to male sexual intercourse.
- The immune correlates of risk in the male genital tract remain poorly defined although HIV-specific antibodies may play an important role.
- The profiles of HIV-specific antibodies in seminal fluid was investigated to understand if prior genital inflammation in the presence or absence of antiretroviral- (ARV) treatment modulates genital tract antibody titres in HIV-infected (HIV+) and HIV-exposed seronegative (HIV-) men.

Methods

- Semen from 36 HIV+ and 40 HIV- men was used to measure HIV-specific binding antibodies (gp120, gp41, p66 and p24), isotypes to measure immunoglobulin (Ig) profiles and cytokines using multiplex assays.



Study Participants

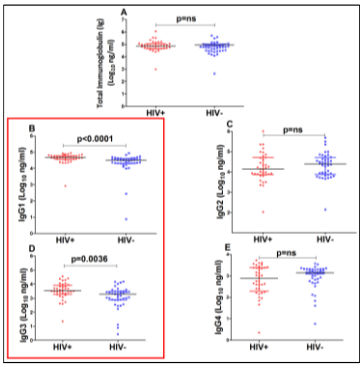
Table 1. Participant demographic profile

Characteristic	HIV-	HIV+ARV-	HIV+ARV+
N	40	25	11
Age, y (median [IQR])	44 (37-51)	39 (34-44)	43 (39-46)
CD4 count, cell/mm3 (median [IQR])	-	391 (278–507)	340 (234–532)
Number of men with detectable HIV RNA in plasma, (N/total [%])	-	25/25 (100)	2/11 (18.2)[b]
Genital tract viral load, RNA copies/mL (median [IQR])	-	1389 (LDL[a]-20 060)	LDL[a] (LDL-LDL)
Number of men with detectable HIV RNA in semen, N/total (%)	-	19/25 (76)	2/11 (18.2)[b]
Number of men with detectable CMV in semen, N/total (%)	6/20	13/25	4/11
Genital tract CMV viral load, DNA copies/mL (median [IQR])	LDL[a] (LDL[a]-800)	374 (LDL[a]-174 025)	LDL[a] (LDL[a]-43 375)

Abbreviations: CMV, cytomegalovirus; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; IQR, interquartile range; LDL, lower than detection level.
[a] LDL, 20 HIV-1 RNA copies/mL
[b] Two participants had plasma viral loads of 740 and 880 copies/mL; an additional 2 participants had seminal viral loads of 414 and 60 200 copies/mL

Results

Semen IgG1 and IgG3 were significantly higher in HIV+ men than HIV- men.



The detection of HIV gp120, gp41, p66 and gag p24 antibodies were higher in the semen of HIV+ than HIV- men (p<0.05 for all).

Table 2. Detectability of HIV antibodies in the semen

	HIV- (n/N)	HIV+ARV- (n/N)	HIV+ARV+ (n/N)
p24	72.5% (29/40)	100% (25/25)	100% (11/11)
p66	82.5% (33/40)	100% (25/25)	100% (11/11)
gp41	2.5% (1/40)	80% (20/25)	100% (11/11)
gp120	2.5% (1/40)	80% (20/25)	90% (10/11)

Detectability is deemed positive if the Mean Fluorescence Intensity (MFI) cut-off value is 100 MFI and above.
If the MFI > 100 MFI, then detectable and value analysed are taken to second level of analysis.

P66 antibody titres inversely correlated with IL-6 in HIV+ARV+ men. Gp41 antibody titres inversely correlated with MIP-1α in HIV+ARV+ men.

Table 3: Semen cytokine association with HIV-specific antibodies observed in HIV+ARV+ (n=11) men

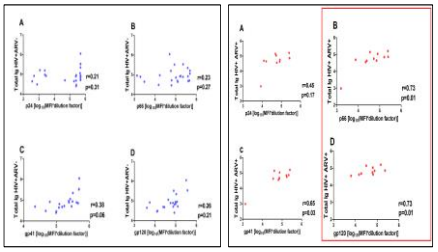
Cytokines	p24		p66		gp41		gp120	
	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value
IL-6	-0,4636	0,1546	**-0,6545**	**0,0336**	-0,4364	0,1826	-0,1636	0,6337
MIP-1α	-0,5000	0,1217	-0,3273	0,3269	**-0,7273**	**0,0144**	-0,0909	0,7964

P24 and gp120 antibody titres correlated significantly with TNF-α in HIV+ARV- men. P24 antibody titres correlated significantly with MIP-1β in HIV+ARV- men.

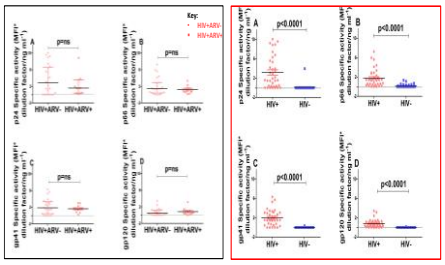
Table 4: Semen cytokine association with HIV-specific antibodies observed in HIV+ARV- (n=25) men

Cytokines	p24		p66		gp41		gp120	
	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value
TNF-α	**0,4832**	**0,0144**	0,3608	0,0764	0,3646	0,0732	**0,4399**	**0,0278**
MIP-1β	**0,4101**	**0,0418**	0,2585	0,2121	0,3001	0,145	0,3537	0,0828

Genital HIV-specific antibodies correlated significantly to total IgG(1-4) in HIV+ARV+ men.



The magnitudes of the genital HIV-specific antibodies was not different in HIV+ARV- compared to HIV+ARV- group indicating that ARVs do not impact on the genital HIV-specific humoral immunity.



Conclusions

- Higher IgG1 and IgG3 subclass profiles in the genital compartment in HIV+ men may indicate HIV- mediated changes to antibody subclasses which likely mediate specific functions like antibody-dependent cellular cytotoxicity or phagocytosis.
- IL-6 and MIP-1α may elicit modulating effects on certain HIV-specific antibodies in HIV+ARV+ men, whilst TNF-α and MIP-1β may modify the levels of certain HIV-specific antibodies in the genital tract in the background of inflammation in HIV+ARV- men.
- Together, these data show that HIV-induced local inflammation can influence humoral immunity and can inform future vaccine research on the immune correlates of risk or protection in male genital tract in the presence or absence of ARVs.

Acknowledgements

The authors would like to thank the college of Health Sciences (University of KwaZulu-Natal, Nelson Mandela Medical School Campus) and The National Research foundation, Dr. D. Archary of CAPRISA (RCA 13101656388) for funding this study. We would also like to thank the participants and the staff at the Empilisweni Clinic, Athlone.

2. Pro-inflammatory Cytokines Impact HIV-Specific Antibodies in the Male Genital Tract

T. Pillay, A J. Olivier, P. Sobia, K. Narain, L J P. Liebenberg, S. Ngcapu, M. Mhlongo, J S. Passmore, C. Baxter and D. Archary.

HIV Research for Prevention (HIVR4P) Conference, Madrid, Spain, 24th October 2018.

Pro-inflammatory Cytokines Impact HIV-Specific Antibodies in the Male Genital Tract

T. Pillay[1], A J. Olivier[2], P. Sobia[1], K. Narain[1], L J P. Liebenberg[1,5], S. Ngcapu[1,5], M. Mhlongo[1], J S. Passmore[1,2,3,4] C. Baxter[1] and D. Archary[1,5]

[1]Centre for the AIDS Programme of Research In South Africa (CAPRISA), University of KwaZulu-Natal, Durban, South Africa, [2]Institute of Infectious Diseases and Molecular Medicine (IDM), University of Cape Town, Cape Town, South Africa, [3]National Health Laboratory Service (NHLS), Cape Town, South Africa, [4]Division of Medical Virology, Department of Pathology, University of Cape Town, South Africa, [5]Department of Medical Microbiology, University of Kwazulu-Natal, Durban, South Africa.

Background

- Local inflammation in the male genital tract may play a crucial role in increasing HIV risk and influencing both the antibody subclasses and isotype switching.
- The association between genital tract immunity and HIV risk remains poorly defined.
- Therefore, we investigated cytokine signatures in relation to antibody profiles in the semen of HIV-uninfected (HIV^-) and HIV-infected (HIV^+) men in the presence and absence of ARVs.

Methods

- HIV-specific binding antibodies (gp120, gp41, p66 and p24), antibody isotypes and cytokines using multiplex assays were measured in the semen of 36 HIV^+ and 40 HIV^- men.

Study Participants

Table 1. Participant demographic profile

Characteristic	HIV^-	HIV^+ARV^-	HIV^+ARV^+
N	40	25	11
Age, years (Median [IQR])	44 [23-58]	40 [30-54]	44 [39-47]
Partner status (n/N):			
Concordant negative	14/40	-	-
Concordant positive	-	13/25	5/11
Discordant negative	21/40	-	-
Discordant positive	-	10/25	6/11
Partner status unknown	5/40	2/25	-
CD4 count, cells/mm3 (Median[IQR])	-	404 [137–737]	264 [179–748]
Plasma viral load, RNA copies/mL (Median [IQR])	-	11000 [LDL-300,000]	LDL [LDL-880]
Semen viral load, RNA copies/mL (Median [IQR])	-	2150 [LDL-135,000]	LDL [LDL-60200]
Number of men with detectable HIV RNA in semen (n/N)	-	19/25	2/11

LDL, lower than detection level <70 HIV-1 RNA copies/mL

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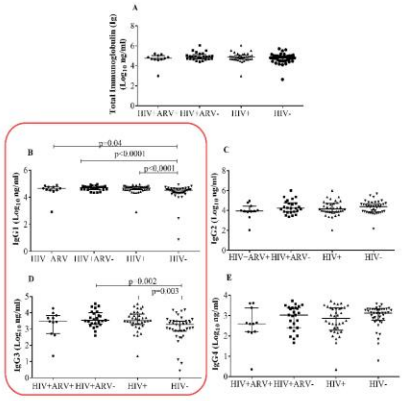
The authors would like to thank the College of Health Sciences (University of KwaZulu-Natal, Nelson Mandela Medical School Campus), Polio Research Foundation (PRF 17/02), National Research Foundation, and Dr. D. Archary of CAPRISA (RCA 13101656388) for funding this study. TP receives support from the South African Department of Science and Technology and the National Research Foundation's Centre of Excellence in HIV Prevention (Grant # UID: 96354). We would also like to thank the participants and the staff at the Empilisweni Clinic, Athlone.

Results

Semen IgG1 and IgG3 were significantly higher in HIV^+ men than HIV^- men.



P66 and gp41 antibody titres correlated inversely with IL-6 and MIP-1α respectively in HIV^+ARV^+ men.

Table 2: Semen cytokine associations with HIV-specific antibodies in HIV^+ARV^+ (n=11) men

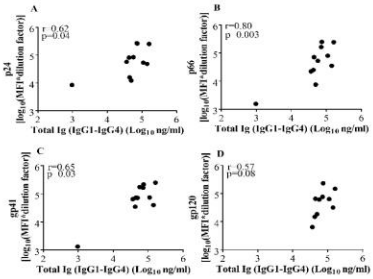
Cytokines	p24		p66		gp41		gp120	
	r value	p value	r value	p value	r value	p value	r value	p value
IL-6	-0,46	0,15	**-0,65**	**0,03**	-0,44	0,19	-0,16	0,63
MIP-1α	-0,50	0,12	-0,33	0,33	**-0,73**	**0,01**	-0,09	0,80

P24 antibody titres correlated directly with TNF-α and MIP-1β, while gp120 antibody titres correlated with TNF-α In HIV^+ARV^- men .

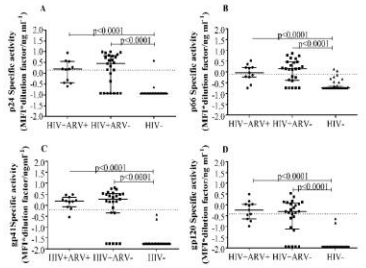
Table 3: Semen cytokine associations with HIV-specific antibodies in HIV^+ARV^- (n=25) men

Cytokines	p24		p66		gp41		gp120	
	r value	p value	r value	p value	r value	p value	r value	p value
TNF-α	**0,48**	**0,01**	0,36	0,08	0,36	0,07	**0,44**	**0,03**
MIP-1β	**0,41**	**0,04**	0,26	0,21	0,30	0,15	0,35	0,08

Genital HIV-specific antibodies correlated significantly to total IgG(1-4) in HIV^+ARV^+ but not in HIV^+ARV^- men.



Genital HIV-specific antibody titres were similar between the HIV^+ARV^+ and HIV^+ARV^- men.



Conclusions

- The IgG1 and IgG3 subclass differences suggest that HIV directly drives the development of these subclasses which in turn can impact antibody function.
- Semen Total Ig may be a good proxy for HIV-specific antibodies in HIV infected individuals on ARVs.
- Semen HIV-specific antibody profiles were not affected by the use of ARVs however, ARVs may impact the pro-inflammatory cytokine and chemokine mileu at the mucosae evidenced by their differential associations with HIV-specific antibodies.
- Together, these data may provide important insight into vaccine induced immunity in combination with ARVs, as pre-exposure prophylaxis, at the site of vulnerability, the male genital tract.

PREVIOUS PUBLICATION

The manuscript entitled, "Semen IgM, IgG1 and IgG3 differentially associate with pro-inflammatory cytokines in HIV-infected men," has been accepted into the journal, Frontiers in Immunology on the 19th December 2018 and is now published online.

Manuscript title: Semen IgM, IgG1 and IgG3 differentially associate with pro-inflammatory cytokines in HIV-infected men.

Authors: Thevani Pillay, Parveen Sobia, Abraham Jacobus Olivier, Kapil Narain, Lenine Liebenberg, Sinaye Ngcapu, Mesuli Mhlongo, Jo-Ann Shelley Passmore, Cheryl Baxter, and Derseree Archary

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Semen IgM, IgG1, and IgG3 Differentially Associate With Pro-Inflammatory Cytokines in HIV-Infected Men

Thevani Pillay[1†], Parveen Sobia[1†], Abraham Jacobus Olivier[2†], Kapil Narain[1], Lenine J. P. Liebenberg[1,3], Sinaye Ngcapu[1,3], Mesuli Mhlongo[1], Jo-Ann S. Passmore[1,2,4,5], Cheryl Baxter[1] and Derseree Archary[1,3]*

[1] Centre for the AIDS Programme of Research in South Africa (CAPRISA), University of KwaZulu-Natal, Durban, South Africa, [2] Institute of Infectious Diseases and Molecular Medicine (IDM), University of Cape Town, Cape Town, South Africa, [3] Department of Medical Microbiology, University of Kwazulu-Natal, Durban, South Africa, [4] National Health Laboratory Service (NHLS), Cape Town, South Africa, [5] Division of Medical Virology, Department of Pathology, University of Cape Town, Cape Town, South Africa

Genital inflammation significantly increases the risk for HIV infection. The seminal environment is enriched in pro-inflammatory cytokines and chemokines. Here, we investigated the interplay between semen cytokines and humoral immunity to understand whether the characteristics of semen antibodies are associated with genital inflammation. In 36 HIV-infected and 40 HIV-uninfected mens' semen, HIV-specific antibodies (gp120, gp41, p66, and p24), immunoglobulin (Ig) subclasses, isotypes and cytokines, using multiplex assays, were measured. Semen IgG1, IgG3, and IgM were significantly higher in HIV-infected compared to HIV-uninfected men ($p < 0.05$). In HIV-uninfected men, pro-inflammatory cytokines IL-6, IL-8, and MCP-1 significantly correlated with IgG1 and total IgG (IgG1+IgG2+IgG3+IgG4) (both $r \geq 0.55$; $p \leq 0.001$). Total IgG in HIV-infected men correlated to HIV-specific antibodies in the semen irrespective of antiretroviral (ARV) use. In HIV-infected, ARV-treated men, p66 and gp41-specific antibodies were inversely correlated with IL-6 and MIP-1α (both $r \geq -0.65$, $p \leq 0.03$). In HIV-infected, ARV-naïve men, p24 and gp120-specific antibodies correlated significantly with pro-inflammatory TNF-α ($r \geq 0.44$, $p \leq 0.03$), while p24 antibodies correlated significantly with chemokine MIP-1β ($r = 0.45$; $p = 0.02$). Local cytokines/chemokines were associated with the mucosal-specific Ig subclasses which likely effect specific antibody functions. Together, these data inform on mucosal-specific immunity that may be elicited in the male genital tract (MGT) in future vaccines and/or combination HIV prevention strategies.

Keywords: semen, HIV, HIV-specific antibodies, immunoglobulins, cytokines, genital inflammation

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ABBREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity
ADCP	Antibody Dependent Cellular Phagocytosis
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
ARV	Antiretroviral
Basic FGF	Basic Fibroblast Growth Factor
BAMA	Binding Antibody Multiplex Assay
BCR	B Cell Receptor
bNAbs	Broadly Neutralizing Antibodies
βNGF	Nerve Growth Factor
BV	Bacterial Vaginosis
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CAP004	CAPRISA 004
CAP008	CAPRISA 008
CD40L	CD40 Ligand
cDNA	Complimentary Deoxyribonucleic Acid
CI	Confidence Interval
CTACK	Cutaneous T Cell Attracting Chemokine
CVL	Cervicovaginal Lavage
DMPA	Depot medroxyprogesterone Acetate
DNA	Deoxyribonucleic Acid
EDC	(1-Ethyl-3 (3-Dimethylaminopropyl) Carbodiimide Hydrochloride
Fab	Fragment Antigen Binding

Fc	Fragment Crystallization
FcRn	Neonatal Fc Receptor
GCLP	Good Clinical Laboratory Practice
GCSF	Granulocyte Colony Stimulating Factor
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
Gp	Glycoprotein
Groα	Growth Regulated Oncogene Alpha
HESN	Highly Exposed Seronegative
HIV	Human Immunodeficiency Virus
HGF	Hepatocyte Growth Factor
HLA	Human Leukocyte Antigen
HSV	Herpes Simplex Virus
Ig	Immunoglobulin
IFNα2	Interferon Alpha 2
IFN β	Interferon Beta
IFNγ	Interferon Gamma
IL	Interleukin
IL1α	Interleukin 1 Alpha
IL1β	Interleukin 1 Beta
IP10	Interferon Inducible Protein 10
IQR	Interquartile Range
LLOD	Lower Limit of Detection
LPS	Lipopolysaccharide
MCP1	Monocyte Chemoattractant Protein 1

MCP3	Monocyte Chemoattractant Protein 3
MFI	Mean Fluorescent Intensity
MIG	Monokine Induced By Interferon Gamma
MIF	Macrophage Migration Inhibitory Factor
MIP1α	Macrophage Inflammatory Protein 1 Alpha
MIP1β	Macrophage Inflammatory Protein 1 Beta
MIP3α	Macrophage Inflammatory Protein 3 Alpha
MPER	Membrane Proximal Region
NK	Natural Killer
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PrEP	Pre-Exposure Prophylaxis
PRRS	Pathogen Recognition Receptors
PDGF-ββ	Platelet Derived Growth Factor
RANTES	Regulated On Activation, Normal T Cell Expressed And Secreted
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SA-PE	Streptavidin Phycoerythrin Conjugate
SCF	Stem Cell Factor
sCD40L	Soluble CD40 Ligand
SCGFβ	Stem Cell Growth Factor Beta
SDF1α	Stromal Cell Derived Factor 1 Alpha
SIV	Simian Immunodeficiency Virus
STI	Sexually Transmitted Infection

SOPs	Standard Operating Procedures
Sulfo-NHS	N-Hydroxysulfosuccinimide)
TCR	T Cell Receptor
TFH	T Follicular Helper
TGF B	Transforming Growth Factor Beta
Th	T Helper
Th1	Type 1
Th2	Type 2
TNFα	Tumor Necrosis Factor Alpha
TRAIL	Tumour Necrosis Factor Related Apoptosis Inducing Ligand
VEGF	Vascular Endothelial Growth Factor

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ABSTRACT

Background: Sexual transmission of HIV across the mucosal surface remains the main route of infection in women. There are several biological and immunological predictors that may enhance mucosal HIV susceptibility, especially in women in high HIV incidence areas. Young women with genital inflammation are at a heightened risk of HIV acquisition. Local inflammation in the female genital tract may play a crucial role in altering the antibody isotypes and IgG subclass profiles that may prevail. Discerning whether this altered immunity may be a consequence of risk or protection against HIV infection may be important in both vaccine-induced and passive immunity studies. Thus, the antibody isotypes, IgG subclasses and cytokine signatures in women with and without genital tract inflammation who acquired HIV infection was investigated.

Methods: Cervicovaginal lavages (CVL) from HIV seroconverters (n=61) and HIV-uninfected women (n=61) and matching plasma samples from the same HIV seroconverters (n=66) and HIV-uninfected women (n=66) who were enrolled in the CAPRISA 004 phase 2 trial and CAPRISA 008 tenofovir gel implementation trial was used to measure HIV-specific binding antibodies (p66, p24, gp41 and gp120), antibody isotypes to measure immunoglobulin profiles and cytokines using multiplex assays.

Results: At the pre-HIV infection time point, HIV-infected women had significantly higher mucosal total IgG, IgG1 and IgM titres compared to HIV-uninfected women ($p \leq 0.05$ for all). Prior to HIV infection, women who subsequently became infected and had genital inflammation had significantly higher mucosal IgM titres ($p=0.05$) compared to HIV-uninfected women who had no genital inflammation. At 3 and 6 months post-infection, HIV-infected women had higher HIV-specific activities for p66, p24, gp41 and gp120 in the genital tract ($p<0.05$ for all) compared to HIV-uninfected women. Additionally, HIV-infected women with genital inflammation had significantly higher HIV-specific activities for mucosal p66, p24, gp41 and gp120 compared to women without genital inflammation ($p<0.05$) at 3 and 6 months. In HIV-infected women, HIV-specific activities for p66, p24, gp41 and gp120 evolved over time in the genital compartment ($p \leq 0.05$ for all). Similarly, at 3 and 6 months post-infection, plasma-specific responses also evolved over time for p66, p24, gp41 and gp120 in HIV-infected women and HIV-infected women with genital inflammation ($p<0.05$ for all). In HIV-infected women without genital inflammation, significant and positive correlations were seen for p66 (at baseline) ($r=0.45$, $p=0.02$) and p24- (at 6 months) ($r=0.46$, $p=0.04$) between the systemic and the genital compartments. At baseline, at least 5 of the 9 pro-inflammatory cytokines and chemokines (MIP1β, MIP1α, IL1β, IL6, TNFα) were positively and strongly associated with IgG1, IgG2, IgG3, IgG4, IgA and IgM in the genital tract ($\beta \leq 0.51$ ng/ml for all; $p<0.05$ for

all). Additionally, mucosal IP10 and IL18 were significantly associated with increased p66- (IP10: β=0.23 MFI*dilution factor/ngml^{-1}, p=0.03; IL18: β=0.38 MFI*dilution factor/ngml^{-1}, p=0.003) and p24-specific (IP10: β=0.20 MFI*dilution factor/ngml^{-1}; IL18: β=0.26 MFI*dilution factor/ngml^{-1}, p=0.02 for both) activities respectively, in the genital tract. Prior to HIV infection, IP10 was significantly associated with increased p66-specific responses (β=0.25 MFI*dilution, p=0.05), whilst IL8 and MCP1 was negatively associated with gp41 (IL8: β=-0.64 MFI*dilution factor, p=0.002) and gp120-specific (IL8: β=-0.47 MFI*dilution factor, p=0.01; MCP1: β=-0.24 MFI*dilution factor, p=0.02) responses in the systemic compartment.

C**onclusion:** Pro-inflammatory cytokines and chemokines showed strong associations with antibody isotypes and IgG subclasses, as well as HIV-specific antibodies in the female genital tract. Together, these data suggest that the local genital tract cytokine milieu may impact the antibody isotypes and specificities in the mucosal compartment, which are important considerations for vaccine induced and passive immunity studies.

INTRODUCTION

In 2017, an estimated 36.9 million people were living with HIV, 1.8 million people became newly infected and 940 000 people died from Acquired Immunodeficiency Syndrome (AIDS)-related illnesses globally (UNAIDS, 2017). Sub-Saharan Africa bears the highest burden of HIV infection accounting for 66% people living with HIV in 2017 (UNAIDS, 2018a). Women in particular, bear a disproportionate burden of HIV infection accounting for the majority of new infections in South Africa in 2017 (UNAIDS, 2018a). Additionally, it is estimated that women are twice as likely to be HIV-infected than men (Ramjee and Daniels, 2013). Importantly, there are many factors (immunological, biological, anatomical and physiological) that contribute to HIV susceptibility and further increase HIV infection rates globally.

Factors that increase risk for HIV acquisition in women include, among others, the mucosal and epithelial barrier integrity, semen viral load, immune activation, availability of sub-mucosal T cell targets for infection, the presence of other sexually transmitted infections (STIs), vaginal hygiene practices and the increased mucosal surface area of the female genital tract (Kaul et al., 2015). Further, Masson et al., (2015) have shown that women with genital inflammation, defined as having 5 of 9 key pro-inflammatory cytokines [interleukin (IL): IL6, IL8, IL1α, interleukin 1 beta (IL1β), tumor necrosis factor-alpha (TNFα), interferon-gamma inducible protein 10 (IP10), monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein 1 alpha (MIP1α), and macrophage inflammatory protein 1 beta (MIP1β)] elevated in the genital tract, had more than a three-fold increased HIV risk compared to women who did not have genital inflammation. Additionally, further studies have shown that in HIV-uninfected women, elevated genital pro-inflammatory cytokines correlated with increased numbers of CD4 target T cells (Kaul et al., 2015, Nkwanyana et al., 2009) and with a proteomic profile indicative of mucosal epithelial cell damage (Arnold et al., 2016). In both HIV-infected men and women, elevated mucosal pro-inflammatory cytokines have been shown to correlate with increased viral loads both in the semen (Gianella et al., 2012, Olivier et al., 2014, Sheth et al., 2005, Politch et al., 1997) and in the vaginal secretions (Gumbi et al., 2008) respectively.

The transudation of HIV-1 specific antibodies from the systemic circulation into secretions in the lower female genital tract has been described in detail by others (Archary et al., 2016, Johansson and Lycke, 2003, Mkhize et al., 2016). Many factors may affect the transudation of antibodies across the mucosa, including vaginal pH (Gupta et al., 2013, Li et al., 2011), the type of antibody glycosylation (Ackerman et al., 2013a), and heterogeneity among individuals. The impact of genital tract inflammation on the isotype, subclass and HIV-binding antibody profile, and the transudation of antibodies into the mucosal compartment are

important areas not fully understood. Genital tract inflammation may have important consequences in the transudation dynamics of various HIV-specific antibodies, isotypes or Ig subclasses in active vaccination or passive immunization strategies.

Given the diverse biological and immunological factors that govern antibody development and transudation either during vaccination or natural infection, understanding whether genital inflammation may skew or alter these profiles, at the site of vulnerability, is important. In populations with pre-existing genital tract inflammation, the alteration of the antibody profiles may have consequences for protection against HIV infection in both vaccine and passive immunity studies. Therefore, this study aimed to characterize these humoral immune parameters in this cohort of women with and without genital tract inflammation who acquired HIV infection. In the context of natural infection, this study investigates how genital inflammation may alter the HIV-specific antibody profiles, and the transudation dynamics at both the isotype and IgG subclass level.

1. LITERATURE REVIEW

1.1 HIV epidemiology

Since the start of the epidemic in 1981, more than 70 million people globally have been infected by the Human Immunodeficiency virus (HIV) with approximately 35 million people having succumbed to the disease (UNAIDS, 2018b). The global adult HIV prevalence rate was 0.8% in 2017 (Figure 1), with 1.8 million people newly infected in 2017 (UNAIDS, 2018b). Sub-Saharan Africa bears the brunt of the HIV epidemic and accounts for 19.6 million people living with HIV (UNAIDS, 2018b). To curb HIV infection rates and alter the trajectory of the HIV epidemic, a safe and efficacious vaccine remains a public health priority, however the development of such a vaccine remains a global challenge.

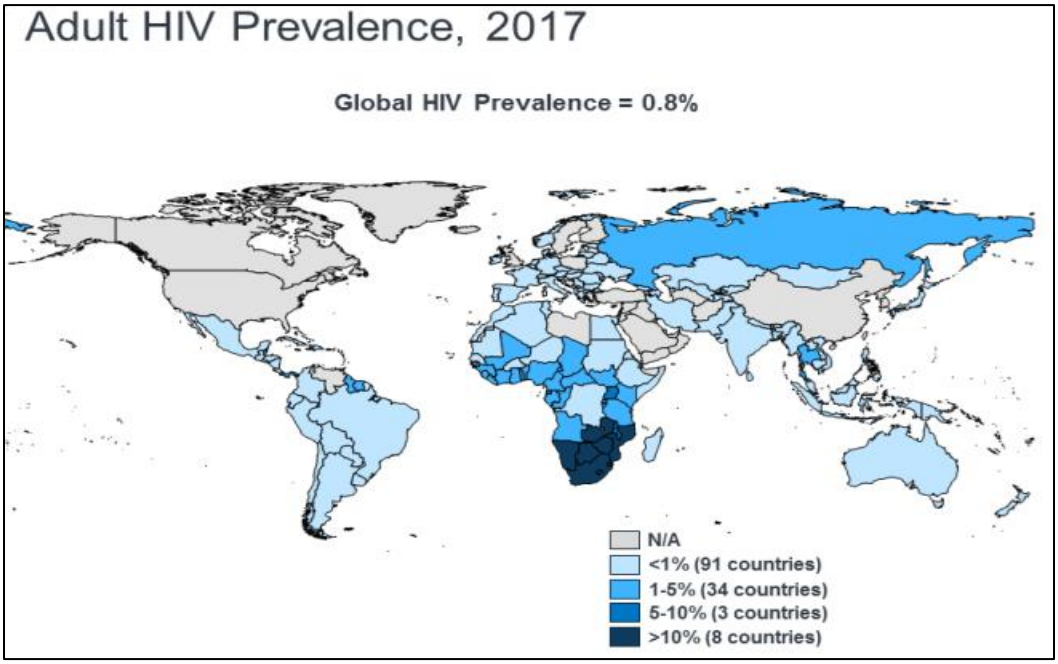


Figure 1: World map illustrating the global adult HIV prevalence in 2017 (Figure adapted from: Kaiser Family Foundation based on UNAIDS).

Young women and adolescent girls are particularly vulnerable to HIV infection in sub-Saharan Africa [as reviewed in (Abdool Karim et al., 2015)]. Compared to men, women aged 15-24 years, have about three-fold higher HIV rates, and acquire HIV infection at least 5-7 years earlier [as reviewed in (Abdool Karim et al., 2017, Shisana et al., 2009)]. KwaZulu-Natal remains the epicenter of the HIV epidemic in South Africa (Shisana et al., 2009), and despite the implementation of pre-exposure prophylaxis (PrEP) (Fonner

et al., 2016), young women remain the most challenging group to protect in an effort toward an AIDS-free generation.

1.2 Behavioural and social risk factors that predispose women to HIV acquisition

Several risk factors predispose young women and adolescent girls to HIV acquisition, these include behavioural, biological, socioeconomic factors (Butler and Eng, 1997) and cultural practices (Sovran, 2013). The lack of education and sexual/reproductive health care services (Butler and Eng, 1997), gender norms (Gupta, 2002, Jewkes et al., 2003), and poverty and violence (Gupta, 2002, Murphy et al., 2006, Higgins et al., 2010, Ackermann and Klerk, 2002) increase the risk for HIV acquisition in women. Intergenerational sexual partnering is an important contributing factor to the HIV epidemic in high-risk populations in South Africa (de Oliveira et al., 2017, Muula, 2008). Although behavioural changes may mitigate the risk of HIV in women, it is unlikely that behaviour alone can alter the trajectory of the epidemic.

1.3 Biological risk factors that predispose women to HIV acquisition

Aside from behavioural factors, there are also several biological risks factors associated with HIV acquisition. Women are more susceptible to HIV risk compared to their male counterparts (Ramjee and Daniels, 2013). This is possibly due to having a greater mucosal surface area, that, coupled with virus exposure and damage to the mucosal epithelia, further increases the risk for HIV acquisition (Abbai et al., 2016). Additional factors that contribute to HIV acquisition include, vaginal insertive practices (Low et al., 2011), the use of particular contraceptive methods (Baeten et al., 2007), STIs [as reviewed in (Abdool Karim et al., 2015, Coombs et al., 2003)], and an inflammatory environment in the female genital tract (Masson et al., 2015).

1.4 HIV-1 Genomic structure

HIV is an enveloped retrovirus which belongs to the genus lentivirus [as reviewed in (Rajarapu, 2014)]. HIV, composed of two copies of single stranded RNA, is primarily made up of nine genes which encode nine polyproteins which are subsequently processed into fifteen protein subunits (Watts et al., 2009). Of these nine genes, *gag*, *pol* and *env* namely, have the information required to produce structural proteins to be used for new virion production. The remaining six genes are *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*, which code for regulatory or accessory proteins and determine how HIV is able to infect new target cells, produce new viral copies, and affect and impact disease progression [as reviewed in (Frankel and Young, 1998)].

1.5 HIV envelope structure

The HIV-1 envelope spikes represent the HIV surface antigens and is made from a glycoprotein (gp) 160 precursor and processed into a trimer containing three gp120 and three gp41 subunits (Figure 2), which each make up the heterodimer [as reviewed in (Munro and Mothes, 2015)]. The envelope interacts with the CD4 binding site on gp120 to initiate the binding process (Kwong et al., 1998). Susceptible CD4 helper T cells and macrophages bear either co-receptors, CXCR4 or CCR5 on their surfaces, which facilitate the binding between this co-receptor and the CD4 receptor by gp120 (Chan and Kim, 1998). The interaction between gp120 and the CD4 receptor on the target cell leads to a conformational rearrangement of gp120, and further engagement between gp120 (V3 loops present on its surface) (Figure 2) and the CCR5/CXCR4 co-receptor binding site, facilitates viral entry into the cell, following gp41 membrane fusion activity [as reviewed in (Munro and Mothes, 2015)].

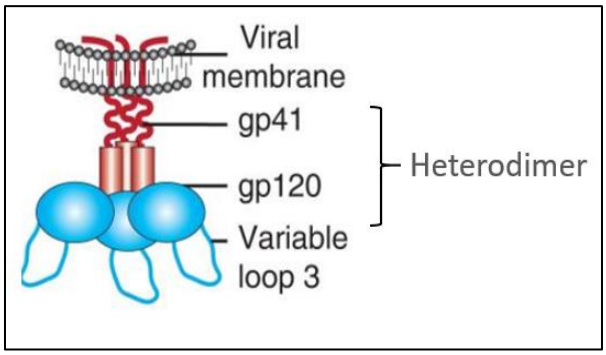


Figure 2: Diagram of the gp120 and gp41 heterodimer [adapted from (Wilen et al., 2012)]. This diagram illustrates the heterodimer structure comprising 3 subunits each of gp120 and gp41, which mediates the binding and fusion of HIV into a target cell.

1.5.1 Glycoprotein 41 (gp41)

Gp41 makes up a subunit of the HIV-1 envelope protein complex and has several sites necessary for infection of host cells within its ectodomain (Mao et al., 2012). This transmembrane protein is a potential target in HIV vaccines as a result of its importance in host cell infection. In a free virion, fusion peptides that reside at the amino termini of gp41 are hidden within the envelope protein complex (Mao et al., 2012). A non-covalent bond with gp120 stabilizes this inactive non-fusogenic state. Gp120 rearranges to expose the binding sites for the co-receptors (CCR5 or CXCR4) (Mao et al., 2012). A cascade of conformational changes is induced within gp120 when it engages with the target cell with subsequent changes in the gp41 subunits. The gp41 core folds into a six-helical coiled structure and exposes the hidden gp41 hydrophobic fusion peptides that are inserted in the membrane of the host cell, thus allowing fusion to occur.

1.5.2 Glycoprotein 120 (gp120)

On the crystal structure of the gp120 core, there is an outer domain and inner domain which is held together by a bridging sheet. Non-covalent bonds with gp41 ensures that gp120 is anchored to the viral membrane (Zhu et al., 2008). The heterodimer facilitates attachment to and entry into the host cell (Zhu et al., 2008). The CD4 binding site, which is found above the bridging sheet, is positioned between the inner and outer domains of the pocket, and these domains are central for CD4 and co-receptor binding (Yoon et al., 2010). Binding between gp120 and a chemokine receptor takes place in the bridging sheet (Liu et al., 2003). Upon binding between the CD4 receptor and gp120, the structure of gp120 is altered and this permits exposure of the bridging sheet, and a more stabilized conformation (Thali et al., 1993) (Sattentau et al., 1993). However, the heterodimer exists as open and closed conformations and reveals different faces to the immune system. Additionally, the evolving glycan shield of gp120 further evades immune recognition by extensive glycosylation [as reviewed in (Crispin et al., 2018, Pantophlet and Burton, 2006)]. Gp120 is also becoming increasingly important in its role in HIV pathogenesis [as reviewed in (Acharya et al., 2015)].

1.5.3 P66 reverse transcriptase (p66 RT)

HIV-1 reverse transcriptase (RT) is encoded in the gag-pol Pr160 precursor protein (Mulky and Kappes, 2005). During the course of, and after viral particle assembly, a 66-kDa RT subunit is produced by viral protease (PR) cleavage of Pr160, and a 51-kDa RT subunit is produced by further cleavage of the C-terminal domain of p66 (Mulky and Kappes, 2005). These two subunits dimerize in the virion to form the functional RT p66 and p51 heterodimer (di Marzo Veronese et al., 1986). The structural and functional characteristics of p66 and p51 are distinct (Mulky and Kappes, 2005).

1.5.4 Protein of 24 (p24)

Two identical single-stranded RNA molecules of HIV virus are enclosed by a viral nucleocapsid protein or p24 [as reviewed in (Rajarapu, 2014)]. P24 makes up most of the viral core, and is detected just before seroconversion (Sabin et al., 2001). Each HIV-1 virus contains between 1500 to 3000 p24 molecules, making it the most abundant viral protein (Summers et al., 1992, Vogt and Simon, 1999). P24 is present at high amounts in blood during the acute and chronic stages of HIV, which renders it a good diagnostic marker, and for monitoring disease progression, evaluation of antiretroviral therapy (ART) and blood donor screening (Allain et al., 1987, Wolf et al., 1988, Fiebig et al., 2003, Petersen et al., 1994).

1.6 HIV-1 life cycle

HIV-1 enters a host cell through the interaction of the CD4 receptor on the target cell and the surface envelope glycoprotein (gp120). The viral membrane and cellular membrane undergo fusion through this

viral envelope glycoprotein gp120/CD4 receptor engagement and the exposed CCR5 or CXCR4 co-receptor binding sites within the V3 crown motif of gp120. (Coffin et al., 1997). Thereafter, co-receptor binding occurs which induces a conformational change within the transmembrane glycoprotein (gp41) region (Sarafianos et al., 2009). Reverse transcriptase (RT) which is enclosed inside the viral core is transcytosed to the cytoplasm of the target cell following membrane fusion. The core is modified by a process of un-coating to facilitate reverse transcription of the viral RNA into the host cell's DNA (Sarafianos et al., 2009). Integration of viral RNA by the reverse transcriptase enzyme copies the viral RNA into complimentary DNA (cDNA) (Coffin et al., 1997). Splicing of viral DNA into the host's DNA by an integrase enzyme occurs within the nucleus to generate the provirus [as reviewed in (Turner and Summers, 1999)]. Messenger RNA, which has the information required for the production of HIV proteins, is transported out of the nucleus of the cell to the endoplasmic reticulum where translation of mRNA into HIV viral proteins occur [as reviewed in (Rajarapu, 2014)]. The new HIV proteins along with viral RNA migrate toward the cells' surface along with envelope and attachment spikes (gp41 and gp120) in preparation for packaging and budding of the new virions [as reviewed in (Rajarapu, 2014)]. Viral protease ensures cleavage of the immature virions into the actual matrix, capsid and nucleocapsid proteins. The various structural components then assemble to produce a mature HIV virion and buds out of the host cell [as reviewed in (Gelderblom, 1997)]. Mature virions are capable of infecting new target cells, and this continuing process ultimately leads to the dysfunction and collapse of the host's immune system [as reviewed in (Rajarapu, 2014)].

1.7 Immune response to HIV infection

The immune system is divided into the innate and adaptive immune system. Cells of the innate immune system, such as natural killer (NK) cells, macrophages, neutrophils and mast cells respond to a breach in the mucosal barrier by HIV, and is the first line of defence against invading HIV [as reviewed in (Vivier et al., 2011)]. Adaptive immunity, defined as the second line of defence, can be further divided into humoral and cell-mediated immunity. In response to HIV infection, the humoral immune response is mediated by antibodies produced by B cells, mostly targeted to cell-free HIV. Therefore, in the context of HIV infection, it is important to understand the developmental process of B cells and antibody responses.

1.7.1 B cell development and their response to antigens

Within a quiescent population of B cells, each B cell expresses a B cell antigen receptor (BCR) with a unique specificity [as reviewed in (Tobón et al., 2013)]. The interaction of a BCR with a specific antigen generates several intracellular signals leading to activation, differentiation, and plasma cell expansion and, ultimately memory B cell formation [as reviewed in (Tobón et al., 2013)].

The process of B cell development [as reviewed in (Bonilla and Oettgen, 2010, Fuxa and Skok, 2007, LeBien and Tedder, 2008)] firstly involves the production of B lymphocytes, which occurs from stem cells present in the bone marrow. B lymphocytes are derived from the early lymphoid progenitor which then passes to the common lymphoid progenitor, and subsequently produces natural killer and dendritic cells, and secondly produces the common lymphoid-2 progenitor that is responsible for the B cell lineage [as reviewed in (Tobón et al., 2013)]. B cells are able to pass through many distinct developmental processes in the bone marrow [as reviewed in (Tobón et al., 2013)].

B lymphocytes are activated when the antigen binds to receptors on the B cell surface. The BCR complex is generated during B cell development as B cells acquire their antigen specificity and follows a program of differential surface antigen expression and sequential heavy and light chain rearrangements [as reviewed in (Tobón et al., 2013)]. This BCR complex, which is initially immunoglobulin M (IgM), determines the cell maturation stage [as reviewed in (Tobón et al., 2013)]. B cells then exit the bone marrow as immature transitional B cells because they transition from the primary lymphoid tissues to the secondary lymphoid tissues. The final stage of maturation and positive and negative selection occurs in the spleen (secondary lymphoid tissue), where the resulting B cells can either be fully matured, anergic or deleted [as reviewed in (Tobón et al., 2013)]. Fully mature B cells, which are characterized by high levels of IgD and intermediate levels of IgM on their cell surface, recirculate between the lymph, blood and the secondary lymphoid tissues. Mature B cells leave the B cell zone and transition into the T cell zone of the lymph node, where they recognize T cells [as reviewed in (Tobón et al., 2013)]. T cells become important for B cell activation during an antigen encounter.

B cells are activated in the secondary lymphoid tissues either by T-cell dependent or T-cell independent activation pathways. T-cell dependent B cell activation involves the activation of both T cells and B cells, upon an antigen encounter (Figure 3). T cells provide ‘help’ to B cells and aid in maturation by two co-stimulatory signals [as reviewed in (Chaplin, 2010)]. Antigens that activate these T cells and B cells are able to establish immunoglobulin (Ig) responses [as reviewed in (Chaplin, 2010, Wu et al., 2016)]. A B cell captures, internalizes and processes the antigen intracellularly via the B cell receptor (BCR), and present the peptides on the cell surface of a B cell via the HLA class II molecule, to the T cell receptor (TCR) on the T cell surface, and constitutes the first signal of T cell help (Figure 3) [as reviewed in (Chaplin, 2010)]. Uptake of the antigen intracellularly influences increased class II expression and CD80 and CD86 expression given off from the B cell (Figure 3) (Wu et al., 2016). A second signal is reciprocated by the T cell after the interaction between the HLA class II molecule and the TCR. The CD40 ligand (CD40L)

expressed on the surface of the T cell, binds to the CD40 receptor found on the surface of the B cell and initiates B cell proliferation and differentiation (Figure 3) [as reviewed in (Chaplin, 2010, Wu et al., 2016)], and interleukin 2, (IL2), interleukin 4 (IL4) and interleukin 5 (IL5) secreted by T cells further aids in the activation of the B cell. Signaling between the CD40L on a T cell and CD40 receptor on a B cell are important for isotype/class switching [as reviewed in (Chaplin, 2010)].

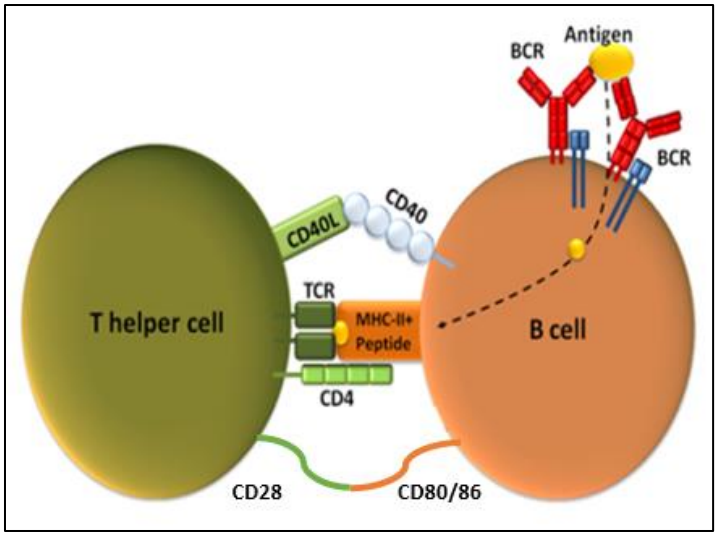


Figure 3: T-cell dependent B cell activation [adapted from (Wu et al., 2016). T-cell help to B cells involves two signals, the first being the presentation of the peptide on the B cell surface via HLA class II molecule to the T-cell receptor on a T cell. The second signal occurs when the CD40L on the T cell binds to the CD40 receptor on the B cell and initiates proliferation and differentiation. These two signals constitute the T-cell dependent B cell activation response to an antigen.

B cells can also be activated in the absence of T cell help, and constitutes T-cell independent B cell responses [as reviewed in (Chaplin, 2010)]. Antigens that stimulate B cells in the absence of T cells include bacterial lipopolysaccharide (LPS) and other polymeric proteins and polysaccharides [as reviewed in (Chaplin, 2010)]. There are two subgroups of T-cell independent antigens, these are thymus-independent antigen I (TI-1) and thymus-independent antigen II (TI-2). TI-1 antigens function independently of BCR specificity, and can induce proliferation and differentiation of B cells without directly stimulating B cells. Whilst, TI-2 antigens mediate cross-linking a number of BCRs and cross activation of these receptors to induce B cell proliferation, differentiation and antibody production [as reviewed in (Maddaly et al., 2010).

1.7.2 Isotype switching, memory B cells and plasma cells

Isotype switching is induced by cytokines derived from T cells [as reviewed in (Chaplin, 2010)]. IL10 expressed by T cells induces switching to IgG1 and IgG3, interferon gamma (IFNγ) of Th1 cells induces switching to IgG2, and transforming growth factor beta (TGFβ) induces switching to IgA [as reviewed in (Chaplin, 2010)]. As B cells undergo isotype switching, somatic hypermutation occurs, whereby random mutations in the antigen-binding regions of the heavy and light chains occur [as reviewed in (Chaplin, 2010)]. Such mutations can result in decreased antigen affinity and cause B cell death. Or an increase in antigen affinity results in B cells producing high affinity antibodies preferentially selected for proliferation [as reviewed in (Chaplin, 2010)]. Clonal expansion and somatic hypermutation occur in the germinal

centers of secondary lymphoid tissues [as reviewed in (Chaplin, 2010) (Schmidlin et al., 2009)]. Memory B cells activate upon re-exposure to its specific antigen and is involved in the secondary immune response [as reviewed in (Chaplin, 2010) (Tangye and Tarlinton, 2009)]. The importance of B cell memory becomes particularly essential in the development of vaccines against pathogenic infections and diseases [as reviewed in (Chaplin, 2010)].

On the other hand, B cells which differentiate and proliferate into plasma cells have the ability to produce antibodies. Each B cell has one unique specificity on its plasma membrane which is specific for a single antigen. A plasma cell produces a single antibody isotype or subclass, each with a unique antigen binding site. Plasma cells are unable to further proliferate and differentiate after antibody secretion, and many undergo apoptosis, or some may survive in the bone marrow for months or years and continue antibody secretion into the blood [as reviewed in (Alberts et al., 2002b)].

1.7.3 What are antibodies?

Antibodies, also known as immunoglobulins, are Y-shaped protein molecules produced by B cells. Antibodies usually appear during a primary immune response as part of the adaptive arm of immunity. An antibody can form a complex with an antigen by binding to a complementary site on the pathogen, resulting in disabling the pathogen and providing signals for other immune defenses.

