IMPACT OF PRE-EXISTING HOST CYTOKINE PROFILE AND PATHOGEN EXPOSURE ON SUBSEQUENT HIV-1 ACQUISITION IN A HIV-1 HIGH RISK POPULATION IN COASTAL KENYA

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A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in Immunology of Pwani University

JANUARY, 2022

DECLARATION

This thesis is my original work and has not been presented in any other University or any other Award.

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DEDICATION

This thesis is dedicated to my doting parents and the Fwambah family at large, my greatest support system.

ACKNOWLEDGEMENTS

I thank God for guiding me every step of this journey.

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ABSTRACT

Background: Pathogenic bacterial, viral, parasitic and fungal infections comprise the biggest burden of disease in sub-Saharan Africa (sSA). Constant exposure to these infections has been reported to lead to an altered immune response, which has been associated with reduced vaccine responses and increased risk of acquisition of secondary infections. Whether altered immune responses increase susceptibility to HIV-1 acquisition in the sSA setting remains less well documented. This study hypothesized that exposure to common pathogens results in elevated immune activation, leading to an increased risk of HIV-1 acquisition.

Methods: A case-cohort study design was used. High-risk HIV-1 negative volunteers were followed up longitudinally. Cases and controls were defined as volunteers who acquired and those who did not acquire HIV-1 infection during a 3-month follow up, respectively. For cases, plasma samples collected 3(+/-1) months before HIV-1 infection were identified. Controls were matched 2:1 to cases by gender, age, risk group and follow-up time from enrolment into the cohort. Immune activation was determined by measuring 37 analytes using the Meso-Scale Discovery platform. IgG antibody levels against two common pathogens in the region, malaria and cytomegalovirus (CMV) were also measured using enzyme-linked immunosorbent assay (ELISA). Immune activation markers and pathogen exposure levels were then compared between cases and controls. Analytes that were significantly different between cases and controls were carried forward to principal component analysis (PCA) to delineate clustering patterns to help explain underlying related biological pathways. Further, principal components were taken forward to linear regression models to determine their association with HIV-1 acquisition.

Results: Overall, 141 samples from cases (n=47) and controls (n=94) were analyzed. Amongst the cytokines measured, MIP-1β (p=0.04), Eotaxin-3 (p=0.02), IL-12p70

(p=0.04), IL-2 (p=0.02), and IL-4 (p=0.008) were significantly elevated among cases compared to controls, while VEGF-D (p=0.049) was significantly lower in cases. Using PCA, analytes were disentangled into four clusters: PC1 (comprising VEGF, MIP-1β, VEGF-C and IL-4), PC2 (comprising MCP-1β, IL-2 and IL-12p70), PC3 (comprising VEGF-D) and PC4 (comprising Eotaxin-3). In regression analysis, PC1 (suggestive of a Th2 profile, p=0.004) and PC3 (suggestive of tissue repair function, p=0.04) were independently associated with HIV-1 acquisition. Further, there were no significant differences between cases and controls on IgG antibody titres against malaria (p=0.47) and CMV (p=0.68). Although IL-1α (p=0.03), IL-5 (p=0.02), and IL-10 (p=0.02) were significantly higher in malaria seropositive than malaria seronegative volunteers, exposure to malaria and CMV were not significantly associated with the observed immune activation.

Conclusion: An altered cytokine profile was observed in cases compared to controls, over similar follow-up duration. Th2 and tissue repair pathways may play a role in increasing and decreasing susceptibility to HIV-1 acquisition, respectively. While malaria and CMV are common in this region, neither was associated with the observed cytokine profile implying that these two infections may not be the only drivers of the immune activation profile observed. The cytokine signal identified in this study warrants comprehensive systems analysis approaches to identify factors that may predispose one to HIV-1 acquisition, particularly in Africa where constant exposure to many infections results in immunomodulation.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral Therapy
AU	Arbitrary Units
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
cDC	Conventional Dendritic Cells
CI	Confidence Interval
CMV	Cytomegalovirus
CSW	Commercial Sex Worker
CXCL	Chemokine (C-X-C motif) ligand
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESCRT	Endosomal Sorting Complexes Required for Transport
FGF	Fibroblast Growth Factor
Flt-1	Fms-Related Tyrosine Kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperrazineethanesulfonic acid
HIV-1	Human Immunodeficiency Virus-1
HRP	Horse-radish Peroxidase
HSV	Herpes Simplex Virus
IAVI	International AIDS Vaccine Initiative
IFN-γ	Interferon-γ
IL-10	Interleukin-10
IL-12 p70	Interleukin-12 p70

IL-12/23 p40	Interleukin-12/23 p40
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-16	Interleukin-16
IL-17A	Interleukin-17A
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
iNKT	Invariant Natural Killer T cell
IP-10	Interferon Inducible Protein-10
IQR	Interquartile Range
ITAC	IFN-inducible T cell α Chemoattractant
KMO	Kaiser Meyer Olkin
LAG-3	Lymphocyte activation gene-3
LIMS	Laboratory Information Management System
LLOQ	Lower Limit of Quantification
MACS	Magnet-assisted Cell Sorting
MCP-1	Monocyte Chemoattractant Protein-1
MCP-4	Macrophage Chemoattractant Protein-4
MDC	Macrophage-Derived Chemokine
MIP-1α	Macrophage Inflammatory Protein-1α

MIP-1β	Macrophage Inflammatory Protein-1β
MSD	Meso-Scale Discovery
MSM	Men who have Sex with Men
MSM-E	Men who have Sex with Men Exclusively
MSM-W	Men who have Sex with Men and Women
NaCl	Sodium Chloride
NK	Natural Killer
OD	Optical Density
OPD	o-Phenylenediamine dihydrochloride
PBS	Phosphate-Buffered Saline
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death-1
pDC	Plasmacytoid Dendritic Cells
PHIS	Pooled Hyper Immune Sera
PIGF	Placental Growth Factor
Prot.	Protocol
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Medium
STI	Sexually Transmitted Infection
TARC	Thymus-and Activation-Regulated Chemokine
TB	Tuberculosis

TBE	Tris-Borate-EDTA
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
Tie-2	Tyrosine Kinase Receptor-2
TNF-α	Tumor Necrosis Factor- α
TNF-β	Tumor Necrosis Factor- β
ULOQ	Upper Limit of Quantification
UNAIDS	United Nations Acquired Immune Deficiency Syndrome
VEGF-A	Vascular Endothelial Growth Factor-A
VEGF-A	Vascular Endothelial Growth Factor-A
VEGF-C	Vascular Endothelial Growth Factor-C
VEGF-D	Vascular Endothelial Growth Factor-D
VEGFR-1	Vascular Endothelial Growth Factor Receptor-1
WSM-E	Women who have Sex with Men Exclusively

CHAPTER ONE: INTRODUCTION

Main problem

HIV-1 prevalence and incidence is substantially higher in sub-Saharan Africa than in the West (UNAIDS, 2021). It is crucial to understand the mechanisms underlying this phenomenon so as to optimize intervention strategies in the African context.

Past findings

Infectious diseases have been shown to be the biggest contributors to sub-Saharan Africa's disease burden (World Health Organization, 2020a). These are caused by pathogenic bacterial, viral, parasitic and fungal infections that are characterized by acute or chronic inflammatory conditions (Bonagura & Rosenthal, 2020). Constant exposure to these infections has been reported to lead to an altered immune responses, by chronic immune activation or immunosuppression, both measured by altered cytokine profiles (Stelekati & Wherry, 2012). Immune activation can affect immune responses either positively or negatively. For instance, a helminth infection, the immune response shifts to a Th2 response and is denoted by hyporesponsiveness and anergy (C. Chen et al., 2005). It is possible that hyporesponsiveness does protect against severity to various infections. For instance, a study from Senegal reported that children co-infected with chronic *Schistosoma haematobium* and malaria exhibited lower *Plasmodium falciparum* parasitemia, compared to those who only had malaria (Lemaitre et al., 2014). On the other hand, immune activation may have detrimental effects. For example, a study conducted on a murine model reported an increased predisposition to secondary *Streptococcus pneumoniae* infection in mice with a prior influenza infection (Sun & Metzger, 2008). Influenza is associated with elevated levels of Th1 cytokine, IFN-γ, reported to compromise the phagocytic ability of macrophages to clear *Streptococcus pneumoniae* in the early stages of the Streptococcal infection (Sun & Metzger, 2008). Immune activation may also affect vaccine responses. Acute malaria in Nigerians was found to attenuate

immune responses to *Salmonella typhi* vaccine (Williamson & Greenwood, 1978). Vaccines found to be protective in other settings have reduced responses in Africa (Grassly et al., 2015). The yellow fever 17D vaccine stimulated immune response in a Swiss cohort, whereas lower responses were observed in a Ugandan cohort due to prior immune activation in the Ugandan cohort (Muyanja et al., 2014).