1.7.4 Diverse functions of antibodies

Antibodies are diverse and can be characterized by the functions that they elicit, this can either be by direct neutralization or engagement of effector cell function through the fragment crystallizable (Fc) receptors. Direct neutralization allows for the fragment antigen-binding (Fab) arm of the antibody to lock directly to the specific antigenic site directly inactivating the pathogen and preventing the pathogen from interacting with healthy cells (Figure 4A) [as reviewed in (Klasse and Sattentau, 2002, Huber and Trkola, 2007)]. The discovery of broadly neutralizing antibodies (bNAbs) that target epitopes spanning the HIV envelope has added optimism to the design of a protective novel vaccine against HIV infection [as reviewed in (Wibmer et al., 2015)]. Some of the HIV-specific bNAbs involved in eliciting direct neutralization function displays great breadth, through the coverage of a wide range of HIV reference strains, and potency, where very small amount of the antibody is need to be effective [as reviewed in (Moore and Williamson, 2016)]. These bNAbs (mostly isolated from HIV-infected individuals) targets specific sites on the HIV envelope including the CD4 binding site antibodies (VRC01,VRC07 and N6) (Walker et al., 2011, Huang et al., 2016), V1V2-specific binding antibodies (PG9, PG16 and CAP256) (Walker et al., 2009, Moore et al., 2011), V3-specific binding antibodies (PGT121) (Walker et al., 2011), gp120/gp41 interface neutralizing antibodies (PGT151)

(Falkowska et al., 2014), and membrane proximal external region (MPER) neutralizing antibodies (2F5, 4E10, 10E8) (Oakes et al., 2018, Chen et al., 2014, Binley et al., 2004). The CAP256 antibody, isolated from a woman who participated in the CAPRISA 002 acute infection study, demonstrated great breadth and potency defined by having a low IC50 titre (Moore et al., 2011). CAP256 neutralized 76% of heterologous viruses and had a subtype bias towards subtype C and A viruses over subtype B viruses (Moore et al., 2011). More recently, the CAP256-VRC26.25 antibody that was isolated from the same CAP256 antibody lineage, displayed 70% neutralization breadth against subtype C viruses, with a remarkably low amount of antibody required to effect neutralization, making this antibody highly potent (Doria-Rose et al., 2016). Two V2-specific bNAbs, PGDM1400 and CAP256-VRC26.25 (Doria-Rose et al., 2014, Doria-Rose et al., 2016), was effective in preventing SHIV infection when passively infused in SHIV-challenged rhesus macaques (Julg et al., 2017). Another preclinical non-human primate study showed modest potency and neutralization breadth against SHIV reference strains when passively infused with monoclonal antibodies (VRC01, NIH45-46, 45-46G54W, 45-46m2, 3BNC117, 12A12, 1NC9, 8ANC195, 10–1074, PGT121, and PGT126) (Shingai et al., 2014). The identification of such bNAbs that confers a degree of protection against HIV are an attractive prevention modality and are currently being evaluated alone and in combinations in passive immunization studies in humans [as reviewed in (Morris and Mkhize, 2017, Julg et al., 2017, Huang et al., 2016)].

Whilst direct neutralization against pathogens is an efficient mode of antibody-mediated defence, non-neutralizing antibodies have also been explored to define additional effector functions used for viral clearance through various mechanisms, i.e. complement lysis, opsonization and phagocytosis/antibody dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC).

Complement lysis is achieved when the Fab arm of the antibody binds to the surface of the pathogen, and the Fc region binds to the first complement component (Figure 4B). This activating complement cascade allows the first complement component to activate a pool of enzymes which induce the lysis of the organism via the membrane attack complex which causes perforation and ultimately cytolysis of the pathogen or organism [as reviewed in (Kopf et al., 2002)].

ADCP, also described as opsonization and phagocytosis, uses neutrophils, monocytes and macrophages to destroy the pathogens. The Fab arm of the antibody binds to the antigen and forms an antibody-antigen complex. The antibody is then recognized by the phagocytic cell, which has specific receptors on their surface (Figure 4C). The phagocytic cell, either macrophages or neutrophils, bind to the antibody via their Fc receptor, and the pathogen is then eliminated via phagocytosis. Here, the antibody acts as an opsonin,

which forms a bridge between the phagocytic cell and the pathogen to ensure phagocytosis of the pathogen. IgG is abundant and binds easily to the Fc receptor on the surface of phagocytic cells, making opsonization and phagocytosis the primary mechanisms for viral clearance [as reviewed in (Mayr et al., 2017, Huber and Trkola, 2007)].

ADCC also uses effector cells, such as NK cells or eosinophils to carry out destruction of the pathogen (Figure 4D). In this case, the Fab arm of the antibody binds to the pathogen or foreign antigen on the infected cell, thus leaving the Fc region free to bind to receptors on the NK cell. This binding complex thus signals the degranulation of NK cells and releases perforin or granzymes which disrupt the cell membrane of the infected target cell, and results in the lysis of the pathogen or infected cell (Sinclair et al., 1988).

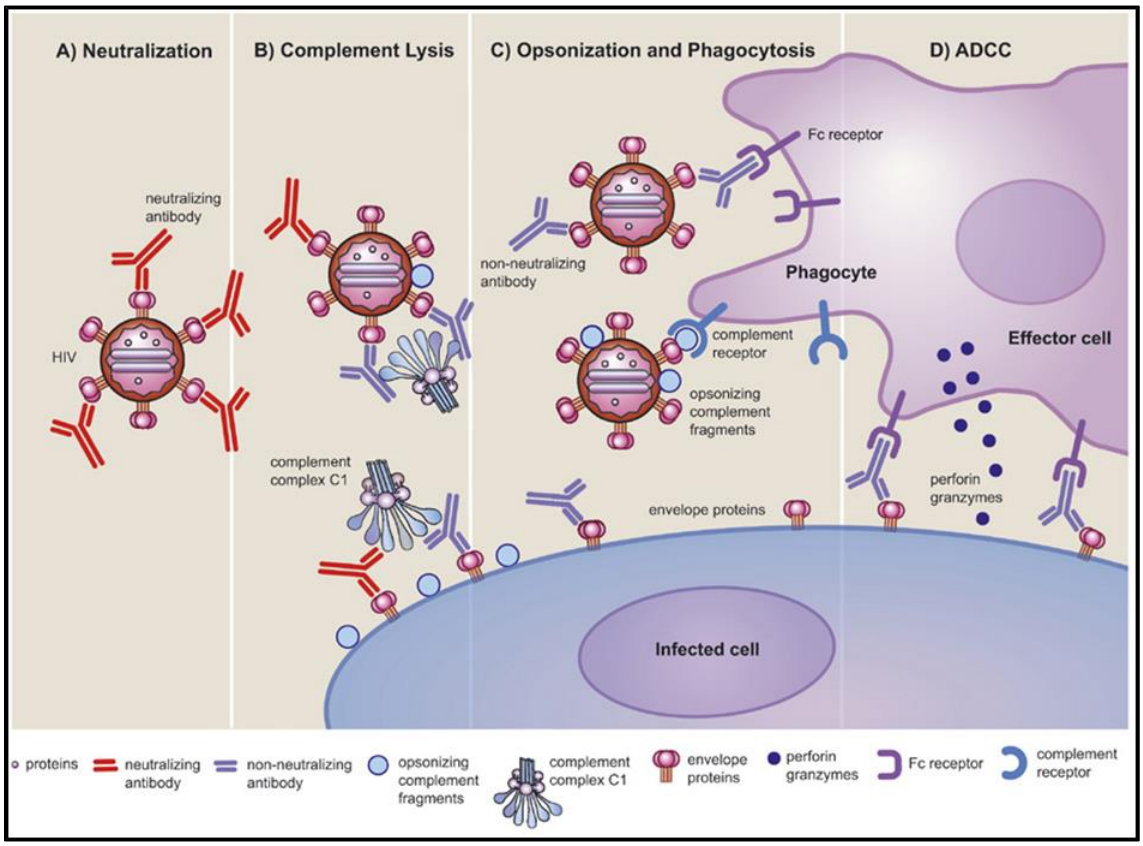


Figure 4: Diverse functions of antibodies [adapted from (Huber and Trkola, 2007)]. (A) Direct neutralization of a pathogen, (B) complement-mediated lysis of free virus by an antibody, (C) opsonization and phagocytosis of a pathogen, and (D) ADCC against infected target cells.

1.7.5 Antibody isotypes and subclasses (IgG1-IgG4)

The five main antibody isotypes are IgA, IgD, IgE, IgM and IgG. IgA is a dimeric antibody found in mucosal surfaces (respiratory tract and the gut), with monomeric (approximately 90%) IgA1 predominantly found in serum, while locally produced polymeric IgA2 is present in external secretions. The secretory component of IgA (sIgA) offers protection against immunoglobulin degradation by proteolytic enzymes and can therefore survive harsh environments like the gastrointestinal tract, thus playing an important role in protective immunity and the prevention of pathogen colonization [as reviewed in (Woof and Kerr, 2006)]. IgD serves as an antigen receptor on B cells that have not been exposed to antigens (Geisberger et al., 2006). IgD causes the activation of basophils and mast cells for the production of antimicrobial factors (Chen et al., 2009). IgE effectively binds to allergens thereby releasing histamine from basophils and mast cells involved in allergy (Galli and Tsai, 2012). IgM is a monomeric antibody and is expressed on the B cell surface, it is a pentamer in a secreted form, which has a high avidity (Geisberger et al., 2006). IgM is the largest antibody compared to other antibody isotypes and presents during the acute infection stage in response to initial antigen exposure. IgG offers the most antibody-mediated immunity against pathogens (Twigg III, 2005).

IgG is the predominant class found in the serum, and in the male (Haimovici et al., 1997, Wolff et al., 1992) and female (Johansson and Lycke, 2003) genital tracts and non-mucosal tissues. IgG plays a role in protective immunity against pathogens and toxins [as reviewed in (Roopenian and Akilesh, 2007)]. IgG is the only antibody that is actively transferred from mother to offspring and confers short-term passive immunity (Morphis and Gitlin, 1970, Brambell, 1966). The neonatal Fc receptor (FcRn) carries out this specific transportation of IgG across the placenta (Simister and Rees, 1985, Simister and Mostov, 1989), and the interaction between the Fc receptor and IgG shows the IgG transport mechanism [as reviewed in (Roopenian and Akilesh, 2007)]. The FcRn prolongs the IgG antibody half-life in serum, thus ensuring high concentrations of this antibody in the circulation [as reviewed in (Roopenian and Akilesh, 2007)]. Recent studies have shown that FcRn in the vaginal epithelium has the ability to facilitate bidirectional transportation of IgG (Li et al., 2005, Ye et al., 2011, Gupta et al., 2013) between the lumen and sub-luminal space in the genital tract for immune protection.

1.7.6 HIV-1 specific IgG subclass responses

The IgG antibody has four subclasses, IgG1, IgG2, IgG3 and IgG4 (Figure 5). IgG1 antibody in HIV-1 binds to Fc region and mediates ADCC of HIV-1 infected cells (Figure 5A) . IgG1 predominate as anti-Env antibodies in HIV infection (Klasse and Blomberg, 1987, McDougal et al., 1987b, Khalife et al., 1988, Mathiesen et al., 1989, Mergener et al., 1987, Sundqvist et al., 1986) during the acute and chronic stages,

and has the broadest response to gag, env and pol proteins (Wilson et al., 2004). IgG1 mediates antiviral functions, through the binding of the Fc receptors on NK cells to facilitate ADCC of HIV infected cells (Ljunggren et al., 1988) and IgG1 can effectively directly neutralize HIV [as reviewed in (French et al., 2017)]. IgG1 is normally the most abundant subclass, but a lack of IgG1 is seen in a variety of primary and secondary antibody deficiencies such as hypogammaglobinemia (Vidarsson et al., 2014). IgG1 deficiencies in combination with other IgG subclass deficiencies are associated with recurrent infections underscoring their importance in infection control and immunity (Jefferis and Kumararatne, 1990).

At various stages throughout HIV-1 infection, IgG2 can be detected (Chiodi et al., 1989, Lambotte et al., 2009), but is less abundant than the other IgG subclasses (Figure 5B) (Klasse and Blomberg, 1987, McDougal et al., 1987a, Khalife et al., 1988). The lack of IgG2 antibodies correlated to the progression to AIDS (Lal et al., 1991) while the detection of IgG2 antibodies in long-term non-progressors correlated with control of viral load (Ngo-Giang-Huong et al., 2001).

IgG3 is the second most predominant IgG subclass (Broliden et al., 1989), and enhanced flexibility of the immunoglobulin hinge region provides IgG3 with a greater *in vitro* neutralizing ability (Figure 5C). During acute HIV infection, anti-gag IgG3 antibodies appear and then declines (Wilson et al., 2004). Previous studies have shown higher levels of IgG3 were found in HIV-infected individuals with higher viral loads and enhanced B-cell dysfunction compared to healthy individuals (Béniguel et al., 2004). IgG3 has a shorter half-life (Morell et al., 1970), and is particularly effective in the induction of ADCC and ADCP (Vidarsson et al., 2014).

The HIV-1 specific IgG4 antibody is found more readily in chronically HIV-1 infected patients (Figure 5D) (Tomaras and Haynes, 2009). IgG4 antibodies arise in response to chronic antigenic stimulation in the setting of chronic parasite exposure and may become more dominant subclass in this setting (Vidarsson et al., 2014).

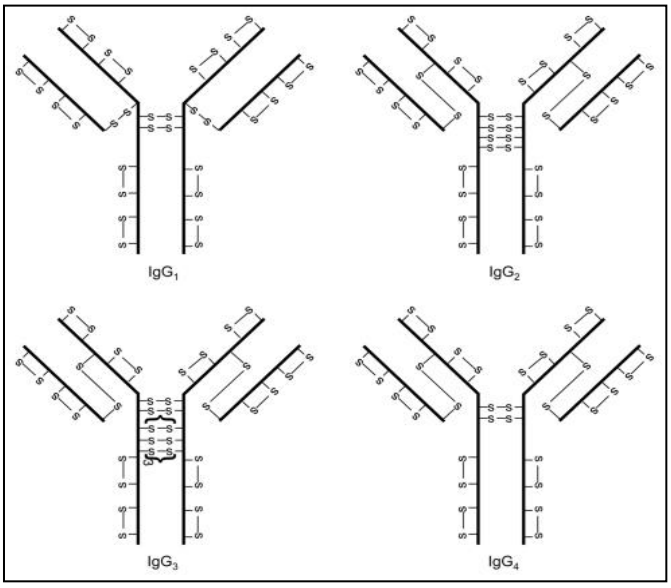


Figure 5: Subclasses of IgG (IgG1, IgG2, IgG3 and IgG4) [adapted from (Liu and May, 2012). The Fragment antigen binding (Fab) arm and Fragment crystallizable (Fc) part of the antibody is linked by the flexible hinge region. Among the IgG subclasses, the length and flexibility of the hinge varies, and as a result this affects the possible conformations of the Fab arms relative to the Fc domain as well as to each other.

1.7.7 HIV-specific antibody responses

HIV-specific antibody responses can be studied at a proteome and epitope level [as reviewed in (Gallerano et al., 2015)]. At a proteomic level, it is possible to identify which HIV proteins are capable of eliciting antibody responses and at an epitope level, it is possible to determine which sites of a protein are bound by antibodies [as reviewed in (Gallerano et al., 2015)]. A mixture of antibody isotypes and many HIV-1 specificities constitute a humoral immune response to HIV-1 throughout infection [as reviewed in (Gallerano et al., 2015)].

Progression of HIV through the early acute phase of infection can be marked by antibody responses to the proteins from the *gag, pol* and *env* genes, in addition to the detection of viral RNA and p24 proteins [as reviewed in (Gallerano et al., 2015)]. The initial response to HIV-1 are anti-gp41 IgM antibodies, thereafter class switching to IgG and IgA occurs (Tomaras et al., 2008). The p24-specific IgG is present at approximately 18 days post-infection [as reviewed in (Gallerano et al., 2015)]. Antibody responses to gp41 and gp120 occur at 13 and 28 days respectively after the presence of detectable viral RNA levels [as reviewed in (Gallerano et al., 2015)]. However, these binding antibodies have no detectable effect on

viremia (Tomaras et al., 2008) and do not exert any selective immune pressure on the envelope (Keele et al., 2008).

One of the approaches to prevent HIV infection at the portal of entry and block productive viral replication in the reproductive tract of women can be through antibody secretion at this mucosal portal of HIV entry (Devito et al., 2000, Belyakov et al., 2004, Smith et al., 2014). In the context of infection, preclinical non-human primate models infected intravenously with live attenuated virus SIVmac239Δnef elicited plasma B cells produced antibodies that were gp41 trimer specific. Histological examination of the macaque female genital tract showed that plasma B cells were present in the submucosa and ectopic tertiary lymphoid follicles of the ectocervix and vagina, and that the gp41-trimer specific IgGs through the FcRn were found lining the vaginal epithelium (Li et al., 2014, Zeng et al., 2016). Subsequent studies have confirmed that the gp41 trimer specific antibodies produced through SIV gp41 trimer immunogens were present both systemically and found complexed to the FcRn in the vaginal epithelium (Voss et al., 2016), indicating a possible role for these HIV specific antibodies to elicit effector functions at the mucosal portal of entry. In humans, mucosal Env-specific antibodies in highly-exposed seronegative (HESN) women correlated with protection (Tudor et al., 2009, Kaul et al., 2001, Seaton et al., 2014), while in the HPTN 035 microbicide trial, mucosal gp120-specific IgAs correlated with protection in these HESN women (Seaton et al., 2014). The immune correlates of protection for the RV144 vaccine trial showed that plasma V1/V2-specific IgG correlated with protection in the presence of low Env-specific IgA (Bonsignori et al., 2012, Pollara et al., 2014). The functional immune responses showed that V1/V2 IgG1 and IgG3 mediated superior ADCC and ADCP activities (Yates et al., 2014) indicating the possible role of circulating HIV-specific antibodies conferring protection. However, whether these antibodies conferred protection through transudation or local production in the genital tract remains undefined as no mucosal samples were taken from participants in the RV144 vaccine trial.

Additionally, HIV-1 specific antibodies have been shown to transudate from the systemic circulation to the female genital tract (Letvin et al., 2011) and 90% of the HIV-specific antibodies found circulating correlated significantly with those in the genital tract (Archary et al., 2016). Vaginal pH, heterogeneity among individuals and the type of antibody glycosylation (Hessell et al., 2007), are a few of the many factors which affect antibody transudation across the mucosa [as reviewed in (Horton and Vidarsson, 2013)]. The transudation from the systemic compartment to the female genital tract highlight the importance of locally produced or transduced antibody responses in the female genital tract to prevent infection (Zhou and Ruprecht, 2014, Sholukh et al., 2015, Neutra and Kozlowski, 2006).

1.8 Antibodies as correlates of protection

Extracellular pathogens travel to their target cells or tissues via the bloodstream, and most vaccines confer protection from these pathogens through the stimulation of antibodies (Plotkin, 1999). Pathogens can also reach their target cells by secreting toxins that can be neutralized by antitoxins, however, there are some pathogens that replicate on mucosal surfaces that become susceptible to antibodies which diffuse from serum (Plotkin and Plotkin, 2008). Antibodies serve as correlates of protection, and one way of demonstrating this principle is by administering them passively by means of an injection, or to detect a protective effect of maternal antibodies in the newborn baby [as reviewed in (Zinkernagel and Hengartner, 2006)]. Vaccines which have been effective and worked against diseases include smallpox, diphtheria, tetanus, pertussis, *Haemophilus influenzae* type b (Hib) infection, pneumococcus infection, hepatitis A, hepatitis B, varicella, measles, rubella, polio, and rabies (Plotkin and Plotkin, 2008). Locally secreted IgA or transcytosed IgG antibodies found on mucosal surfaces can confer protection against pathogenic organisms on that surface and against pathogens that colonize the mucosa before systemic invasion (Plotkin and Plotkin, 2008). The main goal of vaccines in HIV infection is to induce neutralizing antibodies that block infection (Gunn and Alter, 2016). However, antibodies cannot confer protection from pathogens by neutralization alone, most often recruitment of additional antibody effector functions (complement activation and phagocytic clearance) are needed to kill pathogens against which they are directed (Gunn and Alter, 2016). In the context of antibody protection in the female genital tract, Mkhize et al (2016) and later Archary et al (2016) showed transudation dynamics of IgG from the systemic compartment into the genital secretions, and that the induction of systemic HIV-specific bNAbs can prevent viral replication at the portal of entry (Mkhize et al., 2016, Archary et al., 2016).

1.9 The female genital tract

The female genital tract is divided into the lower and upper tracts. The lower tract comprises the ectocervix and vagina, which is lined by squamous epithelium tissue, and the upper tract comprises the endocervix, fallopian tubes and endometrium lined by a single layer of columnar epithelium tissue [as reviewed in (Kaushic et al., 2010, Hickey et al., 2011, Horbul et al., 2011)]. The female genital tract is vulnerable to HIV infection and is the main portal of entry to invading microorganisms and STIs (Ganz, 2002, Mowat, 2003, Nagler-Anderson, 2006, Shacklett et al., 2009). Despite its vulnerability to infection, the female genital tract has particular innate immune defences that act as a primary barrier. The innate immune defence in the female genital tract consists of the secretion of mucus, includes the production of antimicrobial factors, and soluble proteins such as cytokines (Pitman and Blumberg, 2000, Mowat, 2003, Shacklett et al., 2009, Masson et al., 2014). Additionally, the mucosal immune system normally tolerates the commensal microbes present (Shin and Kaul, 2008), and any microbial dysbiosis can lead to a disruption of local

immunity. Anatomical, biological, immunological and behavioural factors all impact on HIV risk in women.

1.9.1 Epithelial cell damage, genital tract inflammation and immune activation

A healthy reproductive tract is generally not permissive to external pathogens and is characterized by having an intact epithelial barrier with a predominance of *Lactobacilli* (Figure 6) [as reviewed in (Burgener et al., 2015)]. *Lactobacilli* plays a role in metabolising glycogen to lactic acid in the lower reproductive tract [as reviewed in (Danielsson et al., 2011)], thus lowering pH levels, creating a hostile environment for microbial growth thereby preventing pathogen invasion [as reviewed in (Valenti et al., 2018, Aroutcheva et al., 2001)]. However, a microbial imbalance that exists in the female genital tract may facilitate easy pathogen invasion and a breach in the mucosal barriers. Sexual intercourse alone can cause microabrasions or tears to the vaginal epithelium increasing the risk for HIV acquisition (Fraser et al., 1999).

HIV can migrate through epithelial cells allowing pathogen translocation (Nazli et al., 2010) via microabrasions or gaps in the epithelial barrier (Figure 6) [as reviewed in (Burgener et al., 2015)], mediation of transcytosis via the FcRn, or through paracellular movement between epithelial cells (Rodriguez-Garcia et al., 2013). In addition, genital tract inflammation has come under the spotlight as a factor that further increases the risk for HIV infection (Arnold et al., 2016).

The body triggers an immune response to the invading pathogen, and the first responders that are recruited to the site of breach include myeloid target cells, such as macrophages, neutrophils, and dendritic cells (Figure 6) [as reviewed in (Burgener et al., 2015)]. These myeloid cells express pattern recognition receptors (PRRs) on their surface, including toll-like receptors (TLRs) [as reviewed in (Alberts et al., 2002a, Schroder and Tschopp, 2010)], which are designed to detect viral or bacterial ligands. These innate cells, via their PRRS, initiate an antigen-specific adaptive immune response and release soluble pro-inflammatory cytokines [as reviewed in (Kumar et al., 2011, Takeda et al., 2003). The secretion of pro-inflammatory cytokines such as IL1, IL8, and IL22 enhance inflammation of the epithelial barrier likely leading to a leakier epithelium (Figure 6) [as reviewed in (Burgener et al., 2015)]. In addition, these cytokines signal CD4 T cells to the breached site, and CD4 T cells become activated. The level of activation of CD4 T cells are measured through the expression of surface markers such as CCR5, CD25, CD38, CD69, CD71, CD95 and HLA-DR (Imlach et al., 2001, Mueller et al., 2001, Li et al., 2012). Therefore, activated CD4 T cells have an upregulated expression of the surface CCR5 co-receptor to which HIV uses opportunistically to bind and gain entry to further replicate and cause viral dissemination.

Another subset of CD4 T-cells, T-helper 17 (Th17) cells that are particularly important in maintaining mucosal barrier integrity also contribute to pathogen clearance at mucosal surfaces. However, the loss of Th17 cells at mucosal surfaces has been linked to inflammation [as reviewed in (Guglani and Khader, 2010, Stieh et al., 2016)]. In contrast, Stieh et al (2016) identified Th17 cells as being the primary targets of SIV during vaginal transmission. Thus, an increase of Th17 cells at the mucosal surface may increase vulnerability for HIV infection and may emphasize its role in HIV pathogenesis [as reviewed in (Klatt and Brenchley, 2010)].

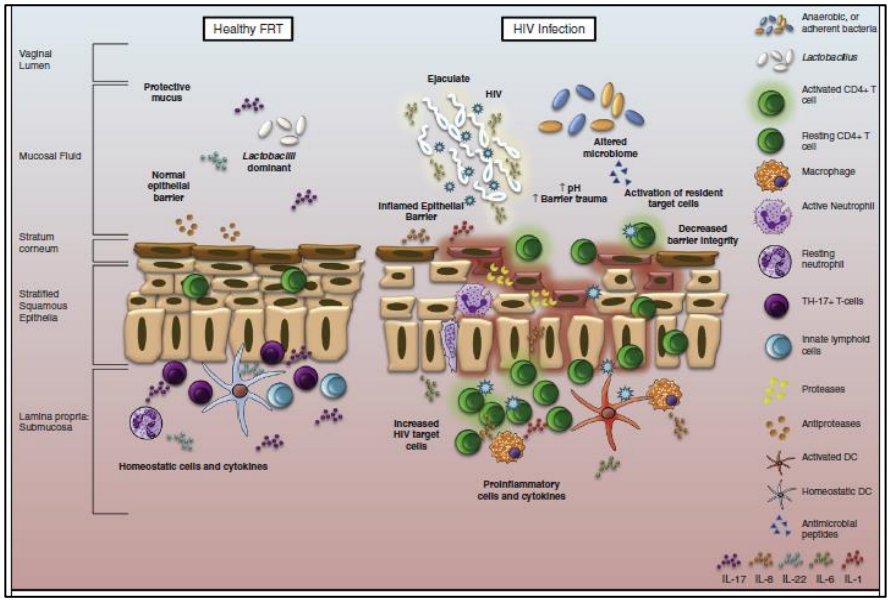


Figure 6: Proposed mechanisms of epithelial barrier damage in the female reproductive tract [adapted from (Burgener et al., 2015)]. In a healthy reproductive tract, there is an intact epithelial barrier which is generally free of invading pathogens. However, in the presence of HIV infection, there is opportunity for microbial pathogens to attach to epithelial cells or innate immune cells (neutrophils, dendritic cells and macrophages) and cause an inflamed environment through the secretion of pro-inflammatory cytokines. Another consequence of this is disruption of the vaginal microbiome with increased barrier trauma and pH levels.

1.9.2 Sexually transmitted infections (STIs) and bacterial vaginosis (BV)

Inflammation is a double edged sword that can afford the clearance of STIs on one hand, but on the other hand facilitate deeper penetration of pathogens into the tissues thereby resulting in further immune activation and inflammation and ultimately epithelial barrier damage (Svanborg et al., 1999, McGee et al., 1999). STIs are the major cause for the recruitment of immune cells and the upregulation of inflammatory cytokines in the genital mucosa [as reviewed in (Kalichman et al., 2011)]. STIs are often persistent or recurrent and in rare cases, few women are able to clear an infection in the absence of antimicrobial treatment in the presence of bacterial STIs [as reviewed in (Golden et al., 2000)]. STIs can either be

symptomatic, where symptoms present soon after exposure, or asymptomatic, with no signs or symptoms of an infection often making their diagnoses difficult (Connolly et al., 2002).

Herpes simplex virus (HSV) is a common STI, of which there are two major groups HSV-1 (which commonly cause cold sores) and HSV-2 (Lou et al., 2012). HSV-2 itself elicits a very pro-inflammatory response leading to increased HIV susceptibility (Kaul et al., 2008). In addition, HSV-2 primarily causes genital ulcers, leading to a compromised epithelial barrier which facilitates HIV infection [as reviewed in (Abdool Karim et al., 2015, Freeman et al., 2006)]. Other STIs which can increase the risk for HIV acquisition include *Human papilloma virus* (HPV), *Chlamydia trachomatis*, *Neisseria gonorrhoea* and *Trichomonas vaginalis* [as reviewed in (Coombs et al., 2003)].

Apart from STIs, there are other conditions such as BV that can further predispose women to increased HIV acquisition. BV has been shown to directly increase genital tract inflammation leading to increased HIV susceptibility (Mirmonsef et al., 2012). BV is characterized by the lack of *Lactobacillus* species leading to a dysbiotic mucosal environment in the vagina that favours the growth of BV-associated organisms (Cook et al., 1992), such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera* Type 1 and Type 2, *Bacteroides* species, *Mobiluncus* species, *Mycoplasma* species, and *Ureaplasma urealyticum*. Symptomatically, BV is characterized by a thin homogeneous white discharge. The gold standard for diagnosing BV is by gram staining of vaginal smears and is based on a Nugent scoring system (Beverly et al., 2005, Nugent et al., 1991). A score of 7 to 10 is consistent with a BV diagnosis (Beverly et al., 2005, Nugent et al., 1991). Another method of BV diagnosis is through the use of an Amsel test, which should fulfil three of any of the following four criteria, (1) abnormal vaginal discharge, (2) a vaginal pH of more than 4.5, (3) a positive amine odour test, (4) and the microscopic confirmation of clue cells (Cook et al., 1992, Amsel et al., 1983).

An upregulation and down-regulation of pro-inflammatory cytokines may occur in women who have BV (Yudin et al., 2003, Sturm-Ramirez et al., 2000, Ryckman et al., 2008). In the CAPRISA 004 trial, which tested the safety and efficacy of tenofovir gel, HIV acquisition was reduced by approximately 39% overall, and by 54% in women with high gel adherence (Abdool Karim et al., 2010). However, Klatt et al (2017) showed that the tenofovir gel was only able to reduce HIV incidence by 18% in women with a *non-Lactobacillus* dominant vaginal microbiome, while in women with a *Lactobacillus* dominant vaginal microbiome, HIV incidence was reduced by 61%. In addition, the amount of tenofovir detected in the mucosa was also lower in *non-Lactobacillus* dominant women (29.8%), compared to the *Lactobacillus* dominant women (46.2%) (Klatt et al., 2017). The *in vitro* mechanism proposed for the stark differences in

tenofovir gel efficacy was that *Gardnerella vaginalis* (the predominant microbe responsible for BV) metabolized the tenofovir thereby undermining the efficacy of tenofovir and leading to increased HIV risk (Klatt et al., 2017). Besides the increased HIV acquisition risk associated with STIs and BV, onward transmission of HIV has been associated in the background of STIs and BV through increased viral shedding in the genital tract secretions leading to the increased infectiousness of HIV-infected individuals (Herold et al., 2013).

1.9.3 Vaginal (douching) and contraception

In addition to biological factors, there are also behavioural risks factors such as vaginal douching and contraceptive use which contribute to HIV acquisition and an inflamed genital tract. Vaginal douching is a common practice in some southern African countries (Maleche and Day, 2011). Vaginal practices, including douching (Low et al., 2011), involves the insertion of drying agents including various household chemicals, alum, herbs, powders, creams or a dry cloth to create a tight, "hot" and dry vagina often perceived as desirable and highly pleasurable to their male partners (Zhang et al., 1997). These practices create an environment susceptible to tearing of the mucosal epithelia through friction during sexual intercourse. These microabrasions or tears disrupts the commensal vaginal bacteria leading to microbial dysbiosis (Hilber et al., 2007, Myer et al., 2005, McClelland et al., 2006). These drying agents can disrupt the natural pH level leading to inflammation, thereby increasing the risk of acquiring HIV.

Depot medroxyprogesterone acetate (DMPA) is one of the most common contraceptive choices in South Africa (Darroch and Singh, 2013) and some studies have shown that its use is associated with higher HIV risk [as reviewed in (Brind et al., 2015)], while other studies showed no such associations (Kleinschmidt et al., 2007, Myer et al., 2007, Kiddugavu et al., 2003). The underlying mechanism for the increased HIV risk is not well defined. However, some studies have found that DMPA causes vaginal thinning and reduced density of intracellular junction proteins in the stratified epithelial layer (Chandra et al., 2013, Ildgruben et al., 2003, Miller et al., 2000, Wieser et al., 2001, Wira et al., 2011). Vaginal epithelial cell thinning permits easy viral entry (Hel et al., 2009) and contact between HIV target cells within the cervicovaginal mucosa and HIV viral particles entering the vaginal lumen. Efficient transmission of SIV was shown in macaques that were vaginally challenged, and the mechanism purported is that the prior use of high-dose DMPA caused sufficient vaginal epithelium thinning leading to efficient vaginal SIV transmission (Abel et al., 2004, Marx et al., 1996, Trunova et al., 2006, Wieser et al., 2001).

1.10 Cytokines in response to inflammation and HIV susceptibility

Cytokines have a pleiotropic nature and local cytokines produced in response to pathogenic organisms may have a variety of effects on local immune and inflammatory responses during mucosal infections (Hedges et al., 1998). Cytokines are grouped according to pro-inflammatory, anti-inflammatory, chemokines, adaptive and growth factors.

The immunologic environment of the female genital tract is distinct from the systemic compartment (Barnabas et al., 2013). Genital inflammation has been shown to contribute to HIV pathogenesis, with high levels of inflammatory cytokines correlating with increased frequency of HIV shedding in the genital tract (Barnabas et al., 2013) and with markers of long-term HIV disease progression, such as CD4 T-cell loss and higher viral load set-point in the plasma (Barnabas et al., 2013).

Genital tract inflammation is defined as having a profile of five of any of the nine inflammatory cytokines above the 75th percentile concentration for each cytokine (MIP1α, MIP1β, IP10, IL8, MCP1, IL1α, IL1β, IL6, and TNFα) in the genital tract (Table 1) (Masson et al., 2015). Masson et al (2015) showed that women with genital tract inflammation (≥5 of 9 inflammatory cytokines elevated) were at significantly increased risk of HIV acquisition. Another study re-affirmed the association of genital tract inflammation and the increased risk for HIV acquisition in women (McKinnon et al., 2018). This study of the same women confirmed that having the median concentrations in the upper quartile for ≥3 of 9; ≥4 of 9; ≥5 of 9; ≥6 of 9 and ≥7 of 9 pro-inflammatory cytokines (IL6, IL8, IL1α, IL1β, TNFα, IP10, MCP1, MIP1α, and MIP1β) across all longitudinal time points defined genital tract inflammation in women (McKinnon et al., 2018). In this study, genital tract inflammation significantly undermined the efficacy of tenofovir gel in preventing HIV in women with genital inflammation compared to women without genital tract inflammation (3% versus 57% respectively) (McKinnon et al., 2018).

Liebenberg et al (2017) showed that mucosa-biased gradients (increased genital tract cytokine levels relative to the plasma cytokine levels) of IP10, MIP1β, IL8, and MCP1, were significantly associated with increased HIV risk. MIP1α and MIP1β recruit CCR5 target cells required for the establishment of HIV infection (Grivel et al., 2011), and IP10 is the ligand for CXCR3 which is expressed on overlapping T-helper type 1 cells as CCR5 (Groom and Luster, 2011). Evidence of elevated CCR5-binding chemokines and regulated on activation, normal T-cell expressed and secreted (RANTES) were also associated with increased HIV risk (Morrison et al., 2014). The table below lists the role of some of the main cytokines that are associated with genital tract inflammation.

Table 1.1: Overview of the cytokines used in the definition of genital tract inflammation (Masson et al., 2015).

Class	Cytokine	Producing cell type	Function/role	Reference
Pro-inflammatory	IL6	Macrophages, T-cells	Mediation of an inflammatory environment, activation, proliferation, apoptosis and differentiation.	(Shah et al., 2011)
Pro-inflammatory	IL1α, IL1β	Activated macrophages	Promotes enhanced inflammatory responses, activation, fever, synthesis of acute phase Proteins.	(Dunn et al., 2001)
Pro-inflammatory	TNFα	Macrophages, monocytes	Occurs during acute inflammation and signals events with cells, which triggers necrosis or apoptosis.	(Alfano and Poli, 2005)
Chemokine	IL8	Tissue and blood cells	Recruits and attracts neutrophils in inflammatory regions.	(Bickel, 1993)
Chemokine	IP10	Leukocytes, activated neutrophils, eosinophils, monocytes, epithelial cells, endothelial cells, and fibroblasts	Regulates innate and adaptive immune responses by affecting the function of activated T cells, NK cells, inflammatory dendritic cells, macrophages, and B cells.	(Liu et al., 2011)
Chemokine	MCP1	Osteoblasts	Recruitment of monocytes, memory T cells, and dendritic cells to the sites of inflammation.	(Carr et al., 1994)
Chemokine	MIP1α, MIP1β	Macrophages	Activates human granulocytes such as neutrophils, eosinophils and basophils, which lead to acute neutrophilic inflammation, and induces the synthesis and release of pro-inflammatory cytokines such as IL1, IL6 and TNFα.	(Wolpe et al., 1988)

1.11 Cytokines regulate B cell responses and antibody isotypes

In addition to cytokines playing a role in inflammatory responses in the genital tract, antibody isotypes and immunoglobulin subclasses may also be altered or skewed owing to the effects of cytokines. Cytokines may impact the type and quality of antibodies that are locally produced or transduced into the genital tract. Upon a pathogen encounter, innate cells (macrophages and dendritic cells) direct phagocytic clearance, and in doing so, these cells also secrete cytokines. And depending on whether a Th1 or Th2 driven-response may arise against an intracellular (viral or bacterial) or extracellular (helminths or parasite) pathogen, the type of antibody produced may also be influenced by type of cytokines secreted during either given response.

During a Th1 response, IL2 and IFNγ are secreted and carry out phagocytic activity, whilst during a Th2 response, IL4, IL5, IL9, IL10 and IL13 are secreted and stimulate high titres of antibody production [as reviewed in (Spellberg and Edwards, 2001, Seder and Paul, 1994, Del Prete et al., 1994)]. IFNγ secreted during a Th1 response (Vazquez et al., 2015) controls class switch recombination from IgM to the IgG1 and IgG3 subclasses [as reviewed in (Spellberg and Edwards, 2001)]. On the other hand, IL4 was shown to enhance B cell activation and drive class switch recombination to IgG2, IgG4 and IgE, driving a Th2 immune response (Vazquez et al., 2015). Thus, cytokines which are secreted during a Th1 or Th2 response play a critical role in protective immunity by helping B cells produce antibodies against foreign pathogens (Naradikian, 2016).

However, in the background of inflammation, we do not understand what the interplay is between how genital tract inflammation may affect the different antibody isotypes or subclass responses both locally, or through the transudation from the systemic into the genital compartment. Although there are several promising biomedical HIV prevention strategies, in the case of active vaccination or passive immunization, we need to understand if genital inflammation can undermine vaccine efficacy or functional antibody responses at the portal of entry, the genital mucosae.

1.12 Strategies for HIV prevention

HIV prevention strategies are of particular importance in women who are unable to negotiate safer sex with their partner [as reviewed in (Abdool Karim et al., 2015)] and more robust methods are needed to reduce the burden of disease. Several HIV prevention strategies have been explored. Topical PrEP was the first to prove the concept that ARVs can reduce HIV transmission (Abdool Karim et al., 2010), yet confirmatory studies such as VOICE (Marrazzo et al., 2015) and FACTS 001 (Rees et al., 2015) failed to confirm the findings, and thus there are no licensed topical products [as reviewed in (Abdool Karim et al., 2015, Abdool Karim et al., 2010)]. Oral PrEP, however, has been shown to reduce the risk of HIV acquisition in a range of populations (Grant et al., 2010, Baeten et al., 2012, Thigpen et al., 2012) and is currently recommended by the World Health Organisation (WHO) as a prevention option. Although PrEP has been successful in lowering HIV risk (Grant et al., 2010, Baeten et al., 2012, Thigpen et al., 2012, Choopanya et al., 2013), young women still remain vulnerable to HIV infection owing to their lack of commitment and adherence to PrEP.

The use of long-acting injectable PrEP (rilpivirine and cabotegravir) and intravaginal rings are promising biomedical HIV prevention strategies that are being developed [as reviewed in (Baxter and Abdool Karim, 2016)]. Long-acting injectable PrEP and intravaginal rings may be more acceptable and feasible for women, however failure to adhere to such prevention methods as prescribed, still pose a challenge in preventing HIV transmission [as reviewed in (Meyers and Golub, 2015)].

A novel HIV prevention strategy being investigated is the use of bNAbs, which have been shown in pre-clinical studies using non-human primate models to prevent SHIV infection, as passive immunization (Julg et al., 2017, Shingai et al., 2014). bNAbs which have been previously tested in preclinical studies and which are currently underway into passive immunity studies in human clinical trials include VRC01, VRC07, PGT121 and CAP256-VRC26.25LS (Julg et al., 2017, Dosenovic et al., 2015, Lynch et al., 2015, Rudicell et al., 2014).

Although there have been several biomedical prevention strategies proposed to prevent HIV infection particularly in women, we still do not understand what the effect of genital tract inflammation may have on the efficacy of various biomedical prevention strategies and what this could mean for vaccine-induced immunity in the genital tract. Efforts which aim to generate appropriate immune responses in the genital tract that could confer protection against HIV is central to the development of a vaccine. Hence, this study investigated the effect of prior genital tract inflammation on antibody isotypes and subclasses, and HIV-specific antibody responses prior to and post-HIV infection in the female genital tract. This study included women enrolled in the CAPRISA 004 phase IIb trial, which assessed the efficacy of the tenofovir gel in a randomized double-blinded placebo control trial, and the CAPRISA 008 1% tenofovir gel implementation trial, based in KwaZulu-Natal, South Africa.

2. HYPOTHESIS

Genital tract inflammation alters the Ig isotype, subclass and HIV-specific binding antibody profiles during natural infection.

3. OBJECTIVES

3.1 PRIMARY OBJECTIVES

3.1.1 To measure the pre-infection and early HIV infection (3 months and 6 months post-infection) immunoglobulin isotypes, IgG subclasses and HIV-1 specific binding antibody profiles in plasma (n=66) and CVLs (n=61) of women who acquired HIV while participating in the CAPRISA 004 and CAPRISA 008 trials (45 women from CAPRISA 004 trial and 21 women from the CAPRISA 008 trial).

3.1.2 To measure the early HIV infection phase (3 months and 6 months post-infection) cytokine profiles in the plasma (n=66) and CVLs (n=61) of the HIV seroconverters for whom pre-infection CVL cytokine data has been collected.

3.2 SECONDARY OBJECTIVES

3.2.1 To determine whether there were associations between the various immunoglobulin isotypes, IgG subclasses or HIV-specific binding antibody profiles with pro-inflammatory cytokines during pre-infection stage and early post-HIV-infection stage in the blood or genital tract.

3.2.2 To determine if the levels of HIV-specific antibodies or IgG subclasses or isotypes during early HIV infection were altered (lower or higher) in the women with pre-infection or post-infection genital tract inflammation compared to women with no genital tract inflammation.

4. METHODS

4.1 Study design

This is a retrospective sub-study of women from the CAPRISA 004 (CAP 004) (BREC Reference: E111/06) (Abdool Karim et al., 2010) and CAPRISA 008 (CAP 008) (BREC Reference: BFC237/010) (Mansoor et al., 2014) studies.

In this sub-study (BREC Reference number: BE0207/17, Appendix II) stored cervico-vaginal lavage (CVL) supernatant (n=61) samples and plasma (n=66) samples were used for each of the women enrolled in the CAP 004 (n=45) and CAP 008 (n=21) trials. The women were matched on the basis of HIV status; HIV-1 seroconverted (cases) or remained HIV-uninfected (controls). All the samples were chosen according to a 1:1 ratio of case: control. The follow-up specimens from these women included matched specimens for the 3 month and 6 month time-points. In the women who seroconverted, the 3 and 6 month specimens were used as the post-infection time-points, whereas the controls were matched in the study for time and remained HIV-uninfected. Baseline samples for each of the women was taken as their last HIV negative test result before becoming HIV-infected. Post-infection time-points were chosen and matched as the closest available sample plus/minus two weeks either side of the 3 months and 6 months follow-up to ensure each case and control were matched accordingly. Controls were matched to each of the cases based on the estimated date of infection, and followed the same criteria as the cases.

4.2 Participant flow diagrams

Genital inflammation was defined as having a profile of ≥5 of the 9 inflammatory cytokines elevated across all timepoints in the study (IL1α, IL1β, IL6, TNFα, IL8, IP10, MCP1, MIP1α, MIP1β) in CVL in this cohort of women, and were at risk of HIV acquisition. The number of women who participated in the CAP 004 and CAP 008 trial who provided CVL and matching plasma samples is provided in Figure 1.

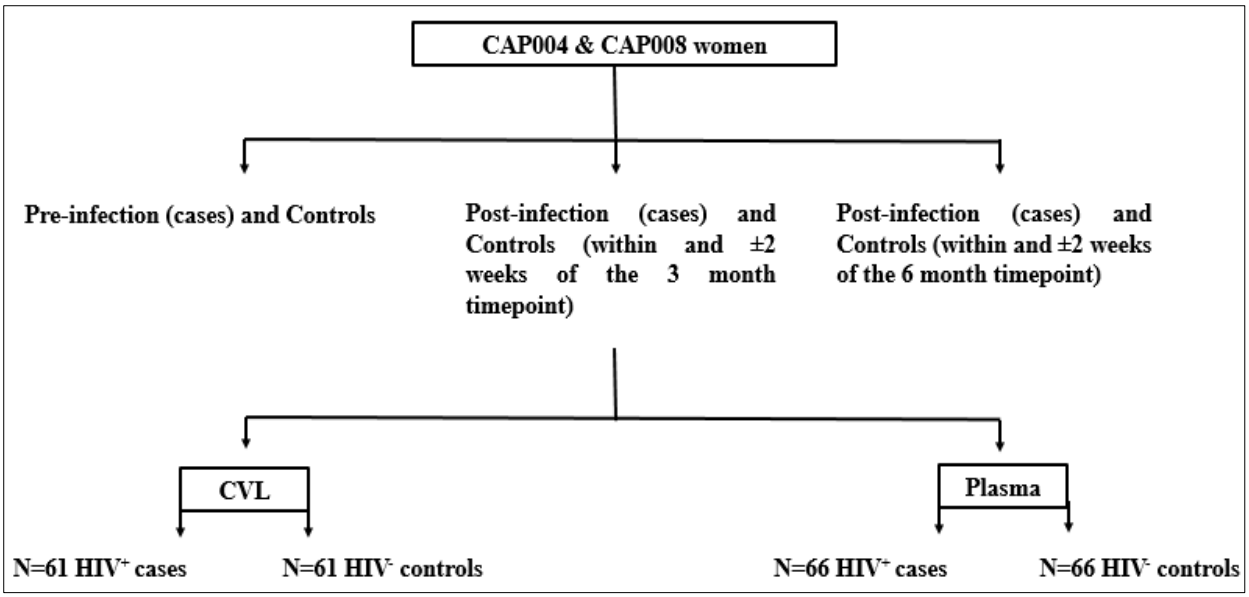


Figure 1: Flow diagram of samples used in the CAP 004 and CAP008 trials.
Collection of CVLs and matching plasma from these women in the CAP 004 trial and CAP 008 trial was before seroconversion, and then follow up visits at 3 months and 6 months. The CVL and matching plasma samples taken from the women who remained HIV-uninfected were taken at the same follow up visits. There were fewer CVL samples available at the time of the study, hence the reduced number of women in the CVL arm compared to the matched plasma arm for the CAP004 and CAP008 trials.

4.3 Specimen collection and processing

4.3.1 Cervico-vaginal lavage (CVL) collection

Participants consented to the specimen collection procedure for genital specimens during the original studies (CAP 004 and CAP 008). A plastic bulb pipette was adapted for dispensing saline and collection of the lavage fluids as follows: the plastic bulb pipettes were cut just below the bulbs and the tips of the pipettes were fixed onto 10ml syringes. A volume of 5mls of sterile phosphate buffered saline (PBS) or normal saline was drawn up through the pipette and passed through a previously lubricated speculum (the speculum was lubricated with water only), after it was inserted into the vagina. The PBS or normal saline was directed toward the cervical os in order to let the saline bathe the cervix and the posterior fornix. The fluids were then aspirated into the same adapted pipette. This procedure of bathing the cervix was repeated three times using the same fluid that was drawn into the pipette to ensure a thorough collection of cervical fluids. The final volume was aspirated and dispensed into a sterile 30ml urine container or 15ml conical tube. The CVL samples were stored on ice and transported to the laboratory for further processing. The time between the collection of the CVL to processing and storage was approximately 5 to 6 hours.

4.3.2 Cervico-vaginal lavage (CVL) specimen processing

The CVL specimen samples were then processed to recover the cellular material and the supernatant fluids for downstream experiments. The CVL specimen was transferred aseptically into a sterile 15ml screw-capped centrifuge tube, and thereafter centrifuged at 400xg (Centrifuge 5810R, Eppendorf, South Africa) for 10 minutes in order to separate the pellet containing the cellular component from the supernatant. The supernatant was aliquoted into cryovials and stored at -70°C. The cell pellet was stored in vapour phase liquid nitrogen at approximately -140°C according to the standard operating procedures (SOPs) for sample storage at CAPRISA. Collection time, the time the samples were processed in the laboratory and the time at which the samples were stored was recorded.

4.3.3 Plasma collection and processing

Participants consented to the blood specimen collection procedure during the original studies (CAP 004 and CAP 008). 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.

4.4 Isotyping assay

Total IgG, IgA and IgM in CVL was quantified using a 6-plex Bio-Plex Pro™ Human Isotyping Panel kit (Bio-Rad, USA) on the Bio-Plex 200 multiplex system (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. Serial dilutions of CVL using PBS was 1:100, 1:50 and mostly 1:10 for samples in the CAP 008 and CAP 004. The principle of the isotyping assay is illustrated in Figure 2.

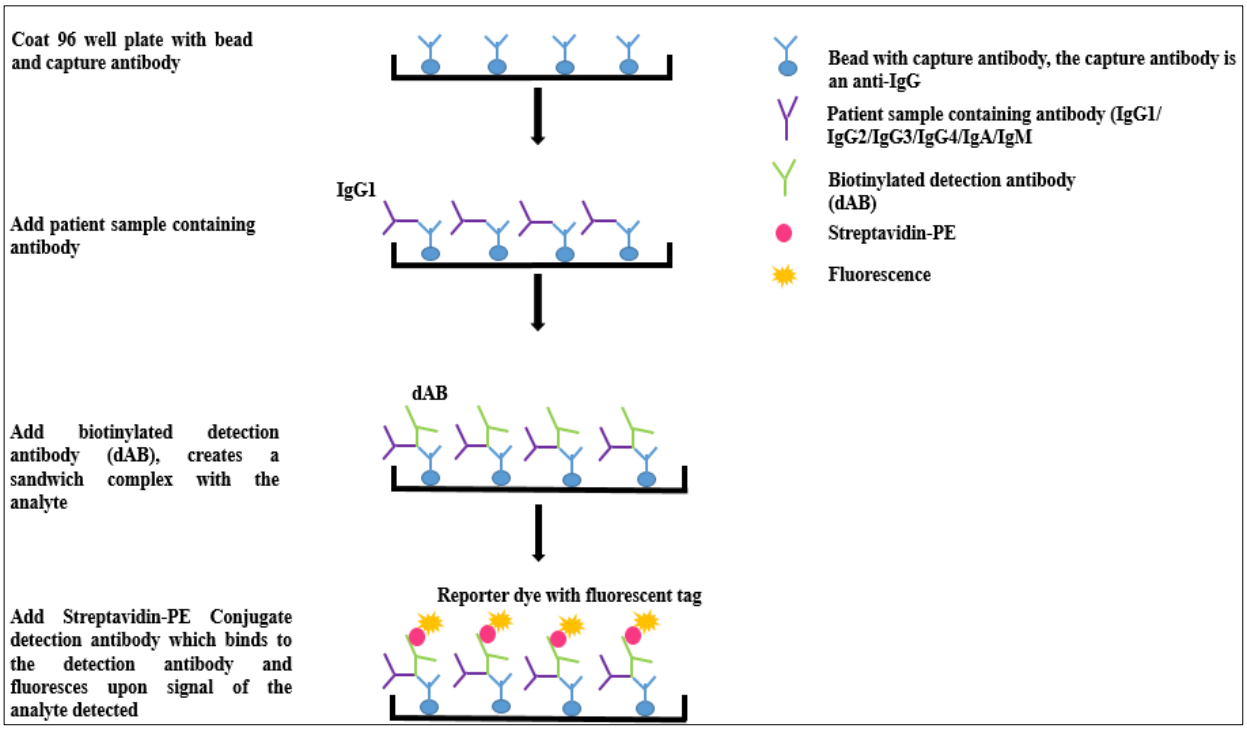


Figure 2: Principle of Bio-Plex sandwich immunoassay used for the isotyping assays.

4.4.1 Initial preparation

The plate layout was planned according to the kit instructions and samples were thawed on ice at -4°C. The Bio-Plex system (Bio-Rad, Hercules, CA) was warmed up for 30 minutes, and thereafter calibrated for a further 10 minutes. Calibration of the machine was done in a MCV (maintenance, calibration and validation) plate by adding 6 drops each of the CAL1 and CAL2 beads provided in a Bio-Plex calibration kit (Bio-Rad, Hercules, CA). A further 70% isopropanol (Appendix I), 10% bleach (Appendix I) and deionized water was dispensed in the required volumes in the MCV plate.

The 10X wash buffer, assay buffer and isotyping diluent were vortexed and brought to room temperature until it was needed. The 1X wash buffer called the isotyping buffer was prepared by adding 60ml of the 10X wash buffer to 540ml deionized water. The Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA) system was then primed using the isotyping (1X) wash buffer. The vial of the kit supplied standard was reconstituted in 781µl of PBS, and the vial of quality controls were each reconstituted in 250µl of PBS and incubated on ice for 30 minutes after a quick vortex of a few seconds. Samples were prepared in a 96 well round bottom plate referred to as the master plate -according to the designed plate

layout (Figure 3). A volume of 15µl of the desired CVL sample was added to 135µl of the assay diluent to each well to result in a 1:10 dilution. The same volume of sample to assay diluent was carried out for all the samples.

Day 1		ISOTYPING EXPERIMENTS FOR CVL								12/Dec/2017	
		Plate 1: CAP008									
1	2	3	4	5	6	7	8	9	10	11	12
A S1	S1	200093 (pre) 1:10 (15+135)	200093 (pre) 1:10 (15+135)	200093/LL812 (6m) 1:10 (15+135)	200093/LL812 (6m) 1:10 (15+135)	120050/LL465 (pre/c) 1:10 (15+135)	120050/LL465 (pre/c) 1:10 (15+135)	200103 (pre) 1:10 (15+135)	200103 (pre) 1:10 (15+135)	200103 (3m) 1:10 (15+135)	200103 (3m) 1:10 (15+135)
B S2	S2	200449(pre/c) 1:10 (15+135)	200449(pre/c) 1:10 (15+135)	200449 (6m/c) 1:10 (15+135)	200449 (6m/c) 1:10 (15+135)	200113 (pre) 1:10 (15+135)	200113 (pre) 1:10 (15+135)	200113 (3m) 1:10 (15+135)	200113 (3m) 1:10 (15+135)	200152/LL568 (pre/c) 1:10 (15+135)	200152/LL568 (pre/c) 1:10 (15+135)
C S3	S3	200129 (pre) 1:10 (15+135)	200129 (pre) 1:10 (15+135)	200154 (pre) 1:10 (15+135)	200154 (pre) 1:10 (15+135)	120059 (pre/c) 1:10 (15+135)	120059 (pre/c) 1:10 (15+135)	200185 (pre) 1:10 (15+135)	200185 (pre) 1:10 (15+135)	200185 (3m) 1:10 (15+135)	200185 (3m) 1:10 (15+135)
D S4	S4	200185/100368 (6m) 1:10 (15+135)	200185/100368 (6m) 1:10 (15+135)	200507/LL656 (pre/c) 1:10 (15+135)	200507/LL656 (pre/c) 1:10 (15+135)	200507 (3m/c) 1:10 (15+135)	200507 (3m/c) 1:10 (15+135)	200231 (pre) 1:10 (15+135)	200231 (pre) 1:10 (15+135)	200231 (3m) 1:10 (15+135)	200231 (3m) 1:10 (15+135)
E S5	S5	200242 (pre) 1:10 (15+135)	200242 (pre) 1:10 (15+135)	200242/100376 (3m) 1:10 (15+135)	200242/100376 (3m) 1:10 (15+135)	200242 (6m) 1:10 (15+135)	200242 (6m) 1:10 (15+135)	200255/LL230 (pre/c) 1:10 (15+135)	200255/LL230 (pre/c) 1:10 (15+135)	200255 (3m/c) 1:10 (15+135)	200255 (3m/c) 1:10 (15+135)
F S6	S6	200256 (pre) 1:10 (15+135)	200256 (pre) 1:10 (15+135)	200264 (pre/c) 1:10 (15+135)	200264 (pre/c) 1:10 (15+135)	200317 (pre) 1:10 (15+135)	200317 (pre) 1:10 (15+135)	200317 (3m) 1:10 (15+135)	200317 (3m) 1:10 (15+135)	200317/100367 (6m) 1:10 (15+135)	200317/100367 (6m) 1:10 (15+135)
G S7	S7	200341 (pre/c) 1:10 (15+135)	200341 (pre/c) 1:10 (15+135)	200341 (3m/c) 1:10 (15+135)	200341 (3m/c) 1:10 (15+135)	200320 (pre) 1:10 (15+135)	200320 (pre) 1:10 (15+135)	200320 (3m) 1:10 (15+135)	200320 (3m) 1:10 (15+135)	200338 (pre) 1:10 (15+135)	200338 (pre) 1:10 (15+135)
H S8	S8	200338/100333 (3m) 1:10	200338/100333 (3m) 1:10	200027 (pre/c) 1:10 (15+135)	200027 (pre/c) 1:10 (15+135)	200614 (pre/c) 1:10 (15+135)	200614 (pre/c) 1:10 (15+135)	CONTROL	CONTROL	BLANK	BLANK

Figure 3: Layout of the of a master plate design for the Isotyping assays. The above 96 well master plate was designed to accommodate standards, control, blanks and samples all in duplicate.

A volume of 150µl of the quality controls was added to the master plate. After the 30 minute incubation period, a fourfold standard serial dilution was prepared as shown in Figure 3.

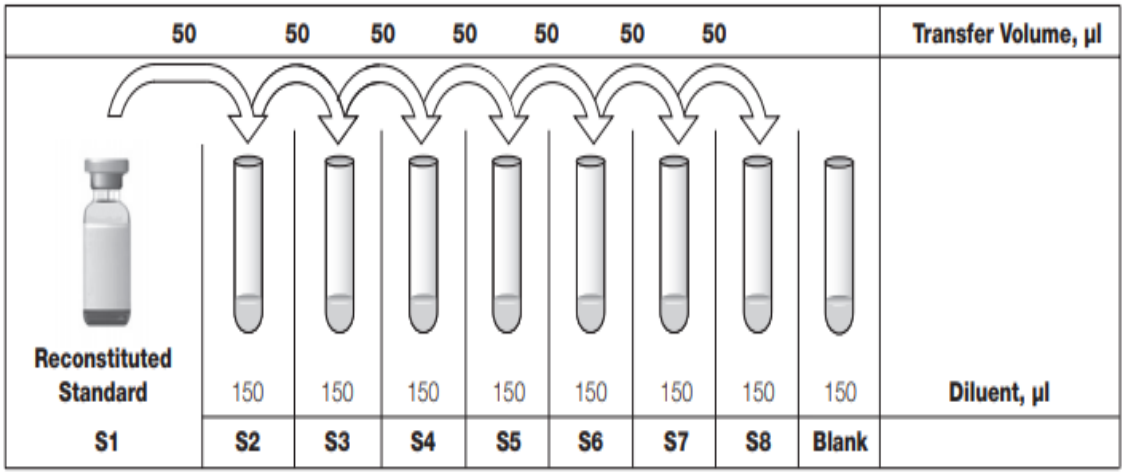


Figure 4: Preparation of a fourfold dilution series (Figure adapted from the Bio-Plex Pro instruction manual).

A volume of 150µl of each of the standards (S1 to S8), and the blank was transferred into their respective wells in the master plate. The coupled beads were vortexed for 30 seconds and 288µl of the 20X beads were added to 5,472µl of assay buffer in a 15ml conical tube to make 1X coupled beads solution.

4.4.2 Running the isotyping assay

The coupled 1X bead solution was vortexed and 50µl was added to each well on the assay plate. The assay plate was washed twice with 100µl of the isotyping wash buffer on the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). A volume of 50µl of the standards, blank, control and samples was then transferred from the master plate into the assay plate and incubated on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm at room temperature for 1 hour. The assay plate was covered with sealing tape and aluminum foil to avoid light exposure as the beads are photosensitive. During the last 10 minutes of the 1 hour incubation period, the detection antibodies were vortexed for 15 seconds and 150µl of the 20X detection antibody was added to 2,850µl of detection antibody diluent in a 15ml conical tube, to make 1X solution. The assay plate was then washed three times with 100µl of the isotyping wash buffer on the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). The prepared 1X detection antibody solution was then vortexed thoroughly and a volume of 25µl was added to each well in the plate. The plate was covered and incubated on the plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm at room temperature for 30 minutes. At this stage, calibration of the Bio-Plex manager software version 6.1 passed and the protocol which contained the standard S1 values and units as provided in the kit was entered into the Bio-Plex 200 manager programme. With the last 10 minutes of the 30 minute incubation period, the 100X streptavidin-PE (SA-PE) was vortexed for 5 seconds and diluted to a 1X solution. A volume of 60µl was added to 5,940µl of assay buffer and transferred into a 15ml conical tube. Once the 30 minute incubation elapsed, the assay plate was washed three times with 100µl of the isotyping wash buffer on the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). Thereafter, 1X streptavidin–PE was vortexed and 50µl was transferred to each well in the assay plate. The plate was then covered and subjected to last incubation step on the plate shaker (Stuart® orbital shaker, UK) for 10 minutes at 850 ± 50 rpm at room temperature. Following this final incubation, the plate was washed three times with 100µl of the isotyping wash buffer, and re-suspended in 125µl of assay buffer. The plate was covered and placed on the plate shaker (Stuart® orbital shaker, UK) for 30 seconds at 850 ± 50 rpm. The sealing tape was removed and the plate was inserted into the Bio-Plex 200 system (Bio-Rad, Hercules, CA) to read at a low RPT which was adjusted to account for a 5,000 gate and 50 bead events.

4.5 Bead coupling procedure for a two-step carbodiimide coupling of protein to carboxylated microspheres

In order to detect HIV specific IgGs, naked polystyrene beads had to be coupled with HIV-1 specific proteins.

4.5.1 Initial preparation

All low binding tubes (USA Scientific 1.5ml micro-centrifuge tubes) were labelled according to the HIV protein and microsphere it was coupled to, and the necessary calculations (Appendix I) were made according to the protocol. The 100mM Monobasic Sodium Phosphate Activation buffer (Appendix I) and Luminex (BAMA) wash buffer (Appendix I) were brought to room temperature and left to shake on the magnetic stir plate (Stuart stir plate, UK) until it was ready for use. The microspheres were protected from light throughout the entire procedure by covering with foil after each step. One of each of the protein-microsphere sets of low binding tubes was used throughout the entire procedure, until it was ready to be transferred into the respective tubes in the end. A doubling reaction was performed throughout the entire experiment.

4.5.2 Microsphere activation

The stock microsphere bead sets: 42 (Bio-Rad, USA), 44 (Bio-Rad, USA), 19 (Bio-Rad, USA), 10 (Bio-Rad, USA) and 53 (Bio-Rad, USA) were vortexed and then sonicated (Ultrasonic CleanernLT-918A, China) for at least 30 seconds to re-suspend the beads into a homogenous mixture to prevent aggregate and clumping of beads that lead to assay and detection inaccuracies and errors.

A volume of 800µl of each microsphere stock was then transferred into low titre binding tubes (USA Scientific micro-centrifuge tube). The stock microspheres were pelleted by micro-centrifugation at 8,000xg (Jouan Centrifuge, France) for 4 minutes. The supernatant was discarded and poured out in the opposite direction of the pellet. The pellet was re-suspended in the small volume of supernatant left over in the tube and vortexed thoroughly. A volume of 200µl of deionized water (dH_2O) was then added to each of the microspheres and vortexed thoroughly and sonicated for 30 seconds to wash the microspheres. The microspheres were pelleted by micro-centrifugation at 8,000xg (Jouan Centrifuge, France) for 4 minutes. The supernatant was removed and the microspheres were washed again with 200µl of dH_2O, and subjected to micro-centrifuguation at 8,000xg (Jouan Centrifuge, France) for 4 minutes. Thereafter 160µl of the activation buffer (100mM Monobasic Sodium Phosphate) was added to the washed microspheres after the supernatant was removed and each of the tubes vortexed. The activation buffer together with the microspheres were then vortexed and sonicated for 30 seconds, and left aside covered in foil. Thereafter 13.1mg of (N-Hydroxysulfosuccinimide) Sulfo-NHS (Thermo Scientific, USA) was measured using the analytical balance (AB104 Mettler Toledo Balance, Switzerland) and dissolved immediately in 262µl of dH_2O to yield a 50mg/ml solution of Sulfo-NHS.

An amount of 10mg was measured and dissolved in 200µl of dH_2O immediately to yield a 50mg/ml solution of EDC [(1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)] (Thermo Scientific, USA). A volume of 20µl of the 50mg/ml Sulfo-NHS and 20µl of the 50mg/ml EDC was added to each of the microspheres and vortexed. The microspheres were incubated on a shaker (Mix Mate, Eppendorf, Germany) for 20 minutes at room temperature and protected from light. The activated microspheres were then pelleted by microcentrifugation at 8,000xg (Jouan Centrifuge, France) for 4 minutes. The supernatant was removed and the microspheres were re-suspended in 500µl of the coupling buffer (PBS) and vortexed and sonicated for 30 seconds. The microspheres were then pelleted by microcentrifugation at 8,000xg (Jouan Centrifuge, France) for 4 minutes. After centrifugation, the activated microspheres were sonicated before washing with 500µl of PBS for the second wash and micro-centrifuged at 8,000xg (Jouan Centrifuge, France) for 4 minutes. The supernatant was removed and the activated washed microspheres were washed in 950µl of PBS that accounted for the volume of antigen that was used. A volume of 1,000µl of PBS was used to wash the blank microsphere (labelled bead set 53).

Numerous washing steps of the microspheres was done to ensure antimicrobials and storage solution was removed. The EDC (Thermo Scientific, USA) and Sulfo-NHS (Thermo Scientific, USA) activated the surface carboxyl groups and yielded a long lived intermediate Sulfo-NHS Ester. All the unreacted EDC and Sulfo-NHS was then removed by several washes to prevent the activation of the carboxyl groups on the protein molecule which result in protein-protein coupling rather than protein-microsphere coupling.

4.5.3 Coupling microspheres to HIV proteins

A volume of 50µl of each of the HIV proteins to achieve a 50µg concentration for a doubling reaction was added to the respective microspheres (p66/bead set 42, gp41/bead set 44, p24/bead set 19, and gp120/bead set 10). The proteins used included gp120 (Jena Bioscience, Germany), gp41 recombinant HIV-1 MN (ImmunoDX, USA), p66 HIV-1 R (Protein Sciences Corporation, USA) and p24 HIV-1/Clade B/C (Immune Technology, USA). The coupling reaction tube was vortexed to mix the microspheres together with the antigens for 30 seconds. The micro-centrifuge tubes were incubated on a shaker (Mix Mate, Eppendorf, Germany) for 2 hours at 1,100 rpm at room temperature and covered with foil to prevent light exposure. After incubation, the coupled microspheres were then subjected to microcentrifugation at 8,000xg for 4 minutes.

The activated microspheres together with the Sulfo-NHS esters on the surface were combined with a protein solution and allowed to mix for 2 hours to ensure that free amines on the protein side chains interacted with the intermediate to form a covalent bond with the microspheres.

4.5.4 Washing and blocking

The supernatant was removed and the pelleted microspheres were flicked in the remaining fluid volume to ensure easy reconstitution. The beads were finally re-suspended in 1ml of the BAMA wash buffer and vortexed for 30 seconds. In order to circumvent bead aggregates, the beads were allowed to mix on the titer plate shaker (Mix Mate, Eppendorf, Germany) for 1 minute at 1,100 rpm to ensure thorough mixing to achieve a homogenous solution. The coupled microspheres were then pelleted by micro-centrifugation at 8,000xg (Jouan Centrifuge, France) for 4 minutes. The supernatant was removed and a second wash using 1ml of the BAMA wash buffer was done. The microspheres were vortexed and allowed to shake on the titer plate shaker (Mix Mate, Eppendorf, Germany) for 1 minute at 1,100 rpm. The coupled microspheres were then pelleted by micro-centrifugation at 8,000xg (Jouan Centrifuge, France) for 4 minutes. The supernatant was removed and the microspheres were re-suspended in 1ml of the BAMA wash buffer and vortexed thoroughly. In separate micro-centrifuge tubes, 1µl of each of the microspheres was added to 19µl of BAMA wash buffer to yield a 1:20 dilution. A volume of 10µl from each of the micro-centrifuge tubes was added to a glass fast reader slide to count the microsphere suspension. A successful coupling reaction resulted in bead counts for each of the protein being more than 7,500 microspheres/µl. Lastly, the remaining volume in each of the primary micro-centrifuge tubes was transferred equally (100µl) to the respective low binding labelled tubes that was initially prepared, and stored at -80°C until ready to use for the BAMA assay.

The coupled microspheres was washed several times in BAMA wash buffer to ensure that any uncoupled hydrophobic patches on the microsphere surface was blocked and any uncoupled protein in solution was removed.

4.6 Binding Antibody Multiplex Assay (BAMA)

HIV-specific antibodies against HIV-1 specific Env and Gag proteins were measured in plasma (1:100; 1:10,000 and 1:100,000 dilutions) and CVL (1:3 and 1:10 dilutions) using a customized HIV-1 binding antibody multiplex assay (BAMA) (Archary et al., 2015, Archary et al., 2016, Haynes et al., 2012, Tomaras et al., 2008, Yates et al., 2011, Yates et al., 2013, Yates et al., 2014). The principle of BAMA is illustrated in Figure 5. All assays were run under good clinical laboratory practice (GCLP)-compliant conditions, and included tracking of positive controls by Levey-Jennings charts. Positivity cut-offs for binding antibody responses in plasma and CVL for antibody-antigen pairs were pre-determined from the available 61 and 60 HIV-1 negative individuals at the time of the study, respectively [Mean Fluorescent Intensity (MFI) + 3 standard deviations)]. Positive controls included titrations of HIV-1+ purified IgG (HIVIG).

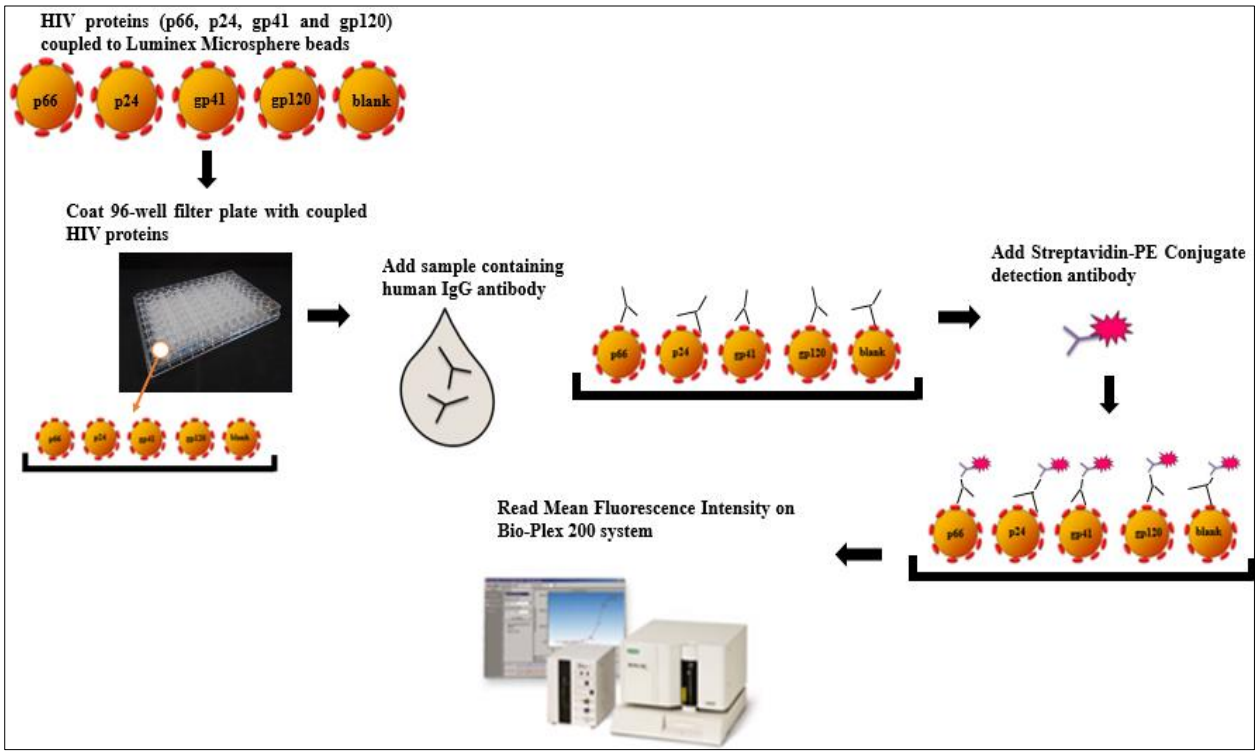


Figure 5: Principle of Customized Binding Antibody Multiplex Assay.

4.6.1 Initial preparation for the BAMA assay

The plate layout was planned and samples were retrieved and thawed on ice and stored at -4°C. Milk blotto/blocking buffer (Appendix I) used as the assay diluent and BAMA wash buffer (Appendix I) were prepared in advance of the assay.

The beads were removed and thawed on ice in a closed ice bucket, protected from light at all times. The BAMA wash buffer and milk blotto were brought to room temperature and left to shake on the magnetic stir plate (Stuart stir plate, UK) until required. The Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA) was primed with BAMA wash buffer. The working bead mixture was prepared in a 15ml conical tube by adding 60µl of each bead set to 5,700µl of BAMA wash buffer. The mixture was covered in foil and vortexed thoroughly. The Bio-Plex system was warmed up for 30 minutes, and thereafter calibrated for a further 10 minutes. Calibration of the machine was performed as mentioned previously.

4.6.2 Running the BAMA assay

The Bio-Rad 96 well vacuum filter plate was pre-incubated with 100µl of BAMA wash buffer in order to wet the filter paper and aspirated thereafter. The working bead mixture initially prepared was vortexed thoroughly again, and 50µl was transferred into each well on the filter plate and covered in foil at all times. The filter plate was left to rotate on a plate shaker (Stuart® orbital shaker, UK) at 220 rpm until needed. Samples were prepared in a 96 well round bottom plate (to facilitate ease of mixing) referred to as the master plate, and according to the designed plate layout and at the respective dilutions (Figure 6). Samples were diluted in assay diluent (milk blotto).

	Plate 1:											
	1	2	3	4	5	6	7	8	9	10	11	12
A	110003 (pre/case)	110003 (pre/case)	110003 (pre/case)	110003 (3m/case)	110003 (3m/case)	110003 (3m/case)	110003/ 100426 (6m/case)	110003/ 100426 (6m/case)	110003/ 100426 (6m/case)	110008 (pre/ control)	110008 (pre/ control)	110008 (pre/ control)
B	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180
C	110008 (6m/ control)	110008 (6m/ control)	110008 (6m/ control)	110012 (pre/case)	110012 (pre/case)	110012 (pre/case)	110012 (3m/case)	110012 (3m/case)	110012 (3m/case)	110012/ 100455 (6m/case)	110012/ 100455 (6m/case)	110012/ 100455 (6m/case)
D	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180
E	120065 (pre/ control)	120065 (pre/ control)	120065 (pre/ control)	120065 (3m/ control)	120065 (3m/ control)	120065 (3m/ control)	110066 (pre/control)	110066 (pre/contr ol)	110066 (pre/contr ol)	110003 (3m/case)	110003 (3m/case)	110003 (3m/case)
F	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180	1:100 3+297	1:10000 3+297	1:100000 20+180
G	HPVIG 500 ug/ml 3+297	HPVIG 83.3 ug/ml 40+200	HPVIG 13.8 ug/ml 40+ 200	HPVIG 2.31 ug/ml 40 + 200	HPVIG 0.38 ug/ml 40 + 200	HPVIG 0.064 ug/m 40 + 200	HPVIG 0.0107 ug/ml 40 + 200	HPVIG .0018 ug/n 40 + 200	HPVIG .0003 ug/n 40 + 200	HPVIG 00005 ug/ 40 + 200	NHS 56 1 : 500 40+160	Blank
H	HIVIG 1:100	HIVIG 1:600	HIVIG 1:3600	HIVIG 1:21600	HIVIG 1:129600	HIVIG 1:777600	HIVIG 1:4665600	HIVIG 1:27993600	HIVIG 1:167961600	HIVIG 1:1007769600	(1:100) 3 +297	Blank

Figure 6: Master plate layout design used for the BAMA assays for the plasma and CVL samples. The above 96 well master plate was designed to accommodate a standard curve, a control which is normal human serum, a blank and samples all in duplicate.

The HIVIG standard curve was performed as per the plate layout, and further serially diluted in assay diluent (milk blotto) by transferring 40µl from the 1st well to the 2nd well, and so on, until the 10th well. Normal human serum (NHS) and milk blotto (blank) was added to the respective wells on the master plate. The filter plate which had the bead mixture was aspirated using the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). A volume of 25µl of the patient samples and control sera, as well as the blank was transferred to the appropriate wells on the 96 well filter plate. It was covered with foil and protected from light, and left to rotate at 220 rpm for 30 to 35 minutes on the plate shaker (Stuart® orbital shaker, UK). During this incubation step, the new protocol was loaded onto the Bio-Plex Manager 6.0 software (Bio-Rad, Hercules, CA). In the remaining last 10 minutes of the incubation, the detection antibodies were prepared in a 15ml conical tube by adding 120µl of Streptavidin-PE (SA-PE) antibody to 11,880µl of milk blotto. The detection antibody was covered in foil and vortexed thoroughly. Once the incubation elapsed, the filter plate was aspirated and washed three times with 100µl of BAMA wash buffer in each well. The detection antibody was vortexed thoroughly again, and 100µl was added to each well on the filter plate and left to rotate for 30 to 35 minutes at 220 rpm on the plate shaker (Stuart® orbital shaker, UK). Once the incubation time elapsed, the filter plate was aspirated and washed three times with 100µl per well of BAMA wash buffer. The beads were then re-suspended in 100 µl of the BAMA wash buffer and left to rotate at 220 rpm on the plate shaker (Stuart® orbital shaker, UK) for 5 minutes. The foil was removed and the filter plate was inserted into the Bio-Plex 200 system to read at a high RPT which was adjusted to account for a 5,000 gate and 50 bead events.

Immunoglobulin (Ig) concentrations were determined by 4-PL logistic regression using the Bio-Plex manager software version 6.1 (Bio-Rad, Hercules, CA). HIV-1 specific activity (SA) was defined as the antigen-specific Mean Fluorescent Intensity (MFI) (adjusted for dilution factor) divided by the total immunoglobulin amount (antigen-specific MFI*dilution/ng/ml total IgG) in order to adjust for individual variation in total Ig recovered when performing CVL. HIV-1 antibody responses were considered positive if they met both antigen-specific positivity criteria and specific activity criteria (mean SA + 3 standard deviations) from a set of 60 seronegative CVLs collected from these women pre-infection. Samples that did not meet the positivity cut-off for specific activity was set to 1/10 of the specific activity cut-off for statistical analysis and visualization purposes.
For mucosal specimens a two-level cut-off is applied. The first level cut-off is based on the raw MFIs and the second level cut-off is based on the specific activity cut-off, which have been based on seronegative individuals in the study.

To determine the first level cut-off (based on the raw MFIs) and the proportion of samples with detectable responses at the three time points, all values that had a MFI above 100 were deemed detectable, while samples with a MFI below 100 were deemed as undetectable responses. For further analysis on magnitude of responses, only detectable (i.e. after cut-off) responses were considered. The MFI values were multiplied by the corresponding dilution factors (Table 2.1), and then divided by Total IgG in order to determine specific activity. A $\log_{10}$ transformation was applied before analysis.

Table 2.1: Dilution factors for CVL IgG, MFI cut-offs and Specific activity cut-offs determined in HIV-uninfected women.

Antigen	Dilution factor for IgG	First level Cut-Off MFI Cut-off	Second level cut-off Specific Activity (MFI*dilution/Total IgG) in the CAP 004 and CAP008 trial
gp41	3	100	-0.55
p24	3	100	-1.45
gp120	3	100	-1.99
p66 (RT)	3	100	-1.05

To determine the second level cut-off (based on specific activity) and the proportion of samples with detectable specific activity at the three time points, all the values which were above 100 MFI and detectable, were then divided by the total IgG. A specific activity pre-determined on seronegative samples was used for the second level of cut-off. If each antigen was above the specific activity cut-off as found in Table 1 and the pre-determined values, then the values were Log_{10} Transformed [Log_{10}(MFI x dilution)/Total IgG].

For plasma specimens, the HIV-specific responses was based on 61 seronegative samples, to which the raw MFIs were averaged and $\log_{10}$ transformed. All women who satisfied the specific activity cut-off as found in Table 2.2, were considered detectable for that antigen.

Table 2.2: Dilution factors for plasma IgG and Specific response cut-offs determined in HIV-uninfected women.

Antigen	Dilution factor for IgG	First level cut-off Specific responses (MFI*dilution/Total IgG) in the CAP 004 and CAP008 trial
gp41	10000	5.28
gag p24	10000	6.64
gp120	10000	5.25
p66 (RT)	10000	6.94

4.7 The 27-Plex Cytokine Assay

The concentrations of 27 cytokines were measured using a 27-plex Bio-Plex Pro™ Human Cytokine Group I 27-Plex Panel (Bio-Rad, USA) on the Bio-Plex 200 multiplex system (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. The principle of the cytokine assay is illustrated in Figure 7. The cytokine panel included the following: basic FGF, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFNγ, IL10, IL12p70, IL13, IL15, IL17A, IL1β, IL1ra, IL2, IL4, IL5, IL6, IL7, IL8, IL9, IP10, MCP1, MIP1α, MIP1β, PDGFββ, RANTES, TNFα and vascular endothelial growth factor (VEGF). Data was collected using Bio-Plex manager software version 6.1, and a 5 PL regression formula was used to calculate sample concentrations from the standard curves. Cytokine levels below the lower limit of detection (LLOD) of the assay was reported as the mid-point between the lowest concentration measured for each cytokine and zero.

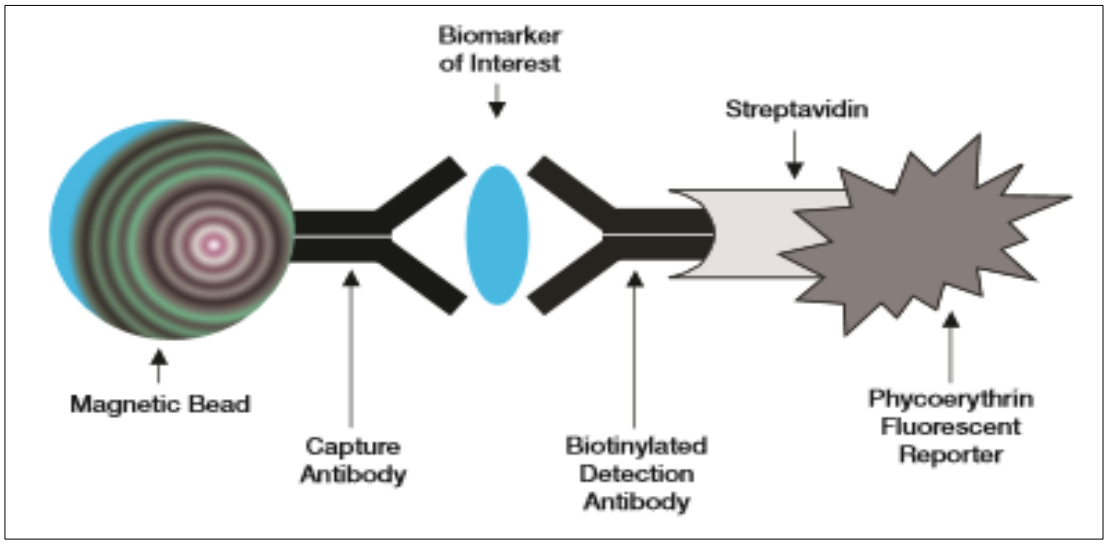


Figure 7: The principle of the cytokine assay (Figure adapted from the Bio-Plex Pro instruction manual).

4.7.1 Initial preparation

The plate layout was planned according to the kit instructions and samples were thawed on ice at -4°C. The Bio-Plex system (Bio-Rad, Hercules, CA) was warmed up for 30 minutes, and thereafter calibrated for a further 10 minutes. Calibration of the machine was performed as mentioned previously.

The 10X wash buffer, assay buffer and sample diluent were vortexed and brought to room temperature until it was needed. The 1X wash buffer called the 27-plex cytokine wash buffer was prepared by adding 60ml of the 10Xwash buffer to 540ml deionized water. The Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA) system was then primed using the 27-plex cytokine (1X) wash buffer. The vial of standards were reconstituted in 500µl of Standard diluent HB, and incubated on ice for 30 minutes after

a quick vortex of seconds. Samples were prepared in a 96 well round bottom plate referred to as the master plate (Figure 8), and according to the designed plate layout. A volume of 25µl of the desired CVL/plasma sample was added to 75µl of the sample diluent to each well to result in a 1:4 dilution. The same volume of sample to sample diluent was carried out for all the samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	S8	S8	3m/control 120065	3m/case 110063	6m/case 120033/100451	3m/control 200020	3m/control 190169	6m/control 190164	3m/control 190161	3m/case 120106
B	S1	S1	S9	S9	3m/case 110044	6m/case 110063/100432	3m/case 120056	6m/control 200020	6m/control 190169	3m/case 200126	3m/case 200248	3m/case 120203/LL780
C	S2	S2	S10	S10	6m/case 110044/100440	pre/control 120061	3m/control 120084	3m/case 190191	3m/case 200075	6m/case 200126/10042	6m/case 200248/100453	6m/case 120203/100346
D	S3	S3	Cntrl	Cntrl	6m/control 110066	6m/control 120061	3m/case 190007	6m/case 190191/100454	3m/control 190013	6m/control 190175	6m/control 200162	3m/case 120232/LL783
E	S4	S4	3m/case 110003	3m/case 110003	3m/case 110062	3m/case 110078	6m/case 190007/100415	6m/control 120090	6m/control 190013	3m/case 200160	3m/case 120009	6m/case 120232/100325
F	S5	S5	6m/case 110003/100426	6m/case 110003/100426	6m/case 110062/100430	6m/case 110078/100439	3m/control 190015	3m/case 190229	3m/case 200091	3m/control 200170	6m/case 120009/100328	6m/case 120239/100327
G	S6	S6	6m/control 110008	6m/control 110008	3m/control 110057	6m/control 110074	3m/case 190032	3m/case 190236	6m/case 200091/100428	3m/case 200176	3m/case 120024	3m/case 120283/LL790
H	S7	S7	3m/case 110012	3m/case 110012	6m/control 110057	3m/case 120033	6m/case 190032	6m/case 190236/100446	3m/control 190164	6m/case 200176/10044	6m/case 120024/100370	6m/case 120283/100347

Figure 8: Layout of the of a master plate design for the 27-plex cytokine assays**.** The above 96 well master plate was designed to accommodate standards, control, blanks and samples all in duplicate.

A volume of 25µl of the plasma serum control diluted in 75µl of sample diluent was added to appropriate well on the master plate. After the 30 minute incubation period a fourfold standard serial dilution was prepared into 9 vials labelled S2 to S10, an additional vial which was labelled as the blank. A volume of 150µl of Standard diluent HB was transferred into vials S2 to S10, and the blank vial. A volume of 128µl from the reconstituted vial of standards was added to the 1st vial with 72µl of Standard Diluent HB, also labelled S1, and 50µl of this volume was transferred into S2. Serial dilutions of 50µl was transferred respectively from S2 into S3, and so on, until S10. After each transfer of volume the vials were properly vortexed and ready for the next transfer. A volume of 150µl of each of the standards (S1 to S10), and the blank was transferred into their respective wells in the master plate. The coupled beads were vortexed for 30 seconds, and 575µl of the 10X beads was added to 5,175µl of assay buffer in a 15ml conical tube, to make 1X coupled beads solution.

4.7.2 Running the 27-plex Cytokine assay

The coupled 1X bead solution was vortexed and 50µl was added to each well on the assay plate. The assay plate was washed twice with 100µl of the 27plex cytokine wash buffer on the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). A volume of 50µl of the standards, blank, control and samples was then transferred from the master plate into the assay plate and incubated on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm at room temperature for 30 minutes. The assay plate was covered

with sealing tape and aluminum foil to avoid light exposure as the beads are photosensitive. During the last 10 minutes of the 30 minute incubation period, the detection antibodies were vortexed for 15 seconds and 300µl of the 10X detection antibody was added to 2,700µl of detection antibody diluent in a 15ml conical tube, to make 1X solution. The assay plate was then washed three times with 100µl of the 27-plex cytokine wash buffer on the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). The prepared 1X detection antibody solution was then vortexed thoroughly and a volume of 25µl was added to each well in the plate. The plate was covered and incubated on the plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm at room temperature for 30 minutes. At this stage, calibration of the Bio-Plex manager software version 6.1 passed and the protocol which contained the standard S1 values and units as provided in the kit was entered into the Bio-Plex 200 manager programme. With the last 10 minutes of the 30 minute incubation period, the 100X streptavidin-PE (SA-PE) was vortexed for 5 seconds and diluted to a 1X solution. A volume of 60µl was added to 5940µl of assay buffer and transferred into a 15ml conical tube. Once the 30 minute incubation elapsed, the assay plate was washed three times with 100µl of the 27-plex cytokine wash buffer on the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). Thereafter, 1X streptavidin–PE was vortexed and 50µl was transferred to each well in the assay plate. The plate was then covered and subjected to last incubation step on the plate shaker (Stuart® orbital shaker, UK) for 10 minutes at 850 ± 50 rpm at room temperature. Following this final incubation, the plate was washed three times with 100µl of the 27-plex cytokine wash buffer, and re-suspended in 125µl of assay buffer. The plate was covered and placed on the plate shaker (Stuart® orbital shaker, UK) for 30 seconds at 850 ± 50 rpm. The sealing tape was removed and the plate was inserted into the Bio-Plex 200 system (Bio-Rad, Hercules, CA) to read at a low RPT which was adjusted to account for a 5,000 gate and 50 bead events.

4.8 IL1 alpha (IL1α) Cytokine assay

The cytokine concentration of IL-1α was measured using a single-plex Human Magnetic Luminex Assay (R&D systems, USA) on the Bio-Plex 200 multiplex system (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. Serial dilutions of CVL and plasma using Calibrator diluent RD6-52 was 1:2 for samples. This kit measured a single analyte coupled to a particular cytokine (IL1α). Data was collected using Bio-Plex manager software version 6.1, and a 5 PL regression formula was used to calculate sample concentrations from the standard curves. Cytokine levels below the lower limit of detection (LLOD) of the assay was reported as the mid-point between the lowest concentration measured for IL1α and zero.

4.8.1 Initial preparation

The plate layout was planned according to the kit instructions and samples were thawed on ice at -4°C. All reagents in the kit were vortexed and brought to room temperature until it was needed. A 1x wash buffer called the IL1α- cytokine wash buffer was prepared for example by adding 20ml of the concentrated wash buffer to 480ml deionized water. The Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA) system was then primed using the IL1α cytokine (1x) wash buffer. The vial of standards was reconstituted in 900µl of Calibrator diluent RD6-52, and incubated at room temperature for 15 minutes with gentle agitation prior to making the dilutions. Samples were prepared in a 96 well round bottom plate referred to as the master plate and according to the designed plate layout (Figure 9). A volume of 50µl of the desired CVL/plasma sample was added to 50µl of the Calibrator diluent RD6-52 to each well to result in a 1:2 dilution. The same volume of sample to Calibrator diluent RD6-52 was carried out for all the samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	S8	S8	3m/control 120065	3m/case 110063	6m/case 120033/100451	3m/control 200020	3m/control 190169	6m/control 190164	3m/control 190161	3m/case 120106
B	S1	S1	S9	S9	3m/case 110044	6m/case 110063/100432	3m/case 120056	6m/control 200020	6m/control 190169	3m/case 200126	3m/case 200248	3m/case 120203/LL780
C	S2	S2	S10	S10	6m/case 110044/100440	pre/control 120061	3m/control 120084	3m/case 190191	3m/case 200075	6m/case 200126/10042	6m/case 200248/100453	6m/case 120203/100346
D	S3	S3	Cntrl	Cntrl	6m/control 110066	6m/control 120061	3m/case 190007	6m/case 190191/100454	3m/control 190013	6m/control 190175	6m/control 200162	3m/case 120232/LL783
E	S4	S4	3m/case 110003	3m/case 110003	3m/case 110062	3m/case 110078	6m/case 190007/100415	6m/control 120090	6m/control 190013	3m/case 200160	3m/case 120009	6m/case 120232/100325
F	S5	S5	6m/case 110003/100426	6m/case 110003/100426	6m/case 110062/100430	6m/case 110078/100439	3m/control 190015	3m/case 190229	3m/case 200091	3m/control 200170	6m/case 120009/100328	6m/case 120239/100327
G	S6	S6	6m/control 110008	6m/control 110008	3m/control 110057	6m/control 110074	3m/case 190032	3m/case 190236	6m/case 200091/100428	3m/case 200176	3m/case 120024	3m/case 120283/LL790
H	S7	S7	3m/case 110012	3m/case 110012	6m/control 110057	3m/case 120033	6m/case 190032	6m/case 190236/100446	3m/control 190164	6m/case 200176/10044	6m/case 120024/100370	6m/case 120283/100347

Figure 9: Layout of the of a master plate design for the IL1α cytokine assays. The above 96 well master plate was designed to accommodate standards, control, blanks and samples all in duplicate.

A volume of 50µl of the plasma serum control diluted in 50µl of Calibrator diluent was added to appropriate well on the master plate. After the 15 minute incubation period a threefold standard serial

dilution was prepared into 9 vials labelled S1 to S10, an additional vial which was labelled as the blank. A volume of 200µl of Calibrator diluent RD6-52 was transferred into vials S2 to S10, and the blank vial. A volume of 1,000µl from the reconstituted vial of standards was added to the 1st vial, also labelled S1, and 100µl of this volume was transferred into S2. Serial dilutions of 100µl was transferred respectively from S2 into S3, and so on, until S10. After each transfer of volume the vials were properly vortexed and ready for the next transfer. A volume of 150µl of each of the standards (S1 to S10), and the blank was transferred into their respective wells in the master plate. The coupled beads were vortexed for 30 seconds, and 500µl of the microparticle cocktail was added to 5ml of Diluent RD2-1 in a mixing bottle (provided), to make the coupled beads solution.

4.8.2 Running the IL1α Cytokine assay

A volume of 50µl of the standards, blank, control and samples was transferred from the master plate into the assay plate. The coupled bead solution was vortexed and 50µl was added to each well on the assay plate, and incubated on a plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm at room temperature for 2 hours. The assay plate was covered with sealing tape and aluminum foil to avoid light exposure as the beads are photosensitive. During the last 10 minutes of the 2 hour incubation period, the detection antibodies were vortexed for 15 seconds and 500µl of the biotin antibody cocktail was added to 5ml of Diluent RD2-1. This was prepared in the mixing bottle (provided). The assay plate was then washed three times with 100µl of the IL1α cytokine wash buffer on the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). The prepared detection antibody solution was then vortexed thoroughly and a volume of 50µl was added to each well in the plate. The plate was covered and incubated on the plate shaker (Stuart® orbital shaker, UK) at 850 ± 50 rpm at room temperature for 1 hour. At this stage, the standard S1 value and unit as provided in the kit was entered into the Bio-Plex 200 manager programme. With the last 10 minutes of the 1 hour incubation period, the streptavidin-PE (SA-PE) was vortexed for 5 seconds and diluted. A volume of 220µl was added to 5.35ml of the IL1α wash buffer and transferred into a 15ml conical tube (wrapped with aluminum foil). Once the 1 hour incubation elapsed, the assay plate was washed three times with 100µl of the IL1α cytokine wash buffer on the Bio-Plex Pro™ Wash station (Bio-Rad, Hercules, CA). Thereafter, the prepared streptavidin–PE was vortexed and 50µl was transferred to each well in the assay plate. The plate was then covered and subjected to last incubation step on the plate shaker (Stuart® orbital shaker, UK) for 30 minutes at 850 ± 50 rpm at room temperature. Following this final incubation, the plate was washed three times with 100µl of the IL1α cytokine wash buffer, and re-suspended in 100µl of assay buffer. The plate was covered and placed on the plate shaker (Stuart® orbital shaker, UK) for 2 minutes at 850 ± 50 rpm. The sealing tape was removed and the plate was inserted into the Bio-Plex 200 system (Bio-Rad, Hercules, CA) to read at a low RPT which was adjusted to account for a 5,000 gate and 50 bead events.

4.9 Statistical Analysis

Parametric paired T-tests were used to determine the differences between cases and controls for HIV-specific responses, HIV-specific activities, Isotypes and IgG subclasses. The non-parametric Kruskal-Wallis test was used to determine the differences in the HIV-specific responses, HIV-specific activity, isotype and IgG subclasses between women with genital tract inflammation versus women who did not have inflammation, and Dunn's Post-test was used to adjust for multiples comparisons between each of the groups. Conditional regression analyses was used to determine cytokine concentrations, antibody isotypes and IgG subclasses in the genital tract and plasma at the pre-infection time point. Pearson's correlation was used to check the compartment relationship between the genital tract and the blood for the HIV specific Ig responses. Multivariate linear mixed models were used to determine whether inflammation is associated with Ig specific responses. This model was adjusted for STIs and other covariates. Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary).