Knowledge gap

Previous findings have focused on the effect of specific infections on the likelihood of HIV-1 acquisition. Collectively, these observations raise the possibility that the cumulative effect of multiple and diverse ongoing or historical infections on the subsequent immunological responses to a new infection in Africa may be substantial. However, this remains less well documented. Mechanisms by which underlying infections may influence responses to secondary infections or vaccines include: interfering with vaccine uptake or pathogen entry, altering the initial innate immune responses or impeding longer term adaptive immune responses (Bonagura & Rosenthal, 2020; Stelekati & Wherry, 2012).

HIV-1 is a pandemic of global concern and 25.3 million people are living with HIV-1 in sub-Saharan Africa (UNAIDS, 2021a). It is possible that underlying infections within the continent may result in immunomodulation or immune activation that predisposes individuals to HIV-1 acquisition and may be a contributing factor to the higher burden of HIV-1 in sub-Saharan Africa. As individuals on the continent are exposed to a myriad of infections, overall immune activation may be representative of the overall immunological effect of this chronic exposure to underlying infections.

Novel aspect of the study

This study sought to determine whether chronic exposure to infections common in sub-Saharan Africa results in modified immune profiles that predispose one to HIV-1 acquisition. Two common and highly chronic pathogens, malaria and cytomegalovirus

(CMV), were quantified and assessed for correlation to immune activation to determine whether these infections contribute to an altered immune response, that in turn drives elevated HIV-1 acquisition.

1.1 Statement of the problem

Chronic infections are prevalent on the African continent (World Health Organization, 2020b). These include helminths, *Plasmodium falciparum*, *Mycobacterium tuberculosis* among others. Previous studies have suggested that these persistent infections induce immune activation which has been found to influence susceptibility to secondary infections and reduced response to vaccines (Bonagura & Rosenthal, 2020; Stelekati & Wherry, 2012). Whether elevated immune activation observed on the African continent increases susceptibility to HIV-1 acquisition is not known. In addition, if commonly occurring pathogens such as malaria and CMV infections are associated with altered immune responses, such as immune activation, and could contribute to elevated HIV-1 acquisition is not known.

The HIV-1 pandemic also continues to be a global public health concern, especially in sub-Saharan Africa where the population bears 67% of the global HIV-1 burden (UNAIDS, 2021b). Whether the disproportionately large burden of chronic infections in Africa alters the immune environment predisposing one to HIV-1 acquisition or affecting vaccine responses remains uncertain. This study seeks to understand whether altered cytokine milieu, likely due to pre-exposure to common pathogens, are associated with subsequent HIV-1 acquisition in high-risk populations living in malaria endemic regions in coastal Kenya.

1.2 Justification

Substantial variation has been observed in HIV-1 prevalence and incidence between Africa and the West (Grassly et al., 2015). Immune activation as a result of chronic

exposure to numerous infections may be one of the attributable factors for this. Africa bears the greatest burden of the HIV-1 pandemic (UNAIDS, 2021b) with 450,000 new cases reported in 2021 (UNAIDS, 2021b). The high burden of HIV-1 disease may be driven by socioeconomic, viral and host factors amongst others. It is possible that the altered cytokine milieu resulting from immune activation may predispose individuals to HIV-1 acquisition and accelerated disease progression. Since measuring all underlying infections can be limited by tests availability to common infections, cytokine milieu is a good proxy of the overall immune activation.

This study aims to improve our understanding on the impact of pre-existing immunostatus and pathogen exposure on subsequent HIV-1 acquisition.

1.3 Objectives

1.3.1 General objective

To determine whether baseline cytokine profile and chronic exposure to infections common in sub-Saharan Africa predisposes one to HIV-1 acquisition.

1.3.2 Specific objectives

1. To measure the cytokine milieu in HIV-1 high-risk volunteers in Coastal Kenya prior to HIV-1 acquisition and establish if this is associated with HIV-1 acquisition.
2. To measure exposure to two common pathogens, malaria parasites and CMV, in HIV-1 high-risk volunteers in Coastal Kenya and establish if this exposure is associated with HIV-1 acquisition.
3. To establish if exposure to malaria parasites and CMV, two common pathogens in this environment, is the key driver of the observed immune activation in objective 1.

1.4 Null Hypothesis

The baseline immune status and chronic exposure to common pathogens does not increase susceptibility to HIV-1 acquisition.

1.5 Scope of study

A previously-conducted prospective cohort study comprising of high-risk HIV-1 negative volunteers residing in Africa, who were followed longitudinally under the International AIDS Vaccine Initiative (IAVI)'s protocol B was used. Volunteers were termed to be at high risk of HIV-1 acquisition based on engaging in risky behaviours such as, having multiple sexual partners, men having sex with men and engaging in commercial sex work. During the sample collection time, an annual HIV-1 incidence rate ranging from 6-7% in MSM and 2-3% in female sex workers was reported for the cohort (Price et al., 2020). In more recent time, high risk individuals have been shown to account for 65% of new HIV-1 infections in 2020, which translates to 975,000 new cases (Avert, 2020). Plasma samples were taken every 3 months for HIV-1 testing and storage. Volunteers who acquired HIV-1 infection transferred for follow up under IAVI's protocol C through to antiretroviral therapy (ART) initiation. Cryopreserved plasma samples collected from Kilifi in Coastal Kenya at 3 months (+/-1) prior to HIV-1 acquisition were used in this study. This time point is close to the estimated date of infection; therefore, it is reflective of the immune environment right before infection. Cytokine profiles were characterized using the Meso-Scale Discovery platform. Malaria and CMV pathogen exposure were quantified using schizont extract and anti-CMV IgG ELISA respectively.

CHAPTER TWO: LITERATURE REVIEW

2.1 Immune responses to infections

Innate immune responses

The immune system is instrumental in protecting the host from invading pathogens which pose a threat to normal host functions. In order to detect and eliminate invading pathogens, the host mounts innate and adaptive immune responses (Chaplin, 2010). The innate immune response is non-specific and can target a wide range of pathogens while the adaptive immune response is slower to develop and effect but specific to the pathogenic microbe (Abul et al., 2016).

Pathogens enter the body through the skin or mucosal surfaces and invade tissues. Here, they reproduce and spread, consequently leading to damage of host tissues. The appropriate immune response is elicited, depending on the nature of the pathogen. The pathogen is detected by sentinel cells residing in these tissues. These immune cells include phagocytes such as macrophages and dendritic cells which are part of the innate response and distinguish the microbe by pathogen-associated molecular patterns (Chaplin, 2010). This sends a signal to the immune cells that the microbe is foreign and needs to be destroyed. The innate immune cells become activated and launch an attack on the invader. The pathogen may be destroyed in several ways. Phagocytes ingest and chemically destroy the pathogen through phagocytosis, or may release extracellular toxic granules onto the pathogen if the pathogen is too large to ingest (Sompayrac, 2015). In addition to this, these cells secrete cytokines which communicate to other immune cells to travel to the site of infection and reinforce the immune response for elimination of the pathogen (Murphy & Weaver, 2016). Other components of the innate immune system include natural killer (NK) cells, neutrophils, complement system, mast cells, basophils and eosinophils (Sompayrac, 2015). At this point, the innate immune response may be sufficient to eliminate the pathogen and contain the infection.

Adaptive immune responses

However, sometimes the innate immune response may not be successful because some pathogens have evolved mechanisms which enable them to evade the innate immune response. These evasive pathogens continue replicating and pathogen antigens increase which triggers the adaptive immune system (Murphy & Weaver, 2016). Macrophages and dendritic cells present antigens to naïve B and T lymphocytes, allowing the latter to assess the antigens. In the occasion of an infection, the antigens presented are non-self, therefore a clone of lymphocytes specific to the antigen becomes activated and proliferates. This is referred to as clonal expansion. Also, differentiation of that particular lymphocyte occurs, thus generating a pool of lymphocytes that are specific to the antigens presented (Abul et al., 2016; Chaplin, 2010). The adaptive immune response comprises of B and T lymphocytes/cells which bear cell surface receptors that confer recognition of specific antigens. T cells comprise of $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells are key cytokine producers as they act as the instructors of other immune cells by producing cytokines which elicit an immune response specific to the invading pathogen (Zhang & An, 2007). Through cytokine signaling, appropriate immune cells are recruited to the site of infection for destruction of the specific pathogen. For instance, in response to a fungal infection, naïve $CD4^+$ T cells differentiate into Th17 cells and secrete IL-17 which is a cytokine that plays an important role in anti-fungal immunity. $CD8^+$ T cells kill infected cells by bridging with and forming pores in the membrane of infected cells and releasing toxic granules leading to cell death, hence preventing further spread of the pathogen. Alternatively, $CD8^+$ T cells can induce programmed death to the infected cells (apoptosis). Therefore, $CD8^+$ T cells are crucial in defence against intracellular pathogens such as viruses (Sompayrac, 2015). B cells express B cell receptors on their cell surfaces which when crosslinked by its cognate antigen, activates the B cell. These cells then proliferate and secrete antibodies specific to the cognate antigen. Antibodies neutralize

the free antigen by binding to the pathogen and inhibiting its infectivity through aggregation. Secondly, antibodies can mark cells for destruction by neutrophils and macrophages through opsonization. Antibodies also aid NK cells in lysing infected cells through antibody-dependent cellular cytotoxicity. The classical complement pathway relies on antibodies binding to pathogens and eventual destruction of the pathogen or infected cell (Forthal, 2015) (Figure 1).