5. RESULTS

5.1 Baseline Demographics

In this sub-study of women from the CAP004 trial, the demographic and clinical characteristics of 45 HIV-infected (HIV^+) and 45 HIV-uninfected (HIV^-) South African women are presented in Table 3.1. Of these women, 38% (n=17) were assigned to the 1% tenofovir gel arm, while 62% (n=28) were assigned to the placebo gel. The majority of the HIV-infected (89%) and HIV-uninfected women (80%) reported being in a stable relationship, with no significant difference ($p>0.05$) at first sexual debut [median age 17 years (16-18 years) and 18 years (17-19 years) respectively]. Depo-Provera was the commonest contraceptive method used by HIV-infected (76%) and HIV-uninfected (73%) women, with no significant differences ($p>0.05$) between these women. HSV-2 was the predominant STI found in 67% of the HIV-infected and 53% of the HIV-uninfected women.

As mentioned previously, genital tract inflammation is defined as having a profile of five of any of the nine inflammatory cytokines above the 75th percentile concentration for each cytokine (MIP1α, MIP1β, IP10, IL8, MCP1, IL1α, IL1β, IL6, and TNFα) elevated across all timepoints in the genital tract. When the 45 HIV-infected women were further stratified according to the presence or absence of genital inflammation (GI), n=6 women were classified as HIV^+GI^+, and n=35 were classified as HIV^+GI^-. When the 45 HIV-uninfected women were further stratified according to the presence or absence of genital inflammation (GI), n=7 women were classified as HIV^-GI^+, and n=33 were classified as HIV^-GI^-. When the women were stratified according to inflammation status, the samples for 4 and 5 of HIV-infected and HIV-uninfected women respectively, were unavailable at the time of this sub-study and therefore the total numbers for these groups are n=41 and n=40 respectively (Table 3.1).

Of the HIV^+GI^+ women, 33% (n=2) were assigned to the 1% tenofovir gel arm, while 67% (n=4) were assigned to the placebo gel. Of the HIV^+GI^- women, 43% (n=15) were assigned to the 1% tenofovir gel arm, while 57% (n=20) were assigned to the placebo gel. All of the HIV^+GI^+ women (100%, n=6) and most of the HIV^+GI^- women (89%, n=31) reported being in a stable relationship. Age at first sexual debut of the HIV^+GI^+ and HIV^+GI^- women were the same [median age 17 years (16-18 years). Depo-Provera was the commonest contraceptive method used by HIV^+GI^+ (100%) and HIV^+GI^- (69%) women. Positive HSV-2 status (which was available at the time of the study) was found in 50% of the HIV^+GI^+ and 71% of the HIV^+GI^- women (Table 3.1).

Of the HIV^-GI^+ women, 43% (n=3) were assigned to the 1% tenofovir gel arm, while 57% (n=4) were assigned to the placebo gel. Of the HIV^-GI^- women, 39% (n=13) were assigned to the 1% tenofovir gel arm, while 61% (n=20) were assigned to the placebo gel. Most of the HIV^-GI^+ women (57%, n=4) and HIV^-GI^- women (82%, n=27) reported being in a stable relationship. Age at first sexual debut of the

HIV^{-}GI^{+} and HIV^{-}GI^{-} women was similar [median age 18 years (17-19 years). The use of Depo-Provera was the commonest contraceptive method used by HIV^{-}GI^{+} (86%) and HIV^{-}GI^{-} (73%) women. Positive HSV-2 status (which was available at the time of the study) was found in 29% of the HIV^{-}GI^{+} and 58% of the HIV^{-}GI^{-} women (Table 3.1).

Table 3.1: Baseline characteristics of women in the CAP004 trial.

Demographics	**HIV$^+$ (N=45)**	**HIV$^-$ (N=45)**	**p-value**	**HIV$^+$GI$^+$ (N=6)**	**HIV$^+$GI$^-$ (N=35)**	**HIV$^-$GI$^+$ (N=7)**	**HIV$^-$GI$^-$ (N=33)**
Age in years (Median) (IQR)	**22 (20-25)**	**22 (20-27)**	**0.21**	**20.5 (20-22)**	**22 (20-25)**	**21 (20-22)**	**23 (20-28)**
Highest level of Education completed:			>0.99				
Primary school not complete % (n)	7 (3)	2 (1)		17 (1)	6 (2)	-	3 (1)
Primary school complete % (n)	2 (1)	2 (1)		-	3 (1)	-	3 (1)
High school not complete % (n)	49 (22)	58 (26)		83 (5)	47 (17)	57 (4)	58 (19)
High school complete % (n)	33 (15)	38 (17)		-	31 (11)	43 (3)	36 (12)
Tertiary education incomplete % (n)	7 (3)	-		-	9 (3)	-	-
Tertiary education complete % (n)	2 (1)	-		-	3 (1)	-	-
Relationship status:			0.47				
Married % (n)	2 (1)	11 (5)		-	-	14 (1)	12 (4)
Stable partner % (n)	89 (40)	80 (36)		100 (6)	89 (31)	57 (4)	82 (27)
Casual partner % (n)	2 (1)	2 (1)		-	3 (1)	-	3 (1)
Other % (n)	7 (3)	7 (3)		-	9 (3)	29 (2)	3 (1)
Sexual Behaviour:							
Age (yrs) of first sex act (Median) (IQR)	17 (16-18)	18 (17-19)	0.07	17 (16-18)	17 (16-18)	18 (17-19)	18 (17-19)
Number of lifetime partners (Median) (IQR)	2 (1-4)	2 (2-3)	0.92	2 (1-2)	2 (1-4)	2 (2-3)	2 (1-3)
Number of vaginal sex acts in the last 30 days [Median (IQR)]	4 (3-8)	6 (4-12)	0.09	4 (2-11)	4 (3-8)	16 (4-17)	6 (3-9)

Contraceptive use:			>0.99				
Depo-Provera % (n)	76 (34)	73 (33)		100 (6)	69 (24)	86 (6)	73 (24)
Oral contraceptive % (n)	7 (3)	16 (7)		-	9 (3)	-	15 (5)
Nur-isterate % (n)	18 (8)	7 (3)		-	23 (8)	14 (1)	6 (2)
Tubal ligation % (n)	-	4 (2)		-	-	-	6 (2)
Condom use:			0.50				
Always % (n)	36 (16)	27 (12)		50 (3)	37 (13)	14 (1)	30 (10)
Sometimes % (n)	64 (29)	73 (33)		50 (3)	63 (22)	86 (6)	70 (23)
STI Testing-Positive only:							
HSV-2 % (n)	67 (30)	53 (24)		50 (3)	71 (25)	29 (2)	58 (19)
***Median months to infection (IQR)**	12 (7-16)	20 (18-24)		13 (10-18)	13 (8-16)	19 (11-22)	21 (18-25)
Treatment arm:			>0.99				
Placebo % (n)	62 (28)	62 (28)		67 (4)	57 (20)	57 (4)	61 (20)
Tenofovir % (n)	38 (17)	38 (17)		33 (2)	43 (15)	43 (3)	39 (13)

$p<0.05$ were statistically significant. IQR, interquartile range. HSV-2, Herpes simplex virus-2. BV, Bacterial vaginosis. HIV^+ and HIV^- women were further stratified according to the presence (HIV^+GI^+ and HIV^-GI^+) or absence (HIV^+GI^- and HIV^-GI^-) of genital inflammation. When the women were stratified according to inflammation status, the samples for 4 and 5 of HIV^+ and HIV^- women respectively, were unavailable at the time of this sub-study and therefore the total numbers for these groups are n=41 and n=40.

* Time (median months) to infection from enrolment.

In this sub-study of women from the CAP008 trial, the demographic and clinical characteristics of 21 HIV-infected (HIV^+) and 21 HIV-uninfected (HIV^-) South African women are presented in Table 3.2. In this sub-study, all of the women used the 1% tenofovir microbicide gel. The majority of the HIV^+ (76%) and HIV^-women (95%) reported being in a stable relationship, with no significant difference ($p>0.05$) in median age at first sexual debut of 17 years (16-18 years) and 18 years (15-19 years), respectively. Depo-Provera was the commonest contraceptive method used by HIV^+ (62%) and HIV^- (67%) women. with no significant differences ($p>0.05$) between these women. Of all the STIs tested, HSV-2 was the predominant STI with 86% and 91% in the HIV^+ and HIV^-groups, respectively. Additionally, there was no significant difference ($p>0.05$) in the median BV score of 2 (range 0-7) and 3 (range 1-6) between HIV^+ and HIV^- women respectively.

When the 21 HIV^+ women were further stratified according to the presence or absence of genital inflammation (GI), n=4 women were classified as HIV^+GI^+, and n=15 were classified as HIV^+GI^-. When the 21 HIV^- women were further stratified according to the presence or absence of genital inflammation (GI), n=2 women were classified as HIV^-GI^+, and n=17 were classified as HIV^-GI^-. When the women were stratified according to genital inflammation status, the samples for 2 of the HIV^+ and 2 of the HIV^- women were unavailable at the time of this sub-study and therefore the total numbers for each of these groups is n=19 (Table 3.2).

The questionnaires that were used to assess the demographic characteristics of the women from the CAP004 trial versus the CAP008 trial were different, therefore the respective tables contain data that show the different parameters measured for or enquired after.

Table 3.2: Baseline characteristics of women in the CAP008 trial.

Demographics	HIV^+ (N= 21)	HIV^- (N= 21)	p-value	HIV^+GI^+ (N=4)	HIV^+GI^- (N=15)	HIV^-GI^+ (N=2)	HIV^-GI^- (N=17)
Age in years (median)	27	27	0.88	30.5	27	23.5	27
(IQR)	(25-33)	(25-33)		(28-35)	(25-31)	(23-24)	(26-30)
Highest level of education completed:			0.09				
Less than primary % (n)	48 (10)	19 (4)		25 (1)	47 (7)	-	24 (4)
Primary complete % (n)	5 (1)	10 (2)		25 (1)	-	-	6 (1)
High school complete % (n)	43 (9)	67 (14)		50 (2)	47 (7)	50 (1)	71 (12)
Tertiary complete % (n)	5 (1)	5 (1)		-	7 (1)	50 (1)	-
Participants partner information:							
Age of regular/stable partner in years [Median (IQR)]	32 (28-37)	31 (27-37)	0.79	33.5 (31-39)	32 (28-37)	26 (26-26)	32 (29-37)
HIV status of regular/stable partner			0.79				
Positive % (n)	-	5 (1)		-	-	-	6 (1)
Negative % (n)	67 (14)	57 (12)		75 (3)	73 (11)	50 (1)	53 (9)
Unknown % (n)	33 (7)	33 (7)		25 (1)	27 (4)	-	41 (7)
No answer % (n)	-	5 (1)		-	-	50 (1)	-
Relationship status:			0.41				
Married % (n)	10 (2)	5 (1)		25 (1)	7 (1)	-	6 (1)
Stable partner % (n)	76 (16)	95 (20)		25 (1)	87 (13)	100 (2)	94 (16)
Casual partner % (n)	14 (3)	-		50 (2)	7 (1)	-	-
Sexual behaviour:							
Age in years of first sex act [Median (IQR)]	17 (16-18)	18 (15-19)	0.77	16 (15-17)	17 (17-18)	19 (18-19)	18 (15-19)
Number of lifetime partners [Median (IQR)]	4 (2-5)	2 (1-4)	0.11	4 (3-5)	3 (2-5)	3 (2-4)	2 (1-4)
Number of vaginal sex acts in the last 30 days [Median (IQR)]	4 (3-5.5)	5 (3-9)	0.19	7 (5.3-10)	4 (2.5-5)	6 (5-7)	5 (3-10)
Contraceptive use:			0.63				
Depo-Provera % (n)	62 (13)	67 (14)		50 (2)	60 (9)	100 (2)	65 (11)
Oral contraceptive % (n)	19 (4)	10 (2)		50 (2)	13 (2)	-	12 (2)
Nur-isterate % (n)	19 (4)	10 (2)		-	27 (4)	-	6 (1)
Other % (n)	-	14 (3)		-	-	-	18 (3)
Condom use:			0.25				
Always % (n)	27 (6)	43 (9)		25 (1)	27 (4)	50 (1)	41 (7)
Sometimes % (n)	52 (11)	48 (10)		50 (2)	53 (8)	50 (1)	47 (8)
Never % (n)	19 (4)	10 (2)		25 (1)	20 (3)	-	12 (2)
STI Testing- Positive only:							
Gonorrhoea % (n)	10 (2)	-		25 (1)	-	-	-
Chlamydia % (n)	10 (2)	-		-	13.3 (2)	-	-
Trichomonas % (n)	10 (2)	5 (1)		-	13.3 (2)	-	5.9 (1)
Mycoplasma % (n)	10 (2)	5 (1)		-	13.3 (2)	-	5.9 (1)
HSV-2 % (n)	86 (18)	91 (19)		75 (3)	86.7 (13)	100 (2)	88.2 (15)
BV [Median (IQR)]	2 (0-7)	3 (1-6)	0.99	1 (0-2)	2 (2-7)	4 (3-6)	3 (0-4)
Time (months) to infection from enrolment [Median (IQR)]	13 (6-17)	24 (23-25)		10 (5-16)	12 (6-17)	21 (19-22)	24 (23-25)

$p<0.05$ were statistically significant. IQR, interquartile range. HSV-2, Herpes simplex virus-2. HIV^+ and HIV^- women were further stratified according to the presence (HIV^+GI^+ and HIV^-GI^+) or absence (HIV^+GI^- and HIV^-GI^-) of genital inflammation. When the women were stratified according to inflammation status, the samples for 2 of the HIV^+ and 2 of the HIV^- women were unavailable at the time of this sub-study and therefore the total numbers for these groups are n=19 for each group.

All participants' data for the various analytes measured in the women from the CAP004 and the CAP008 trials were included together in all subsequent analyses.

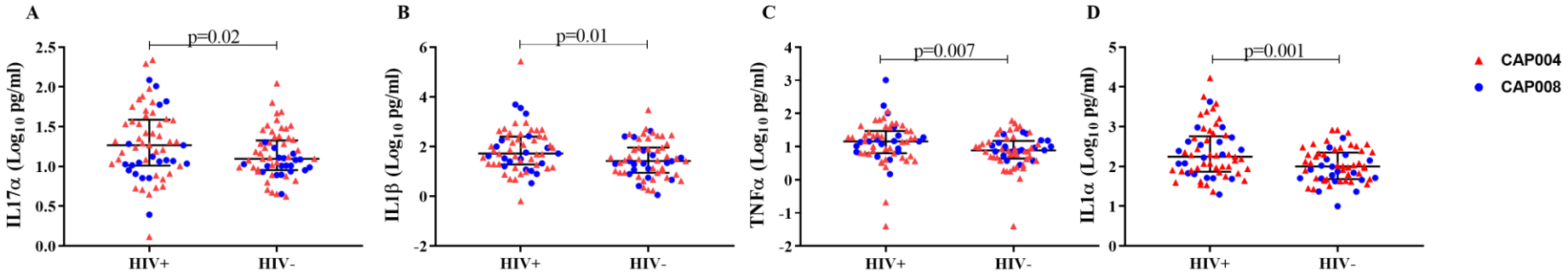
5.2 Mucosal cytokine profiles between HIV-infected and HIV-uninfected women

To assess genital inflammation, 48 cytokines were measured at baseline (this is the pre-infection time point) in HIV^+ women and in the matched controls (HIV^-) from both CAP004 and CAP008 studies in the CVL. Subsequently, at 3 and 6 months, we further measured 28 cytokines in the CVL for both studies in both groups of women that included the HIV^+ women and (HIV) controls to understand if the genital inflammation persisted at those time points (Figure 1). Of the 48 cytokines measured at baseline in the women who became HIV^+ (Supplementary Table 1), higher median cytokine concentrations were found for the majority of the cytokines compared to HIV^- women. Likewise, of the 28 cytokines measured at 3 and 6 months post-infection (Supplementary Table 1), HIV^+ women maintained higher median cytokine concentrations for the majority of the cytokines compared to HIV^- women. Overall, at baseline, 18 cytokines (IL17α, IL1β, TNFα, IL1α, IFNγ, IL13, IL4, IL2Rα, IL8, IP10, CTACK, Groα, IL16, MCP3, MIG, HGF, IL3, and SDF1α) (Figure 1A-1R) were significantly higher ($p<0.05$) in HIV^+ (n=61) compared to HIV^- (n=60) women. Of these 18 cytokines, 5 of the pro-inflammatory cytokines and chemokines [IL1β (Figure 1B) and TNFα (Figure 1C) and IL1α (Figure 1D), IL8 (Figure 1I) and IP10 (Figure 1J)] satisfied the definition of genital inflammation as previously reported (Masson et al., 2015). At 3 months, three adaptive cytokines and growth factors (IL15, IL5 and VEGF) (Figure 1S-1U) were significantly higher ($p<0.05$) in HIV^+ (n=45) compared to HIV^- (n=19) women. At 6 months, four adaptive cytokines and growth factors (IL15, GMCSF, PDGFββ and VEGF (Figure 1V-1Y) were significantly higher ($p<0.05$) in HIV^+ (n=36) compared to HIV^- (n=25) women.

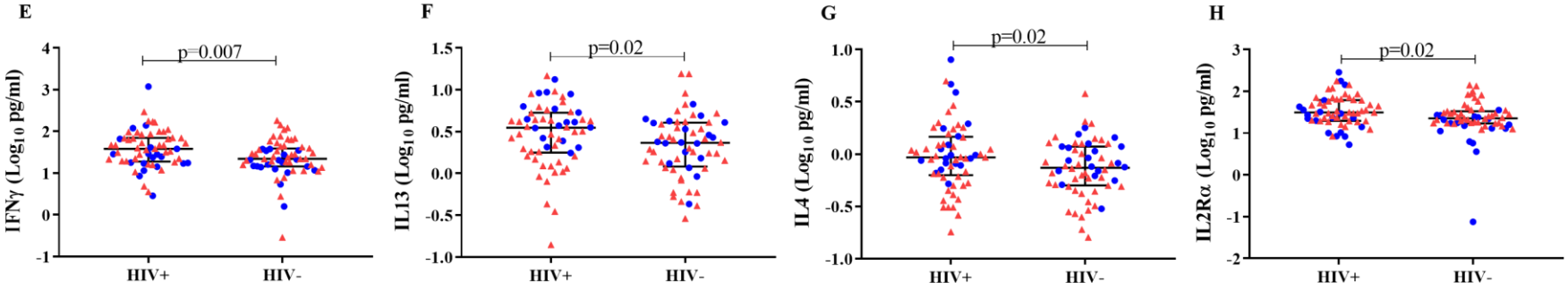
Thereafter, HIV^+ and HIV^- women were further stratified according to the presence ($HIV^+G.I^+$ and $HIV^-G.I^+$) or absence ($HIV^+G.I^-$ and $HIV^-G.I^-$) of genital inflammation based on the definition of having ≥ 5 of the 9 cytokines above the 75^{th} median percentile, taking the baseline, 3 month and 6 month time points into account. This was the classification used going forward for the subsequent analyses including the IgG subclasses, Ig isotypes and the HIV-specific activities and HIV-specific responses in the genital tract and plasma.

Baseline

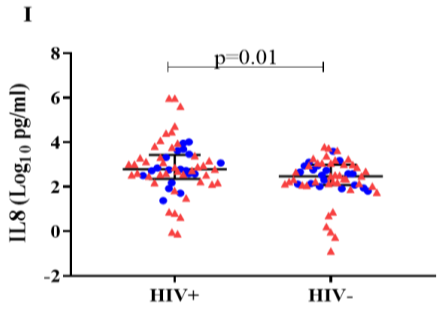
Pro-inflammatory cytokines

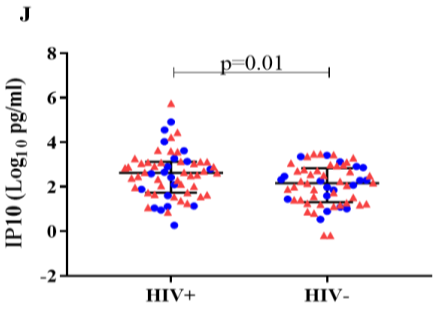


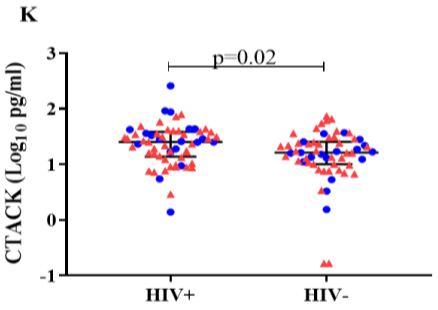
Adaptive cytokines

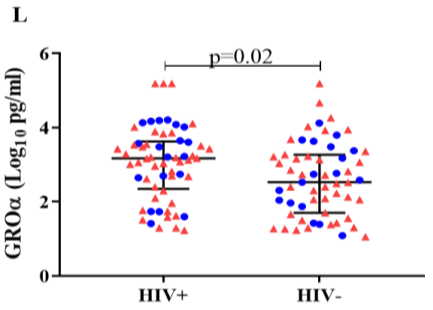


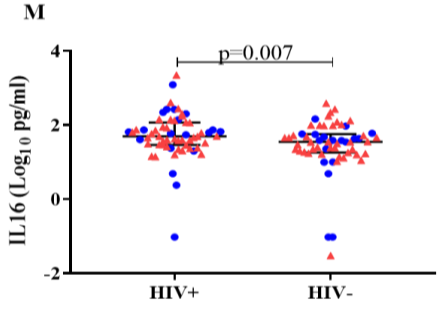
Chemokines

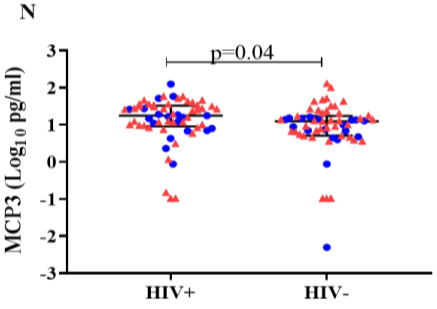


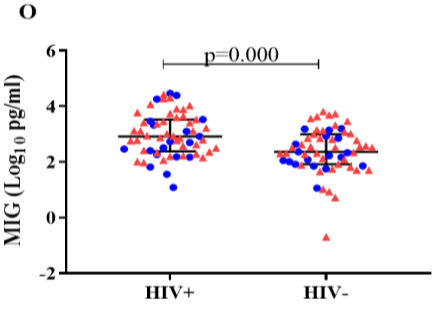




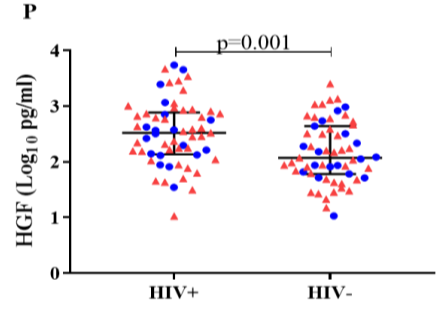


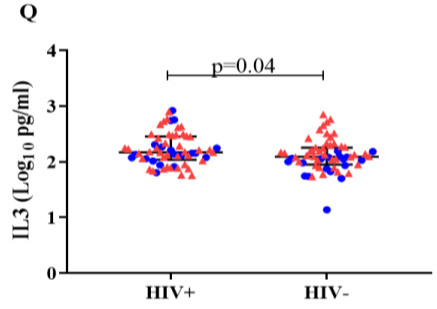


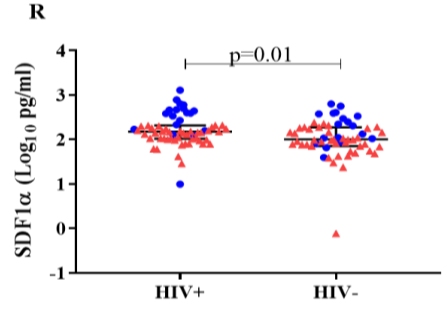




Growth factors







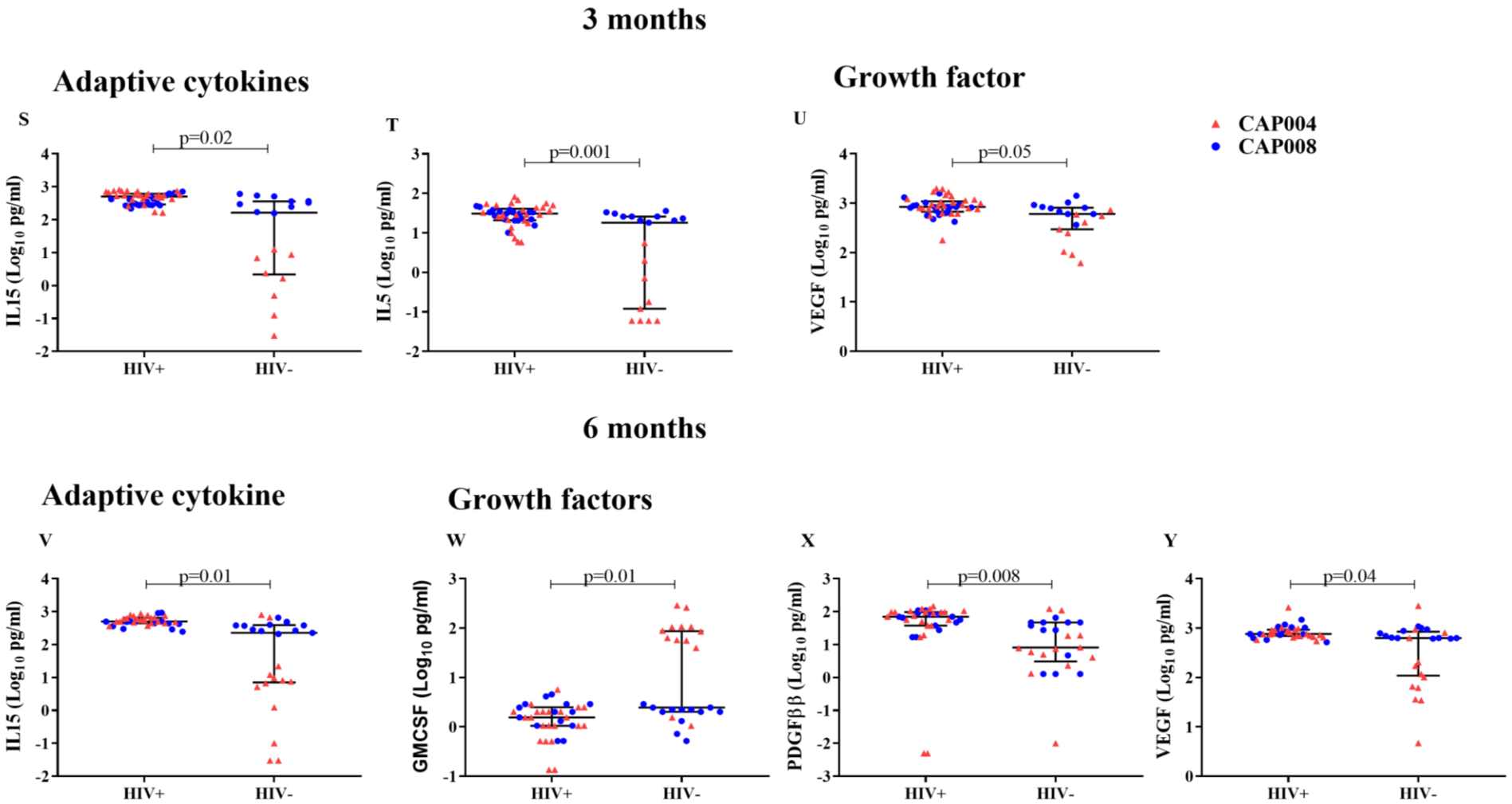


Figure 1: Longitudinal analyses of mucosal cytokine profiles between cases and controls in the CAP004 and CAP008 trials at baseline, 3 and 6 months. Paired T tests were used to compare between groups and $p<0.05$ were considered statistically significant. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, HIV^+ represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

5.3 Antibody isotypes and IgG subclass profiles in the female genital tract

5.3.1 At baseline HIV-infected women (pre-HIV-infection) had significantly higher IgG1 titres compared to HIV-uninfected women- intergroup analyses

To assess the levels of antibody isotypes and IgG subclasses in the female genital tract, quantification of IgG subclasses (IgG1, IgG2, IgG3 and IgG4), IgA and IgM were performed and compared between HIV^+ and HIV^- women at baseline, 3 and 6 months (Table 3.3). At baseline, Total Ig titres were significantly higher in HIV^+ [median 4.50 log_{10} ng/ml (IQR 4.33-4.61 log_{10} ng/ml)] compared to HIV^- [(median 4.40 log_{10} ng/ml (IQR 4.18-4.61 log_{10} ng/ml); p=0.05] women. (Table 3.3). In addition, subclass differences were evident at baseline (Table 3.3) in HIV^+ [4.12 (4.02-4.19)] compared to HIV^- [4.05 (3.90-4.18)] (p=0.04) women for IgG1. In addition, significantly higher IgM titres were found in the HIV^+ [3.05 (2.78-43.54)] compared to HIV^- [2.89 (2.55-3.39)] (p=0.05) women at baseline. The median titres of total IgG, IgG subclasses, IgA and IgM were similar between HIV^+ and HIV^- women at the 3 and 6 months (Table 3.3). When the HIV^+ and HIV^- women were further stratified for inflammation (Figure 2), IgM was higher at baseline in HIV^+GI^+ [3.42 (2.86-4.03)] compared to the HIV^-GI^- [2.83 (2.52-3.33)] (p=0.05) women (Figure 2S).

Table 3.3: Antibody isotypes and subclasses between cases and controls in the CAP004 and CAP008 trials.

	Baseline			3 months			6 months		
Antibody isotype/ subclass	**HIV^+ [Median (IQR)] N= 53**	**HIV^- [Median (IQR)] N= 61**	**p-value**	**HIV^+ [Median (IQR)] N=46**	**HIV^- [Median (IQR)] N=17**	**p-value**	**HIV^+ [Median (IQR)] N=36**	**HIV^- [Median (IQR)] N=23**	**p-value**
Total IgG	**4.5 (4.33-4.61)**	**4.40 (4.18-4.61)**	**0.05**	4.35 (4.04-4.47)	4.38 (3.93-4.47)	0.68	4.15 (3.88-4.45)	4.23 (3.94-4.41)	0.36
IgG1	**4.12 (4.02-4.19)**	**4.05 (3.90-4.18)**	**0.04**	4.07 (3.88-4.15)	4.08 (3.77-4.11)	0.96	4.01 (3.78-4.12)	4.08 (3.82-4.12)	0.86
IgG2	3.77 (3.56-4.32)	3.68 (3.41-4.14)	0.17	3.43 (2.81-3.71)	3.56 (3.13-3.70)	0.16	3.00 (2.29-3.49)	3.17 (2.62-3.52)	0.15
IgG3	3.61 (3.16-3.87)	3.54 (3.13-3.81)	0.52	3.40 (2.90-3.73)	3.55 (2.83-3.87)	0.82	3.33 (2.25-3.78)	3.09 (2.74-3.42)	0.08
IgG4	3.50 (2.91-3.90)	3.21 (2.80-3.85)	0.16	3.46 (2.95-3.84)	3.56 (2.72-3.80)	0.82	3.12 (2.35-3.55)	3.25 (2.82-3.70)	0.19
IgA	4.08 (3.93-4.25)	3.93 (3.85-4.15)	0.08	4.04 (3.91-4.20)	4.07 (3.901-4.23)	0.58	3.95 (3.74-4.12)	4.05 (3.94-44.21)	0.90
IgM	**3.05 (2.78-3.54)**	**2.89 (2.55-3.39)**	**0.05**	3.00 (2.35-3.37)	2.70 (2.103.11)	0.60	2.52 (1.93-3.12)	2.48 (2.09-2.93)	0.73

Abbreviations: IQR, interquartile range; Ig, immunoglobulin. At baseline, HIV^+ represents women who subsequently became HIV^+. $p<0.05$ was considered statistically significant.

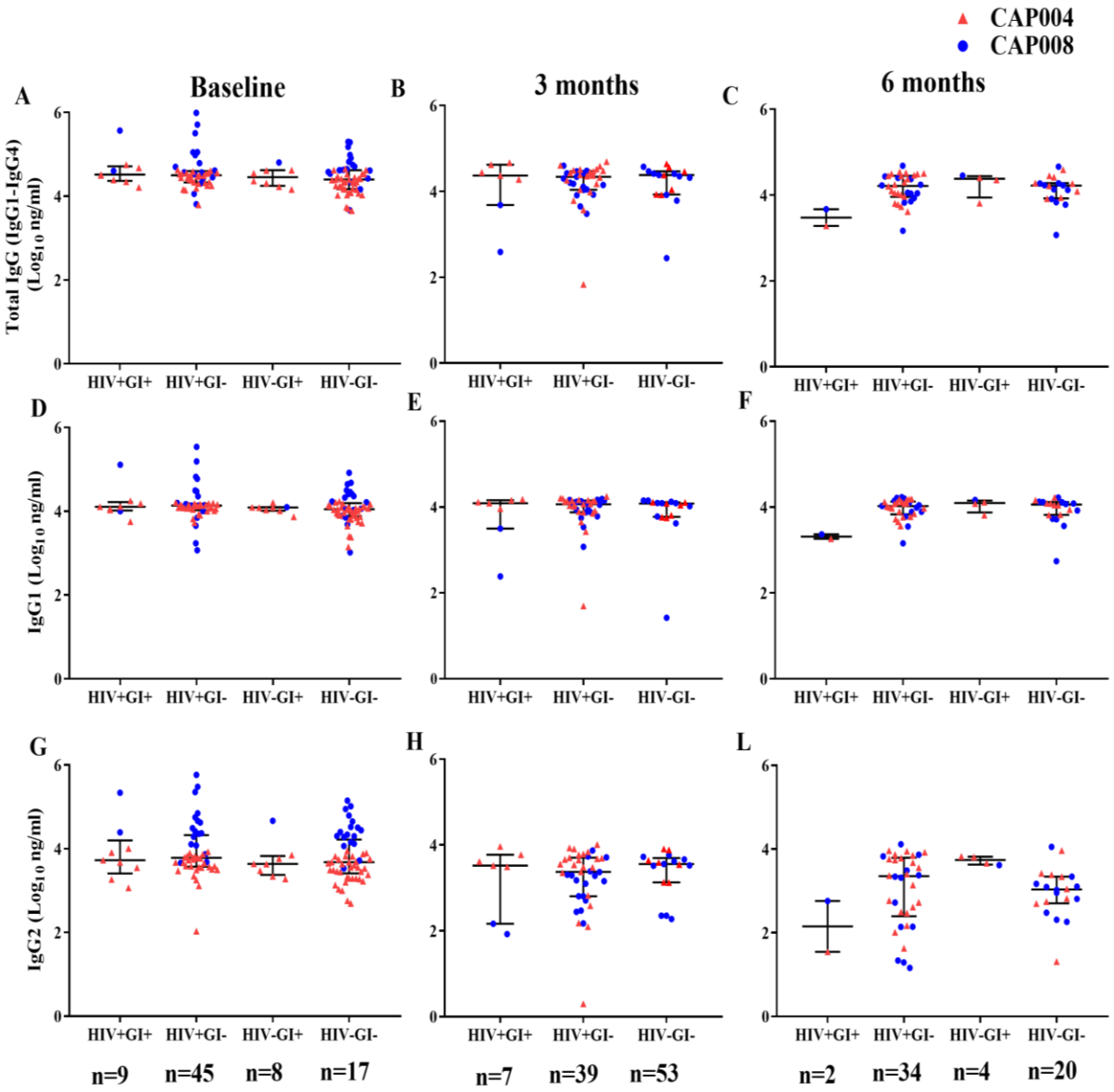


Figure 2.1: Comparison of the Total IgG (sum of IgG1to IgG4), IgG subclasses IgG1 and IgG2 in CVL from HIV^+GI^+, HIV^+GI^-, HIV^-GI^+ and HIV^-GI^- women. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Kruskal Wallis test was used to compare between groups and $p<0.05$ were considered statistically significant. At baseline, HIV^+GI^+ and HIV^+GI^- represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial. Numbers of women in each category varied from baseline to 6 months as follows, at baseline [HIV^+GI^+ (n=9), HIV^+GI^- (n=45), HIV^-GI^+ (n=8) and HIV^-GI^- (n=17)] (A, D, G, J, M, P, and S), at 3 months [HIV^+GI^+ (n=7), HIV^+GI^- (n=39) and HIV^-GI^- (n=53)] (B, E, H, K, N, Q and T) and at 6 months [HIV^+GI^+ (n=2), HIV^+GI^- (n=34), HIV^-GI^+ (n=4) and HIV^-GI^- (n=20)] (C, F, I, L, O, R, and U).

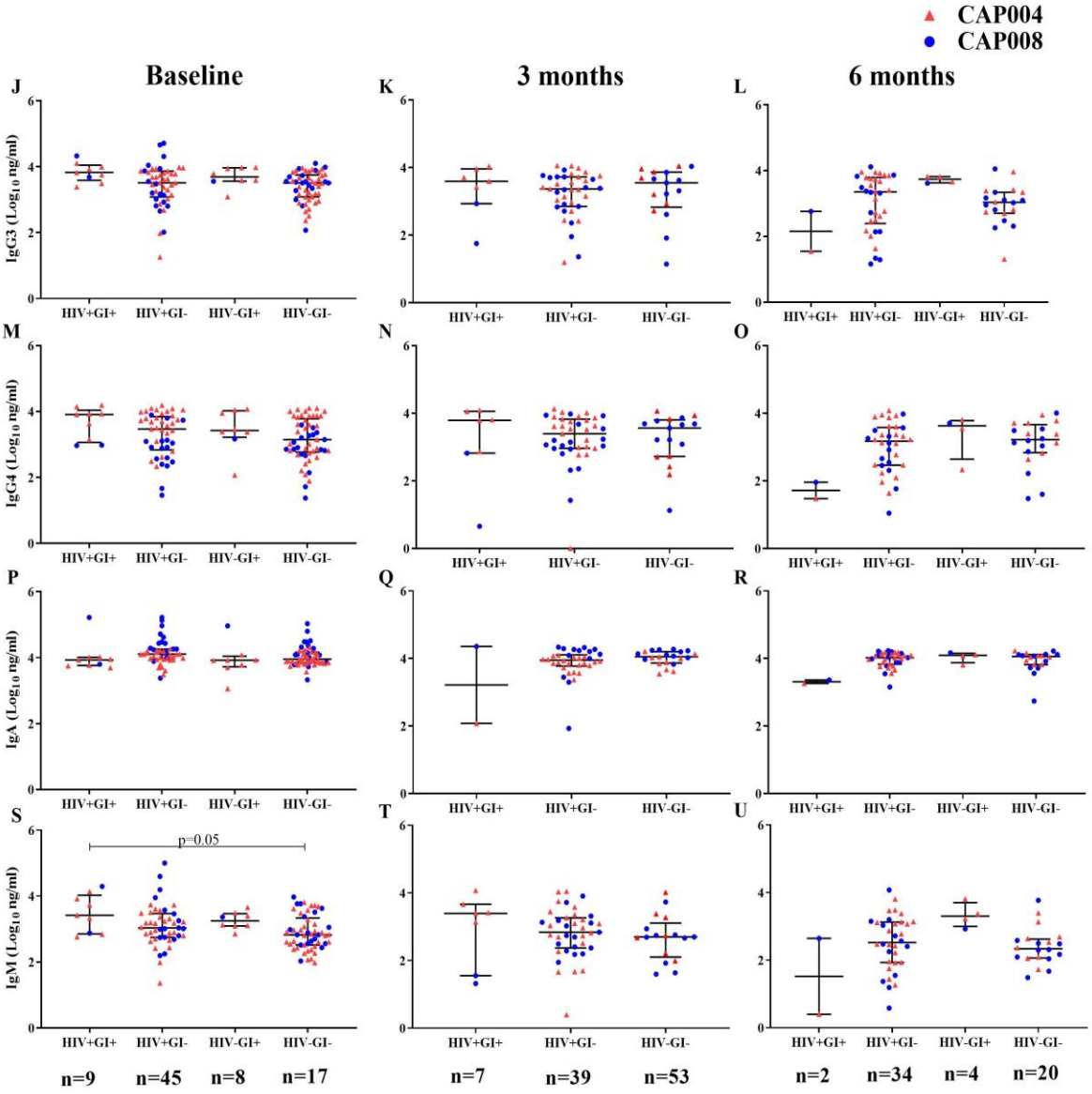


Figure 2.2: Comparison of IgG subclasses, IgG3, IgG4, IgA and IgM in CVL from $HIV^{+}GI^{+}$, $HIV^{+}GI^{-}$, $HIV^{-}GI^{+}$ and $HIV^{-}GI^{-}$ women Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Kruskal Wallis test was used to compare between groups and $p<0.05$ were considered statistically significant. At baseline, $HIV^{+}GI^{+}$ and $HIV^{+}GI^{-}$ represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial. Numbers of women in each category varied from baseline to 6 months as follows, at baseline [$HIV^{+}GI^{+}$ (n=9), $HIV^{+}GI^{-}$ (n=45), $HIV^{-}GI^{+}$ (n=8) and $HIV^{-}GI^{-}$ (n=17)] (A, D, G, J, M, P, and S), at 3 months [$HIV^{+}GI^{+}$ (n=7), $HIV^{+}GI^{-}$ (n=39) and $HIV^{-}GI^{-}$ (n=53)] (B, E, H, K, N, Q and T) and at 6 months [$HIV^{+}GI^{+}$ (n=2), $HIV^{+}GI^{-}$ (n=34), $HIV^{-}GI^{+}$ (n=4) and $HIV^{-}GI^{-}$ (n=20)] (C, F, I, L, O, R, and U).

5.3.2 Genital tract baseline (pre-HIV-infection) IgG subclasses and isotypes were significantly higher than the subsequent time points- intragroup analyses

The levels of antibody isotypes and IgG subclasses were evaluated longitudinally in the HIV^+ and HIV^- women based on their genital inflammation status (Figure 3 and Figure 4). Irrespective of genital inflammation status, the total IgG, IgG subclasses (IgG1, IgG2, IgG3 and IgG4), IgA and IgM profiles were assessed in HIV^+ and HIV^- women at baseline, at 3 and at 6 months (Figure 3 and Figure 4).

In the HIV^+ women, total IgG were significantly higher at baseline [median 4.50 log_{10} ng/ml (IQR 4.34-4.61 log_{10} ng/ml)] compared to 3 and 6 months [median 4.35 log_{10} ng/ml (IQR 4.04-4.47 log_{10} ng/ml) and median 4.15 log_{10} ng/ml (IQR 3.88-4.45 log_{10} ng/ml) respectively; $p<0.0001$ for all] (Figure 3A). Similarly, IgG1 was significantly higher at baseline [4.12 (4.02-4.19)] than at 3 or at 6 months [4.07 (3.88-4.15) and 4.01 (3.78-4.12) respectively; $p<0.005$ for all] (Figure 3B). In addition, IgG2 was significantly higher at baseline [3.77 (3.56-4.32)] than at 3 or at 6 months [(3.43 (2.81-3.71) and 3.00 (2.29-3.49 respectively, $p<0.005$ for all] (Figure 3C). IgG3 was significantly higher at baseline [3.61 (3.16-3.87)] than at 3 or at 6 months [3.40 (2.90-3.73) and 3.33 (2.25-3.78) respectively, $p=0.001$] (Figure 3D). IgG4 was also significantly higher at baseline [3.50 (2.91-3.90)] than at 3 or at 6 months [3.46 (2.95-3.84) and 3.12 (2.35-3.55) respectively, $p=0.003$] (Figure 3E). IgA was significantly higher at baseline [4.08 (3.93-4.25)] than at 3 or at 6 months [(4.04 (3.91-4.20) and 3.95 (3.74-4.12) respectively, $p<0.005$ for all] (Figure 3F). IgM was significantly higher at baseline [3.05 (2.78-3.54)] than at 3 or at 6 months [3.00 (2.35-3.37) and 2.52 (1.93-3.12) respectively, $p<0.05$ for all] (Figure 3G).

A similar trend was seen in HIV^- women, Total IgG was significantly higher at baseline [4.40 (4.18-4.62)] than at 3 or at 6 months [4.38 (3.93-4.47) and 4.23 (3.94-4.41) respectively, $p=0.0006$] (Figure 4A). IgG1 was significantly higher at baseline [4.05 (3.90-4.18)] than at 3 or at 6 months [4.08 (3.77-4.11) and 4.08 (3.82-4.12)] respectively, $p=0.03$] (Figure 4B). IgG2 was significantly higher at baseline [3.68 (3.41-4.14)] than at 3 or at 6 months [3.56 (3.13-3.70) and 3.17 (2.62-3.52) respectively, $p<0.05$] (Figure 4C). IgG3 was significantly higher at baseline [3.54 (3.13-3.81)] than at 3 or at 6 months [3.55 (2.83-3.87) and 3.09 (2.74-3.42) respectively, $p=0.002$] (Figure 4D). IgM was significantly higher at baseline [2.89 (2.55-3.39)] than at 3 or at 6 months [2.70 (2.10-3.11) and 2.48 (2.09-2.93) respectively, $p=0.002$] (Figure 4G).

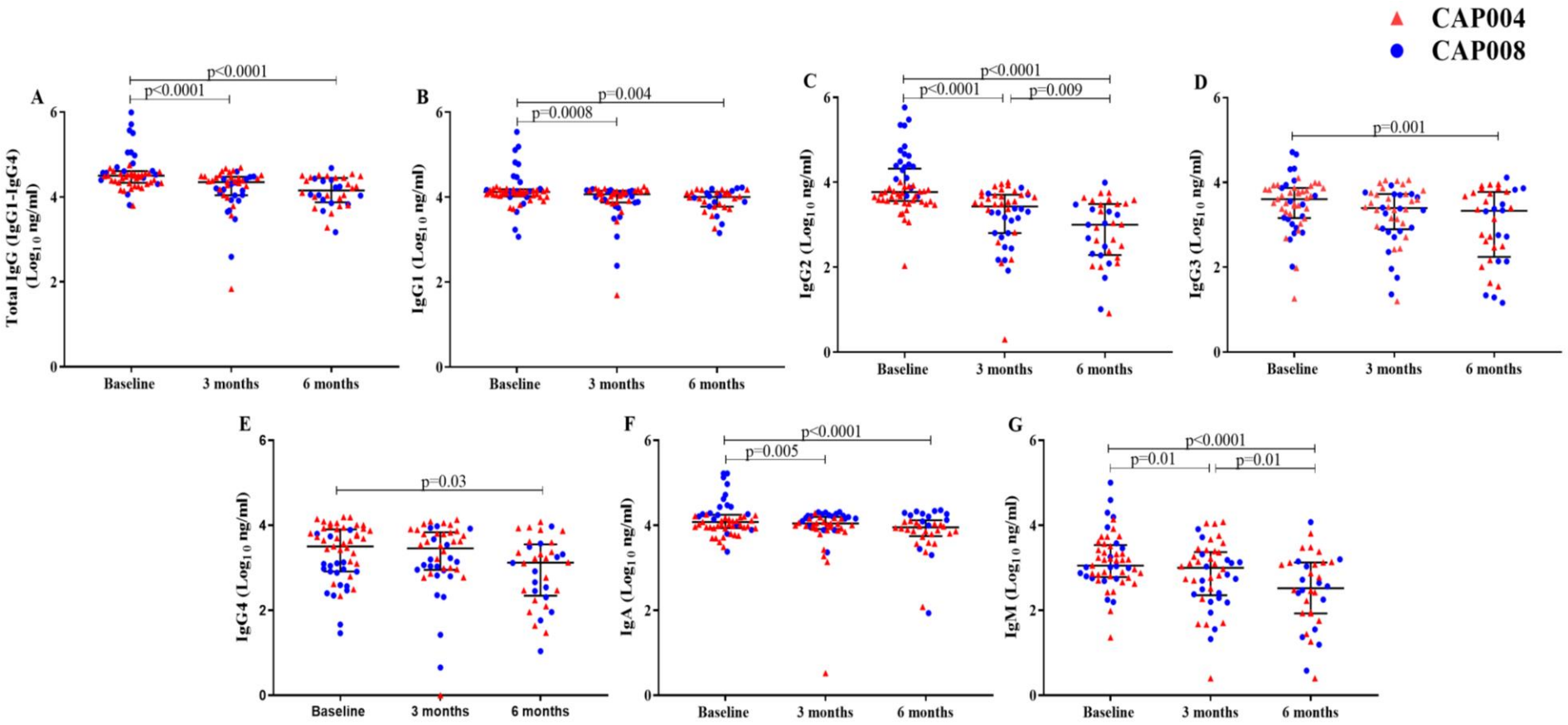


Figure 3: Comparison of the total Immunoglobulins (IgG1-IgG4) (A), IgG1 (B), IgG2 (C), IgG3 (D), IgG4 (E) (IgG1-IgG4) subclasses, IgA (F) and IgM (G) in CVL from HIV$^+$ women at baseline (n=53), at 3 months (n=46) and at 6 months (n=36). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. At baseline, HIV$^+$ represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

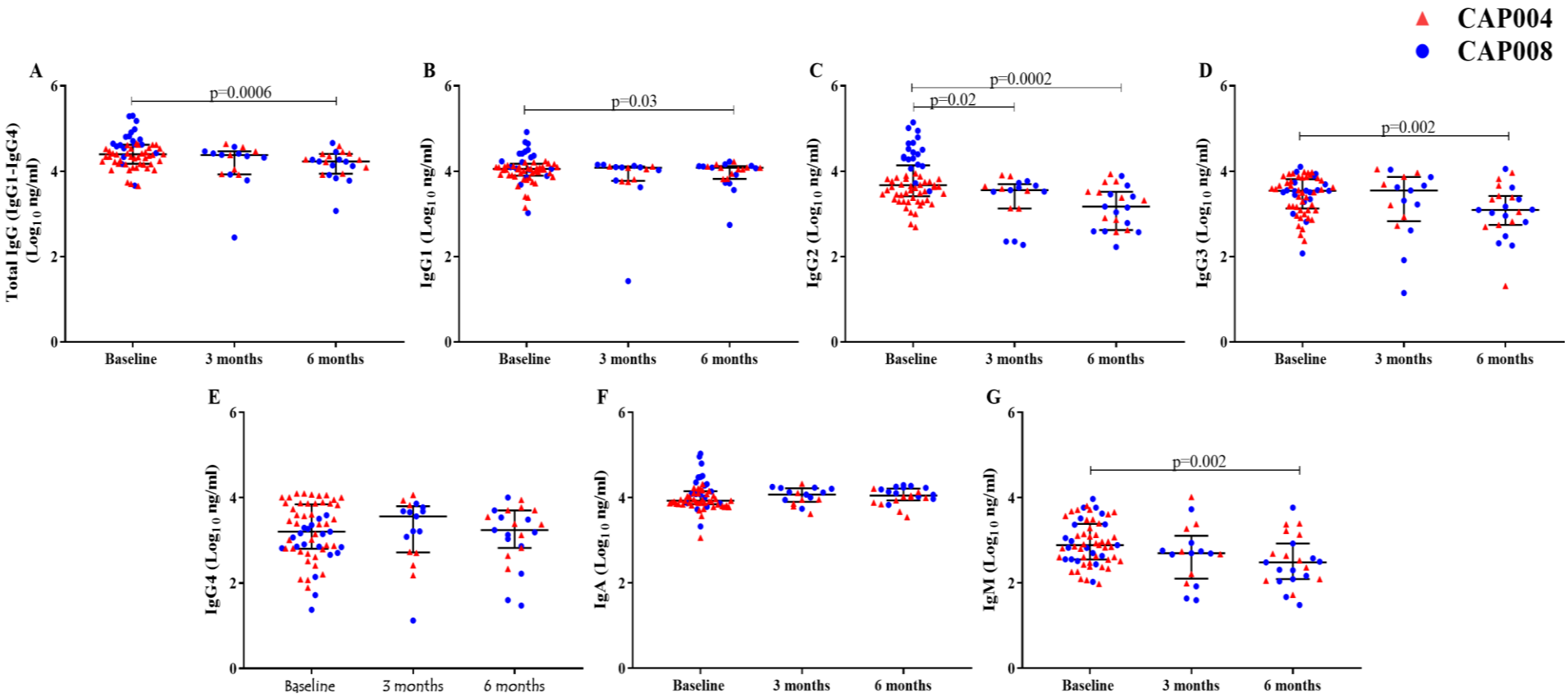


Figure 4: Comparison of the total Immunoglobulins (IgG1-IgG4) (A), IgG1 (B), IgG2 (C), IgG3 (D), IgG4 (E) (IgG1-IgG4) subclasses, IgA (F) and IgM (G) in CVL from HIV$^-$ women at baseline (n=61), at 3 months (n=17) and at 6 months (n=23). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

5.3.3 HIV-infected women without genital inflammation had significantly higher mucosal IgG subclasses and isotypes at baseline compared to their subsequent time points- intragroup analyses based on inflammation

The levels of antibody isotypes and IgG subclasses were evaluated longitudinally (baseline, 3 and 6 months) in the HIV^+ and HIV^- women based on their genital inflammation status. Although no differences were seen in the women who had genital inflammation, irrespective of HIV infection status, (HIV^+GI^+, Figure 5 and HIV^-GI^+, Figure 7), there were significant isotype and subclass differences for HIV^+GI^- (Figure 6) and HIV^-GI^- women (Figure 8). In the HIV^+GI^- women, total IgG was significantly higher at baseline [4.45 (4.19-4.57)] than at 3 or at 6 months [4.30 (4.04-4.46)] and 4.15 (3.96-4.44) respectively, $p<0.0005$ for all] (Figure 6A). IgG1 was also significantly higher at baseline [4.09 (4.01-4.17] than at 3 months or at 6 months [4.07 (3.89-4.45) and 4.03 (3.83-4.13) respectively, $p<0.05$ for all] (Figure 6B). Significantly higher IgG2 was present at baseline [3.79 (3.58-4.11) compared to 3 months [3.48 (2.82-3.77)] and at 6 months [3.04 (2.36-3.58)]; $p<0.005$ for all (Figure 6C). At 6 months, only IgG4 showed a significant decrease from 3 months [3.54 (2.99-3.91) and 3.24 (2.47-3.68) respectively, $p=0.03$] (Figure 6E). IgA similarly showed significant decreases from baseline [4.08 (3.95-4.24)] to 3 months and 6 months [4.06 (3.93-4.19) and 3.97 (3.78-4.12) respectively, $p<0.01$ for both] (Figure 6F). Furthermore, IgM also significantly decreased from baseline [3.18 (2.80-3.73)] compared to 3 and 6 month levels [3.03 (2.41-3.45) and 2.75 (1.95-3.24) respectively, $p<0.02$ for all comparisons] in HIV^+GI^- women (Figures 6G).

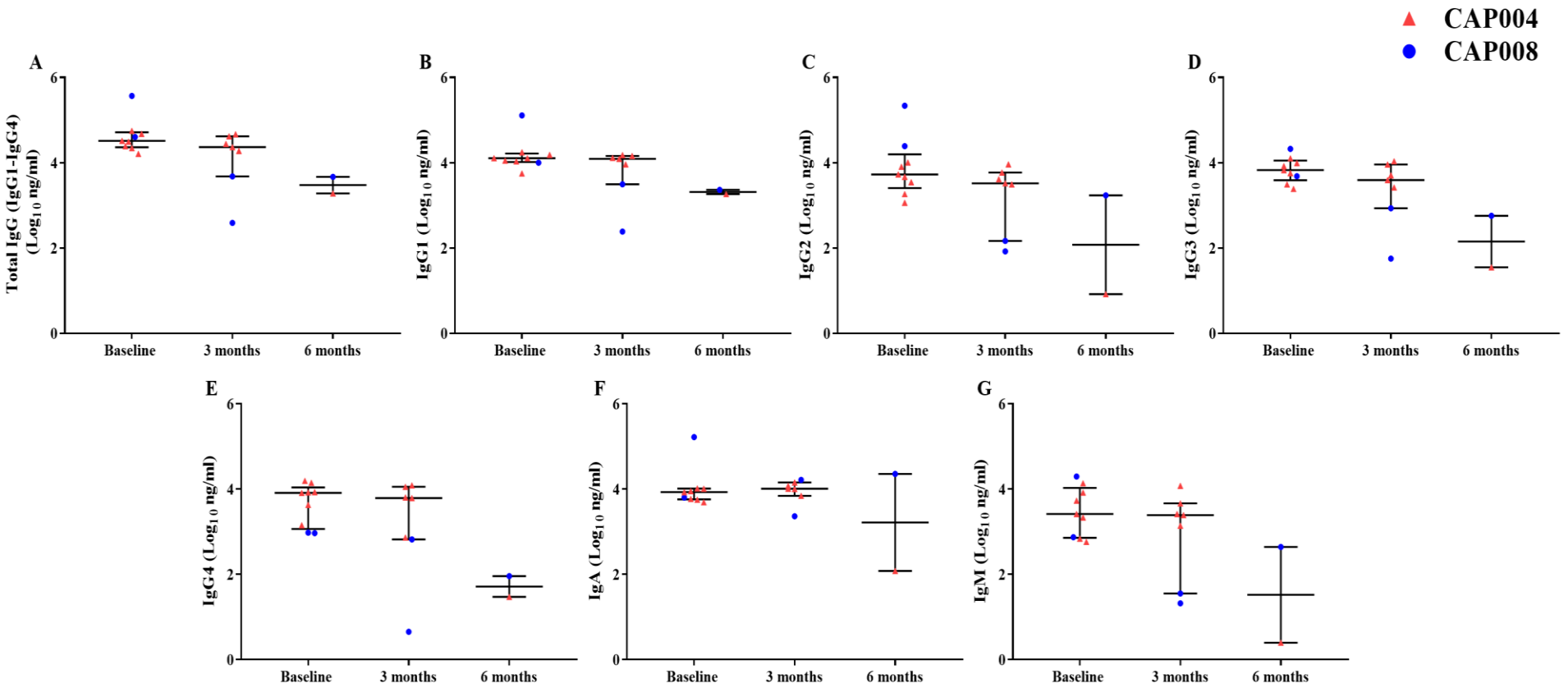


Figure 5: Comparison of the total Immunoglobulins (IgG1-IgG4) (A), IgG1 (B), IgG2 (C), IgG3 (D), IgG4 (E) (IgG1-IgG4) subclasses, IgA (F) and IgM (G) in CVL from HIV^+GI^+ women at baseline (n=9), at 3 months (n=7) and at 6 months (n=2). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. At baseline, HIV^+GI^+ represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

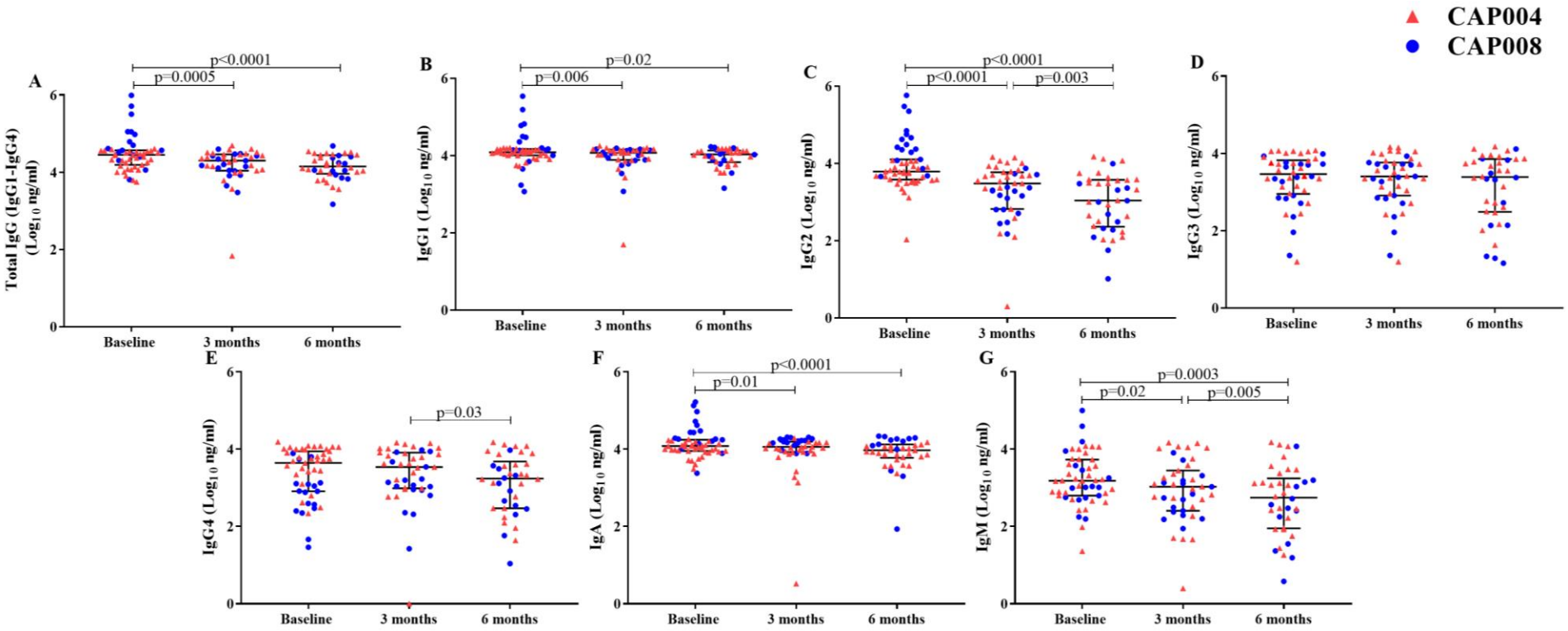


Figure 6: Comparison of the total Immunoglobulins (IgG1-IgG4) (A), IgG1 (B), IgG2 (C), IgG3 (D), IgG4 (E) (IgG1-IgG4) subclasses, IgA (F) and IgM (G) in CVL in CVL from $HIV^{+}GI^{-}$ women at baseline (n=51), at 3 months (n=43) and at 6 months (n=38). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. At baseline, $HIV^{+}GI^{-}$ represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

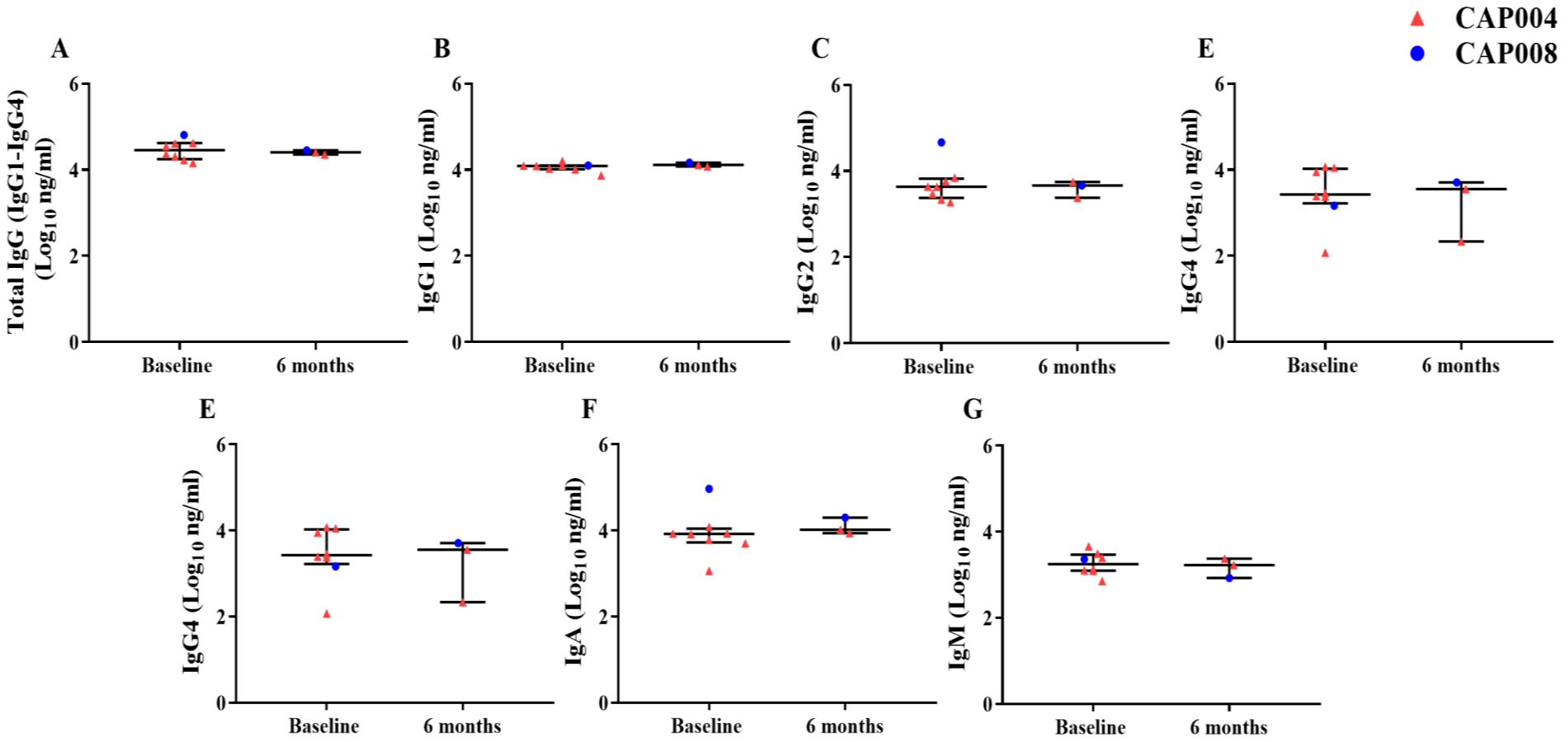


Figure 7: Comparison of the total Immunoglobulins (IgG1-IgG4) (A), IgG1 (B), IgG2 (C), IgG3 (D), IgG4 (E) (IgG1-IgG4) subclasses, IgA (F) and IgM (G) in CVL from HIV^{-}GI^{+} women at baseline (n=8) and at 6 months (n=3). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

5.3.4 In healthy women without genital inflammation, mucosal IgG1, IgG4 and IgA remained unchanged while significant decreases in total IgG, IgG2, IgG3 and IgM were seen from baseline to 6 months

In HIV^-GI^- women, total IgG was significantly higher at baseline [4.40 (4.17-4.62)] than at 6 months [4.22 (3.98-4.28)] (p=0.0006) (Figure 8A). Similarly, IgG3 was significantly higher at baseline [3.51 (3.09-3.73)] than at 6 months [3.07 (2.72-3.38)] (p=0.002) (Figure 8D) and IgM showed significant decreases at baseline [2.83 (2.52-3.25)] compared to 6 months [2.43 (2.09-2.68)] (p=0.002) (Figure 8G) in HIV^-GI^- women. IgG2 was the only subclass to show consistently significant decreases from baseline [3.68 (3.40-4.25)] to 3 months and 6 months [3.56 (3.13-3.70) and 3.16 (2.64-3.52) and at 6 months respectively, $p<0.005$ for all] (Figure 8C). In contrast, IgG1 (Figure 8B), IgG4 (Figure 8E) and IgA (Figure 8F) remained unchanged over time.

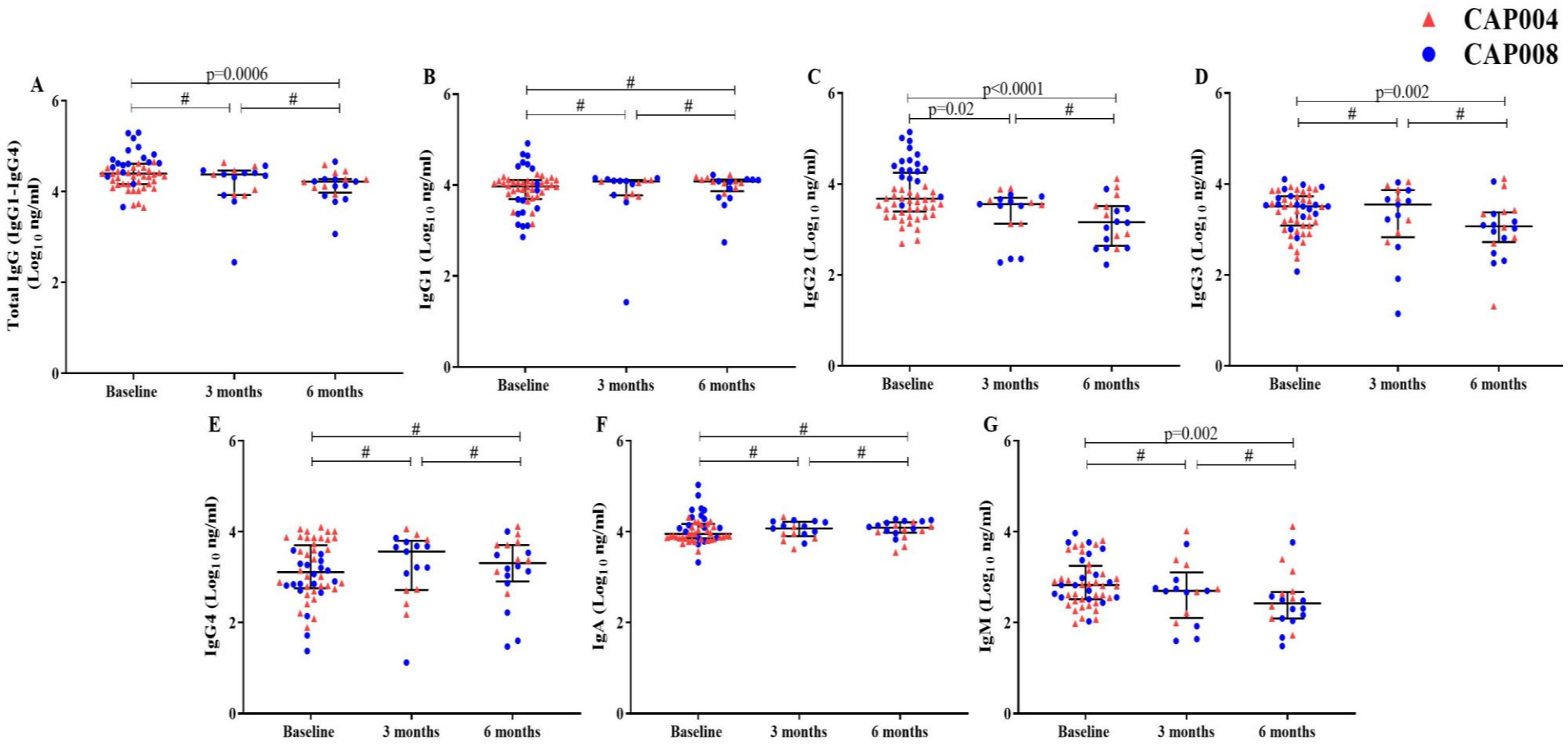


Figure 8: Comparison of the total Immunoglobulins (IgG1-IgG4) (A), IgG1 (B), IgG2 (C), IgG3 (D), IgG4 (E) (IgG1-IgG4) subclasses, IgA (F) and IgM (G) in CVL from HIV^{-}GI^{-} women at baseline (n=57), at 3 months (n=17) and at 6 months (n=20). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. P-values that were not statistically significant are indicated by a #. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

5.4 Mucosal HIV-specific antibody activities in women

5.4.1 HIV-infected women with genital inflammation had significantly higher HIV-specific activities compared to women without genital inflammation - intergroup analyses

To control for the inter-subject heterogeneity for genital specimen recovery, the HIV-IgG-specific activity was normalized against the total IgG (IgG1+IgG2+IgG3+IgG4) in the CVL specimens.

HIV-specific activities for all four antibody-specificities were higher in HIV^+ women compared to HIV^- women at 3 months ($p<0.0001$). At 3 months, p24-specific activity was significantly higher in HIV^+ women [median -0.38 Log_{10} (MFI*dilution factor ng ml^{-1}) (IQR -1.44 to -0.09 Log_{10} (MFI*dilution factor ng ml^{-1})] compared to HIV^- women [median -1.89 Log_{10} (MFI*dilution factor ng ml^{-1}) (IQR -2.50 to -1.43 Log_{10} (MFI*dilution factor ng ml^{-1})] ($p=0.009$) (Figure 9E). Additionally, significantly higher gp41-specific activity was found in HIV^+ [-0.75 (-1.25 to -0.24)] than in HIV^- women [-3.17 (-3.51 to -2.61)] ($p=0.002$) (Figure 9H). Gp120 also showed significantly higher median specific activity in HIV^+ [-2.80 (-4.63 to -1.92)] than in HIV^- [-4.63 (-4.63 to -4.63)] ($p=0.02$) women (Figure 9K). Additionally, at 6 months p66 was significantly higher in HIV^+ [-0.46 (-0.79-0.12)] than in HIV^- [-2.00 (-2.39 to -1.13)] ($p<0.0001$) women (Figure 9C). P24-specific activities were similarly significantly higher in HIV^+ [0.06 (0.03-1.05)] than in HIV^- [-2.28 (-2.56 to -1.72)] ($p<0.0001$) (Figure 9F). Gp41-specific activities were significantly higher at 6 months in HIV^+ [-0.07 (-0.56-0.51)] than in HIV^- [-3.17 (-3.40 to -3.00)] ($p<0.0001$) (Figure 9I) women.Gp120-specific activities were also significantly higher in HIV^+ [-1.49 (-2.29 to -0.931)] than in HIV^- [-4.63 (-4.63 to -3.58)] ($p<0.0001$) (Figure 9L) were also significantly higher in HIV^+ compared to HIV^- women.

When the HIV^+ and HIV^- women were further stratified according to GI status, p24-specific activity at baseline was significantly higher in HIV^+GI^+ [-1.73 (-2.43 to -1.44)] compared to HIV^+GI^- [-2.42 (-2.80 to -2.04)] ($p=0.03$) women (Figure 10D). At 3 months, p24- (Figure 10E), gp41- (Figure 10H) and gp120-specific activities (Figure 10K) were notably higher in HIV^+GI^+ and HIV^-GI^- compared to HIV^-GI^- women ($p<0.05$). Additionally, HIV-specificities to all four HIV-specific antibodies were higher in HIV^+GI^- ($p<0.0001$ for all) compared to HIV^-GI^- women (Figure 10C, Figure 10F, Figure 10I and Figure 10L) at 6 months.

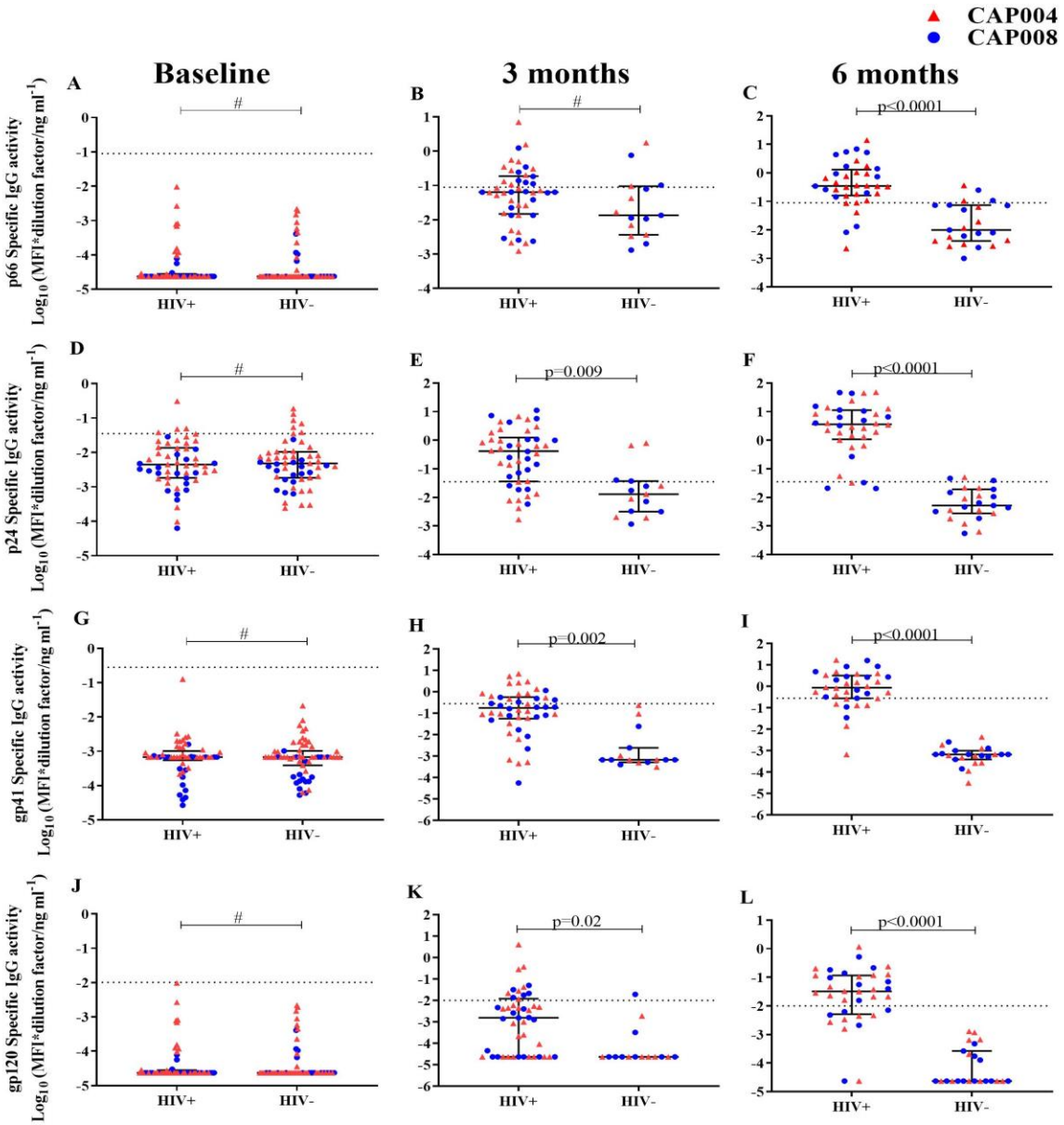


Figure 9: HIV-specific activity Log_{10} (MFI*dilution factor/ngml^{-1}) in CVL from HIV^{+} and HIV^{-} women for p66 (A, B and C), p24 (D, E and F), gp41 (G, H and I) and gp120 (J, K and L). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon tests were used to compare groups and $p<0.05$ were considered statistically significant. P-values that were not statistically significant are indicated by a #. All values falling below the detectable specific activities [based on average CVL specific activities of (n=60) HIV^{-} women] are reflected on or below the dotted lines. At baseline, HIV^{+} represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial. Numbers of women in each category varied from baseline to 6 months as follows, at baseline [HIV^{+} (n=53) and HIV^{-} (n=59)], 3 months [HIV^{+} (n=46) and HIV^{-} (n=15)] and 6 months [HIV^{+} (n=36) and HIV^{-} (n=23)].

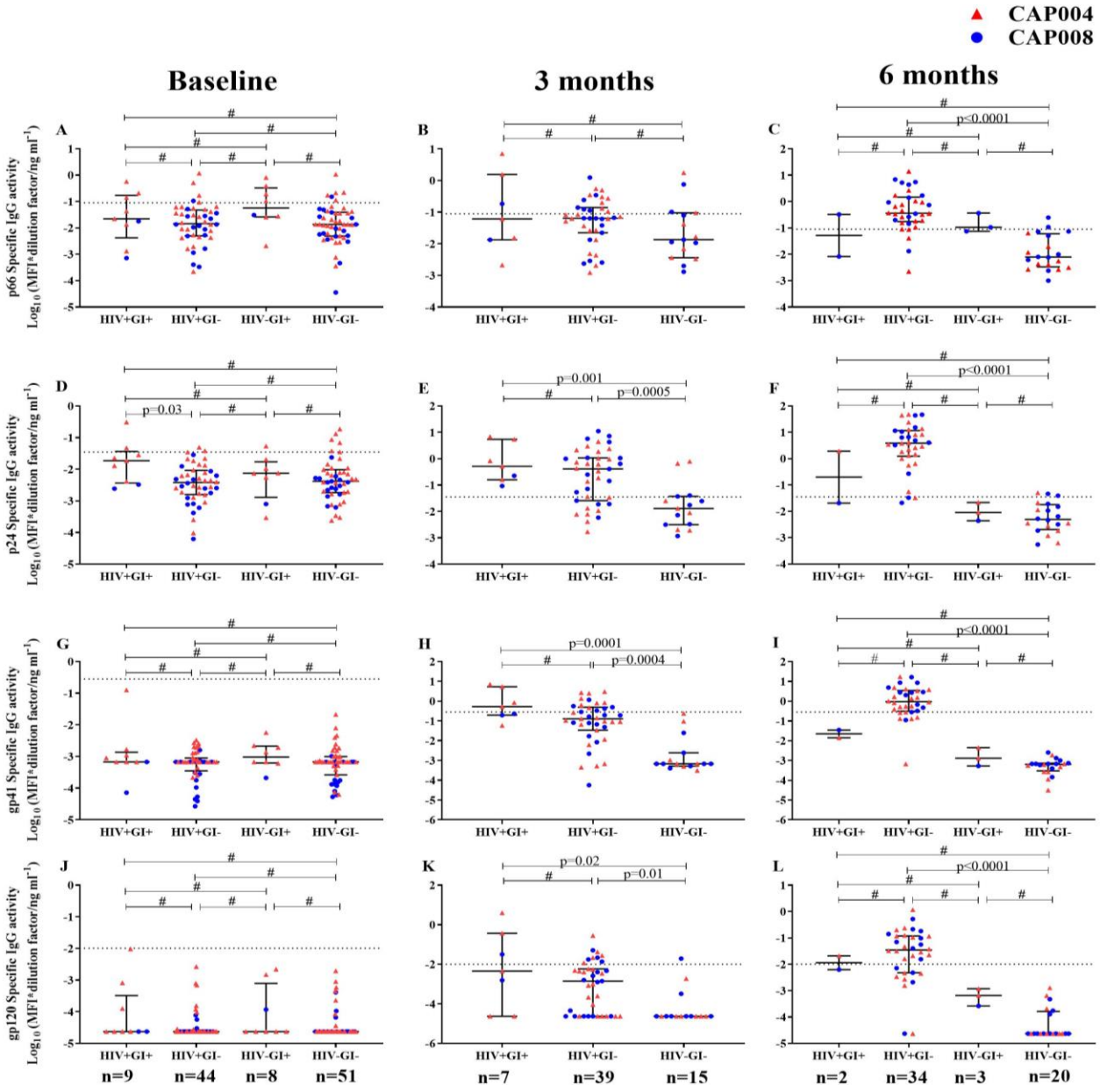


Figure 10: HIV-specific activity Log_{10} (MFI*dilution factor/ngml^{-1}) in CVL from HIV^{+}GI^{+}, HIV^{+}GI^{-}, HIV^{-}GI^{+} and HIV^{-}GI^{-} women for p66 (A, B and C), p24 (D, E and F), gp41 (G, H and I) and gp120 (J, K and L). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon tests were used to compare groups and $p<0.05$ were considered statistically significant. P-values that were not statistically significant are indicated by a #. All values falling below the detectable specific activities [based on average CVL specific activities of (n=60) HIV^{-} women] are reflected on or below the dotted lines. At baseline, HIV^{+} represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial. Numbers of women in each category varied from baseline to 6 months as follows, at baseline [HIV^{+}GI^{+} (n=9), HIV^{+}GI^{-} (44), HIV^{-}GI^{+} (n=8) and HIV^{-}GI^{-} (n=51)], at 3 months [HIV^{+}GI^{+} (n=7), HIV^{+}GI^{-} (n=39) and HIV^{-}GI^{-} (n=15)], and at 6 months [HIV^{+}GI^{+} (n=2), HIV^{+}GI^{-} (n=34), HIV^{-}GI^{+} (n=3) and HIV^{-}GI^{-} (n=20)].

5.4.2 HIV-specific activities significantly increase in HIV-infected women over time- intragroup analyses

HIV-specific activities were evaluated longitudinally in HIV^+ and HIV^- women. Significantly higher p66- (Figure 11A), p24- (Figure 11B), gp41- (Figure 11C) and gp120-specific activities (Figure 11D) ($p<0.05$ for all) were found only in HIV^+ women at baseline, 3 and 6 months and unsurprisingly not in HIV^- (Supplementary Figure 1A-D) women at baseline, 3 and 6 months. When the women were further stratified according to their genital inflammation status, a similar trend was seen only in HIV^+GI^- women ($p<0.0001$) (Figure 12A- D). There were no significant differences in HIV-specific activities in the HIV^+GI^+ (Supplementary Figure 2), HIV^-GI^+ (Supplementary Figure 3) and HIV^-GI^- (Supplementary Figure 4) women.

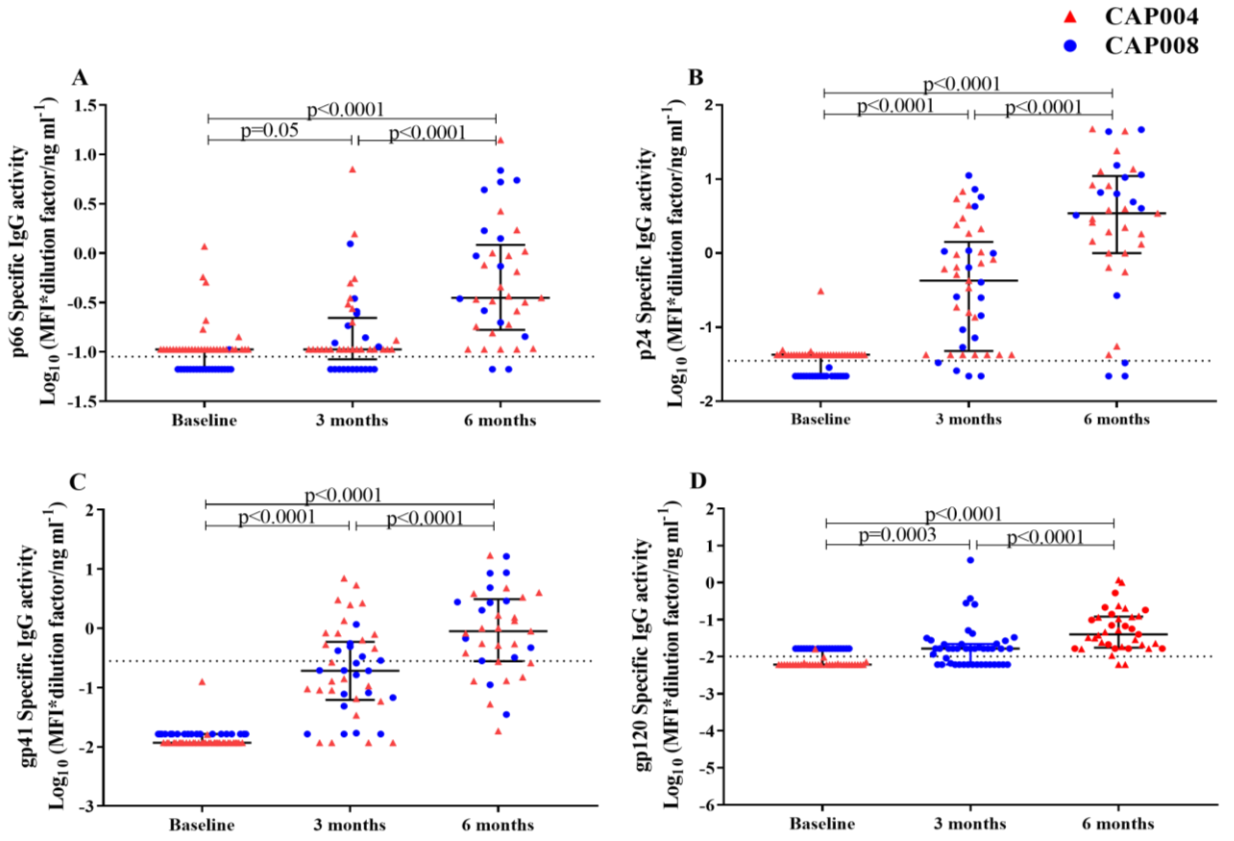


Figure 11: HIV-specific activity Log_{10} (MFI*dilution factor/$ngml^{-1}$) in CVL for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV^+ women at baseline (n=52), at 3 months (n=45) and at 6 months (n=37). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific activities [based on average CVL specific activities of (n=60) HIV^- women] are reflected on or below the dotted lines. At baseline, HIV^+ represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

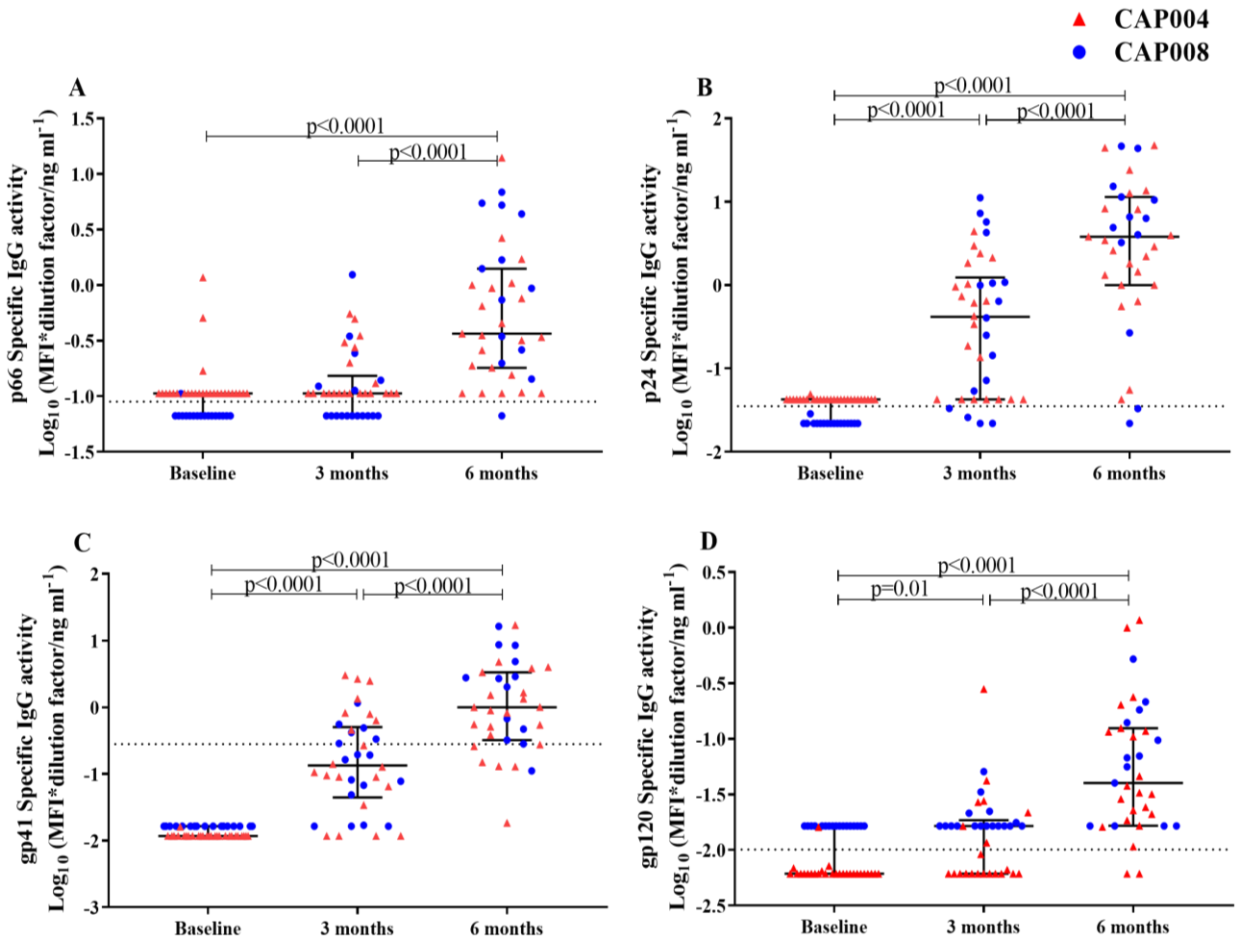


Figure 12: HIV-specific activity Log_{10} (MFI*dilution factor/ngml^{-1}) in CVL for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV^{+}GI^{-} women at baseline (n=44), at 3 months (n=38) and at 6 months (n=35). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific activities [based on average CVL specific activities of (n=60) HIV^{-} women] are reflected on or below the dotted lines. At baseline, HIV^{+}GI^{-} represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

5.5 Systemic HIV-specific antibody responses in women

5.5.1 HIV infection leads to significantly higher systemic HIV-specific responses in HIV-infected women - intergroup analyses

Next, the HIV-specific IgG responses in the plasma were evaluated in HIV^+ and HIV^- women. At 3 months, p66-specific responses were higher in HIV^+ [median 6.67 Log_{10} (MFI*dilution factor) (IQR 6.24-6.91 Log_{10} (MFI*dilution factor)] than in HIV^- [median 5.95 Log_{10} (MFI*dilution factor) (IQR 5.45-6.37 Log_{10} (MFI*dilution factor)] ($p<0.0001$) (Figure 13B) women. At 3 months, p24-specific responses were higher in HIV^+ [7.16 (6.62-7.84] than in HIV^- [5.36 (4.97-5.71] ($p<0.0001$) women (Figure 13E). At 3 months, gp41-specific responses were higher in HIV^+ [7.03 (6.51-7.44)] than in HIV^- [4.00 (4.00-4.46)] ($p<0.0001$) women (Figure 13H). At 3 months, gp120-specific responses were higher in HIV^+ [5.51 (5.00-5.80)] than in HIV^- [4.00 (4.00-4.18)] ($p<0.0001$) women (Figure 13K).

Likewise, at 6 months, p66-specific responses were higher in HIV^+ [7.01 (6.72-7.46)] than in HIV^- [5.92 (5.36-6.46)] ($p=0.0003$) women (Figure 13C). At 6 months, p24-specific responses were higher in HIV^+ [7.40 (6.63-8.10)] than in HIV^- [5.44 (5.11-5.90)] ($p<0.0001$) women (Figure 13F). At 6 months, gp41-specific responses were higher in HIV^+ [7.24 (6.67-7.68)] than in HIV^- [4.08 (4.00-5.03)] ($p<0.0001$) women (Figure 13I). At 6 months, gp120-specific responses were higher in HIV^+ [5.94 (5.36- 6.25)] than in HIV^- [4.00 (4.00-4.60)] ($p<0.0001$) women (Figure 13L).

At 3 months, when the HIV^+ and HIV^- women were further stratified according to genital inflammation status, HIV^+GI^+ and HIV^+GI^- women had significantly higher p66- (Figure 14B), p24- (Figure 14E), gp41- (Figure 14H) and gp120-specific responses (Figure 14K) compared to HIV^-GI^+ and HIV^-GI^- women ($p<0.05$ for all). This trend remained unchanged for p66- (Figure 14C), p24- (Figure 14F), gp41- (Figure 14I), and gp120-specific responses (Figure 14L) between HIV^+GI^+ and HIV^+GI^- compared to HIV^-GI^+ and HIV^-GI^- women at 6 months ($p<0.05$ for all).

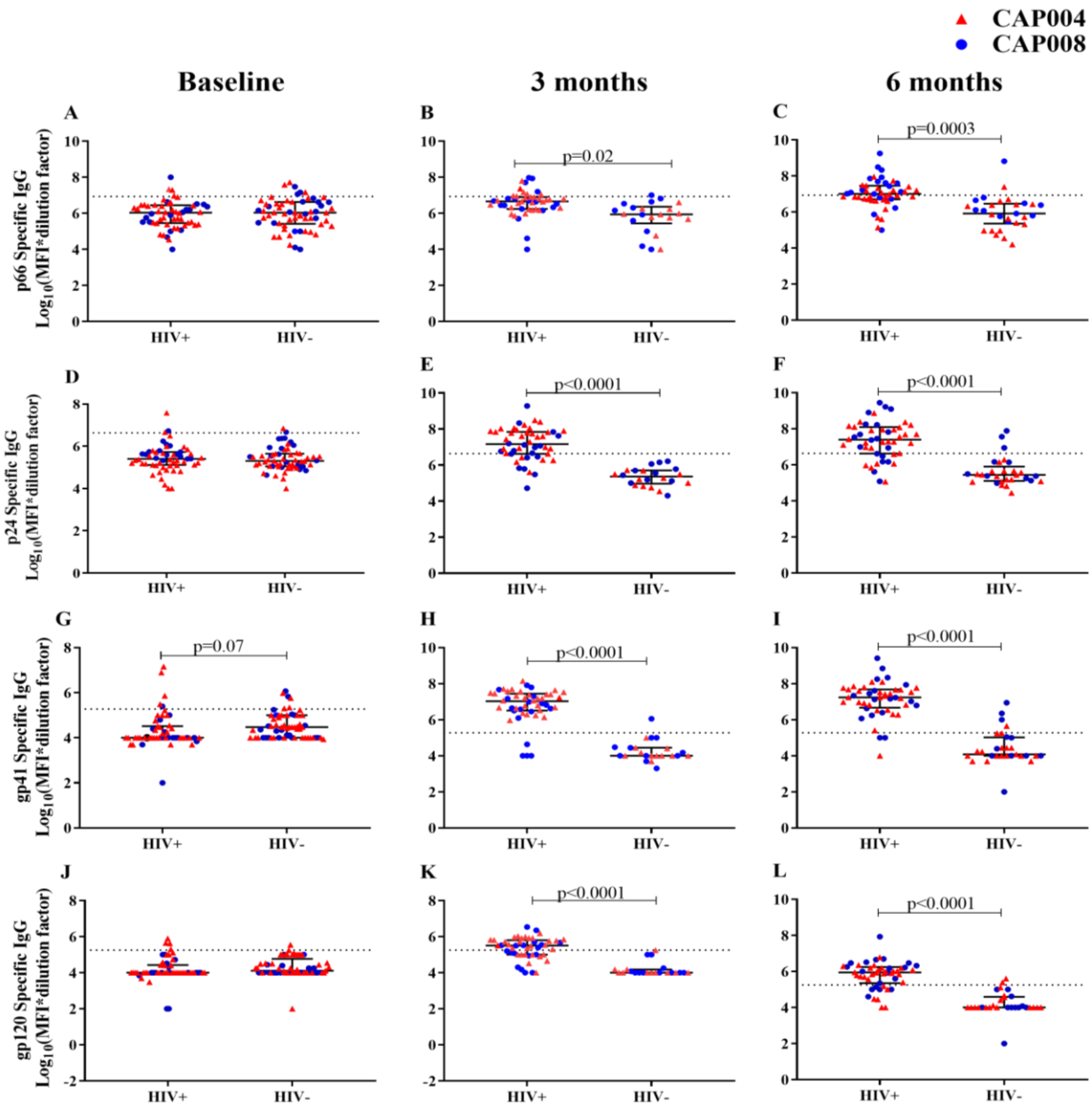


Figure 13: Plasma HIV-specific responses [Log_{10} (MFI*dilution factor)] from HIV^+ and HIV^- women for p66 (A, B and C), p24 (D, E and F), gp41 (G, H and I) and gp120 (J, K and L). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon tests were used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific responses [based on average plasma specific responses of (n=61) HIV^- women] are reflected on or below the dotted lines. At baseline, HIV^+GI^- represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial. Numbers of women in each category varied from baseline to 6 months as follows, at baseline [HIV^+ (n=56) and HIV^- (n=61)], at 3 months [HIV^+ (n=47) and HIV^- (n=22)] and at 6 months [HIV^+ (n=48) and HIV^- (n=29)].