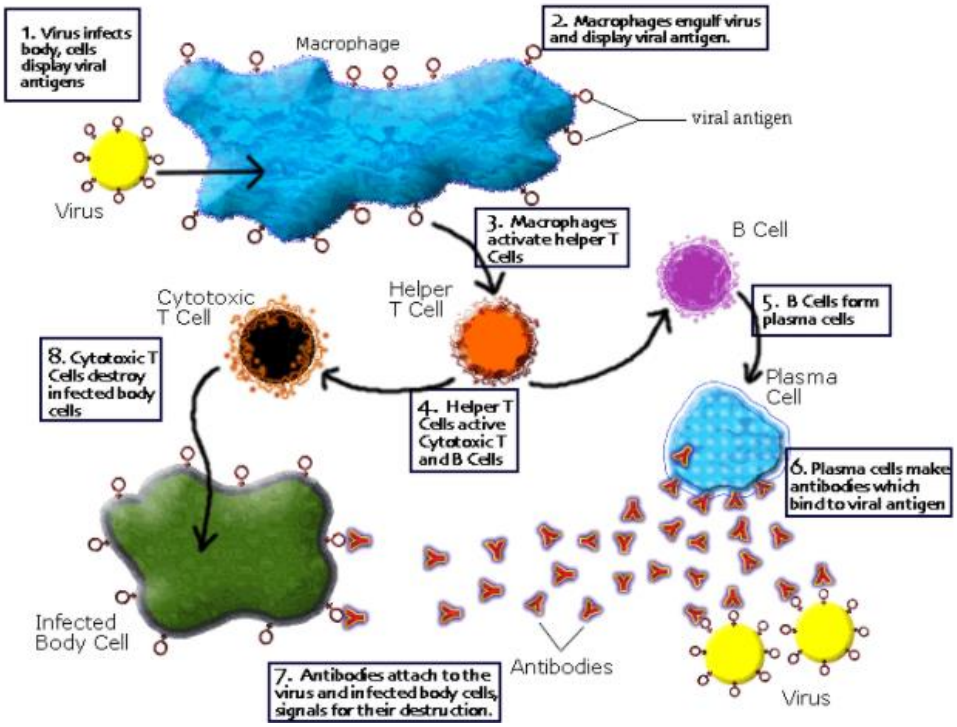


Figure 1: The course of a typical immune response. (Grant, 2012). 1) The pathogen, virus, enters the body and is detected as foreign by a macrophage. 2) The macrophage phagocytoses the virus, processes and presents viral antigens to naïve T cells. 3) T cells are activated by a combination of the presented antigens, costimulatory signals and cytokines. 4) $CD4^+$ T cells secrete cytokines which activate $CD8^+$ T cells. 5) B cells are activated by help of $CD4^+$ T cell and differentiate into plasma cells. 6) Plasma cells secrete antibodies which target the free virus 7) and virus-infected cells. 8) Activated $CD8^+$ T cells kill infected cells.

Immune responses to acute versus chronic infections

Acute infections here refers to infections that one's immune system is able to clear from the system, while a chronic infection is not completely cleared by the immune system and persists within the host (Malyshkin, 2014). In the case of an acute infection, the concerted effort of the innate and adaptive immune response succeeds in clearing the pathogen from the body. The antigen levels reduce over time as a consequence of the immune response. Contraction follows whereby immune cells, particularly antigen-stimulated lymphocytes undergo apoptosis, to restore homeostasis. However, some lymphocytes persist to constitute the memory compartment. This is essential for the induction of a rapid immune response upon secondary exposure to the same pathogenic antigen, which prevents disease (Abul et al., 2016). (Figure 2)

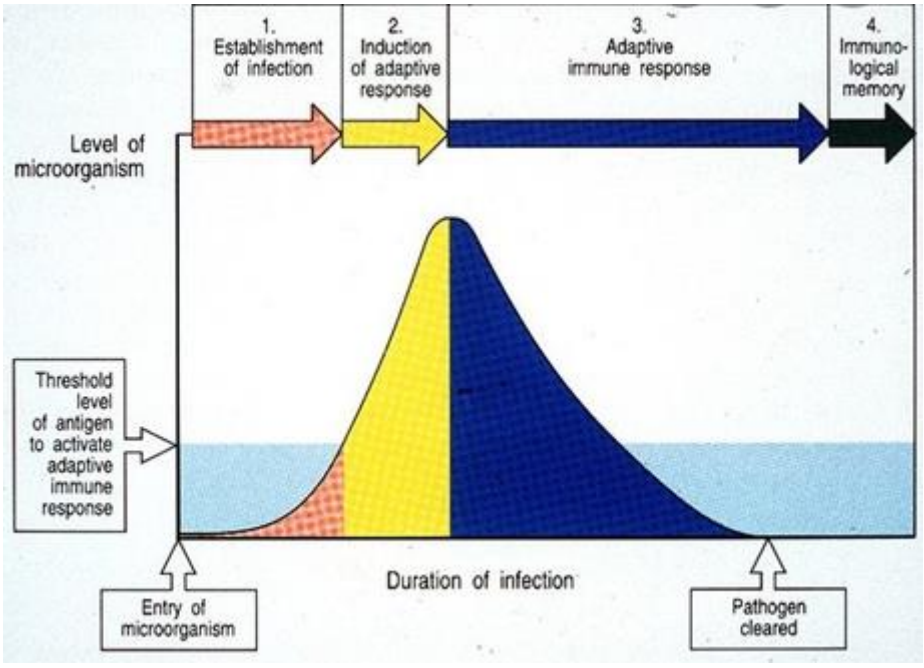


Figure 2: Progression of an acute infection. (Murphy & Weaver, 2015). Entry of a pathogen into the body triggers the innate immune system. If unsuccessful in controlling pathogen replication, the antigen threshold required to induce an adaptive immune response is met. The adaptive immune response takes about a week to develop. Upon clonal expansion and differentiation, B and T cells effect their functions and the pathogen

is successfully cleared from the body over time. A portion of effector cells persist to form memory B and T cells, while the rest die by apoptosis to regain homeostasis.

In contrast, a chronic infection is characterized by the persistence of the infectious agent after primary infection, owing to its ability to form a reservoir within infected cells and achieve latency. Following infection, the pathogen infects host cells and the pathogen is released periodically over time (Boldogh et al., 1996). This study focuses on two chronic infections: chronic malaria and CMV. Malaria and CMV were selected on the basis that they are both chronic infections and therefore their insult on our immunity would be more long-term and hence more reflective of continuous long-term exposure. In addition, these infections are endemic in coastal Kenya and therefore immune activation in this population would most likely be driven or at least partially driven by these two infections.

Chronic malaria is defined as long-term exposure to *Plasmodium falciparum* in malaria semi-immune individuals, comprising of older children and adults residing in malaria-endemic regions (Langhorne et al., 2008). This infection is asymptomatic and patients do not present with symptoms associated with acute malaria such as fever (Bousema et al., 2014). These individuals are protected from severe malaria and death. Primary exposure in the rest of the population follows the acute course of infection as previously described with common malaria symptoms such as fever. If not treated, this could result to death (Langhorne et al., 2008). Long-term exposure to *Plasmodium falciparum,* the causative agent of malaria, has been found to alter the phenotype and function of B and T lymphocytes. In a study conducted in Mali, $CD4^+$ T cells were observed to exhibit high expression of inhibitory receptor, programmed cell death-1 (PD-1) (Butler et al., 2011). Similarly, another study in Coastal Kenya also reported an increased concentration of $CD4^+$ T cells expressing inhibitory receptors, PD-1 and lymphocyte activation gene-3 (LAG-3) (Illingworth et al., 2013). Studies from The Gambia, Mali and Peru also reported

an increased expression of $CD19^{+}$ $CD20^{+}$ $CD10^{-}$ $CD21^{-}$ $CD27^{-}$ atypical memory B cells which are similar to exhausted memory B cells identified in other chronic infections such as HIV-1 and hepatitis C (Nogaro et al., 2011; Portugal et al., 2012; Weiss et al., 2011). These cells exhibit sub-optimal proliferation compared to naive B cells and memory B cells following activation. In addition, they also exhibit high levels of inhibitory receptors (Moir et al., 2008; Wherry et al., 2007). B and T cell exhaustion is associated with lymphocyte unresponsiveness and loss of effector functions (Wherry & Kurachi, 2015), which is detrimental to the control of both the existing chronic infection, as well as heterologous infections.

Human CMV is a chronic infection which has been reported to have a long-term impact on immune system homeostasis (Picarda & Benedict, 2018). Though understudied, CMV is a common infection whose prevalence ranges from 55%-97% among HIV-1 negative adults in sub-Saharan Africa, according to a meta-analysis conducted by Bates and Brantsaeter (Bates & Brantsaeter, 2016). CMV infection occurs in 3 distinct phases. Phase I is primarily an asymptomatic acute infection which is similar to the one described before. This phase is characterized by infection of lymphocytes and activation of NK cells, followed by secretion of type I interferon. Circulating lymphocytes are infected and transport the virus into primary and secondary lymphoid organs, where the infection is spread to other lymphocytes (Taylor, 2003). In this phase, the innate immune response is induced and the adaptive immune system is primed to the newly-encountered virus (Picarda & Benedict, 2018). Studies conducted on murine models show that murine CMV infection is associated with memory-like NK cells similar to adaptive lymphocytes (O'Sullivan et al., 2015). Also, another subset of NK cells, $NKG2C^{hi}$ makes up almost half of the NK cell population in some infected individuals which may compromise the NK cell response to heterologous infections. These cells have been found to remain activated throughout the other phases of infection (Lopez-Vergès et al., 2011). A wide

variety of T and B lymphocyte populations are primed during this phase, however, some human CMV antigens have been reported to induce sub-optimal lymphocyte responses (Dauby et al., 2016; Picarda & Benedict, 2018). At the end of this phase, viremia is controlled and the viral antigens are low in systemic circulation. Nonetheless, the virus is not completely cleared from the body, and hides in infected cells in the lymphoid tissues. Phase II is associated with high-level viral replication and persistence within select localized tissues which lasts for months to years (Picarda & Benedict, 2018). A subset of T cells known as inflationary memory T cells do not contract. Instead, they proliferate during phase II due to continued antigen exposure (Karrer et al., 2003). Antibodies specific to CMV are also highly induced, with a steady increase in IgG in people who are healthy but CMV-infected (Vescovini et al., 2016). At the end of phase II, the immune system is successful in controlling the infection. Phase III is a period of viral latency. Here, viral persistence is contained and host-virus coexistence is established. The viral genome is maintained within select cells in various sites where gene expression is present but restricted, however virus production is lacking. Nonetheless, appropriate conditions within the host e.g. a compromised immune system, can result in reactivation of viral replication (Roizman & Sears, 1987). Spontaneous gene expression during CMV reactivation release and exposure of viral antigens to immune cells and propagates immunomodulation which contributes to immune inflation (Snyder et al., 2008). Memory inflation is the maintenance of a large population of inflationary memory T cells which is unique to CMV infection (Klenerman & Oxenius, 2016). CMV has been reported to have an aging effect on the immune system over time, in the elderly (Pawelec et al., 2010) which may affect immune response to other infections (Figure 3).

Both chronic malaria and CMV impair effector functions of cells of the immune system, thus dysregulating the immune response required to protect the host from the bystander infection as well as secondary infections.

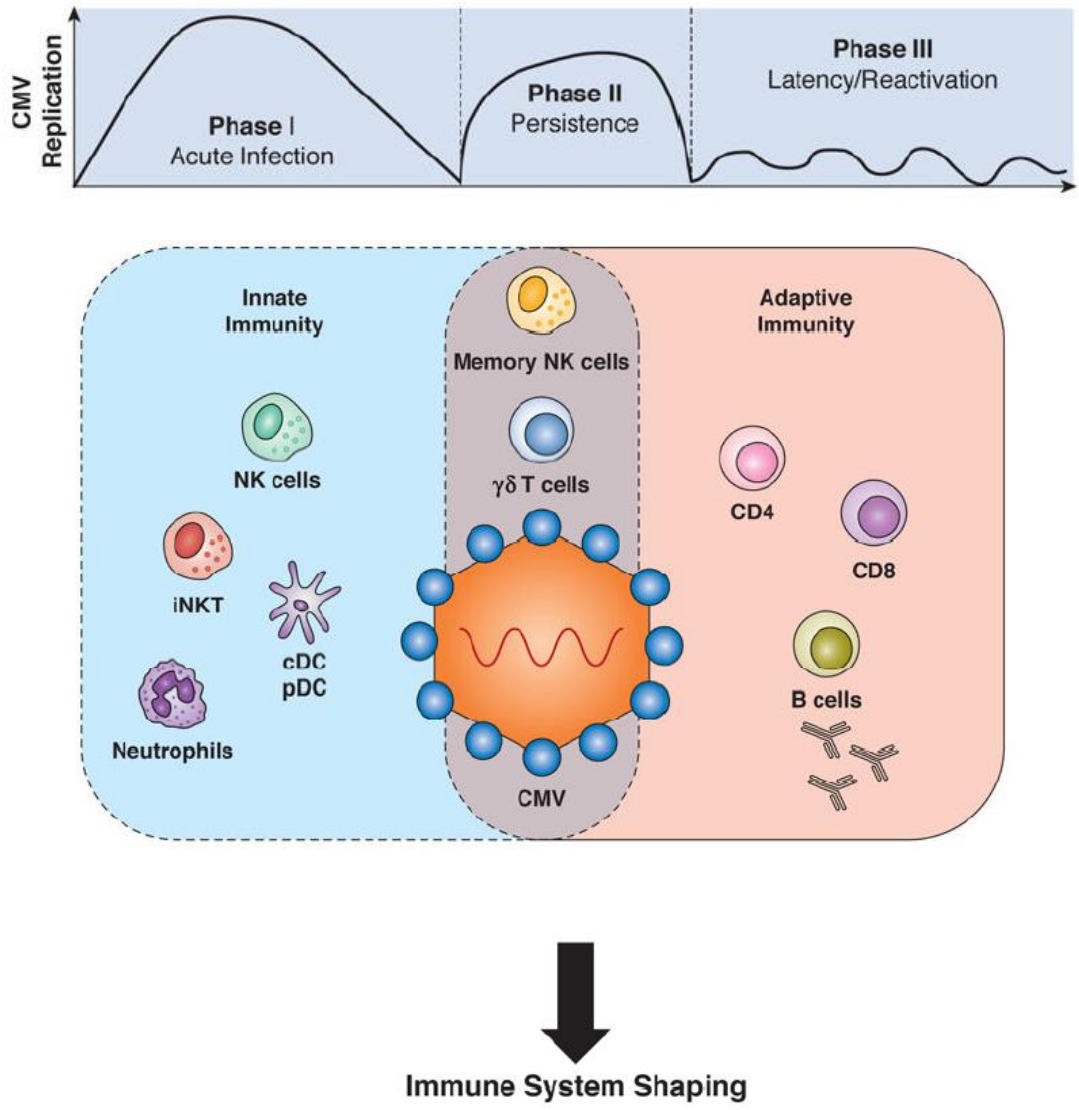


Figure 3: Impact of CMV on the immune system**.** (Picarda & Benedict, 2018). Top panel: The 3 phases of a CMV infection. Phase I) First-time exposure to CMV and infection of circulating lymphocytes. Induction of the immune system and control of viremia. Phase II) Long-term persistent viral replication within localized tissues which continues to remodel the immune system. Phase III) Viral replication across many sites that promotes immune inflation over a lifetime. Bottom panel: Different cells of the innate and adaptive systems are dysregulated during the 3 phases of a CMV infection, the image shows those that are directly impacted.

2.2 Impact of immune activation on secondary infections

Several *in vivo* and *in vitro* studies have been conducted to elucidate the effect of bystander infections on secondary infections.

Tables 1 and 2 represent summaries of studies conducted to determine the effect of specific underlying infections on susceptibility to or protection from secondary infections.

Table 1: Studies representing the impact of immune activation caused by primary infections on secondary infections. [↑-Elevated levels, ↓-Decreased levels. *C. rodentium*-*Citrobacter rodentium, P.falciparum- Plasmodium falciparum, M. tuberculosis*-*Mycobacterium tuberculosis, , S. pneumoniae-Streptococcus pneumoniae, S. aureus*-*Staphylococcus aureus, S. mansoni- Schistosoma mansoni,* HTLV- Human T-lymphotropic virus].