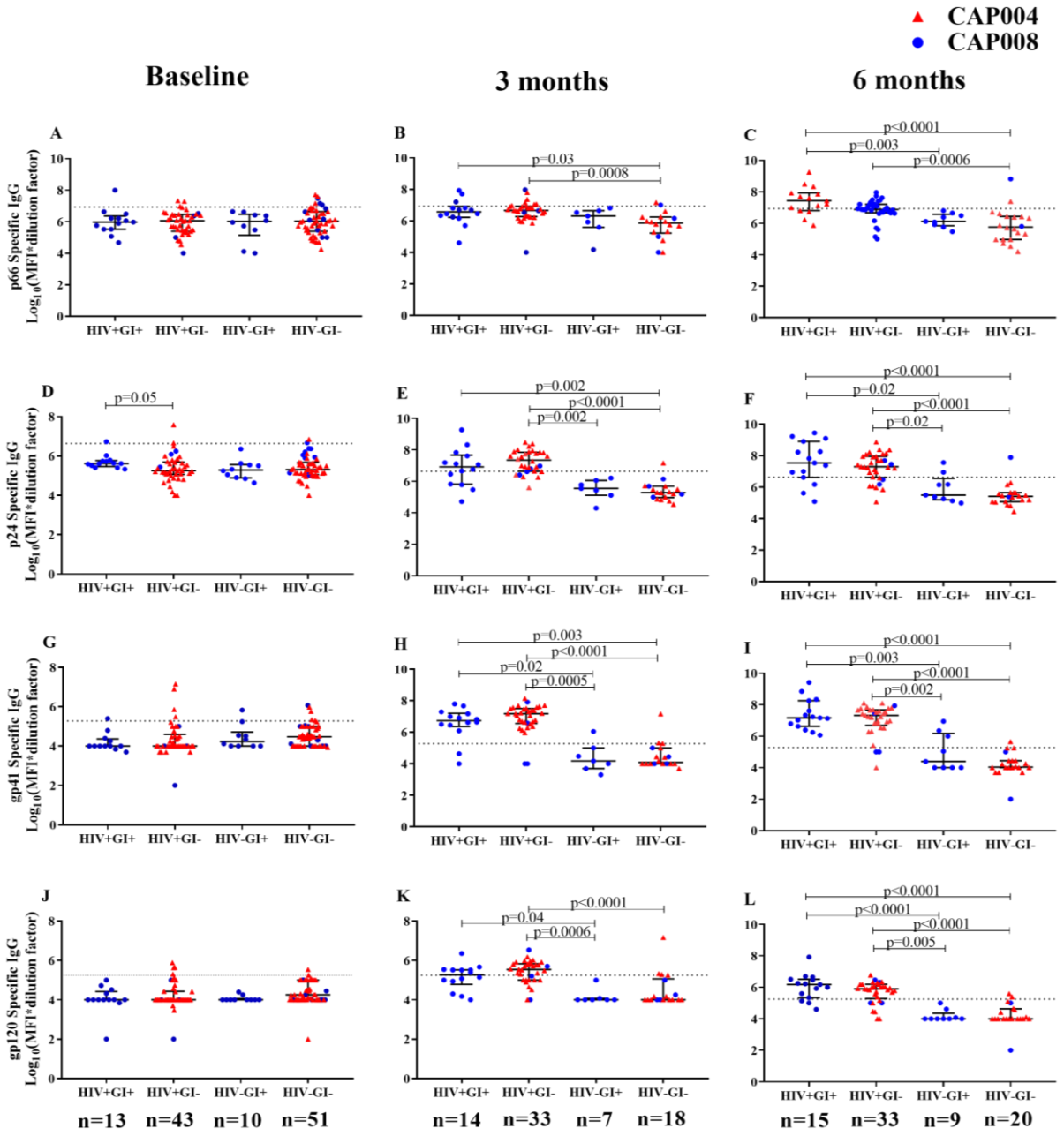


Figure 14: Plasma HIV-specific responses [Log_{10} (MFI*dilution factor)] from HIV^+GI^+, HIV^+GI^-, HIV^-GI^+ and HIV^-GI^- women for p66 (A, B and C), p24 (D, E and F), gp41 (G, H and I) and gp120 (J, K and L). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon tests were used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific responses [based on average plasma specific responses of (n=61) HIV^- women] are reflected on or below the dotted lines. At baseline, HIV^+GI^- represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial. Numbers of women in each category varied from baseline to 6 months as follows, at baseline [HIV^+GI^+ (n=13), HIV^+GI^- (n=43), HIV^-GI^+ (n=10) and HIV^-GI^- (n=51)], at 3 months [HIV^+GI^+ (n=14), HIV^+GI^- (n=33), HIV^-GI^+ (n=7) and HIV^-GI^- (n=18)], and at 6 months [HIV^+GI^+ (n=15), HIV^+GI^- (n=33), HIV^-GI^+ (n=9) and HIV^-GI^- (n=20)].

5.5.2 Plasma HIV-specific responses significantly increase in HIV-infected women over time-intragroup analyses

HIV-specific responses were evaluated longitudinally in HIV^+ and HIV^- women. Significantly higher p66- (Figure 15A), p24- (Figure 15B), gp41- (Figure 15C) and gp120-specific responses (Figure 15D) ($p<0.05$ for all) were found in HIV^+ at baseline, 3 and 6 months. As expected, there were no significant HIV-specific responses in HIV^- women, except for p24 ($p=0.02$) (Figure 16B). When the women were further stratified according to their genital inflammation status, a similar trend was seen in HIV^+GI^+ (Figure 17) and HIV^+GI^- women (Figure 18) ($p<0.05$ for all). There were no significant differences in HIV-specific responses in the HIV^-GI^+ (Supplementary Figure 5) and HIV^-GI^- women (Supplementary Figure 6).

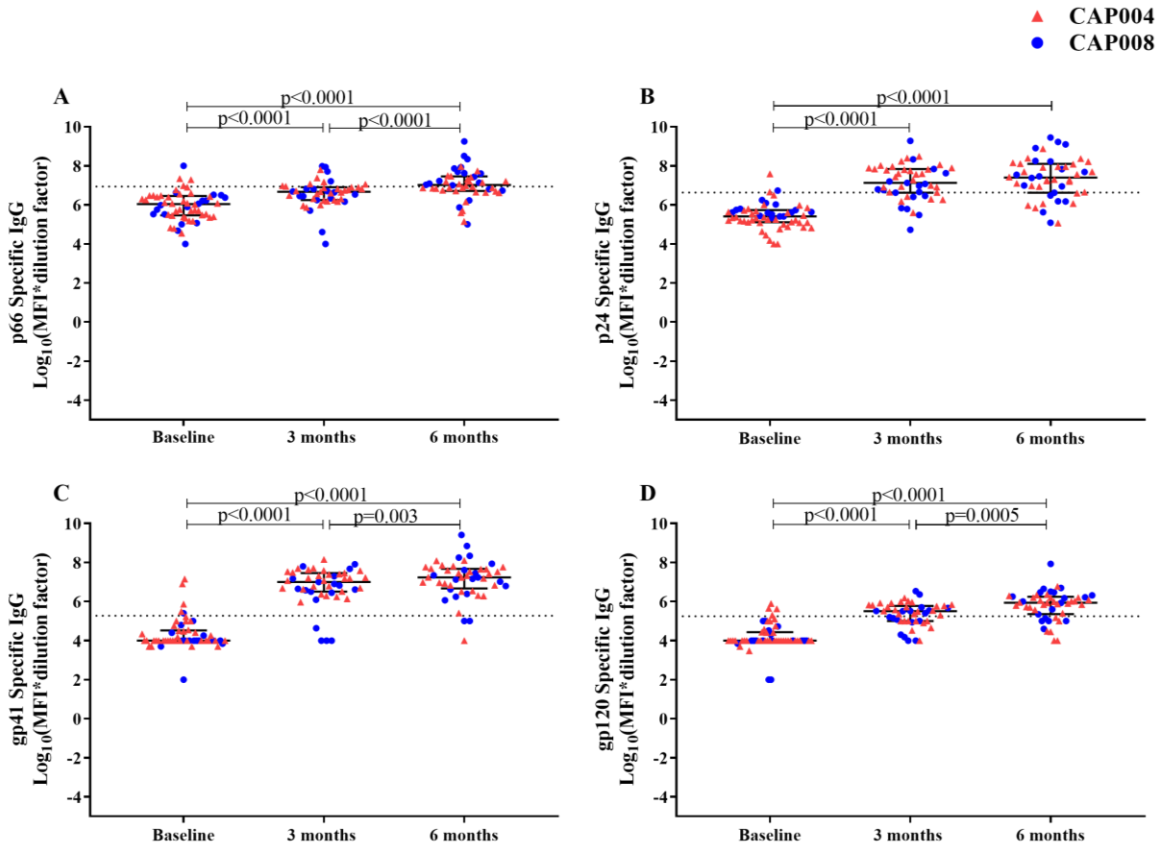


Figure 15: Plasma HIV-specific responses [Log_{10} (MFI*dilution factor)] for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV^+ women at baseline (n=57), at 3 months (n=48) and at 6 months (n=48). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific responses [based on average plasma specific responses of (n=61) HIV^- women] are reflected on or below the dotted lines. At baseline, HIV^+GI^- represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

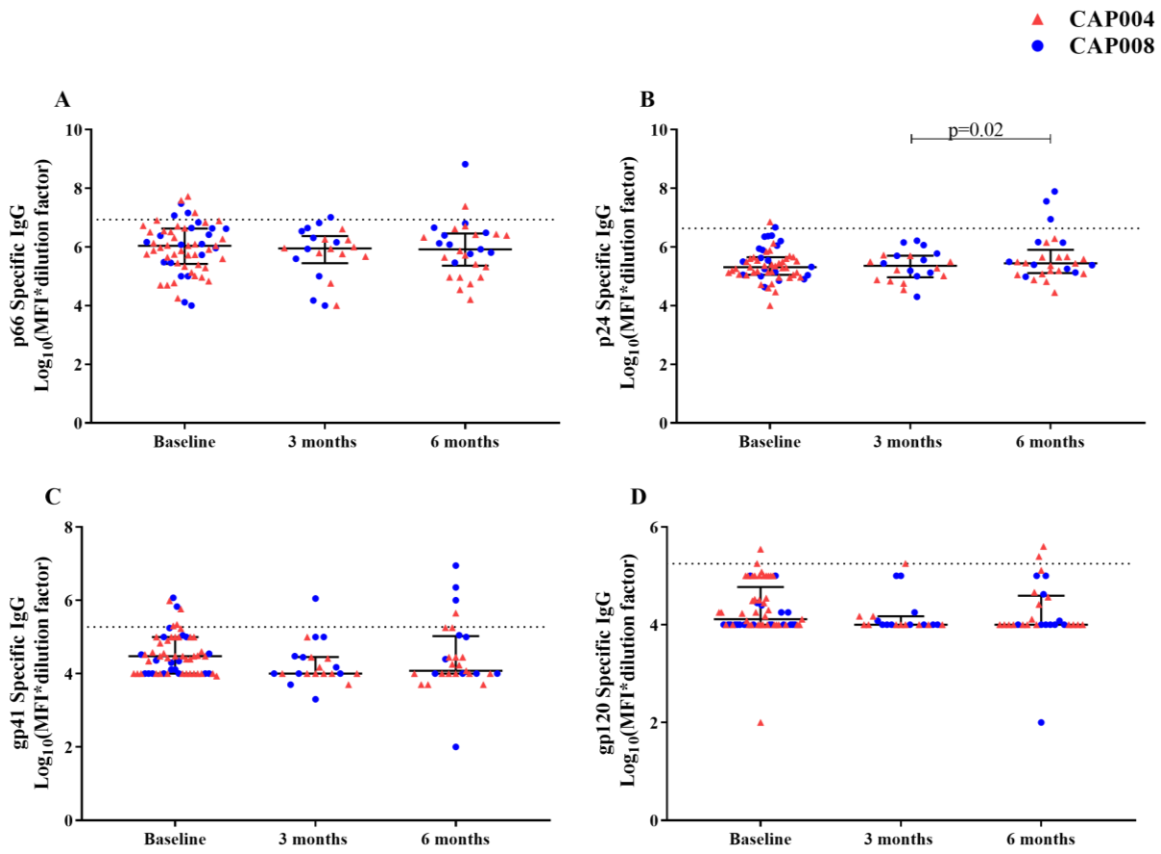


Figure 16: Plasma HIV-specific responses [Log_{10} (MFI*dilution factor)] for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV^- women at baseline (n=61), at 3 months (n=22) and at 6 months (n=29). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific responses [based on average plasma specific responses of (n=61) HIV^- women] are reflected on or below the dotted lines. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

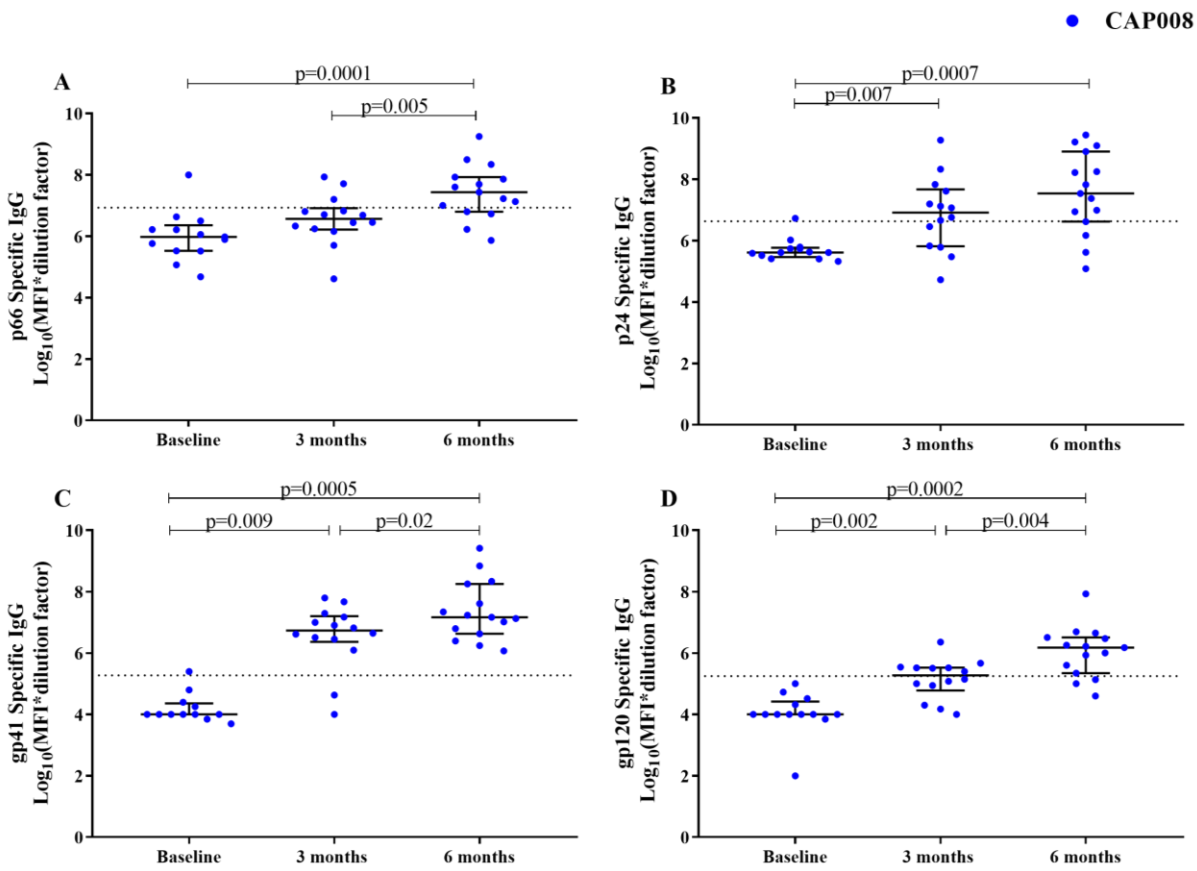


Figure 17: Plasma HIV-specific responses [Log10 (MFI*dilution factor)] for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV+GI+ women at baseline (n=13), at 3 months (n=14) and at 6 months (n=15). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific responses [based on average plasma specific responses of (n=61) HIV- women] are reflected on or below the dotted lines. At baseline, HIV+GI- represents women who subsequently became HIV infected. Blue circles represent women from the CAP008 trial.

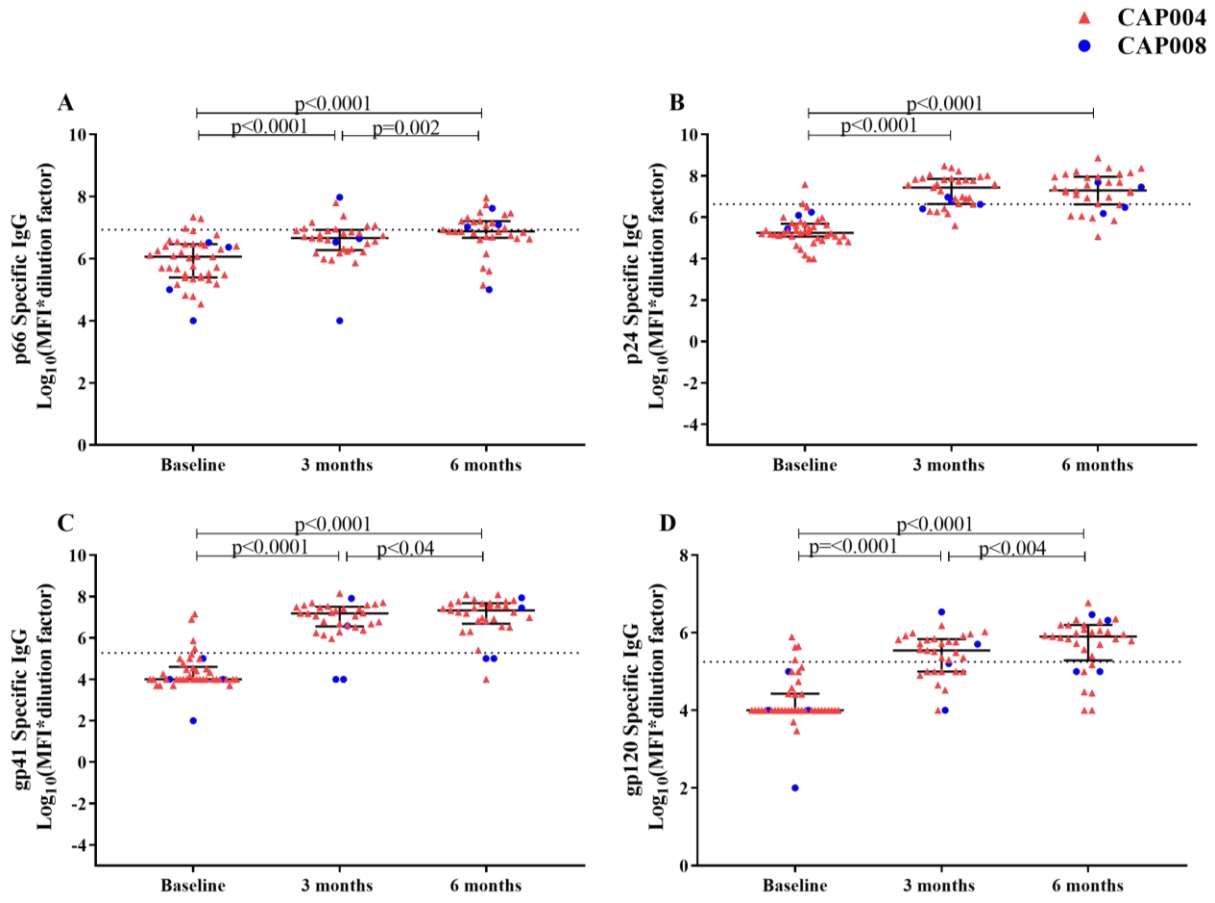


Figure 18: Plasma HIV-specific responses [Log_{10} (MFI*dilution factor)] for p66 (A), p24 (B), gp41 (C) and gp120 (D) from $HIV^{+}GI^{-}$ women at baseline (n=47), at 3 months (n=37) and at 6 months (n=39). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific responses [based on average plasma specific responses of (n=61) HIV^{-} women] are reflected on or below the dotted lines. At baseline, $HIV^{+}GI^{-}$ represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

5.6 In the absence of genital inflammation, p66 and p24-specific responses directly correlate between the systemic and the genital compartments

Next, the relationship between HIV-specific responses in the plasma and the genital tract were investigated longitudinally according to inflammation status. At baseline, there was a significant and positive correlation in HIV^+ women for p66 (r=0.45, p=0.002) (Figure 19A). This trend continued over time in the same HIV^+ women at 3 months, with more significant and positive correlations seen for p66 (r=0.42, p=0.006), p24 (r=0.50, p=0.0001), gp41 (r=.046, p=0.002) and gp120 (r=0.37, p=0.02) (Figure 19E-Figure 19H). At 6 months, there was also significant and positive correlations seen for p66 (r=0.39, p=0.03), p24 (r=0.55, p=0.001), gp41 (r=.040, p=0.03) and gp120 (r=0.44, p=0.01) (Figure 19I-Figure 19L).

When these women were further stratified according to inflammation, significant and positive correlations were seen in the absence of genital inflammation in HIV^+GI^- women for p66 (at baseline) (r=0.45, p=0.02) (Figure 20A) and p24- (at 6 months) (r=0.46, p=0.04) (Figure 20J).

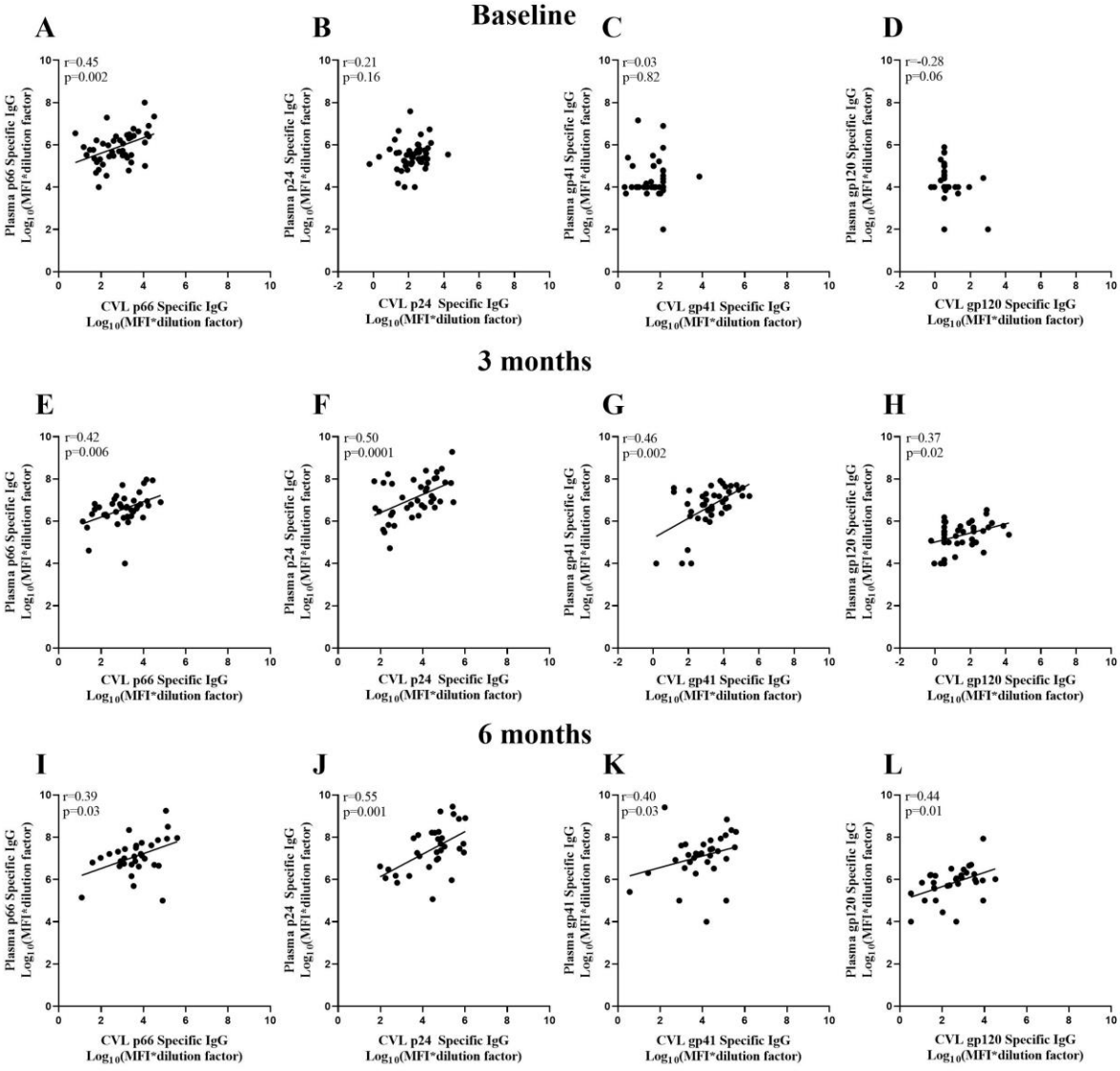


Figure 19: Correlations of HIV-specific responses in the plasma and CVL of HIV$^+$ women for p66 (A, E & I), p24 (B, F & J), gp41 (C, G & J) and gp120 (D, H & L). Pearson's correlations were used to check the compartment relationship between the plasma and the CVL for the HIV specific IgG responses. At baseline, HIV$^+$ represents women who subsequently became HIV infected.

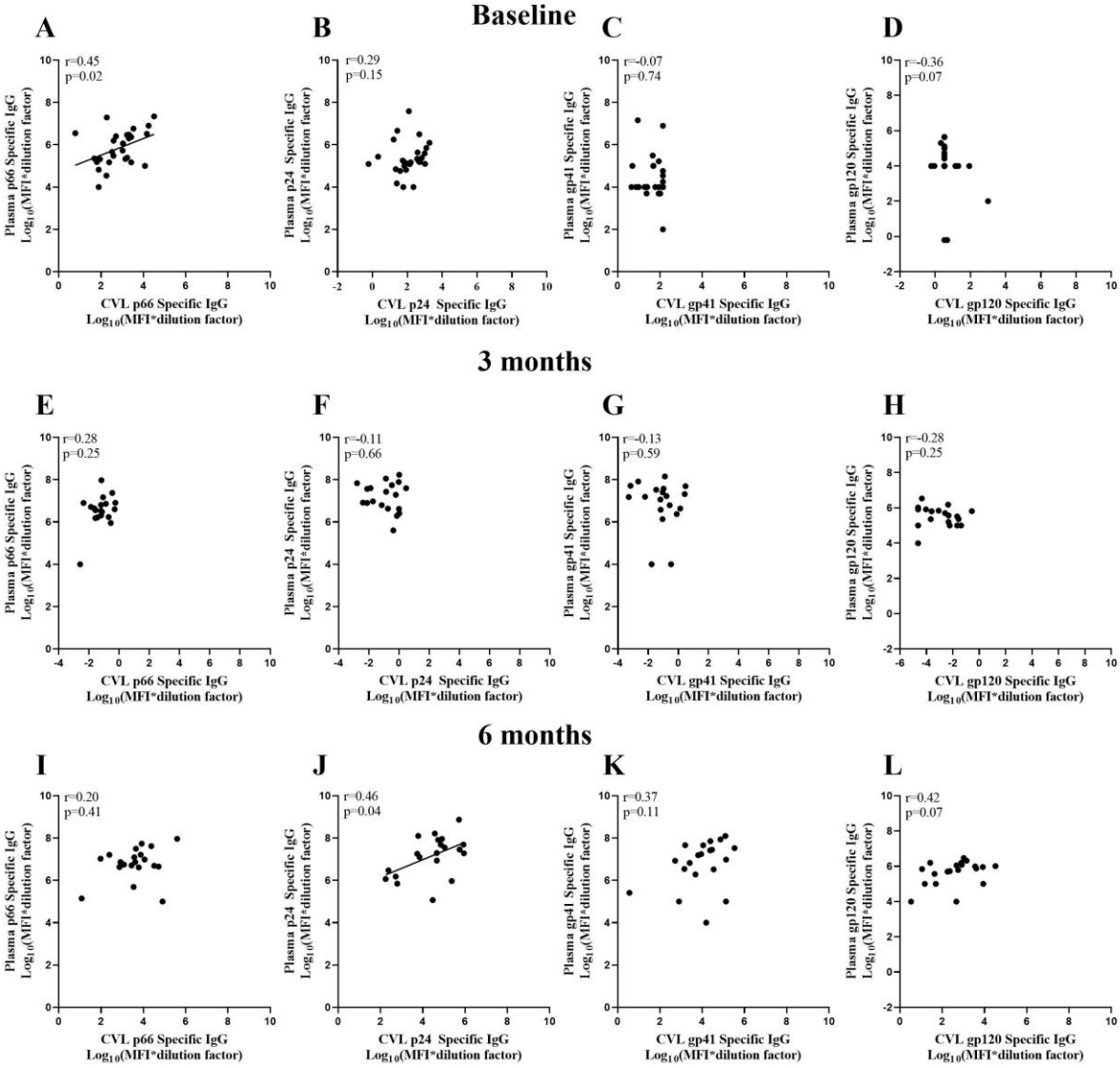


Figure 20: Correlations of HIV-specific responses in the plasma and CVL of $HIV^{+}GI^{-}$ women for p66 (A, E & I), p24 (B, F & J), gp41 (C, G & J) and gp120 (D, H & L). Pearson's correlations were used to check the compartment relationship between the plasma and the CVL for the HIV specific IgG responses. At baseline, $HIV^{+}GI^{-}$ represents women who subsequently became HIV infected.

5.7 Baseline mucosal cytokine signatures predict HIV acquisition risk

In order to determine the predictors of HIV acquisition risk, various cytokine profiles, antibody isotypes, IgG subclasses and HIV-specific antibodies were investigated (Table 3.4). Conditional regression analyses were performed at a univariate and multivariate level. In the univariate analysis, HIV acquisition increased more than four-fold with CTACK [OR, 4.33; (95% CI, 1.14-16.43); p=0.03] and MIG [OR, 2.11; (95% CI, 1.18-3.77); p=0.012]. After adjusting for age, sexual debut, frequency of condom use, number of vaginal sex acts, tenofovir use and HSV-2, higher levels of IFNγ [OR, 3.89; (95% CI, 1.14-13.32); p=0.03], CTACK [OR, 4.83; (95% CI, 1.13-20.74); p=0.03], MIG [OR, 2.50; (95% CI, 1.18-5.28); p=0.02] and SDF1α [OR, 7.63; (95% CI, 1.17-49.77); p=0.03] were associated with increased HIV acquisition risk. Apart from these chemokines and growth factors, none of the antibody isotypes, IgG subclasses and HIV-specific antibodies were associated with HIV acquisition risk before and after adjusting for the multiple variables.

Table 3.4: Mucosal cytokine signatures, antibody isotypes, IgG subclasses and HIV-specific antibody profiles associated with HIV acquisition risk

	Univariate Analysis				Multivariate Analysis			
Analyte	**Odds Ratio (OR)**	**95% CI Lower**	**95% CI Upper**	**P value**	**Adjusted Odds Ratio (AOR)**	**95% CI Lower**	**95% CI Upper**	**P value**
IL12p70	1.44	0.71	2.95	0.31	1.43	0.59	3.48	0.43
IL17α	1.35	0.47	3.86	0.58	1.80	0.52	6.24	0.35
IL1β	1.31	0.80	2.14	0.28	1.14	0.62	2.08	0.67
IL6	1.10	0.60	2.02	0.76	1.35	0.62	2.92	0.45
TNFα	2.01	0.87	4.67	0.10	2.38	0.91	6.22	0.08
IL12p40	2.04	0.57	7.37	0.28	2.36	0.59	9.48	0.23
IL18	1.46	0.76	2.79	0.26	1.70	0.81	3.53	0.16
IL1α	2.19	0.99	4.86	0.05	2.13	0.84	5.39	0.11
MIF	0.98	0.57	1.70	0.95	1.50	0.71	3.19	0.29
TNFβ	2.86	0.84	9.77	0.09	3.00	0.75	12.01	0.12
TRAIL	1.09	0.68	1.74	0.73	1.23	0.71	2.12	0.46
IL10	1.15	0.50	2.63	0.74	1.10	0.41	2.97	0.84
IL1Rα	0.94	0.30	2.95	0.91	1.35	0.38	4.79	0.64
IFNγ	2.39	0.86	6.64	0.09	**3.89**	**1.14**	**13.32**	**0.03**
IL13	2.02	0.72	5.68	0.18	2.00	0.61	6.54	0.25
IL15	0.82	0.49	1.38	0.46	1.31	0.61	2.81	0.49
IL2	0.95	0.62	1.45	0.80	1.18	0.70	1.99	0.53
IL4	3.35	0.70	16.05	0.13	4.36	0.66	29.03	0.13
IL5	1.10	0.66	1.83	0.72	1.32	0.72	2.43	0.37
IL2Rα	4.08	0.97	17.06	0.05	3.91	0.86	17.69	0.08
Eotaxin	1.09	0.69	1.71	0.72	1.15	0.63	2.08	0.66
IL8	1.35	0.90	2.02	0.15	1.45	0.91	2.31	0.12
IP10	1.36	0.88	2.10	0.17	1.42	0.84	2.39	0.19
MCP1	0.84	0.34	2.08	0.71	0.75	0.26	2.15	0.59
MIP1α	1.63	0.71	3.73	0.25	1.98	0.69	5.71	0.21
MIP1β	0.94	0.48	1.85	0.85	0.96	0.42	2.18	0.91
RANTES	1.14	0.66	1.95	0.64	1.27	0.69	2.32	0.45
CTACK	**4.33**	**1.14**	**16.43**	**0.03**	**4.83**	**1.13**	**20.74**	**0.03**
Groα	1.21	0.78	1.87	0.39	1.45	0.83	2.53	0.19
IFNα2	3.18	0.58	17.51	0.18	3.00	0.52	17.31	0.22
IL16	1.76	0.85	3.64	0.13	2.11	0.92	4.83	0.08
MCP3	1.83	0.83	4.06	0.13	1.87	0.79	4.39	0.15
MIG	**2.11**	**1.18**	**3.77**	**0.01**	**2.50**	**1.18**	**5.28**	**0.02**
Basic FGF	2.14	0.48	9.64	0.32	3.57	0.52	24.45	0.20
GCSF	1.12	0.65	1.93	0.69	1.27	0.63	2.60	0.50
GMCSF	1.03	0.19	5.54	0.97	0.77	0.11	5.35	0.80
IL7	1.59	0.73	3.43	0.24	1.77	0.71	4.40	0.22
IL9	0.93	0.46	1.90	0.85	1.00	0.45	2.23	0.10
PDGFββ	1.36	0.58	3.18	0.48	1.70	0.61	4.75	0.31
HGF	2.00	0.91	4.41	0.08	2.62	0.93	7.39	0.07
IL3	4.33	0.91	20.52	0.07	5.54	0.94	32.68	0.06
LIF	2.04	0.53	7.80	0.30	2.21	0.50	9.77	0.30
MCSF	1.37	0.57	3.31	0.48	0.98	0.35	2.73	0.97
SCF	1.25	0.78	2.01	0.35	1.28	0.71	2.29	0.41
SCGFβ	1.03	0.76	1.41	0.83	1.12	0.78	1.59	0.55
SDF1α	3.30	0.80	13.61	0.10	**7.63**	**1.17**	**49.77**	**0.03**
βNGF	1.27	0.77	2.09	0.34	0.68	2.27	0.477	0.65
VEGF	1.54	0.69	3.43	0.29	1.55	0.60	3.97	0.36
Total IgG	2.31	0.47	11.40	0.30	2.32	0.38	14.39	0.37
IgG1	1.84	0.47	7.17	0.38	1.50	0.32	7.13	0.61
IgG2	1.23	0.33	4.56	0.76	1.03	0.23	4.72	0.97
IgG3	0.73	0.28	1.89	0.52	0.62	0.21	1.83	0.38
IgG4	1.31	0.58	2.98	0.51	1.18	0.45	3.09	0.73
IgA	4.08	0.72	23.07	0.11	5.06	0.61	41.81	0.13
IgM	1.36	0.58	3.18	0.48	1.28	0.49	3.35	0.61
p66	1.07	0.63	1.82	0.80	1.00	0.56	1.79	0.99
p24	0.67	0.32	1.42	0.30	0.50	0.20	1.23	0.13
gp41	0.96	0.33	2.83	0.94	0.85	0.22	3.30	0.82
gp120	0.92	0.40	2.12	0.84	0.94	0.35	2.51	0.90

Abbreviations: IQR, interquartile range; OR, odds ratio; AOR, adjusted odds ratio; CI, confidence interval; IL, interleukin; TNF, tumor necrosis factor; MIF, macrophage migration inhibitory factor; TRAIL, TNF-related apoptosis-inducing ligand; IFNγ, interferon gamma; IP10, interferon-γ inducible protein 10; MCP1, monocyte chemoattractant protein 1; MIP1, macrophage inflammatory protein 1; RANTES, regulated on activation, normal T expressed and secreted; CTACK, cutaneous T cell-attracting chemokine; Groα, growth-regulated oncogene alpha; MCP3, monocyte chemoattractant protein 3; MIG, monokine induced by gamma; Basic FGF, Basic fibroblast growth factor; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte macrophage colony-stimulating factor; PDGFββ, platelet-derived growth factor beta; HGF, hepatocyte growth factor; LIF, Leukemia inhibitory factor; MCSF, macrophage colony-stimulating factor; SCF, stem cell factor; SCGFβ, stem cell growth factor beta; SDF1α, stromal cell-derived factor 1 alpha; βNGF, nerve growth factor beta; VEGF, vascular endothelial growth factor; gp, glycoprotein. $p<0.05$ were considered statistically significant. The model was adjusted for age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use.

5.8 The relationship between cytokines, antibody isotypes, IgG subclasses and HIV-specific antibodies in the genital tract

5.8.1 Prior to HIV infection, mucosal inflammatory cytokines directly associate with antibody isotypes, IgG subclasses and HIV-specific activities

To determine whether genital inflammation alters or skews the antibody isotypes, and IgG subclasses, a linear mixed model analyses was performed. The univariate mixed model was adjusted for HIV infection status, and the multivariate mixed model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, and HSV-2.

Overall, the antibody isotypes and IgG subclasses (IgG1-IgG4) (Figure 21-Figure 24) were significantly associated with the majority of the cytokines (pro-inflammatory cytokines, chemokines, growth factors, adaptive cytokines and anti-inflammatory cytokines) at the pre-HIV infection time point in the univariate analyses. After adjusting for the variables (HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, and HSV-2), the antibody isotypes and IgG subclasses (IgG1-IgG4) remained significantly associated with most of the cytokines (pro-inflammatory cytokines, chemokines, growth factors, adaptive cytokines and anti-inflammatory cytokines) at the pre-HIV infection time point.

Most notably, of the 9 pro-inflammatory cytokines and chemokines that was used in the definition of genital inflammation, at least 6 of these cytokines were significantly associated with the IgG subclasses (IgG1-IgG4) and antibody isotypes in the multivariate analyses (Figure 21- Figure 24). Total IgG was positively associated with IL8, IP10, MIP1β, MIP1α, IL1β, IL1α, IL6 and TNFα in the multivariate analysis, for example, for every 1 pg/ml increase in IL8, there was a 0.09 ng/ml ($p=0.03$) increase in total IgG (Figure 21B). IgG1 was positively associated with IL8, IP10, MCP1, MIP1β, MIP1α, IL1β, IL6 and TNFα in the multivariate analyses (Figure 21D). IgG2 was positively associated with IP10, MIP1β, MIP1α, IL1β, IL1α, IL6 and TNFα in the multivariate analyses (Figure 22B). IgG3 was positively associated with IP10, MIP1β, MIP1α, IL1β, IL6 and TNFα in the multivariate analyses (Figure 22D). IgG4 was positively associated with IL8, IP10, MIP1β, MIP1α, IL1β, IL1α, IL6 and TNFα in the multivariate analyses (Figure 23B). IgA was positively associated with MCP1, MIP1β, MIP1α, IL1β, IL6 and TNFα in the multivariate analyses (Figure 23D). IgM was positively associated with IL8, IP10, MIP1β, MIP1α, IL1β, IL6 and TNFα in the multivariate analyses (Figure 24B).

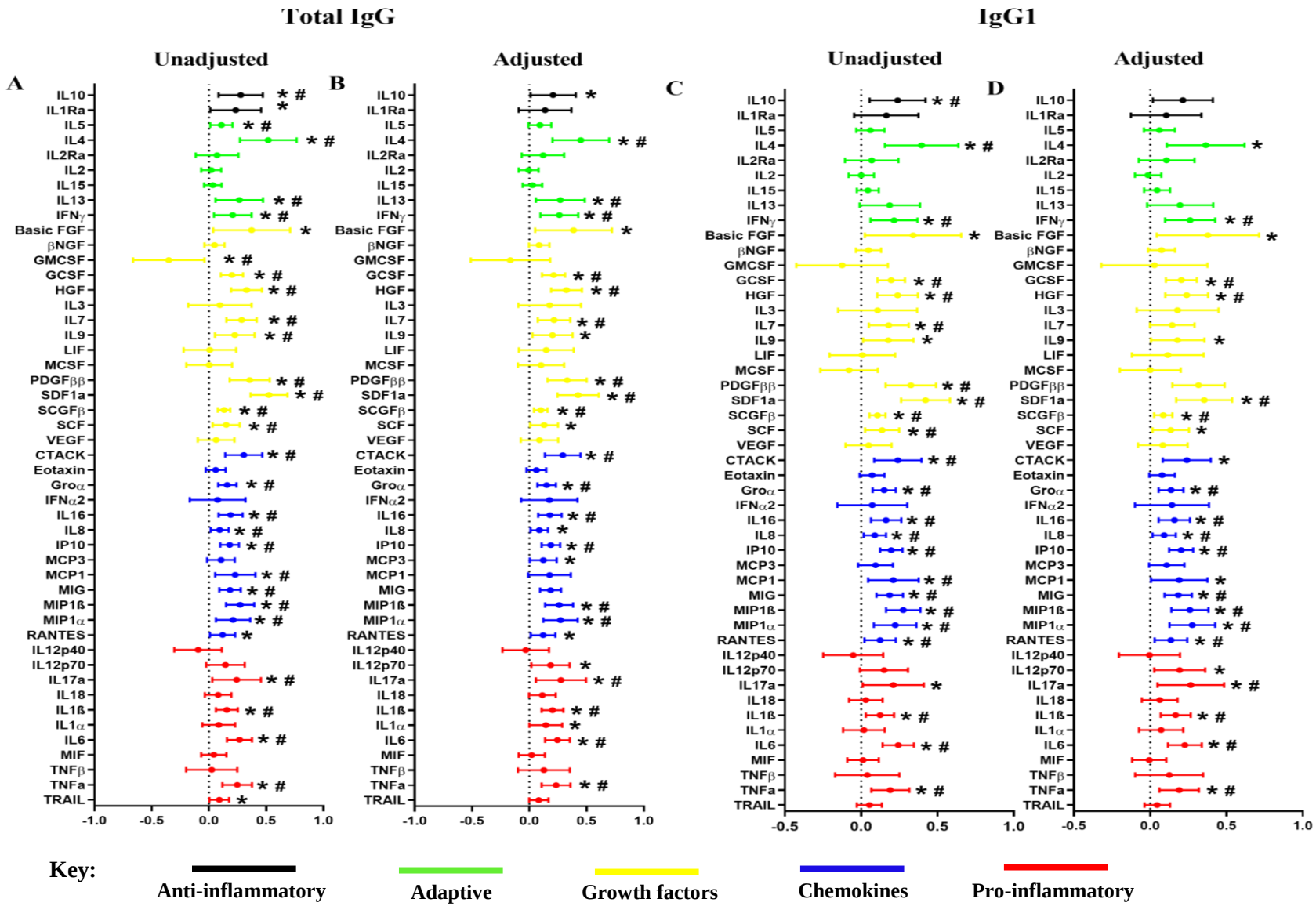


Figure 21: Association between genital cytokines and Total IgG (A & B) and IgG1 (C & D). β-coefficients, p-values and corresponding false discovery values were determined using linear regression models. The model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use. β-coefficients are indicated by shaded circles and error bars indicate 95% confidence intervals. P-values <0.05 are represented by *, and those p-values that are significant after false discovery rate adjustment are represented by #. (Refer to the raw data attached at the end of the thesis).

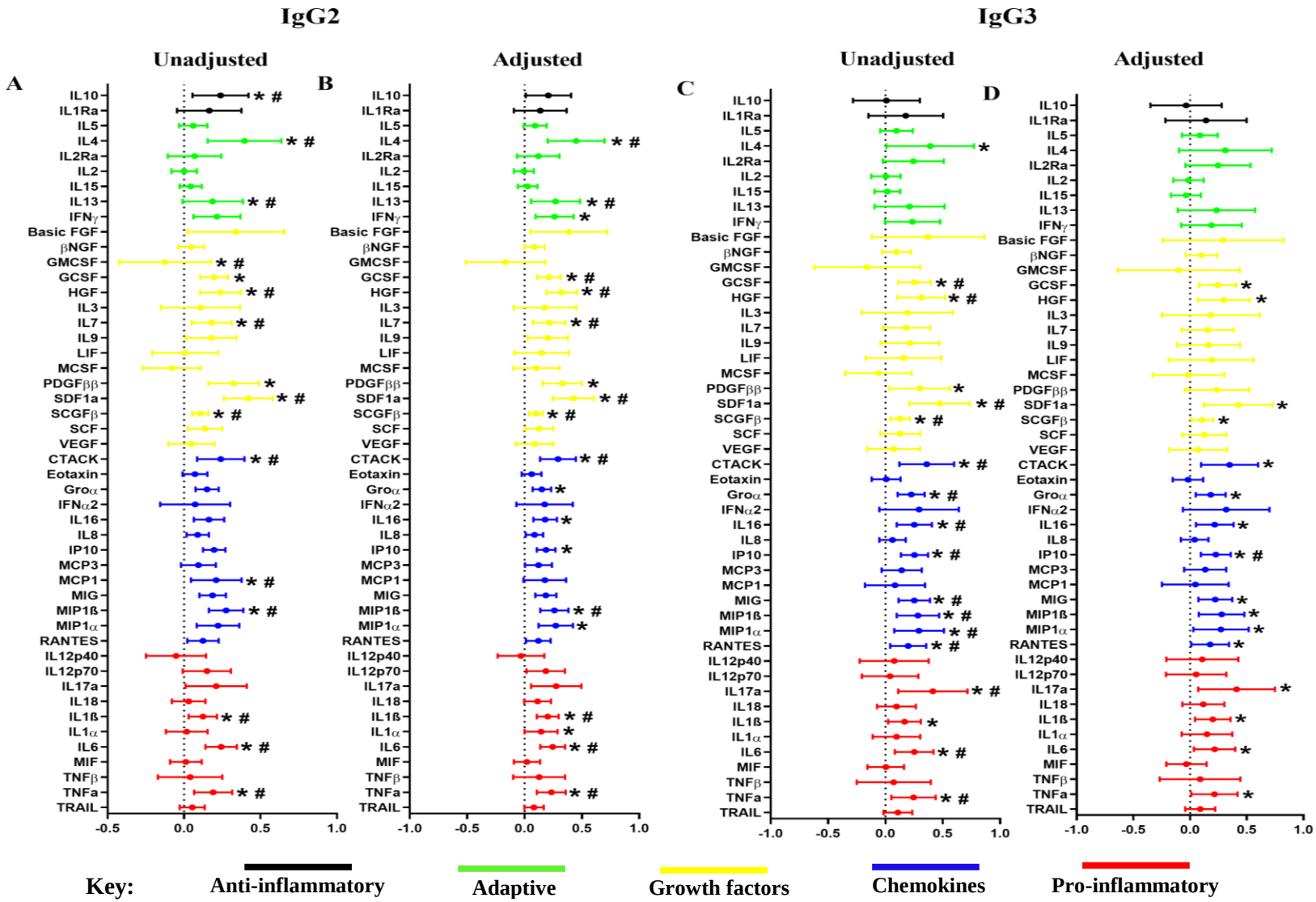


Figure 22: Association between genital cytokines and IgG2 (A & B) and IgG3 (C & D). β-coefficients, p-values and corresponding false discovery values were determined using linear regression models. The model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use. β-coefficients are indicated by shaded circles and error bars indicate 95% confidence intervals. P-values <0.05 are represented by *, and those p-values that are significant after false discovery rate adjustment are represented by #. (Refer to the raw data attached at the end of the thesis).