Primary infection	**Secondary infection**	**Effect of primary infection on cytokine levels**	**Effect on secondary infection**	**References**
Helminths	*C. rodentium*	↑IL-4, IL-5, IL-10 ↓IFN-γ, IL-17	Increased predisposition to *C. rodentium* infection	(C.-C. Chen et al., 2006; C. Chen et al., 2005; Su et al., 2011)
Helminths	*P. falciparum*	↓CXCL9, IL-12p35, IL-12p40	Aggravates acute malaria Protective effect on chronic malaria	(Lemaitre et al., 2014; Nacher et al., 2002; Sokhna et al., 2004)

Primary infection	**Secondary infection**	**Effect of primary infection on cytokine levels**	**Effect on secondary infection**	**References**
Helminths	*P. falciparum*	↓CXCL9, IL-12p35, IL-12p40	Aggravates acute malaria Protective effect on chronic malaria	(Lemaitre et al., 2014; Nacher et al., 2002; Sokhna et al., 2004)
Helminths	*M. tuberculosis*	↑IL-10 ↓IFN-γ	Increased damage to tissues by *M. tuberculosis*	(Resende et al. 2007)
Plasmodium	*M. tuberculosis*	-	Accelerated disease progression of *M. tuberculosis*	(Hawkes et al., 2010)
Measles	Pneumonia	↓IL-4, IL-10, IL-12, IL-13	Increased susceptibility to pneumonia	(Carsillo et al., 2009)
Influenza virus	*S. pneumoniae*	↑IFN-γ	Increased risk of *S. pneumoniae* infection. At latter stages, ↑IFN-γ inhibits macrophage ability to clear *S. pneumoniae*	(Sun & Metzger, 2008)

Primary infection	**Secondary infection**	**Effect of primary infection on cytokine levels**	**Effect on secondary infection**	**References**
Influenza virus	*S. aureus*	↑IFN-α and β ↓IL-15, CXCL1, MIP-2	Increased susceptibility to *S. aureus*	(Shahangian et al. 2009; Small et al. 2010)
Unrelated infections	Measles	-	Diminishes antibodies specific to other unrelated infections	(Mina et al., 2019)
Viral hepatitis	*Plasmodium*	↑IL-10	Reduced parasitemia and development of asymptomatic malaria	(Andrade et al., 2011)
HTLV	*S. mansoni*	↑IFN-α and β ↓IL-4, IL-5, IL-13	Increased susceptibility to *S. mansoni*	(Porto et al., 2004)
M. tuberculosis	*Plasmodium*	↑IFN-γ	The higher the IFN-γ levels, the lower the susceptibility to *Plasmodium*	(Page et al., 2005)

The mechanisms by which these infections potentially alter the immune response may occur at different stages (Figure 4). First, the chronic/underlying infection may enhance pathogen or vaccine entry for instance by breaching the anatomical barrier that prevents pathogen invasion. For instance, sexually transmitted infections (STIs) presenting with ulceration damage the skin which may increase the risk of HIV-1 acquisition (Ward & Rönn, 2010). Furthermore, infections may also reduce production of antimicrobial compounds by the innate immune cells (Stelekati & Wherry, 2012). They may also promote pathogen replication. For example, *Candida albicans* has been reported to promote HIV-1 replication, following HIV-1 entry into the body (De Jong et al., 2008). Another possibility is that the underlying infection may inhibit antigen presentation capabilities necessary for activation of lymphocytes. Perhaps the infection may favor the differentiation of either Th1 or Th2 cells and inhibit the other. Consequently, this may modify cytokine production, lower cytotoxicity and dysregulate B cell responses. For instance, a bystander helminth infection favors a Th2 cytokine response which has been reported to exacerbate a subsequent *Mycobacterium tuberculosis* infection (Resende et al., 2007). Lastly, memory development and maintenance may also be affected by these chronic infections. For instance, *Heligmosomoides polygyrus* infection inhibits differentiation of effector $CD8^+$ T cells to memory $CD8^+$ T cells specific for heterologous pathogens (Stelekati et al., 2014; Stelekati & Wherry, 2012) (Figure 4).

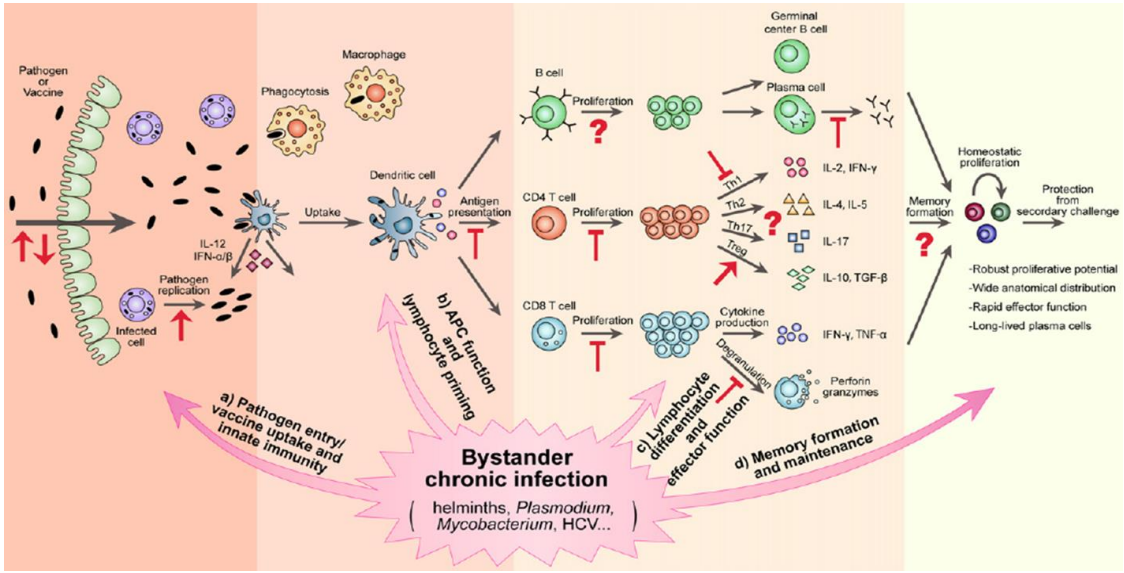


Figure 4: Stages of the immune response that may be altered by a chronic infection. (Stelekati & Wherry, 2012). Chronic infections may a) damage the skin or epithelial layer, easing entry of other pathogens/vaccine, b) compromise antigen processing and presentation, c) alter proliferation, differentiation and effecting of lymphocyte functions or d) inhibit formation and maintenance of long-term memory.

2.3 Effect of bystander infections on HIV-1 susceptibility

To determine if chronic infections have an impact on acquisition of a secondary infection, HIV-1 has been selected as a proof of concept. HIV-1 offers a unique opportunity in that a defined moment of infection can be estimated, allowing for a clear definition of a period prior to and post HIV-1 infection. In addition, the disproportionately higher burden of HIV-1 infection in sub-Saharan Africa, warrants a better understanding of potential factors that would predispose individuals in this setting to HIV-1 acquisition.

2.4 HIV-1 Epidemiology (general epidemic and specifically the epidemic in Africa)

In 2021, 37.7 million people worldwide were living with HIV-1 whereas 1.5 million people were newly infected (UNAIDS, 2021b). Sub-Saharan Africa is the region mostly affected by the virus, having 25.3 million people living with the virus. This comprises

67% of the global HIV-1 burden. Yet, the region only bears a disproportionate 13.3% of the world population (UNAIDS, 2021b).

2.5 HIV-1 Transmission

Following transmission of the virus into a susceptible host, HIV-1 gains entry into the cells and immediately begins the process of replication. The primary target cells of HIV-1 are the $CD4^+$ T cells, although HIV-1 may infect any cell with a CD4 receptor. (Berger et al., 1999; Eckert & Kim, 2001; Ray & Doms, 2006). HIV-1 accesses $CD4^+$ T cells by either being transported to them by dendritic cells e.g., in lymphoid organs or interacting with them firsthand, in the epithelial layers (Swanstrom & Coffin, 2012).

During entry, one specific glycoprotein gp120, attaches itself to the membrane of the cell by binding to the CD4 receptor. The virus then interacts with chemokine coreceptors, CCR5 and/or CXCR4 which leads to a change in conformation that cannot be reversed. Fusion then occurs by pore formation and the viral core is deposited inside the cell's cytoplasm (Simon et al., 2010). The viral core breaks apart and releases the viral genome which is then reverse transcribed by host reverse transcriptase to DNA. Meanwhile, HIV-1 integrase together with host DNA repair enzymes, inserts the viral reverse transcribed viral DNA into areas in the host's genome that are actively transcribed (Scherdin et al., 1990; Schröder et al., 2002; Mitchell et al., 2004). This converts the cell into a virus-producing system. Viral DNA expression produces proteins that are packaged close to the cell membrane, ready for dispatch. New virions are ejected from the cell via the vesicular sorting pathway (ESCRT-1, 2, 3) which facilitates the budding of endosomes into multivesicular bodies (Simon et al., 2010).