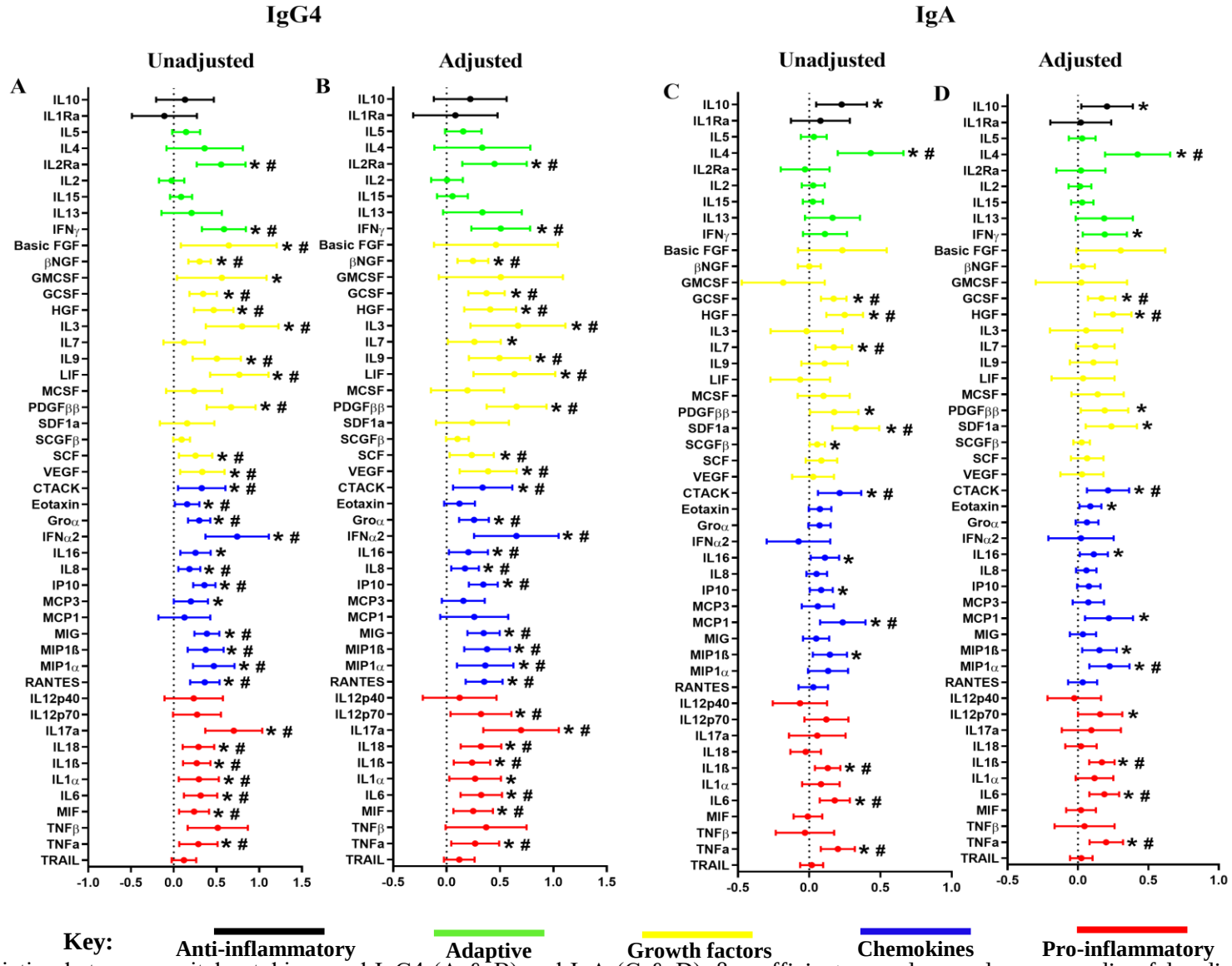


Figure 23: Association between genital cytokines and IgG4 (A & B) and IgA (C & D). β-coefficients, p-values and corresponding false discovery values were determined using linear regression models. The model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use. β-coefficients are indicated by shaded circles and error bars indicate 95% confidence intervals. P-values <0.05 are represented by *, and those p-values that are significant after false discovery rate adjustment are represented by #. (Refer to the raw data attached at the end of the thesis).

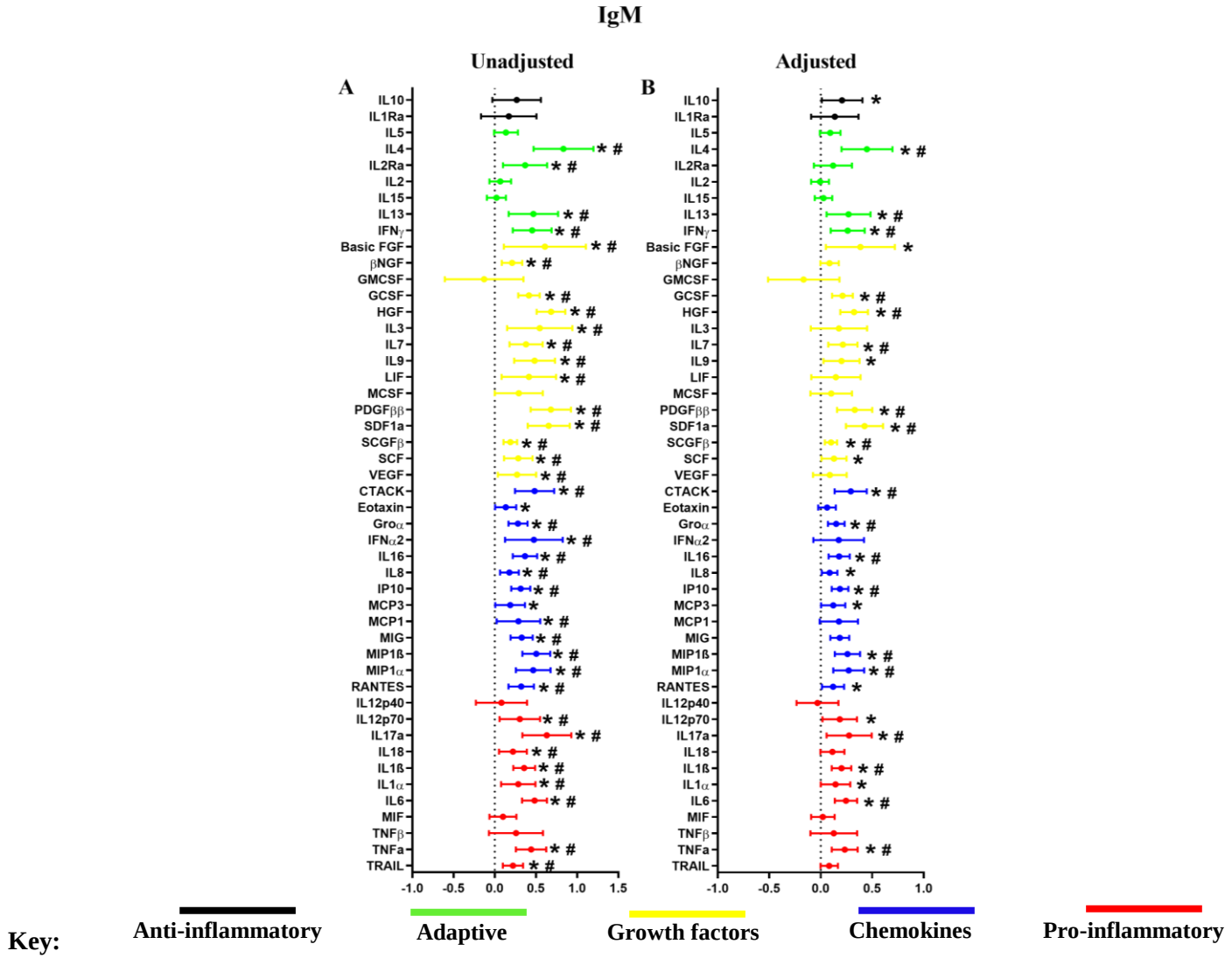


Figure 24: Association between genital cytokines and IgM (A & B). β-coefficients, p-values and corresponding false discovery values were determined using linear regression models. The model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use. β-coefficients are indicated by shaded circles and error bars indicate 95% confidence intervals. P-values <0.05 are represented by *, and those p-values that are significant after false discovery rate adjustment are represented by #. (Refer to the raw data attached at the end of the thesis).

5.8.2 Mucosal IP10 and IL18 significantly associated with increased p66- and p24-specific activities in the genital tract

To determine whether genital inflammation alters the levels of HIV-specific antibodies, a linear mixed model analysis was performed. The univariate mixed model was adjusted for HIV infection status, and the multivariate mixed model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, and HSV-2.

After adjusting for the variables, p66 specific IgG activity was significantly associated with IL2Rα, βNGF, GCSF, IL3, PDGFββ, SCGFβ, IP10, MIP1β, RANTES, IL18 and TNFβ (Figure 25B). For example, every 1 pg/ml increase in IL2Rα, there was a 0.45 MFI*dilution factor/ngml^{-1} (p=0.02) increase in p66 IgG specific activity (Figure 25B). Similarly, p24 specific IgG activity was significantly associated with IL1Rα, GCSF, PDGFββ, SCGFβ, VEGF, IL16, IP10, MIG and IL18 in the multivariate analysis (Figure 25D). In addition, the HIV-specific activities for gp41 and gp120 were positively associated with LIF (Figure 26B) and IL17α (Figure 26D) respectively.

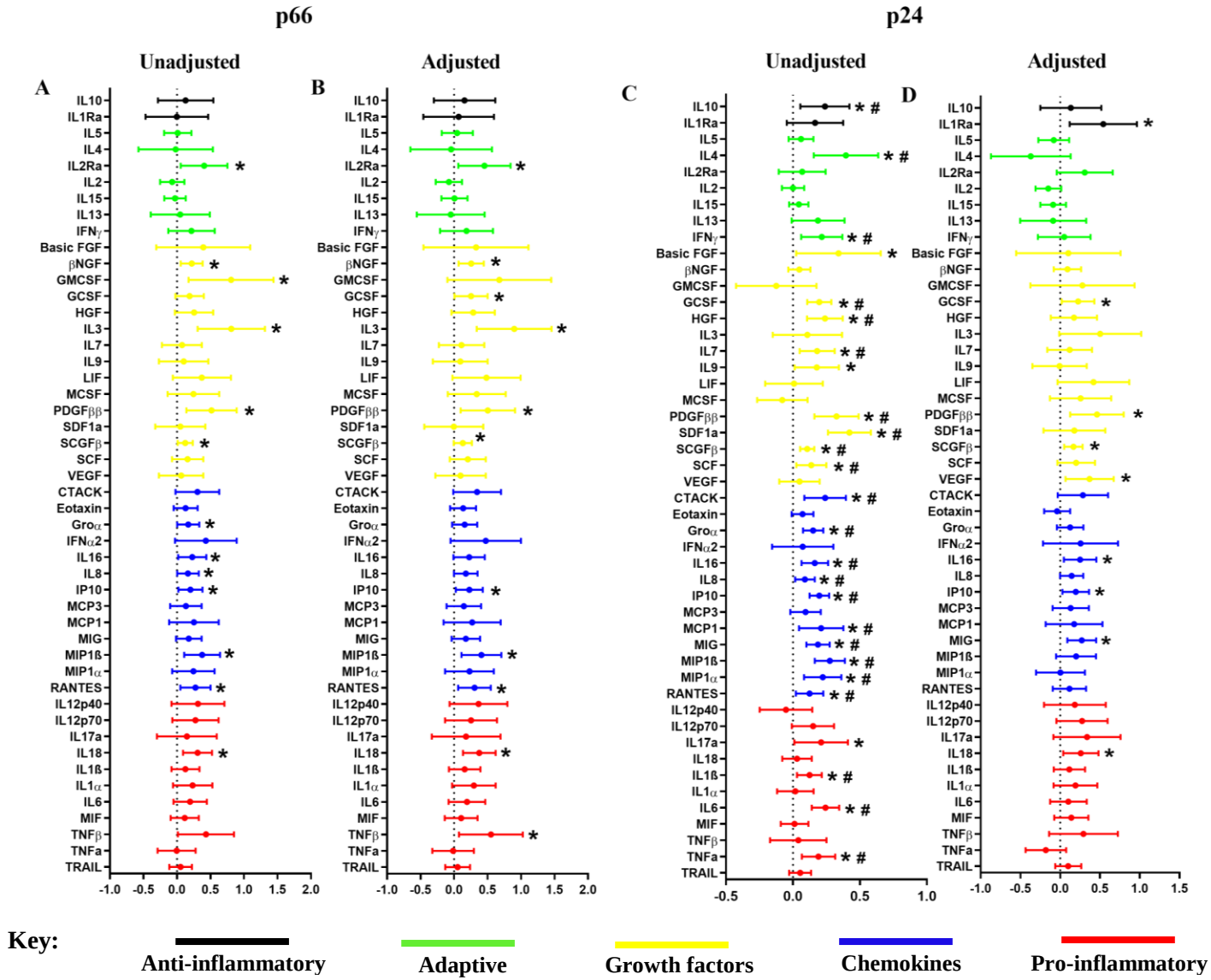


Figure 25: Association between genital cytokines and p66 (A & B) and p24 (C & D). β-coefficients, p-values and corresponding false discovery values were determined using linear regression models. The model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use. β-coefficients are indicated by shaded circles and error bars indicate 95% confidence intervals. P-values <0.05 are represented by *, and those p-values that are significant after false discovery rate adjustment are represented by #. (Refer to the raw data attached at the end of the thesis).

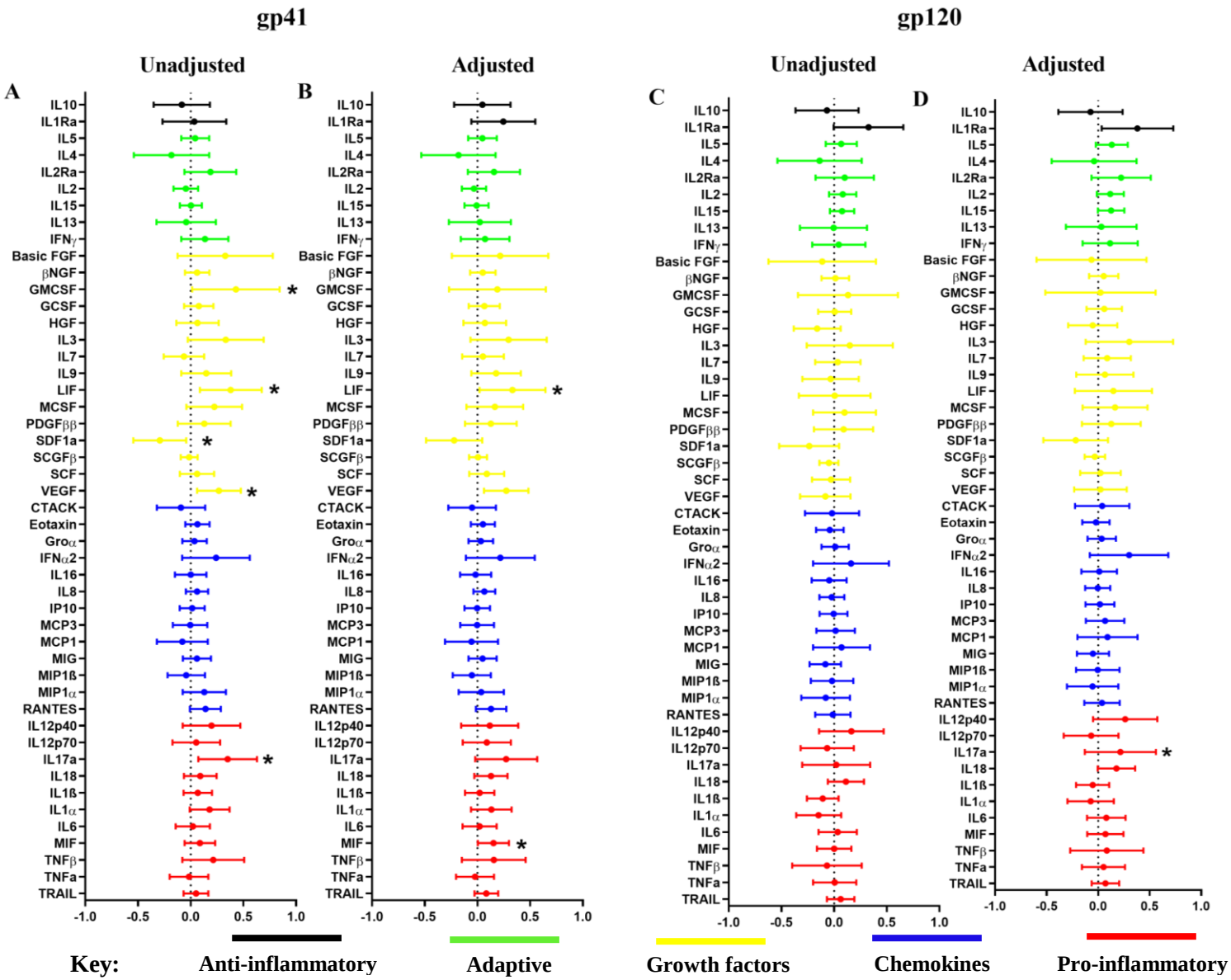


Figure 26: Association between genital cytokines and gp41 (A & B) and gp120 (C & D). β-coefficients, p-values and corresponding false discovery values were determined using linear regression models. The model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use. β-coefficients are indicated by shaded circles and error bars indicate 95% confidence intervals. P-values <0.05 are represented by *, and those p-values that are significant after false discovery rate adjustment are represented by #. (Refer to the raw data attached at the end of the thesis).

5.9 Systemic IP10 was consistently significantly higher over time in HIV-infected compared to HIV-uninfected women

In order to determine the cytokine profiles longitudinally in the systemic compartment of women, 28 cytokines were measured at baseline, at 3 months and at 6 months in HIV^+ and HIV^- women (Supplementary Table 2). Of the 28 cytokines measured at baseline, HIV^+ women had lower median concentrations in their plasma to majority of the cytokines compared to HIV^- women, while at 3 and at 6 months, plasma cytokines were higher. At baseline, IP10 was significantly lower (p=0.005) in the plasma of HIV^+ (n=48) compared to HIV^- (n=29) women (Figure 27A). At 3 months, IP10 (p=0.001) and GMCSF (p=0.05) was significantly higher in HIV^+ (n=43) (Figure 27B and Figure 27C) compared to HIV^- (n=46) women. At 6 months, IP10, MCP1, IL2 and IL7 were significantly higher ($p<0.05$) in HIV^+ (n=47) compared to HIV^- (n=22) women (Figure 27D- Figure 27G).

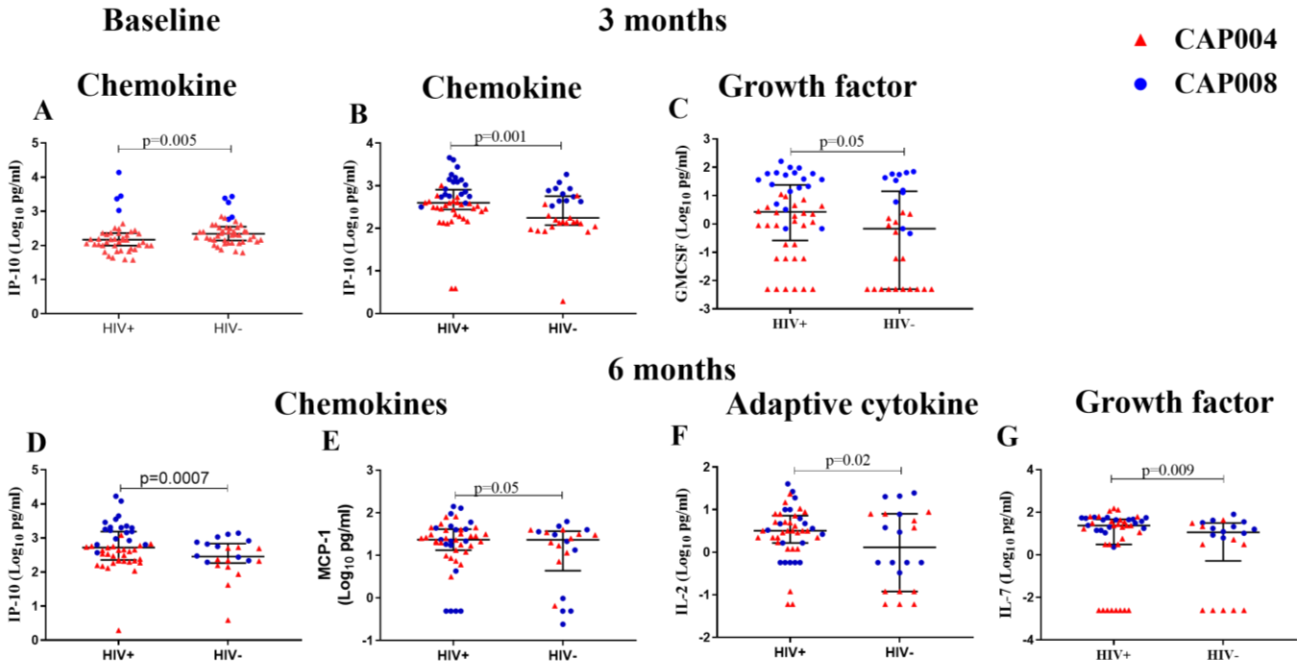


Figure 27: Longitudinal analysis of systemic cytokine profiles between cases and controls in the CAP004 and CAP008 trials. Paired T tests were used to compare between groups and $p<0.05$ were considered statistically significant. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. At baseline, HIV^+GI^- represents women who subsequently became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

5.10 Baseline systemic cytokines IP10 and TNFα predict HIV acquisition risk

In order to determine the predictors of HIV acquisition risk, various cytokine profiles and HIV-specific antibodies in the plasma were investigated (Table 5). Conditional regression analyses were performed at a univariate and multivariate level. In the univariate analysis, the risk of HIV acquisition increased with higher levels of IP10 [OR, 5.52; (95% CI, 1.35-22.51); p=0.02]. After adjusting for age, sexual debut, frequency of condom use, number of vaginal sex acts, tenofovir use and HSV-2, higher levels of IP10 [OR, 6.22; (95% CI, 1.44-26.78); p=0.02], and TNFα [OR, 10.66; (95% CI, 1.14-99.43); p=0.04] were associated with increased HIV acquisition risk (Table 3.5). Apart from these pro-inflammatory cytokines and chemokines being associated with increased HIV risk, none of the HIV-specific antibodies were associated with HIV acquisition risk before and after adjusting for the multiple variables.

Table 3.5: Systemic cytokine signatures and HIV-specific antibody profiles associated with HIV acquisition risk

	Univariable Analysis				Multivariable Analysis			
Analyte	**Odds Ratio (OR)**	**95% CI Lower**	**95% CI Upper**	**P value**	**Adjusted Odds Ratio (AOR)**	**95% CI Lower**	**95% CI Upper**	**P value**
IL1β	0.40	0.14	1.13	0.08	0.47	0.16	1.38	0.17
IL6	1.37	0.54	3.49	0.51	1.35	0.43	4.25	0.61
TNFα	2.95	0.58	14.90	0.19	**10.66**	**1.14**	**99.43**	**0.04**
IL1α	0.78	0.33	1.87	0.58	0.73	0.26	2.03	0.54
IL10	1.19	0.67	2.10	0.56	1.30	0.69	2.44	0.42
IL8	0.46	0.14	1.56	0.21	0.51	0.13	1.93	0.32
IP10	**5.52**	**1.35**	**22.51**	**0.02**	**6.22**	**1.44**	**26.78**	**0.01**
MCP1	0.61	0.27	1.36	0.23	0.49	0.19	1.29	0.15
MIP1α	1.03	0.55	1.94	0.92	1.53	0.67	3.50	0.32
MIP1β	0.97	0.32	2.92	0.95	0.97	0.29	3.31	0.97
IL7	1.35	0.31	5.86	0.69	1.96	0.35	10.82	0.44
p66	0.97	0.55	1.69	0.91	0.99	0.51	1.93	0.98
p24	1.07	0.53	2.17	0.84	0.94	0.42	2.14	0.89
gp41	0.56	0.29	1.07	0.08	0.51	0.26	1.03	0.06
gp120	0.65	0.32	1.32	0.24	0.49	0.20	1.25	0.14

Abbreviations: IQR, interquartile range; OR, odds ratio; AOR, adjusted odds ratio; CI, confidence interval; IL, interleukin; TNF, tumor necrosis factor; IP10, interferon-γ inducible protein 10; MCP1, monocyte chemoattractant protein 1; MIP1, macrophage inflammatory protein 1; gp, glycoprotein. $p<0.05$ were considered statistically significant. The model was adjusted for age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use.

5.11 IL8 and MCP1 are negatively associated with gp41- and gp120-specific responses in the systemic compartment

To determine whether genital inflammation alters the levels of HIV-specific antibodies, a linear mixed model analyses was performed. The univariate mixed model was adjusted for HIV infection status, and the multivariate mixed model was adjusted for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, and HSV-2.

In the univariate analyses, IL8 was significantly and positively associated with a p66-specific IgG response, for every 1 pg/ml increase in IL8, there was a 0.46 MFI ($p=0.02$) increase in p66 (Table 3.6). Additionally, IL6, IP10 and IL7 was significantly and positively associated with p24-specific IgG responses ($p<0.05$) in the systemic compartment (Table 3.6). However, there were significant but negative associations for IL8, TNFα and MCP1 with gp120-specific IgG responses ($p<0.05$) in the systemic compartment (Table 3.6).

After adjusting for the variables, IL8 was significantly but negatively associated with gp41- and gp120 specific IgG responses , for every 1 pg/ml increase in IL8, there was a 0.64 MFI ($p=0.002$) decrease in gp41, and 0.47 MFI*dilution ($p=0.01$) decrease in gp120- specific IgG responses in the systemic compartment (Table 3.7). Another chemokine, MCP1 was significantly but negatively associated with gp120 specific IgG responses in the systemic compartment (Table 3.7).

Additionally, after adjusting for HIV-infection status, age, sexual debut, the number of vaginal sex acts, tenofovir use, and HSV-2, IP10 was significantly and positively associated with a p66-specific IgG response , for every 1 pg/ml increase in IP10, there was a 0.25 MFI ($p=0.05$) increase in a p66-specific IgG response in the systemic compartment (Table 3.7).

Table 3.6: Univariable analysis between genital cytokines and HIV-specific antibodies (p66, p24, gp41 and gp120)

	p66			p24			gp41			gp120		
Cytokines	**β coefficient**	**95% CI**	**p value**	**β coefficient**	**95% CI**	**p value**	**β coefficient**	**95% CI**	**p value**	**β coefficient**	**95% CI**	**p value**
IL1β	0.22	-0.11-0.55	0.19	0.24	-0.04-0.52	0.09	-0.10	-0.40-0.20	0.50	-0.11	-0.36-0.14	0.39
IL6	-0.02	-0.31-0.27	0.91	**0.28**	**0.05-0.51**	**0.02**	-0.09	-0.35-0.17	0.50	-0.13	-0.35-0.08	0.23
TNFα	0.11	-0.31-0.52	0.61	0.21	-0.13-0.56	0.22	-0.26	-0.63-0.11	0.17	**-0.31**	**-0.62 to -0.008**	**0.05**
IL1α	-0.06	-0.40-0.28	0.72	0.04	-0.23-0.32	0.75	-0.16	-0.45-0.15	0.32	-0.18	-0.44-0.09	0.19
IL10	0.08	-0.14-0.29	0.49	0.07	-0.11-0.25	0.46	-0.13	-0.33-0.06	0.18	-0.06	-0.22-0.11	0.50
IL8	**0.46**	**0.06-0.85**	**0.02**	0.05	-0.29-0.39	0.77	**-0.37**	**-0.72 to -0.008**	**0.05**	**-0.39**	**-0.68 to -0.09**	**0.01**
IP10	0.16	-0.02-0.35	0.08	**0.19**	**0.04-0.34**	**0.02**	0.03	-0.13-0.20	0.70	-0.10	-0.24-0.04	0.16
MCP1	0.05	-0.19-0.30	0.67	0.01	-0.20-0.21	0.96	-0.09	-0.31-0.14	0.45	**-0.20**	**-0.38 to -0.02**	**0.03**
MIP1α	0.09	-0.14-0.33	0.44	-0.10	-0.30-0.10	0.31	-0.05	-0.26-0.16	0.66	-0.13	-0.30-0.05	0.16
IL7	0.09	-0.27-0.44	0.63	**0.29**	**0.001-0.58**	**0.05**	-0.06	-0.38-0.26	0.70	-0.22	-0.49-0.04	0.09

Abbreviations: CI, confidence interval; IL, interleukin; TNF, tumor necrosis factor; IP10, interferon-γ inducible protein 10; MCP1, monocytee chemoattractant protein 1; MIP1, macrophage inflammatory protein 1; gp, glycoprotein. p<0.05 were considered statistically significant.

Table 3.7: Multivariable analysis between genital cytokines and HIV-specific antibodies (p66, p24, gp41 and gp120)

	p66			p24			gp41			gp120		
Cytokines	**β coefficient**	**95% CI**	**p value**	**β coefficient**	**95% CI**	**p value**	**β coefficient**	**95% CI**	**p value**	**β coefficient**	**95% CI**	**p value**
IL1β	0.21	-0.16-0.58	0.27	0.15	-0.14-0.43	0.30	-0.15	-0.47-0.18	0.37	-0.09	-0.36-0.19	0.54
IL6	-0.06	-0.40-0.28	0.73	0.18	-0.08-0.43	0.17	-0.16	-0.45-0.13	0.27	-0.08	-0.33-0.17	0.53
TNFα	-0.01	-0.57-0.56	0.98	0.05	-0.38-0.47	0.82	-0.40	-0.88-0.07	0.10	-0.27	-0.68-0.14	0.20
IL1α	-0.05	-0.40-0.31	0.79	-0.07	-0.35-0.21	0.61	-0.20	-0.53-0.12	0.22	-0.19	-0.48-0.10	0.20
IL10	0.04	-0.21-0.28	0.78	0.004	-0.18-0.19	0.97	-0.15	-0.35-0.06	0.16	-0.02	-0.20-0.15	0.79
IL8	0.37	-0.11-0.84	0.13	-0.22	-0.58-0.15	0.24	**-0.64**	**-1.03 to-0.25**	**0.002**	**-0.47**	**-0.82 to -0.13**	**0.01**
IP10	**0.25**	**0.005-0.50**	**0.05**	0.15	-0.04-0.34	0.12	-0.03	-0.25-0.19	0.80	-0.10	-0.28-0.09	0.30
MCP1	0.08	-0.20-0.36	0.56	0.05	-0.16-0.26	0.64	-0.15	-0.39-0.09	0.21	**-0.24**	**-0.44 to -0.04**	**0.02**
MIP1α	0.05	-0.22-0.32	0.70	-0.19	-0.39-0.01	0.06	-0.19	-0.42-0.04	0.11	-0.19	-0.38-0.01	0.06
IL7	-0.003	-0.45-0.44	0.99	0.16	-0.17-0.50	0.33	-0.28	-0.66-0.10	0.14	-0.25	-0.57-0.08	0.14

Abbreviations: CI, confidence interval; IL, interleukin; TNF, tumor necrosis factor; IP10, interferon-γ inducible protein 10; MCP1, monocyte chemoattractant protein 1; MIP1, macrophage inflammatory protein 1; gp, glycoprotein. p<0.05 were considered statistically significant. The model was adjusted for age, sexual debut, the number of vaginal sex acts, tenofovir use, HSV-2 and frequency of condom use.

6. DISCUSSION

The interplay of the factors that contribute to genital tract inflammation (STIs, BV, epithelial barrier damage) and increased HIV acquisition risk have been studied (Masson et al., 2015, Masson et al., 2014, Abbai et al., 2016, Mirmonsef et al., 2012). The characterization of antibody isotypes, IgG subclasses, as well as HIV-specific antibody profiles in the female genital tract (Archary et al., 2016) have also been studied. However, the underlying effect that genital inflammation may have on the antibody isotypes, IgG subclasses and HIV-specific antibody profiles in the female genital tract has not been investigated. In this study, we tested the hypothesis that genital inflammation altered or skewed the mucosal antibody isotypes and subclasses, as well as the HIV-specific antibody profiles in the female genital tract. In this study we found that genital inflammation did in fact skew the antibody isotypes, IgG subclasses and HIV-specific antibody profiles in the female genital tract.

The cytokine profile at baseline showed that 18 cytokines were increased in women who subsequently became HIV-infected compared to women that remained HIV-uninfected. Of these 18 cytokines, 5 pro-inflammatory cytokines and chemokines (IL1β, TNFα, IL1α, IL8 and IP10) satisfied the definition of inflammation (Masson et al., 2015). Additionally, HIV seroconversion was positively associated with a raised genital inflammatory cytokine profile (MIP1α, MIP1β, IL8 and IP10) (Masson et al., 2015), which is consistent with the IL8 and IP10 levels shown in the present study. Data from animal studies showed that following vaginal SHIV exposure, chemotactic IL8 in rhesus macaques was essential to establish SHIV infection (Li et al., 2009). In addition, chemotactic IP10 was found to recruit HIV target cells such as T cells, dendritic cells, macrophages and monocytes and was associated with HIV risk (Stanford and Issekutz, 2003, Liebenberg et al., 2017). These data further affirmed the findings that genital cytokine levels were raised prior to HIV infection, and the occurrence of higher genital concentrations of HIV target cell-recruiting chemokines and a genital pro-inflammatory milieu contribute to HIV acquisition risk in these women (Masson et al., 2015). These data are further verified in a study by Liebenberg et al (2017) showing that raised genital tract IP10, along with other cytokines like MIP1β, IL8 and MCP1 that were significantly associated with increased HIV risk in women. IP10, MIP1β, IL8 and MCP1 all showed a mucosa-biased gradient that strongly predicted HIV infection risk in women (Liebenberg et al., 2017).

At the pre-HIV infection stage (in women who subsequently became infected), the total IgG, IgG1 and IgM antibody titres were significantly higher compared to women who remained HIV-uninfected for the duration of the study. These data indicate that at the pre-HIV infection stage, prior sexual exposures to HIV [as reviewed in (Marfatia et al., 2017, Jain and Mayer, 2014)] may have elicited both the increased IgG1 and IgM titres. Whether these are HIV-specific IgG or IgM antibodies remains an important question.

Inflammation also impacted on IgM antibody titres, where increases were observed in women who were inflamed compared to women who were not inflamed and subsequently became HIV-infected. Naturally occurring IgM present in the umbilical cord blood at birth, is the first antibody to respond to an infection and known to increase during an inflammatory response (Lobo et al., 2012). Therefore these findings underscore the need to investigate increased IgM in the genital tract prior to HIV infection and the possibility that this immunoglobulin may be a marker of risk or protection against HIV in women with genital tract inflammation. Lobo et al (2012) showed that increased IgM titres increase with Th17 cell responses, and inhibits HIV infectivity (Lobo et al., 2008b). IgM dampens inflammation and inhibits leucocyte production of certain pro-inflammatory cytokines, such as TNFα and IL2 (Lobo et al., 2008a). In addition, human serum derived-IgM was shown to inhibit T cell activation and viral entry by binding to CD3 and CD4, and directly blocking the co-receptors CXCR4 and CCR5 from binding to chemokines which in turn inhibited chemotaxis (Lobo et al., 2008a, Lobo et al., 2008b) which in turn reduces targets for HIV infection. Recently Env-specific IgM was shown to protect against mucosal SHIV infections in the macaque model (Gong et al., 2018), underscoring a potential role of Env-specific IgM in humans. However a limitation of this study was that Env-specific IgM was not measured, and whether this antibody can efficiently reduce inflammation and block or inhibit the chemokine receptors on target cells or directly capture and inhibit virions in the mucosal compartment remains unknown.

In the absence or presence of genital inflammation, in the HIV-uninfected woman without genital inflammation the IgG1, IgG4 and IgA titres remained unchanged over time. In contrast, in HIV-infected women without genital inflammation, there were decreased IgG1, IgG2, IgG4, IgA and IgM titres from pre-HIV infection to six months post infection. Whereas IgG3 titres did not differ over time in the HIV-infected women without genital inflammation, our data showed that the IgG3 titres did indeed significantly decrease over time in HIV-uninfected women also without inflammation. These data may indicate that HIV infection alone drives the heterogeneous antibody responses in the genital compartment where some of the antibody isotypes and IgG subclasses may be up- or down-regulated (Haimovici et al., 1997). IgG3 in the serum has been shown to be a marker of recent HIV-infection (Ljunggren et al., 1988, Viana et al., 2018, Wilson et al., 2004, McGowan et al., 2005), however, it may also play a role in the genital compartment to elicit or effect neutralizing and non-neutralizing antibody functionalities (Yates et al., 2014, Chung et al., 2014). The VAX003 trial elicited higher gp120-specific IgG1 and gp120-specific IgG3 antibody titres and higher Fc effector profiles compared to RV144 (Chung et al., 2014). However, despite these findings, VAX003 did not confer protection compared to the RV144 albeit moderate protection levels (31.2% efficacy) (Haynes et al., 2012). These data suggest that neither the quantity of antibody nor the function alone could explain the reduced HIV infections in the RV144 vaccine study compared to the VAX003 (Chung et al.,

2014). Essentially, it is the quality and not the quantity of the antibodies that matter. Among the IgG subclasses, IgG1 and IgG3 have higher affinity towards the FcRn which facilitates their transcytosis across the epithelial cells (Gupta et al., 2013). In comparison to IgG2 and IgG4 subclasses, IgG1 and IgG3 also demonstrate higher affinity for FcγRIIIa on NK cells, which leads to NK cell activation and subsequent ADCC (Bruhns et al., 2009, Smalls-Mantey et al., 2012, Lux et al., 2013). Increased phagocytic activities were found in HIV untreated chronic progressors and HIV controllers due to the differential interaction of the Fc part of the antibody with the FcγRIIb and FcγRIIa receptors expressed by phagocytic cells (Ackerman et al., 2013b). This differential interaction may also be due to the Fc glycosylation of the antibodies (Ackerman et al., 2013b). However, in HIV elite controllers, despite having lower IgG1 and IgG3 titres, potent polyfunctional antibody-mediated responses were found compared to viraemic individuals, further supporting the protective role antibodies may exert in controlling HIV infection (Lai et al., 2014, Ackerman et al., 2016). Therefore, additional studies are required to determine whether these subclass profiles can be fine-tuned to exploit polyfunctional IgG responses at the vulnerable mucosal site, the female genital tract.

In HESN women participating in the HPTN035 microbicide trial, IgA was found to be the predominant isotype in vaginal secretions (Seaton et al., 2014). The gp120-specific IgA responses exerted a protective role in these HESN women (Seaton et al., 2014), which may indicate that the presence of mucosal IgA may be a proxy of protection against HIV exposure in individuals enrolled in HIV prevention trials (Mackelprang et al., 2012). Mucosal Env-specific antibody responses mainly directed towards gp41 (IgA, IgG and IgM) were shown to develop early in HIV infection [as reviewed in (Gallerano et al., 2015, Yates et al., 2013)]. Haimovici et al., (1997) showed that mucosal p24-specific IgA was elevated in women with genital inflammation. Furthermore, elevated titres of anti-HIV IgG antibodies (p24-specific antibodies) were found in the genital tracts of women with a history of heterosexual HIV transmission (Haimovici et al., 1997) and increased gp160-specific IgG activity in women with concurrent STIs (Artenstein et al., 1997).

In the genital mucosa, as expected, women who subsequently became HIV-infected had significantly higher mucosal HIV-specific IgG activities for p66, p24, gp41 and gp120 at three and six months compared to HIV-uninfected women. Additionally, evolving HIV-specific activities were seen over time in the genital tract of HIV-infected women. HIV-infected women without genital inflammation also displayed significantly higher and evolving HIV-specific activities to p66, p24, gp41 and gp120 over time. In contrast, in HIV-infected women with genital inflammation, HIV-specific activities declined from three months to six months. These data suggests that genital inflammation can undermine the specific antibody titres in the

mucosal compartment despite overall increasing titres systemically during HIV infection. A major limitation to these findings is the limited sample size that precluded significant findings in HIV-infected women with genital inflammation. Apart from genital inflammation undermining the HIV-specific antibody responses prevailing in the genital tract, these data have important consequences for vaccine induced immunity at the vulnerable site of exposure which may in turn affect vaccine efficacy.

The HIV-specific responses in the plasma also displayed similar profiles as that of the genital tract, with significantly higher and evolving responses in HIV-infected than in the HIV-uninfected women. It is noteworthy that even in the women who remained HIV-uninfected, two of the women had consistent detectable plasma HIV-specific antibody responses to three HIV-specific proteins: p66, p24 and gp41 despite the assay stringent cut-offs to determine detectability. These data suggest that these women may have had repeated exposures to HIV to develop these antibodies (Schaefer et al., 2005, Mazzoli et al., 1997, Beyrer et al., 1999). We did not anticipate to see the impact of genital inflammation on the HIV-specific antibody responses in the systemic compartment, HIV-infected women with genital inflammation had similar plasma HIV-antibody specific responses for p24, p66, gp41 and gp120 at three months and six months post-infection compared to the HIV-infected women without genital inflammation. Only at baseline, a p24-specific IgG responses in the plasma were significantly higher in HIV-infected women without inflammation compared to HIV-infected women with inflammation. These data suggest that the magnitude and kinetics of HIV-specific responses in the blood were not altered and that the presence of inflammation in the genital tract is more likely to have local effects in the mucosal compartment. Unsurprisingly, irrespective of inflammation status, the plasma HIV-specific responses remained higher in the HIV-infected compared to the uninfected women who mostly had undetectable responses. When the HIV-specific responses were correlated between the compartments in the HIV-infected women, all four plasma HIV-specific antibody responses were positively associated with those in the genital tract. However, when the data was further stratified for inflammation, women without inflammation were more likely to have p66 and p24 responses significantly and positively associated between the compartments, and no such associations were seen for the inflamed women suggesting that genital inflammation can alter these compartmental correlations. Studies have shown that the there is a close concordance between the blood and genital tract for HIV-specific antibody responses (Mkhize et al., 2016) even in the presence of prior topical PrEP in the form of the 1% tenofovir microbicide gel (Archary et al., 2016). However, how genital inflammation may have impacted these cross compartmental associations was not factored into these studies.

Overall, the HIV-specific activities and responses for all four HIV-specific antibodies showed a similar pattern between women who received the topical tenofovir gel and those who used the placebo gel. These data suggests that tenofovir did not impact on the HIV-specific activities and responses in the mucosal and systemic compartments, respectively. Although, previous studies have established that ARVs modulate the avidity of HIV-specific antibodies in patients with primary HIV-infection irrespective of the class of drugs used (Selleri et al., 2007, Laeyendecker et al., 2015, Killian et al., 2006), others have demonstrated that ARVs did not affect antibody binding or maturation kinetics (Adalid-Peralta et al., 2006, Wendel et al., 2013). ARVs, however, are known to reduce immune activation and CD4 T cell loss in macaques with no delay in antibody seroconversion (Curtis et al., 2011, Kersh et al., 2012). Another study in healthy individuals on short-term PrEP of oral tenofovir-emtricitabine (Truvada™) showed that relative to the baseline, the months usage of PrEP significantly lowered the circulating T cell activation and soluble CD27 (CD27 is secreted by activated T cells) profiles (Castillo-Mancilla et al., 2015). However, the other pro-inflammatory cytokines and other soluble markers of inflammation (CD14 and hs-CRP) remained unchanged (Castillo-Mancilla et al., 2015).These data indicate that prior oral PrEP may have a differential immunomodulatory effect on certain markers of systemic immune activation and cytokines causing inflammation in healthy individuals. However, how prior oral PrEP may affect the genital tract immunity and markers of inflammation is not fully understood.

Cytokines have been shown to influence immunoglobulin class switching (Tangye et al., 2002) as well as subclass synthesis (Kawano et al., 1994). Prior to HIV infection, all four IgG subclasses and isotypes in the genital tract were positively and strongly associated with IL8, IP10, MIP1α, MIP1β, IL1β, IL6 and TNFα after adjusting for potential confounders. These data suggest that in the presence of a pro-inflammatory environment, these subclasses and isotypes do prevail. The levels of p66- and p24-specific activities were also shown to be positively and strongly associated with pro-inflammatory cytokines -IP10 and IL18, and growth factors- GCSF, PDGFββ and SCGFβ. IL8 is a chemokine which is responsible for attracting neutrophils to an inflammatory environment (Dinarello, 2000), and may bear a protective role in clearing inflammation in the presence of such antibody isotypes or IgG subclasses prior to HIV infection in the mucosal compartment of women. However, it is uncertain whether this chemotactic profile in the genital secretions of HIV-infected or HIV-uninfected women may be a correlate of risk or protection against HIV acquisition, or alternatively, it may be a reflection of the general immunity that is present in the mucosal compartment of these women. Innate IP10 is produced in response to bacteria, viruses, fungi and parasites (Liu et al., 2011). One study demonstrated that at the primary stage of HIV infection, systemic IP10 increased significantly before the development of clinical symptoms in parallel to viral loads (Stacey et al., 2009). Other studies show that IP10 in plasma was also associated with immune activation (Noel et al.,

2014) and can be used as a predictor of rapid HIV progression (Liovat et al., 2012, Jiao et al., 2012), thus representing an earlier biomarker than that of viraemia or CD4 T cell counts (Liovat et al., 2012). In addition, IP10 was shown to be a useful screening tool to identify individuals with acute HIV infection from other patients with undifferentiated fever in a cohort of HIV-seronegative individuals (Pastor et al., 2017). Macrophage inflammatory proteins have been shown to mechanistically drive cellular resistance to R5-tropic HIV viruses in some elite controllers (Walker et al., 2015).

Furthermore, increased levels of IL1β was also shown to be associated with HIV disease progression and may likely lead to CD4 T cell depletion, increased viral loads and immune dysregulation (Guo et al., 2014). A limitation of the current study is the absence of genital viral load data in HIV-infected women (this was not included as a test in the main trials). Other studies report that genital tract specimens with detectable viral loads had increased levels of IL6 and TNFα than genital tract specimens without detectable viral loads (Mukura et al., 2012), and is consistent with the findings that IL6 and TNFα increase with increased HIV expression (Zara et al., 2004, Poli and Fauci, 1993, Poli et al., 1990).

In the systemic compartment, the univariate analyses showed that plasma gp41- and gp120-specific IgG responses were negatively associated with plasma IL8 and MCP1. After adjusting for potential confounders, p66-specific IgG positively associated with IP10 in the plasma. It is also noteworthy that women who subsequently became HIV-infected, had consistently and significantly higher IP10 levels from the pre-HIV infection to six months post-infection, thus highlighting its putative role as a marker in the blood for HIV acquisition. In fact, the adjusted conditional regression analyses highlighted a more than six-fold increased risk for HIV acquisition with IP10 and TNFα. However, in the context of inflammation in the cervicovaginal mucosa, the limited numbers of samples from each group of women based on their inflammation status precluded a stratified analysis to determine which cytokines may be more strongly influencing which type of antibody in the female genital tract.

A major strength of this study is the availability and investigation of a cohort of women longitudinally from the pre-HIV infection stage to the post HIV infection stage (cases) that were recruited into large scale clinical studies testing a topical form of PrEP as an ARV containing microbicide gel. In addition, the cases and the controls (women who remained uninfected) were effectively matched reducing any selection bias among HIV-infected and HIV-uninfected women who shared similar clinical and demographical profiles. Furthermore, this study was one of the few studies that was able to show the association between inflammatory cytokines and antibody isotypes, IgG subclasses and HIV-specific binding antibodies in the genital tract of women prior to HIV infection. Another strength of the study is the matching plasma and

genital tract specimens which allowed for some of the compartmental correlative analyses. Several limitations also exist within the present study and prevents the investigators from making conclusions about some of the analyses presented. One of the central issues is that we have no functional data to verify the role of HIV-specific antibodies in protection or viral control. Cytokines are also known to degrade over time and this could have also presented a limitation as these were stored samples and may have lower cytokine concentrations than usually expected. Another caveat to the present study is the unavailability of samples which precluded analyses of some of the significant associations between antibody isotypes, IgG subclasses and HIV-specific binding antibodies in women who were HIV-infected with genital inflammation. Owing to the unavailability of samples, some of the statistical tests could not be performed. Data is also lacking regarding the CD4 counts and viral loads of these women, viral loads of sexual partner/s, HIV partner status of some of the women, and their partners' exposure to ARVs or PrEP.

7. CONCLUSIONS

In conclusion, this study provided evidence of higher IgG1 and IgM in the genital tract of women prior to HIV-infection compared to HIV-uninfected women, suggesting that prior sexual exposures to HIV may have elicited both the increased IgG1 and IgM titres. Women who subsequently became HIV-infected had significantly higher and evolving mucosal HIV-specific IgG activities compared to HIV-uninfected women. In the presence of genital inflammation, HIV-specific activities decreased from three to six months in HIV-infected women. These data suggest that although HIV may drive these HIV-specific antibody activities, genital inflammation may also undermine HIV-specific responses prevailing in the genital tract. HIV-specific responses of all four antibody specificities correlated between the systemic and genital compartments in HIV-infected women over time. Upon further stratification into inflammation status, this study showed that p66- and p24-specific responses significantly associated between the compartments in HIV-infected women without genital inflammation. In HIV-infected women with genital inflammation such significant cross compartmental associations were not found, these data may suggest that genital inflammation could impact and undermine these compartmental correlations which otherwise showed strong and significant associations in women without genital inflammation. In addition, this study provided evidence that genital pro-inflammatory cytokines were strongly associated with antibody isotypes and IgG subclasses, suggesting that even in a pro-inflammatory environment, these isotypes and IgG subclasses do prevail. Certain pro-inflammatory cytokines and growth factors were also strongly associated with p66- and p24-specific activities in the genital tract. This study provided evidence of the different antibody isotypes and IgG subclasses, as well as the HIV-specific binding antibody profiles in the mucosal compartment of women during natural infection. Taken together, this study showed the relationship

between genital cytokine signatures and these parameters in the genital tract of HIV-infected and HIV-uninfected women.

7.1 Future Directions

Going forward, it would be important to see if there are qualitative functional differences of antibodies in women with genital tract inflammation compared to those women without genital tract inflammation. Functional studies investigating the ADCC or ADCP may inform us of what the potential differences may be in an inflamed genital tract environment and how this may inform future vaccine studies aimed at protecting this vulnerable mucosal surface. Indeed, if there is scope to investigate the IgM isotype in the future on a larger cohort of women from the pre-HIV infection stage to the post-HIV infection stage in both the blood and genital tract as a potential additional marker of genital inflammation. If the IgM correlates significantly between the compartments then we may be able to confirm its role as a potential new biomarker that may be easier to test for in the blood rather than in the genital tract. This would require large numbers of women. These data and findings may also inform us about the quality of the antibodies and the structural and molecular characteristics of the Fc portion of the antibodies that may be different in the inflamed genital tract compared to the uninflamed genital tract. Additionally, as the genital microbiome plays a key role in also modulating inflammation, investigating the interplay between the microbiome and antibody responses in the genital tract may also provide clues as to what types of antibodies may likely be prevalent in a microbially diverse versus a non-diverse vagina. Together, these future studies may better inform our understanding of biological and immunological factors to mitigate HIV-infection risk among women.

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9. SUPPLEMENTARY

Table S1: Mucosal cytokine profiles between cases and controls in the CAP004 and CAP008 trials at baseline, 3 and 6 months

Cytokine	Baseline (case) [Median (IQR)] N=61	Baseline (control) [Median (IQR)] N=60	p-value	3 months Post-infection (case) [Median (IQR)] N=45	3 month time point (control) [Median (IQR)] N=19	p-value	6 months Post-infection (case) [Median (IQR)] N=36	6 month time point (control) [Median (IQR)] N=25	p-value
IL12p70	1.64 (1.34-1.97)	1.57 (1.24-1.81)	0.07	0.48 (-0.04-0.71)	0.76 (0.48-1.61)	0.32	0.48 (-0.05-0.71)	0.48 (-0.02-1.26)	0.22
IL17α	**1.26 (1.01-1.59)**	**1.09 (0.95-1.33)**	**0.02**	1.39 (1.23-1.62)	1.27 (1.13-1.43)	0.09	1.22 (1.10-1.34)	1.27 (1.09-1.52)	0.32
IL1β	**1.71 (1.27-2.40)**	**1.41 (0.94-1.95)**	**0.01**	1.48 (0.97-2.04)	1.50 (0.86-1.76)	0.42	0.94 (0.56-1.41)	1.09 (0.44-1.79)	0.33
IL6	0.89 (0.54-1.25)	0.77 (0.47-1.26)	0.50	0.60 (0.33-0.85)	0.62 (0.38-0.98)	0.36	0.43 (0.33-0.56)	0.50 (0.26-0.86)	0.25
TNFα	**1.15 (0.80-1.47)**	**0.88 (0.64-1.17)**	**0.01**	1.27 (1.14-1.54)	1.05 (0.77-1.27)	0.07	1.17 (0.95-1.33)	1.04 (0.87-1.26)	0.72
IL12p40	2.57 (2.44-2.71)	2.54 (2.42-2.62)	0.05	-	-	-	-	-	-
IL18	2.44 (1.95-2.82)	2.11 (1.71-2.75)	0.35	-	-	-	-	-	-
IL1α	**2.24 (1.86-2.76)**	**1.99 (1.68-2.35)**	**0.00**	1.59 (0.76-1.89)	1.47 (1.04-2.22)	0.93	1.10 (0.64-1.54)	1.31 (1.09-1.85)	0.44
MIF	3.72 (3.08-3.94)	3.61 (3.13-3.95)	0.59	-	-	-	-	-	-
TNFβ	0.67 (0.46-0.95)	0.63 (0.40-0.82)	0.10	-	-	-	-	-	-
TRAIL	1.57 (0.94-2.15)	1.34 (0.86-1.78)	0.18	-	-	-	-	-	-
IL10	1.54 (1.18-1.72)	1.42 (1.16-1.61)	0.06	1.32 (1.17-1.42)	1.36 (0.95-1.43)	0.72	1.36 (1.24-1.43)	1.22 (1.17-1.33)	0.19
IL1Rα	4.01 (3.84-4.15)	3.99 (3.77-4.24)	0.71	5.00 (4.45-5.39)	4.43 (3.62-4.9)	0.64	5.02 (4.63-5.68)	4.35 (3.53-5.01)	0.19
IFNγ	**1.58 (1.27-1.84)**	**1.34 (1.16-1.59)**	**0.01**	0.99 (0.85-1.11)	1.02 (0.89-1.42)	0.76	1.03 (0.89-1.07)	1.05 (0.99-1.23)	0.13
IL13	**0.54 (0.24-0.73)**	**0.36 (0.08-0.61)**	**0.02**	0.12 (-0.25-0.14)	0.14 (-0.08-0.42)	0.27	-0.03 (-0.10-0.14)	-0.03 (-0.03-0.16)	0.51
IL15	0.27 (-1.2-0.91)	0.47 (-1.1-0.8)	0.35	**2.70 (2.46-2.79)**	**2.21 (0.34-2.56)**	**0.02**	**2.70 (2.64-2.81)**	**2.35 (0.85-2.59)**	**0.01**
IL2	0.41 (0.05-0.67)	0.46 (0.19-0.68)	0.51	0.89 (0.51-1.07)	0.50 (0.04-0.7)	0.48	0.76 (0.52-0.9)	0.60 (0.37-0.73)	0.23
IL4	**-0.03 (-0.20-0.17)**	**-0.13 (-0.20-0.07)**	**0.02**	0.37 (0.14-0.58)	0.15 (-0.30-0.32)	0.13	0.34 (0.15-0.47)	0.13 (-0.26-0.31)	0.43
IL5	-0.13 (-1.20-0.32)	-0.42 (-1.20-0.23)	0.35	**1.49 (1.32-1.61)**	**1.26 (-0.92-1.41)**	**0.00**	1.46 (1.36-1.51)	1.37 (-1.22-1.43)	0.07
IL2Rα	**1.49 (1.29-1.79)**	**1.35 (1.23-1.52)**	**0.01**	-	-	-	-	-	-
Eotaxin	0.86 (-0.70-1.19)	0.64 (-0.40-0.95)	0.19	0.22 (-0.03-0.40)	0.16 (-0.05-0.74)	0.95	0.10 (0.00-0.22)	0.30 (-0.03-0.62)	0.23
IL8	**2.78 (2.35-3.43)**	**2.47 (2.07-2.98)**	**0.01**	3.02 (2.35-3.7)	2.87 (2.43-3.18)	>0.99	2.91 (2.16-3.15)	2.21 (2.00-3.54)	0.55
IP10	**2.62 (1.73-3.12)**	**2.16 (1.31-2.84)**	**0.01**	1.67 (1.42-2.67)	1.54 (1.32-2.04)	0.28	1.89 (1.45-2.47)	1.62 (1.35-2.81)	0.74
MCP1	1.35 (1.12-1.54)	1.28 (1.01-1.46)	0.20	1.19 (0.89-1.52)	1.05 (0.85-1.33)	0.56	0.99 (0.69-1.22)	1.08 (0.68-1.51)	0.85
MIP1α	0.31 (-0.12-0.52)	0.18 (-0.20-0.36)	0.09	0.23 (0.04-0.47)	0.21 (0-0.40)	0.64	0.08 (-0.06-0.23)	0.22 (0.08-0.38)	0.14
MIP1β	1.25 (0.66-1.73)	1.05 (0.64-1.53)	0.49	0.87 (-0.09-1.24)	0.81 (0.33-1.32)	0.27	0.40 (-0.77-0.8)	0.82 (0.26-1.24)	0.17
RANTES	1.06 (0.53-1.33)	0.72 (0.33-1.11)	0.06	1.05 (0.88-1.16)	1.00 (0.48-1.21)	0.49	1.02 (0.94-1.16)	0.97 (0.70-1.11)	0.24
CTACK	**1.40 (1.13-1.59)**	**1.21 (1.00-1.41)**	**0.02**	-	-	-	-	-	-
Groα	**3.17 (2.34-3.63)**	**2.52 (1.70-3.27)**	**0.02**	-	-	-	-	-	-
IFNα2	1.37 (1.25-1.6)	1.32 (1.18-1.42)	0.10	-	-	-	-	-	-
IL16	**1.69 (1.46-2.07)**	**1.54 (1.25-1.76)**	**0.01**	-	-	-	-	-	-
MCP3	**1.24 (0.95-1.52)**	**1.09 (0.70-1.24)**	**0.04**	-	-	-	-	-	-
MIG	**2.91 (2.38-3.52)**	**2.36 (1.91-3.00)**	**-**	-	-	-	-	-	-
Basic FGF	1.41 (1.32-1.55)	1.38 (1.29-1.45)	0.05	1.61 (1.37-1.81)	1.47 (1.15-1.68)	0.58	1.56 (1.37-1.65)	1.41 (1.19-1.57)	0.21
GCSF	2.52 (1.92-2.83)	2.36 (1.68-2.87)	0.28	2.49 (2.34-2.74)	2.32 (1.95-2.68)	0.85	2.46 (2.28-2.62)	2.35 (2.04-2.69)	0.83
GMCSF	1.91 (1.81-2.02)	1.91 (1.80--2.03)	>0.99	0.31 (0.02-0.51)	0.46 (0.30-2.01)	0.41	**0.19 (0.02-0.4)**	**0.39 (0.30-1.94)**	**0.01**

IL7	0.69 (0.21-0.87)	0.50 (0.13-0.76)	0.06	0.69 (-0.06-1.02)	0.13 (-0.13-0.78)	0.39	0.87 (-0.12-1.1)	-0.076 (-0.13-0.67)	0.12
IL9	0.75 (0.47-1.16)	0.66 (0.46-0.88)	0.07	1.26 (1.01-1.56)	1.01 (0.76-1.18)	0.17	1.25 (1.02-1.37)	0.93 (0.64-1.36)	0.28
PDGFββ	1.08 (0.79-1.3)	0.92 (0.65-1.15)	0.06	1.84 (1.44-2.08)	0.99 (0.29-1.67)	0.15	**1.84 (1.57-1.99)**	**0.91 (0.48-1.67)**	**0.01**
HGF	**2.52 (2.13-2.89)**	**2.07 (1.78-2.64)**	**0.00**	-	-	-	-	-	-
IL3	**2.17 (2.03-2.46)**	**2.09 (1.95-2.26)**	**0.04**	-	-	-	-	-	-
LIF	1.27 (1.14-1.57)	1.23 (1.05-1.43)	0.09	-	-	-	-	-	-
MCSF	1.93 (1.70-2.22)	1.91 (1.55-2.13)	0.22	-	-	-	-	-	-
SCF	1.04 (0.72-1.51)	0.91 (0.55-1.27)	0.07	-	-	-	-	-	-
SCGFβ	2.51 (2.06-3.04)	2.38 (0.70-2.84)	0.06	-	-	-	-	-	-
SDF1α	**2.17 (2.01-2.32)**	**2.00 (1.84-2.27)**	**0.01**	-	-	-	-	-	-
Bngf	0.21 (-0.4-0.79)	-0.10 (-0.4-0.3)	0.13	-	-	-	-	-	-
VEGF	2.52 (2.16-2.94)	2.37 (2.02-2.64)	0.08	**2.92 (2.83-3.04)**	**2.78 (2.47-2.91)**	**0.05**	**2.88 (2.84-2.97)**	**2.79 (2.03-2.93)**	**0.04**

Abbreviations: IQR, interquartile range; IL, interleukin; TNF, tumor necrosis factor; MIF, macrophage migration inhibitory factor; TRAIL, TNF-related apoptosis-inducing ligand; IFNγ, interferon gamma; IP10, interferon-γ inducible protein 10; MCP1, moncoyte chemoattractant protein 1; MIP1, macrophage inflammatory protein 1; RANTES, regulated on activation, normal T expressed and secreted; CTACK, cutaneous T cell-attracting chemokine; Groα, growth-regulated oncogene alpha; MCP3, moncoyte chemoattractant protein 3; MIG, monokine induced by gamma; Basic FGF, Basic fibroblast growth factor; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte macrophage colony-stimulating factor; PDGFββ, platelet-derived growth factor beta; HGF, hepatocyte growth factor; LIF, Leukemia inhibitory factor; MCSF, macrophage colony-stimulating factor; SCF, stem cell factor; SCGFβ, stem cell growth factor beta; SDF1α, stromal cell-derived factor 1 alpha; βNGF, nerve growth factor beta; VEGF, vascular endothelial growth factor. $p<0.05$ were considered statistically significant. Blank cells indicate that those specific cytokines were not measured at 3 months and 6 months.

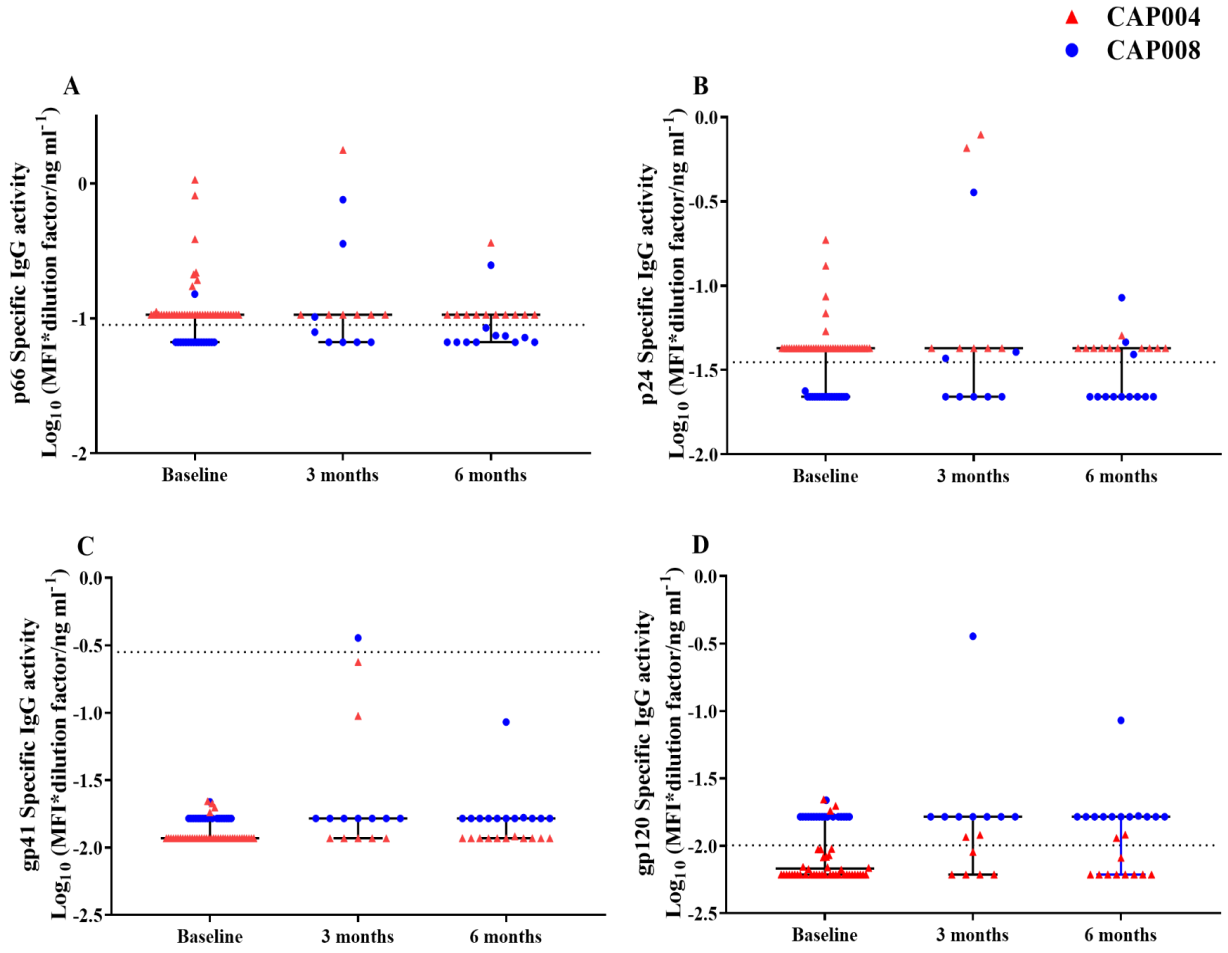

Figure S1: HIV-specific activities Log_{10} (MFI*dilution factor/ngml^{-1}) in CVL for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV^{-} women at baseline (n=60), at 3 (n=15) and at 6 months (n=23). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific activities [based on average CVL specific activities of (n=60) HIV^{-} women] are reflected on or below the dotted lines. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

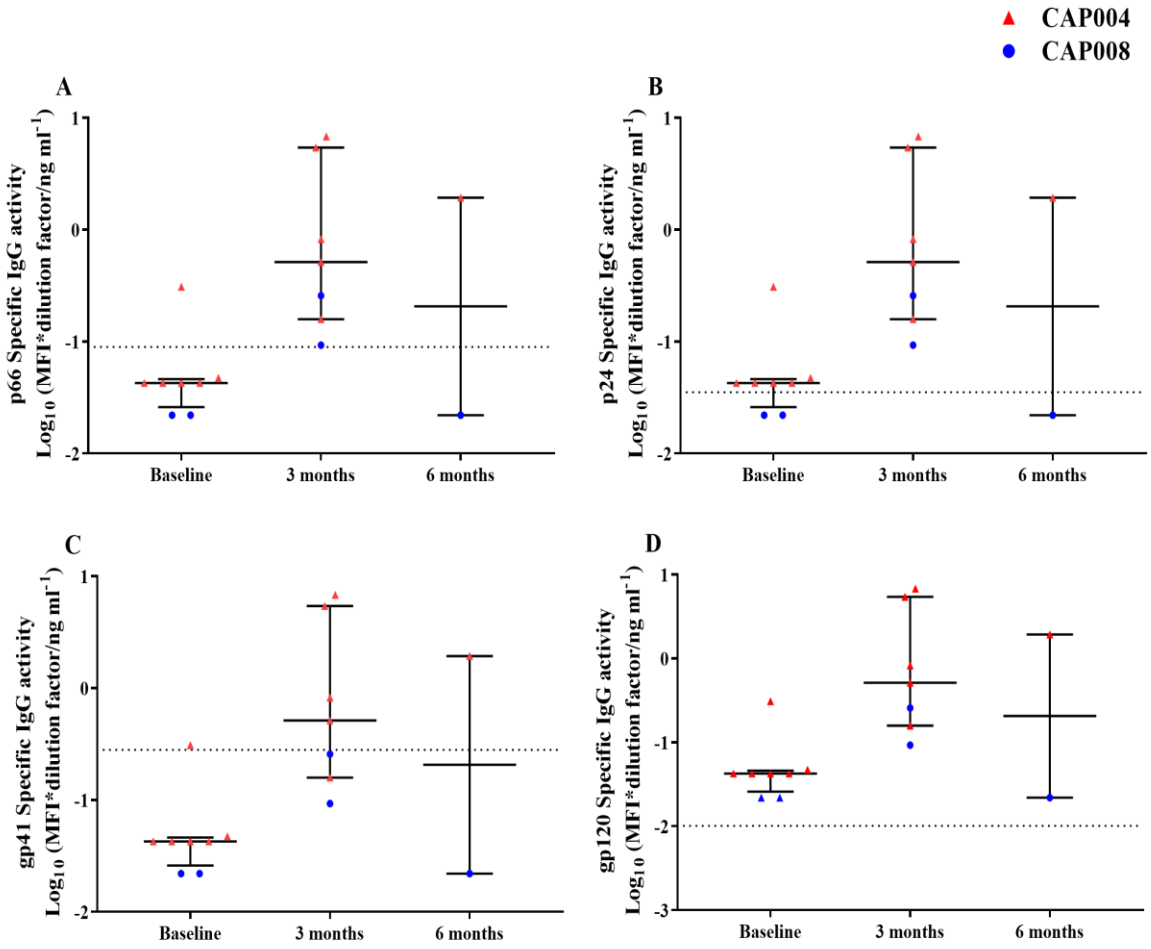

Figure S2: HIV-specific activities Log_{10} (MFI*dilution factor/ngml^{-1}) in CVL for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV^{+}GI^{+} women at baseline (n=8), 3 months (n=7) and 6 months (n=2). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific activities [based on average CVL specific activities of (n=60) HIV^{-} women] are reflected on or below the dotted lines. At baseline, HIV^{+}GI^{+} represents women who became HIV infected. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

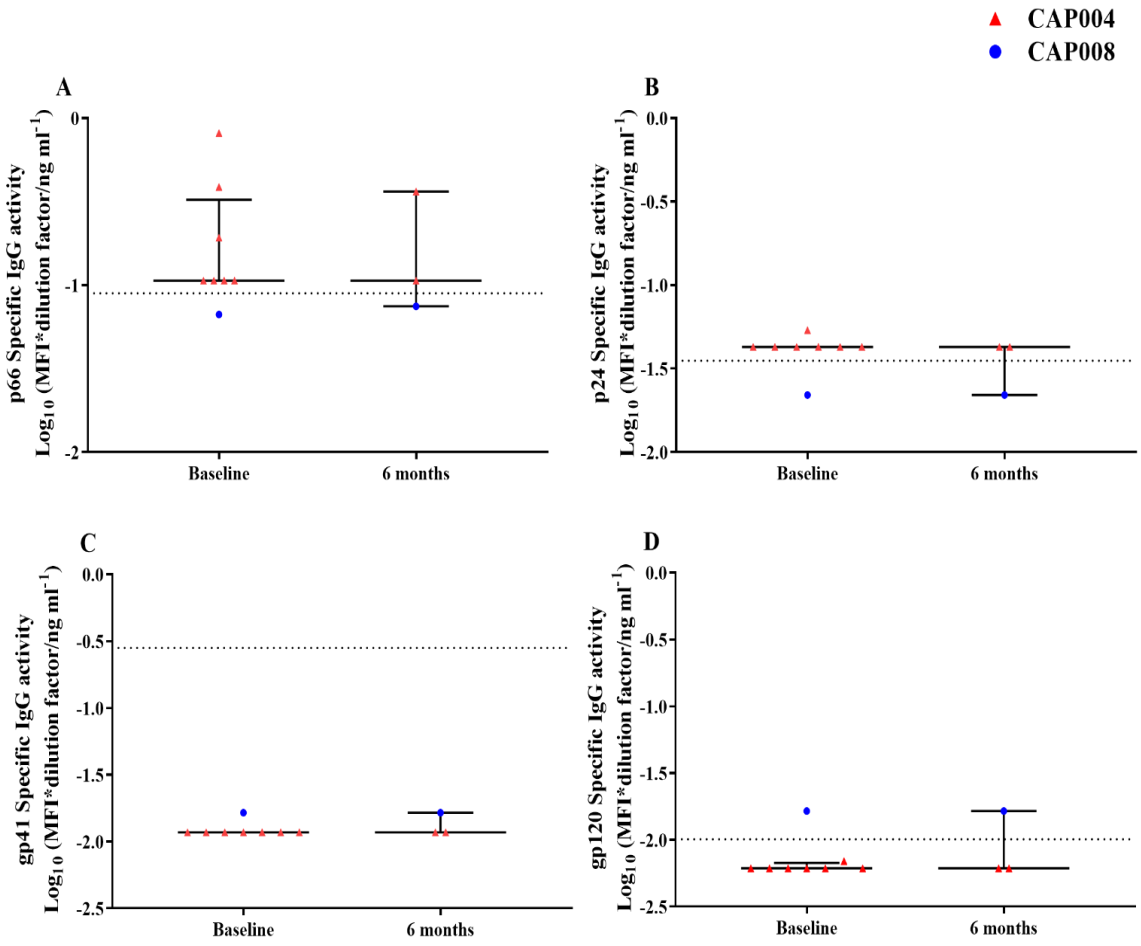

Figure S3: HIV-specific activities Log_{10} (MFI*dilution factor/ngml^{-1}) in CVL for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV^{-}GI^{+} women at baseline (n=8) and at 6 months (n=3). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific activities [based on average CVL specific activities of (n=60) HIV^{-} women] are reflected on or below the dotted lines. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

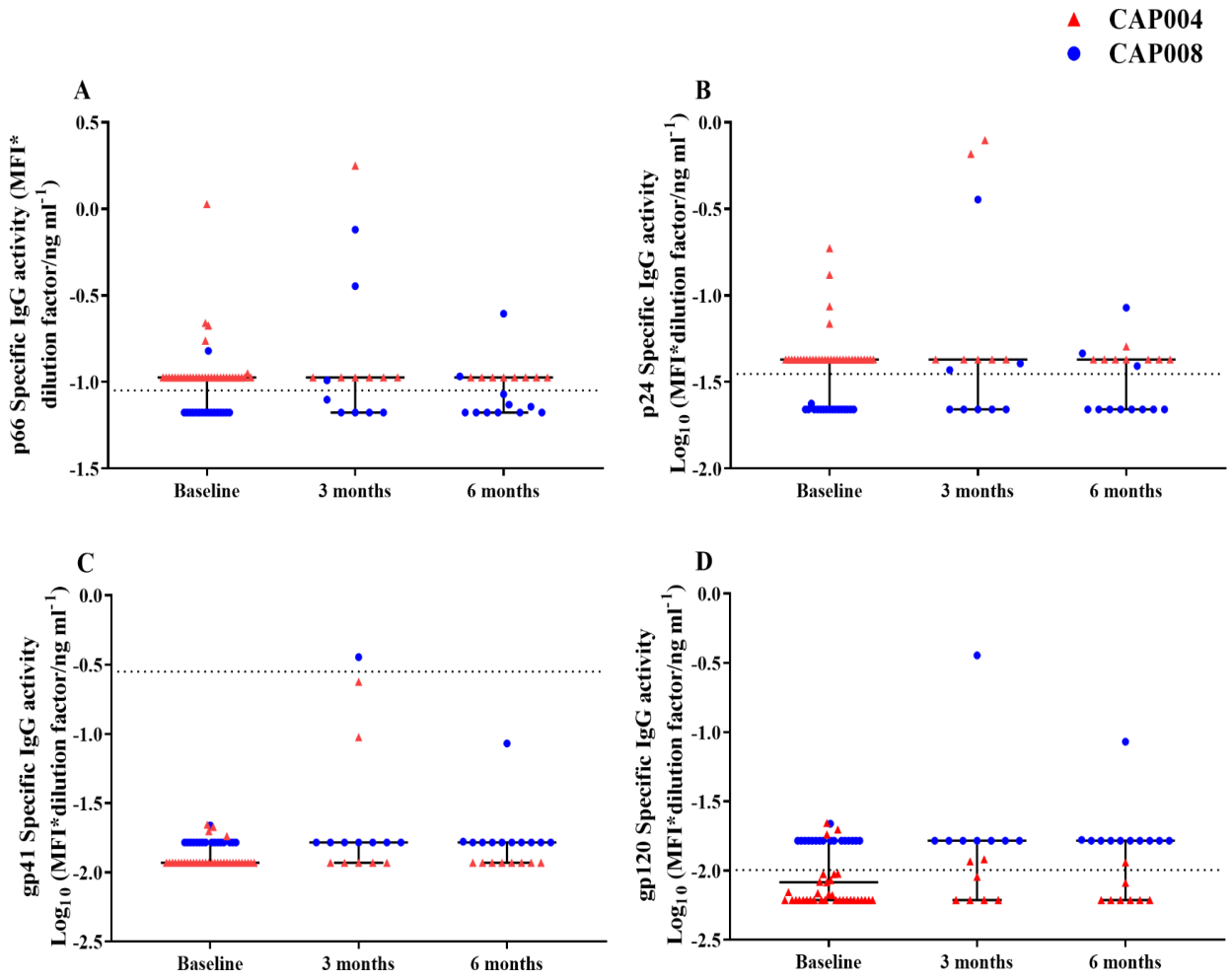

Figure S4: HIV-specific activity Log_{10} (MFI*dilution factor/ngml^{-1}) in CVL for p66 (A), p24 (B), gp41 (C) and gp120 (D) from HIV^{-}GI^{-} women at baseline (n=51), 3 months (n=15) and 6 months (n=19) post-infection. Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable specific activities [based on average CVL specific activities of (n=60) HIV^{-} women] are reflected on or below the dotted lines. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

Table S2: Longitudinal analysis of plasma cytokine profiles between cases and controls in the CAP004 and CAP008 trials.

Cytokine	Baseline (case) [Median (IQR)] N=48	Baseline (control) [Median (IQR)] N=29	p-value	3 months Post-infection (case) [Median (IQR)] N=43	3 month time point (control) [Median (IQR)] N=46	p-value	6 months Post-infection (case) [Median (IQR)] N=47	6 month time point (control) [Median (IQR)] N=22	p-value
IL12p70	1.02 (0.66-1.56)	1.11 (0.97-1.34)	0.88	0.87 (0.42-1.91)	0.84 (-0.16-1.76)	0.99	0.65 (-0.82-1.41)	0.76 (-0.82-1.29)	0.19
IL17α	1.13 (-0.31-1.62)	1.29 (0.25-1.52)	>0.99	0.87 (0.42-1.91)	0.84 (-0.16-1.76)	0.99	0.92 (0.11-1.80)	0.87 (0.32-1.88)	0.32
IL1β	-1 (-2.30 to -0.57)	-2 (-2.30 to -0.65)	0.06	-0.02 (-0.30-0.65)	-0.15 (-0.59-0.61)	0.07	0.01 (-0.37-0.72)	0.01 (-0.39-0.59)	0.30
IL6	0.08 (-0.42-0.53)	-0.04 (-0.53-0.42)	0.25	0.18 (-0.27-1.04)	0.18 (-0.22-1.01)	0.97	0.32 (-0.43-1.10)	0.40 (-0.43-0.99)	0.15
TNFα	0.64 (0.52-0.78)	0.63 (0.38-0.86)	0.23	1.77 (1.55-1.98)	1.60 (1.44-1.95)	0.15	1.71 (1.53-2.05)	1.63 (1.40-1.88)	0.09
IL1α	-0.17 (-0.17 to -0.17)	-0.17 (-0.17 to -0.17)	0.56	-0.16 (-0.82-0.10)	-0.16 (-0.33-0.10)	0.99	-0.16 (-0.29-0.10)	-0.005 (-0.29-0.21)	0.85
IL10	0.33 (-0.62-0.99)	-0.47 (-0.62-0.86)	0.16	0.82 (0.25-1.13)	0.42 (-0.920-1.21)	0.15	0.77 (-0.26-1.34)	0.72 (-0.15-1.06)	0.38
IL1Rα	1.98 (1.76-2.23)	2.07 (1.89-2.25)	0.88	2.23 (2.02-2.37)	2.35 (2.08-2.56)	0.96	2.20 (1.97-2.48)	2.12 (1.96-2.47)	0.33
IFNγ	2.39 (0.93-2.43)	2.36 (2.03-2.68)	0.88	0.77 (0.55-2.17)	0.73 (0.50-2.04)	0.21	0.77 (0.54-2.20)	0.80 (0.28-2.05)	0.09
IL13	0.94 (0.47-1.45)	1.07 (0.77-1.57)	>0.99	0.74 (0.40-1.13)	0.53 (0.23-1.29)	0.37	0.77 (0.40-1.18)	0.83 (0.47-1.25)	0.77
IL15	0.81 (0.013-1.44)	0.01 (0.01-0.32)	0.50	1.22 (0.73-1.46)	1.08 (0.43-1.28)	0.23	0.81 (0.81-1.32)	0.89 (0.61-1.81)	0.97
IL2	0.009 (-0.46-0.68)	0.007 (0.007-0.31)	>0.99	0.62 (0.02-0.90)	0.41 (-0.24-0.84)	0.62	**0.51 (0.22-0.86)**	**0.12 (-0.92-0.90)**	**0.02**
IL4	0.43 (-0.37-0.62)	0.51 (0.43-0.63)	0.88	0.36 (-1.00-0.88)	0.37 (-0.66-0.73)	0.88	0.25 (-0.59-0.83)	0.44 (-1.00-0.77)	0.07
IL5	0.71 (0.04-0.95)	0.96 (0.64-1.12)	>0.99	0.71 (0.04-0.95)	0.96 (0.64-1.12)	>0.99	1.24 (0.82-1.52)	1.12 (0.84-1.59)	0.55
Eotaxin	1.98 (1.39-2.16)	1.99 (1.80-2.22)	>0.99	1.41 (1.17-1.74)	1.39 (1.17-1.76)	0.51	1.34 (1.13-1.83)	1.40 (1.18-1.97)	0.21
IL8	0.33 (-0.10-0.59)	0.43 (-2.00-0.78)	0.65	1.05 (0.77-1.40)	1.06 (0.58-1.27)	0.68	1.10 (0.67-1.36)	0.94 (0.46-1.23)	0.18
IP10	**2.17 (1.99-2.37)**	**2.35 (2.15-2.55)**	**0.01**	**2.60 (2.44-2.91)**	**2.24 (2.08-2.76)**	**0.001**	**2.72 (2.36-3.19)**	**2.46 (2.27-2.84)**	**0.0007**
MCP1	2.16 (1.94-2.24)	2.16 (1.92-2.34)	0.52	1.47 (1.18-1.67)	1.45 (1.04-1.63)	0.30	1.37 (1.12-1.62)	1.36 (0.64-1.57)	**0.05**
MIP1α	0.52 (-0.68-0.80)	0.37 (-0.68-0.82)	0.48	0.29 (0.00-0.72)	0.30 (-0.17-0.68)	0.99	0.34 (-0.01-0.67)	0.29 (-0.18-0.70)	0.21
MIP1β	1.34 (1.15-1.50)	1.43 (1.24-1.49)	0.62	1.69 (1.47-1.87)	1.77 (1.41-1.92)	0.58	1.63 (1.50-1.81)	1.77 (1.56-1.92)	0.93
RANTES	3.26 (3.02-3.73)	3.41 (3.05-3.55)	0.63	3.33 (3.02-8.92)	3.26 (2.82-3.70)	0.28	3.09 (2.93-4.11)	3.28 (2.93-8.92)	0.67
Basic FGF	1.37 (0.66-1.62)	1.41 (1.31-1.68)	0.88	1.52 (1.35-2.02)	1.42 (0.86-1.79)	0.52	1.52 (1.08-1.96)	1.60 (1.27-1.84)	0.64
GCSF	1.84 (0.93-2.05)	1.84 (1.70-2.00)	0.88	1.90 (1.15-2.32)	1.73 (0.19-2.25)	0.57	1.83 (0.81-2.40)	1.78 (0.42-2.21)	0.39
GMCSF	0.09 (-0.29-0.37)	-0.06 (-0.35-0.06)	0.18	**0.43 (-0.58-1.38)**	**-0.17 (-2.30-1.15)**	**0.05**	0.46 (-1.22-1.33)	0.84 (-0.85-1.62)	0.64
IL7	-2.30 (-2.30 to -0.22)	-2.30 (-2.30 to -0.34)	0.12	1.36 (0.49-1.66)	1.25 (-1.06-1.60)	0.92	**1.38 (0.49-1.67)**	**1.05 (-0.28-1.50)**	**0.01**
IL9	1.47 (0.41-1.59)	1.09 (0.92-1.39)	0.63	1.78 (1.55-1.98)	1.70 (1.50-1.99)	0.85	1.75 (1.57-1.94)	1.78 (1.51-2.04)	0.25
PDGFββ	2.14 (1.27-3.04)	2.51 (2.23-2.90)	>0.99	2.36 (1.61-2.99)	2.16 (0.92-2.67)	0.38	2.09 (0.92-2.65)	2.32 (1.23-2.76)	0.58
VEGF	1.37 (1.32-1.93)	1.48 (1.20-1.77)	0.63	1.97 (1.52-2.28)	1.70 (1.32-2.19)	0.45	1.77 (1.47-2.23)	1.60 (1.32-2.24)	0.76

Abbreviations: IQR, interquartile range; IL, interleukin; TNF, tumor necrosis factor; IP10, interferon-γ inducible protein 10; MCP1, moncoyte chemoattractant protein 1; MIP1, macrophage inflammatory protein 1; RANTES, regulated on activation, normal T expressed and secreted; Basic FGF, Basic fibroblast growth factor; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte macrophage colony-stimulating factor; PDGFββ, platelet-derived growth factor beta; VEGF, vascular endothelial growth factor.

$p<0.05$ were considered statistically significant

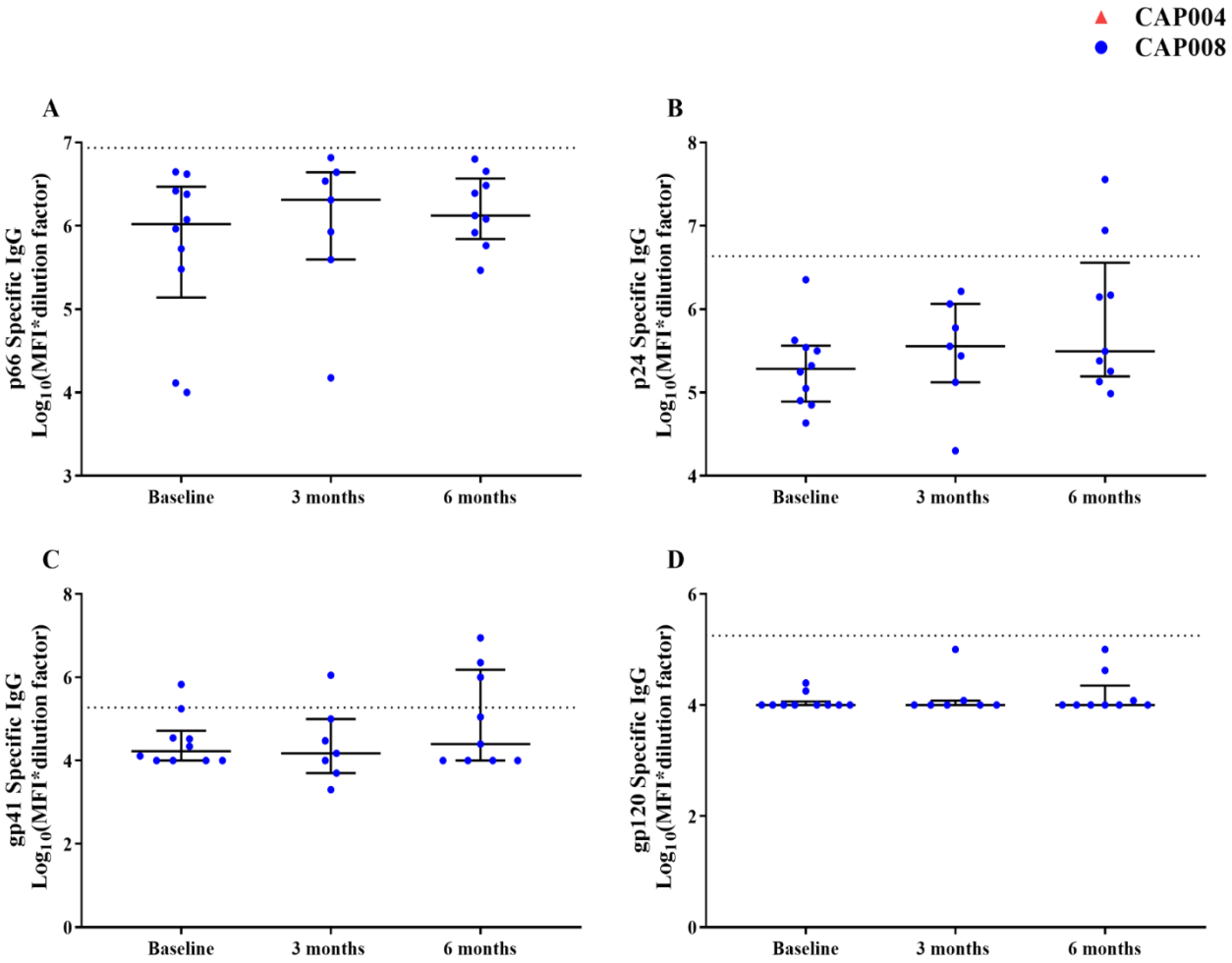

Figure S5: HIV-specific responses [Log_{10} (MFI*dilution factor)] in plasma for (A) p66, (B) p24, (C) gp41 and (D) gp120 from $HIV^{-}GI^{+}$ women at baseline (n=10), at 3 months (n=7) and 6 months (n=9). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable HIV-specific responses [based on average plasma HIV-specific responses of (n=61) HIV^{-} women] are reflected on or below the dotted lines. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

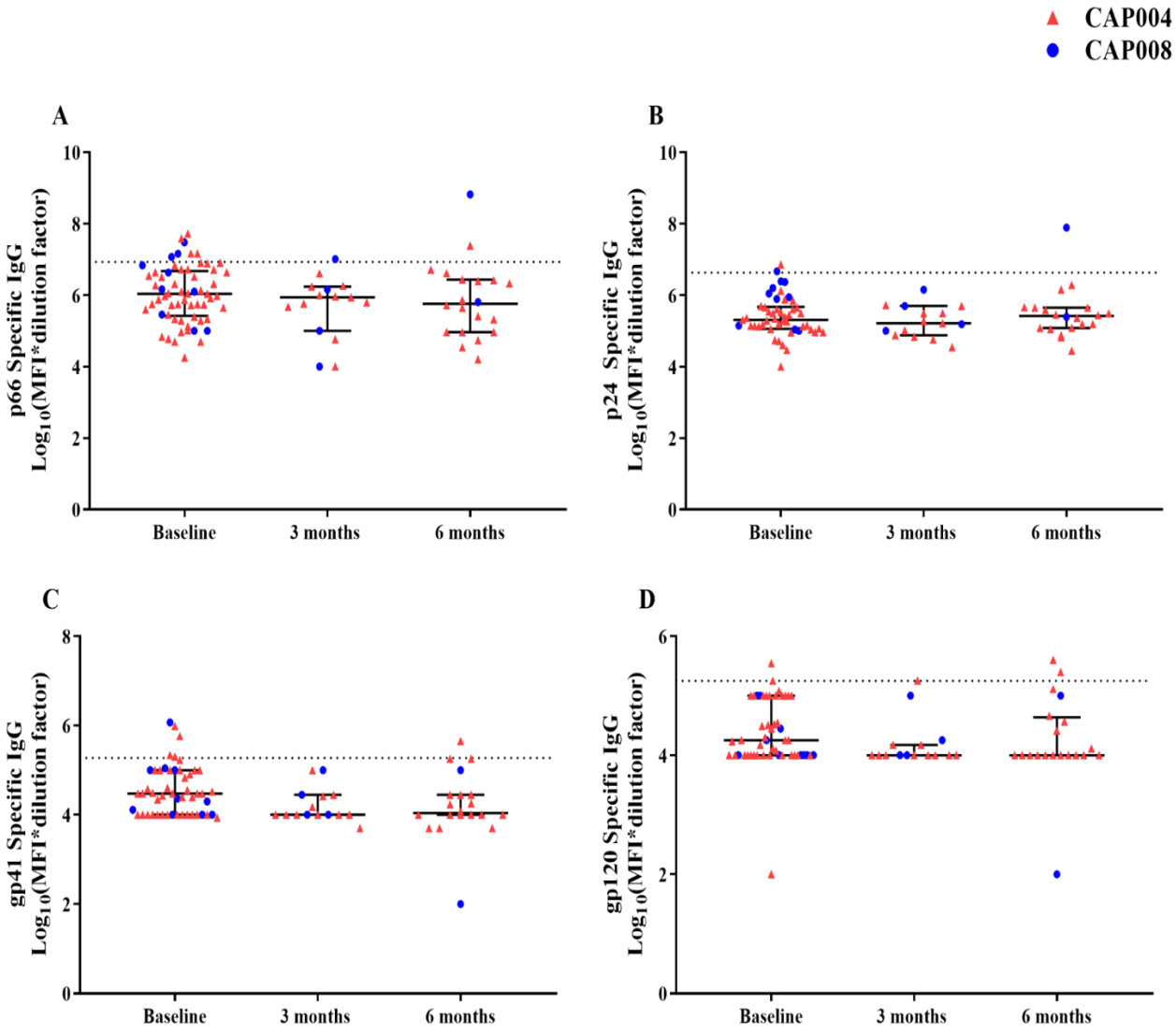

Figure S6: HIV-specific responses [Log_{10} (MFI*dilution factor)] in plasma for (A) p66, (B) p24, (C) gp41 and (D) gp120 from $HIV^{-}GI^{-}$ women at baseline (n=51), at 3 months (n=15) and 6 months (n=20). Each data point represents an individual sample. The scatter dot plot includes the medians and interquartile ranges. The Wilcoxon test was used to compare groups and $p<0.05$ were considered statistically significant. All values falling below the detectable HIV-specific responses [based on average plasma HIV-specific responses of (n=61) HIV^{-} women] are reflected on or below the dotted lines. Red triangles represent women from the CAP004 trial and blue circles represent women from the CAP008 trial.

10. APPENDIX I

10.1 Solutions

10.1.1 70% Isopropanol

175ml of 100% Isopropanol added to 75ml of deionized water (dH_2O).

10.1.2 10% Bleach (NaOH)

167ml of 15% NaOH added to 83ml of deionized water (dH_2O).

10.1.3 100mM Monobasic Sodium Phosphate Activation buffer pH 6.2

Monobasic Sodium Phosphate in Deionized H2O/pH 6.2

Components required	Mass	Molarity
Sodium phosphate dibasic heptahydrate (mw: 268 g/mol)	6.032 g	0.0225 M
Sodium phosphate monobasic monohydrate (mw: 138 g/mol)	10.694 g	0.0775 M

A volume of 800ml of deionized water was added to a 1L glass bottle. To this bottle, 6.032g of Sodium phosphate dibasic heptahydrate was added. Thereafter, 10.694 g of Sodium phosphate monobasic monohydrate to the solution. The solution was adjusted to the desired pH of 6.2 using HCL or NaOH. Lastly, deionized water was added until the volume was 1L.

10. 1.4 Luminex BAMA wash buffer

A volume of 10ml of the 50X luminex wash buffer was added to 490ml of PBS. To this, 0.25g of sodium azide and 250µl of Tween 20 was added to the BAMA wash buffer bottle. A magnetic stirrer was placed inside the flask and allowed to shake until all the components was thoroughly mixed, and then kept overnight in a -20 ̊C fridge.

10.1.5 Milk Blotto

A volume of 95ml of PBS, 5ml goat serum, 50µl tween 20 and 1g of milk powder was added to a tissue culture flask. A magnetic stirrer was placed inside the flask to ensure all the components were thoroughly mixed, and then kept overnight in a -20 ̊c fridge.

10.1.6 HIV Protein Calculations

A volume of 50µg of each antigen (p24 100µg/0.1ml, p66 µg/µl, gp41 100 µg/0.1ml, gp120 100mg/0.1mg) was coupled to their respective mircospheres (bead set 19, bead set 42, bead set 44, and bead set 10). To achieve this desired concentration, a volume of 50µl of each antigen was added to 950µl of PBS in their respective low binding titre tubes.

11. APPENDIX II

11.1 BREC Acceptance letter for the study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

26 April 2017

Ms T Pillay (211534977)
Discipline of Medical Microbiology
School of Laboratory Medicine and Medical Sciences
pillayt92@gmail.com

Dear Ms Pillay

Protocol: The effect of genital tract inflammation HIV-specific binding antibodies, IgG subclass and isotype transudation.
Degree: MMedSc
BREC reference number: BE0207/17

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 10 March 2017.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 19 April 2017 to BREC letter dated 10 April 2017 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval** and may begin as from 26 April 2017.

This approval is valid for one year from **26 April 2017.** 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 **RATIFIED** by a full Committee at its next meeting taking place on **09 May 2017.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor Joyce Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor:
cc administrator:

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2489 Facsimile: +27 (0) 31 260 4609 Email:

12. APPENDIX III

12.1 Previous Publication

Please refer to the publication attached at the end of the thesis.

PILLAY, T., SOBIA, P., OLIVIER, A. J., NARAIN, K., LIEBENBERG, L. J. P., NGCAPU, S., MHLONGO, M., PASSMORE, J.-A. S., BAXTER, C. & ARCHARY, D. 2019. Semen IgM, IgG1, and IgG3 Differentially Associate With Pro-Inflammatory Cytokines in HIV-Infected Men. *Frontiers in Immunology,* 9. doi.org/10.3389/fimmu.2018.03141