Various factors affect HIV-1 pathogenesis, the two major factors are viral phenotype, which is determined by the cell tropism, and secondly, receptors and co-receptors that facilitate viral entry into target cells (Naif, 2013). Cell tropism is defined as the cells

which are preferentially infected by the virus (Berger et al., 1999), which determines the viral strain (X4 or R5 HIV-1 strain) (Naif, 2013). The R5 strain is so named because it infects macrophages, monocytes and T lymphocytes by interacting with the cells' CCR5 coreceptors. For this reason, the R5 strain is also known as the macrophage-tropic (M-tropic) strain (Naif, 2013). On the other hand, the X4 strain, also known as T-cell line tropic (T-tropic) infects T cell lines and T lymphocytes only, using the CXCR4 coreceptor (Naif, 2013) (Figure 5). The binding of chemokine SDF-1 to CXCR4 inhibits the binding of the R4 HIV-1 strain. Likewise, the binding of CCR5 by chemokines MIP-1α, MIP-1β and RANTES, blocks the binding by R5 strain (Levy, 2007). Nonetheless, the initial entry does not cause deadly damage to the host (Eckert & Kim, 2001).

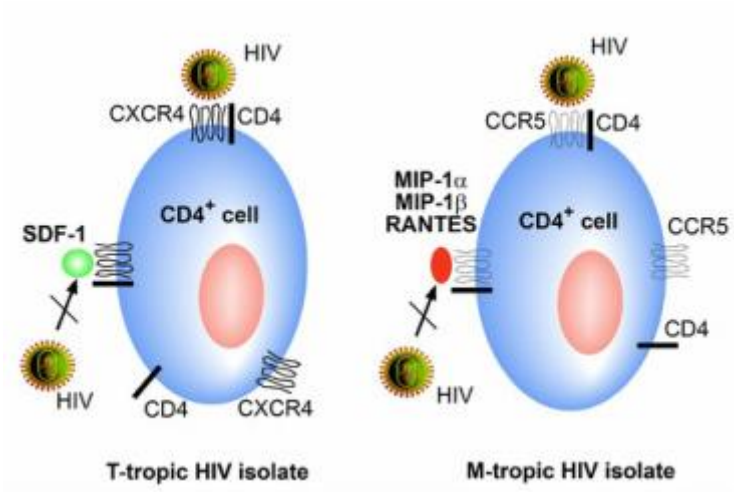


Figure 5: Blocking of HIV-1 coreceptors by natural ligands. Binding of SDF-1 and MIP-1α, MIP-1β, RANTES to CXCR4 and CCR5 respectively thus blocking HIV-1 (Levy, 2007). [SDF-1- Stromal cell-derived factor 1-MIP-1α: Macrophage inflammatory protein-1α; MIP-1β-Macrophage inflammatory protein-1β; RANTES-Regulated on activation, normal T cell expressed and secreted/Chemokine C-C motif ligand 5 (CCL5); CXCR4-C-X-C chemokine receptor 4; CCR5-C-C chemokine receptor type 5].

2.6 Impact of immune activation on HIV-1 acquisition

Table 2 summarizes *in vitro* studies focusing on the impact of immune activation induced by bystander infections on HIV-1 entry and HIV-1 immune response following infection.

2.6.1 *In vitro* studies on the effect of bystander infections on HIV-1 infection

Table 2: *In vitro* studies representing the impact of immune activation caused by primary infections, on HIV-1 entry and HIV-1 immune responses following infection. [*M. tuberculosis- Mycobacterium tuberculosis; N. gonorrhea-Neisseria gonorrhea, C. albicans-Candida albicans; P. falciparum- Plasmodium falciparum;* HSV- Human Simplex Virus; RANTES- Regulated on activation, normal T cell expressed and secreted/ Chemokine C-C motif ligand 5 (CCL5)]

Primary infection	**Secondary infection**	**Cytokine**	**Effect on HIV-1**	**References**
M. tuberculosis	HIV-1	↓RANTES ↑IL-4	Accelerated disease progression	(Gopinath et al. 2000)
N. gonorrhea *C. albicans*	HIV-1	↑TNF-α	Enhanced HIV-1 replication	(De Jong et al., 2008)
P. falciparum	HIV-1	↑IFN-γ ↑CCR5	Increased risk of HIV-1 infection	(Moriuchi et al., 2002; Pisell et al., 2002)
Leishmania	HIV-1	↑IL-4, ↑IL-10	Increased disease progression	(Preiser et al., 1996)
Schistosomiasis	HIV-1	-	Increased risk of HIV-1 acquisition	(Downs et al., 2012; Patel et al., 2021)
Helminths	HIV-1	-	Accelerated disease progression	(Christopher Whalen et al., 1995)

Primary infection	**Secondary infection**	**Cytokine**	**Effect on HIV-1**	**References**
Lymphatic filariasis	HIV-1	-	Increased risk of HIV-1 acquisition	(Kroidl et al., 2016)
Viral hepatitis	HIV-1	-	Accelerated progression and mortality	(Chun et al., 2012)
Genital infections	HIV-1	-	Increased risk of HIV-1 acquisition	(Freeman et al., 2006; Haaland et al., 2009; Horbul et al., 2011)
-	HIV-1	↑Fractalkine ↑ITAC ↑IL-7	Increased susceptibility to HIV-1	(McInally et al., 2021)
-	HIV-1	↑IL-10 ↑CXCL10	Increased risk of HIV-1 acquisition and transmission	(Kahle et al., 2015)

Skewing of cytokine production has been shown to be a risk factor for HIV-1 acquisition as shown in the table above. De Jong et al., reported that genital infections enhanced TNF-α production in vaginal and skin explants which consequently increased HIV-1 replication in Langerhans cells (De Jong et al., 2008). In addition, TNF-α is a proinflammatory cytokine which activates naïve T cells to Th1 cells which are targets of HIV-1 (Xiao et al., 1998). Plasmodium falciparum antigens have been reported to induce increased secretion of IFN-γ in macrophages (Pisell et al., 2002). IFN- γ is a proinflammatory cytokine which skews cytokine production in favour of a Th1 profile (Schoenborn & Wilson, 2007), characterized by activated Th1 $CD4^+$ T cells, which are HIV-1 target cells. McInally et al. demonstrated that an elevation in Fractalkine, IFN-inducible T cell α chemoattractant (ITAC) and IL-7 levels is predictive of eventual HIV-1 acquisition in the seronegative partner in a HIV-1 serodiscordant couple (Mcinally et

al., 2021). Fractalkine is the natural ligand for CX3CR1 expressed on T cells, monocytes and NK cells, inducing proliferation and migration of these cells to the site of infection (Hoshino et al., 2004). Fractalkine therefore avails activated T cells and monocytes for HIV-1 infection. ITAC is an IFN-inducing cytokine which promotes a proinflammatory response thus increasing activated $CD4^+$ T cells, the primary targets of HIV-1 (Swingler et al., 1999). IL-7 is involved in maintaining T cell homeostasis and maintaining T follicular helper cell function. Therefore, Fractalkine, ITAC and IL-7 have all been observed to increase in non-communicable diseases such as fibromyalgia (Bäckryd et al., 2017), oesophageal and colorectal cancers (Bednarz-Misa et al., 2019) and sarcoidosis (Arger et al., 2019). Increased levels of IL-10 and IP-10 were found to be associated with increased risk of HIV-1 acquisition (Kahle et al., 2015). IL-10 is a Th2 cytokine which skews the cytokine response in favour of a Th2 response thus dampening a Th1 response which is involved in anti-HIV-1 immunity (Michael Brown et al., 2005). Similar to ITAC, IP-10 is induced by IFN and attracts T cells and monocytes to sites of infection (Dufour et al., 2002). This chemokine activates and stimulates the trafficking of activated T cells to the site of infection thus facilitating influx of HIV-1 target cells and subsequent infection (Sankapal et al., 2016). Therefore, existing literature suggests that pre-existing cytokine perturbations may play a role in increasing susceptibility to HIV-1 acquisition in those at high-risk of infection.

2.6.2 Epidemiological evidence on effect of bystander infections on HIV-1 susceptibility

The sub-Saharan African population is exposed to a plethora of infectious diseases (World Health Organization, 2020a) and several observational studies have been conducted to unravel the effect of particular infections on HIV-1 acquisition. Genital inflammation/ulceration has been found to increase HIV-1 risk (Haaland et al., 2009;

Hope, 2018; Malhotra et al., 2011; Masson et al., 2015; Passmore & Jaspan, 2018; Roberts et al., 2012; Sabo et al., 2020; Wall et al., 2017). Sexually transmitted infections (STIs) contribute to increased genital inflammation (Levine et al., 1998; Li et al., 2009; Novak et al., 2007). A study conducted on serodiscordant Zambian couples found that STI-positive status of the HIV-1 negative partner was associated with increased likelihood to contract HIV-1 from an HIV-1 infected partner (Wall et al., 2017). Similarly, a study from South Africa reported a 3.2 increased odds of HIV-1 acquisition in women who recorded clinical evidence of genital inflammation as compared to those who did not exhibit genital inflammation (Masson et al., 2015). STIs are a risk factor for HIV-1 infection because STIs which present with skin and mucosal ulceration disrupt the mucosa thus easing the entry of HIV-1 into the mucosa following HIV-1 exposure. Furthermore, STI-induced inflammation triggers the recruitment of activated $CD4^+$ T cells to the genital mucosa which HIV-1 readily infects (Ward & Rönn, 2010).

Sub-Saharan Africa exhibits an ecological overlap between malaria transmission and HIV-1 transmission. A cross-sectional study conducted in Tanzania reported a malaria and HIV-1 prevalence of 12.3% and 7.9% respectively. Malaria infection was associated with 3.12 increased odds of acquiring HIV-1 compared to absence of malaria infection, as quoted in the publication referenced (Nielsen et al., 2006). A multi-site study in East Africa aimed to compare HIV-1 prevalence in low and high malaria transmission settings. It was reported that residents of high malaria transmission settings experience a 2.44-fold increased likelihood of being HIV-1 positive compared to residents of low malaria transmission settings (Cuadros et al., 2011). Conversely, Cuadros et al conducted a similar multi-site study in Ghana, Mali, Burkina Faso, Liberia, Guinea and Cameroon and did not find an association between malaria and HIV-1 transmission within the West African setting (Cuadros et al., 2011). These conflicting results may be attributed to lower HIV-1 prevalence in West Africa than in East Africa. Also, the different HIV-1 subtypes

circulating in the two regions as well as environmental and behavioral differences between the two settings may have contributed to the observed differences. It has been speculated that immune activation resulting from malaria may be one of the factors increasing the risk of HIV-1 acquisition. Compelling evidence from a mouse model have shown that malaria induces activation of T cells not only in systemic circulation, but also at the mucosal sites, namely the gut and genital tract (Chege et al., 2014). Stimulation of peripheral blood mononuclear cells (PBMCs) with malaria antigens induced expression of TNF-α and IL-6 (Xiao et al., 1998), proinflammatory cytokines which activate T cells, the primary targets of HIV-1.

Some studies have also found that *Schistosoma mansoni* (*S. mansoni*) infection also has an ecological overlap with HIV-1 transmission. A study in Tanzania found that *S. mansoni*-infected women have a 6-fold increased likelihood of being HIV-1 positive compared to women who were *S. mansoni* uninfected (Downs et al., 2012). A similar study in Uganda reported that participants who were seropositive for *S. mansoni* soluble egg antigens were more likely to be HIV-1 positive compared to those who were seronegative for *S. mansoni* soluble egg antigens (Stabinski et al., 2011). Likewise, a prospective cohort study from Zambia found that women seropositive for *S. mansoni* showed an increased HIV-1 risk compared to those who were not (Wall et al., 2018). *S. mansoni*-infected women may be at higher risk of HIV-1 acquisition than men because schistosome eggs tend to deposit and accumulate in the female genital organs such as the vulva, vagina and cervix. This leads to long-term bleeding and inflammation within this region resulting into recruitment of $CD4^+$ T cells to this area thus availing these cells to HIV-1 infection (Wall et al., 2018). On the contrary, cross-sectional and cohort studies conducted on a cohort living along the Lake Victoria shores in Uganda reported a lack of association between *S. mansoni* and HIV-1. Nonetheless, this study under-represented females who tested positive for *S. mansoni* [18 (21.42)] which may have underpowered

the ability to detect an association between *S. mansoni* and HIV-1, if *S. mansoni* predisposes only women to HIV-1 infection (Sanya et al., 2015; Ssetaala et al., 2015). The observed association may be attributed to *S. mansoni*-induced gut epithelial damage, which causes microbial translocation into systemic circulation. This increases blood lipopolysaccharide (LPS) levels which induce inflammation and increase the pool of activated T cells which promotes HIV-1 infection. Moreover, inflammation at the gut mucosal site may trigger inflammation at other mucosal sites such as the genital tract (Gill et al., 2010). Helminths also favor a Th2 response which inhibits a Th1 response important for HIV-1 immunity (Brown et al., 2006).

Therefore, it is highly suggestive that endemic infections within the sub-Saharan African setting may be associated with an increased predisposition to HIV-1 acquisition.

2.7 HIV-1 disease progression

During primary infection, the prevalent strain is the R5 strain, though with disease progression, X4 is detected and has been reported to correlate with disease progression and immunodeficiency (Connor et al., 1997). Within the primary and latency period, the infected individual does not show symptoms (clinical latency) but the virus is stealthily replicating in the cells (Swanstrom & Coffin, 2012) (Figure 6). This period is characterized by the direct as well as bystander killing of immune cells and dysregulation of the immune microenvironment to optimize viral replication (Wang et al., 2012). Antibodies specific for HIV-1 in plasma can be detected using serological techniques after three months, post infection (Naif, 2013). HIV-1 progresses slowly and the duration is dependent on host and viral factors (Naif, 2013). The association between the replication of HIV-1 and the immune activation that controls the replication, determines the plasma viral load (Blanchard et al., 1997) and is one of the best markers for disease progression (Saag et al., 1996). Initially, viral load exponentially increases, peaks in about

6 weeks before reducing to a stable level, known as the viral set point, and occurs between 3 and 6 months from infection and is also predictive of disease progression (McMichael et al., 2010). As viral replication continues, the more $CD4^+$ T cells are destroyed, therefore $CD4^+$ T cell count is also a good marker of disease progression (Criteria, 2010). Initiation of antiretroviral therapy was previously pegged on viral load and $CD4^+$ T cell counts. However renewed World Health Organization policies recommend immediate initiation of antiretroviral therapy upon HIV-1 detection (World Health Organization, 2016b). In the absence of antiretroviral therapy, $CD4^+$ T cell count continues to decline crossing a threshold where an individual is said to be in the Acquired immune deficiency syndrome (AIDS) phase (Naif, 2013), marked by extreme immunodeficiency, paving way for opportunistic pathogens to establish infection. There is also an exponential increase in viremia and eventually death (Swanstrom & Coffin, 2012) (Figure 6).

At the time of sample collection, ART initiation was based on T cell count and viral load with initiation in 2003 being in HIV-1-positive patients with 200 T cells/mm^3 (World Health Organization, 2002). This directive was revised in 2010, whereby the threshold was increased to 350 T cells/mm^3 (World Health Organization, 2010), and later to 500 T cells/mm^3 in 2013 (World Health Organization, 2013). However, in 2016, ART is administered on a test and treat basis as recommended by WHO (World Health Organization, 2016), regardless of the clinical stage.

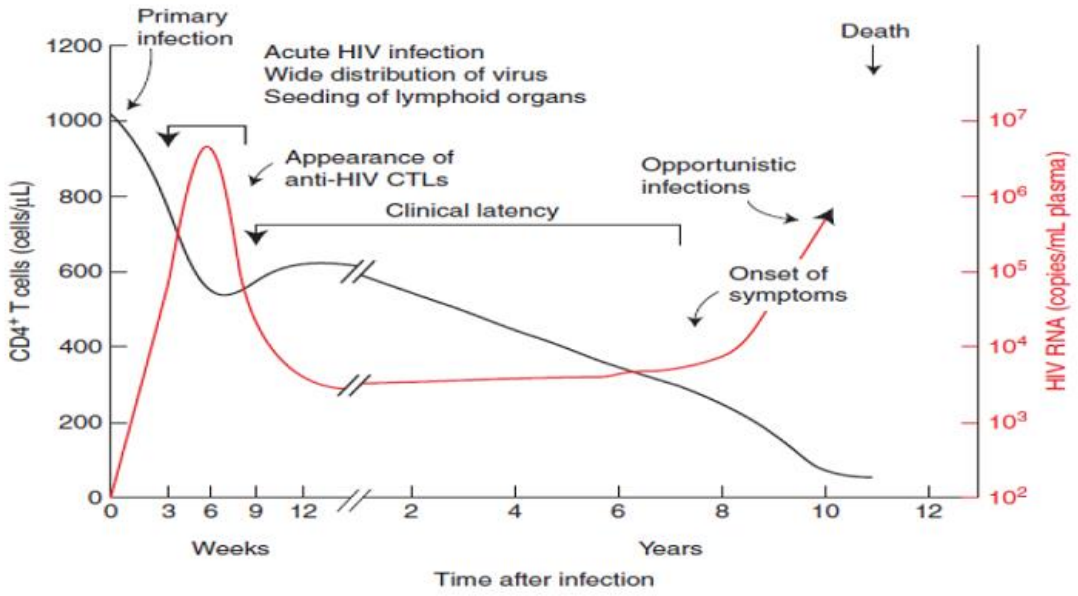


Figure 6: Time course of typical HIV-1 infection. (Swanstrom & Coffin, 2012) Patterns of $CD4^+$ T cell decline and viremia over a period of HIV-1 infection. Red curve represents HIV-1 RNA viral load in plasma. Black curve represents plasma $CD4^+$ T cell count. [CTLs-Cytotoxic T lymphocytes].

HIV-1 accounts for 95% of infections globally (AIDSmap, 2021). HIV-1 progresses much faster to AIDS than HIV-2. Majority of HIV-2 infected individuals remain as long-term non-progressors. It has been reported that immune activation and viral load is much lower in HIV-2 infection, suggesting HIV-2 induces an immune response which limits viral pathology, that is protective against disease progression (Nyamweya et al., 2013).

2.7.1 Epidemiological evidence on effect of bystander infections on HIV-1 disease progression

Besides impacting HIV-1 susceptibility, endemic infections have also been implicated in influencing HIV-1 progression after infection.

Wall et al conducted a study on HIV-1 discordant Zambian couples and reported a hazard ratio of 2.2 in HIV-1 and schistosomiasis co-infected women compared to women who were only HIV-1 infected (Wall et al., 2018). A randomized controlled trial from Kenya

enrolled HIV-1 infected participants with an *Ascaris lumbricoides* infection but albendazole-naïve. The study reported an increase in $CD4^+$ T cell count with treatment of the soil-transmitted helminth with albendazole. (Walson et al., 2008). Correspondingly, Wolday et al., found a strong positive correlation between the number of worm eggs excreted by HIV-1 infected participants and HIV-1 plasma viral load. Three months post helminth treatment translated into a significant decrease in HIV-1 viral load (Wolday et al., 2002). This is reflective of the role of helminths in accelerating HIV-1 disease progression. Conversely, a study from Zambia did not find an association between helminth treatment and low HIV-1 viremia in participants co-infected with intestinal helminths and HIV-1. This study was however limited by the low intensity of helminth infection in the study area (Modjarrad et al., 2005).

A cohort study conducted in Malawi found that HIV-1 infected participants who were diagnosed with malaria exhibited higher viral loads in comparison to those who did not contract malaria (Kublin et al., 2005). A similar study from Malawi also observed that HIV-1 infected participants with acute malaria exhibited higher HIV-1 viral load compared to those who did not have malaria (Hoffman et al., 1999).

Tuberculosis (TB) is often associated with HIV-1 infection. In a retrospective cohort study conducted in the United States, HIV-1 infected participants who presented with active TB had 2.17 higher odds of dying compared to HIV-1 positive individuals who did not have acute TB (C Whalen et al., 1995). These two infections have been reported to potentiate each other (Getahun et al., 2010; Pawlowski et al., 2012; C Whalen et al., 1995). TB is one of the causes of accelerated HIV-1 progression to AIDS (Corbett et al., 2003).

A study in Burkina Faso found that women dually infected with HIV-1 and herpes simplex virus type 2 (HSV-2) who subsequently received HSV-2 treatment reported a

significant reduction in plasma and genital HIV-1 viral load over time (Nagot et al., 2007). Similarly, men who have sex with men (MSM) receiving HSV-1 suppressive valacyclovir exhibited a reduction in seminal HIV-1 viral load in dually infected MSM (Zuckerman et al., 2009). Likewise, an episode of acute bacterial STI in HIV-1 infected commercial sex workers positively correlated with HIV-1 viremia (Anzala et al., 2000). Lastly, a study by Sadiq et al reported a 21% drop in $CD4^+$ T cell count during an episode of early latent syphilis (Sadiq et al., 2005).

Evidently, co-infection with HIV-1 has a significant effect on HIV-1 progression. The immune response induced as a consequence of other infections may promote replication of HIV-1 thus accelerating HIV-1 disease progression.

While previous studies have looked at the impact of individual infections on HIV-1 acquisition, with some showing associations and some not, this study seeks to assess the effect of baseline immune status on HIV-1 infection as this accounts for a potential mirror for a wider variety of infections. Secondly, the impact on the immune system may be long term and linger on even after the pathogen has been cleared but the insult on the immune system may persist predisposing one to HIV-1 acquisition. As a secondary objective, this study seeks to determine if pre-exposure to two common pathogens, malaria and CMV, are responsible for baseline immune profiles and if in themselves, are associated with HIV-1 acquisition.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design

A case-cohort study design was applied using data and samples collected from two cohorts sponsored by IAVI between 2003 and 2011 (Amornkul et al., 2013). These cohorts are part of a collaboration between IAVI and nine clinical research centres in East and Southern Africa (Kenya, Uganda, Rwanda, Zambia and South Africa). Volunteers recruited from the Kenyan site were included.

The first cohort referred to as Protocol B enrolled and followed up monthly HIV-1 high risk volunteers including men who have sex with men (MSM) and commercial sex workers (CSW). Volunteers who acquired HIV-1 infection during follow up despite receiving HIV-1 risk-reduction counseling and testing (Amornkul et al., 2013), were transferred to a secondary cohort, Protocol C, for quarterly follow up and HIV-1 care. HIV-1 infection was determined by HIV-1 specific antibody assays, RNA/PCR assay or the p24 antigen assay. This study identified volunteers with known seroconversion estimated dates and retrospectively selected their sample taken 3(+/-1) month prior to infection. Appropriate controls based on age, gender, risk group, duration in the study follow up were then selected amongst those in Protocol B who remained seronegative.

3.2 Study population

The study comprised of HIV-1 high risk population, including men who have sex with men (MSM) and commercial sex workers (CSW), between 18-49 years of age who were at high risk of HIV-1 acquisition (Kamali et al., 2015; Price et al., 2020).

Ethical approval: This study received ethical approval from Kenya Medical Research Institute Scientific Ethics Review Committee (KEMRI SERU), protocol number SSC 1027 and SSC 894. Written informed consent was obtained before commencement of the study.

3.3 Volunteer selection

Cases were first selected if they had been enrolled into protocol B and tested HIV-1 positive during follow up and had a plasma sample collected at 3 (+/-1) months prior to their estimated date of infection. For cases, plasma samples collected 3 (+/-1) months prior to HIV-1 infection were identified. Controls were matched 2:1 to cases by gender, age, risk group and follow-up time from enrolment into protocol B. For controls, plasma samples were also matched to case samples based on duration from enrolment into protocol B and calendar date of sampling (+/-1 month) (Figure 7). The freezer location coordinates of the specific selected samples were sought from the Laboratory Information Management System (LIMS) software which is a database for all stored cryopreserved samples.

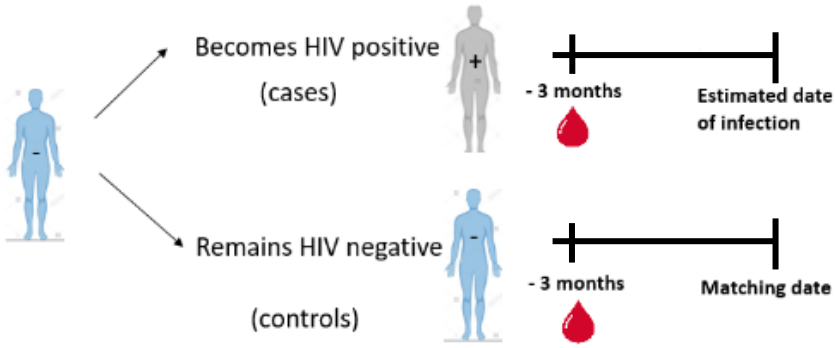


Figure 7: The IAVI cohort study follow up. The course of the case-cohort study and the time point at which plasma samples were analyzed for this study.

3.4 Sample processing

3.4.1 Assaying of cytokine and chemokine profile

To determine the cytokine and chemokine levels at the above time point, the Meso-Scale Discovery (MSD) multi-spot 37-plex assay was used (MesoScaleDiagnostics, LLC). Cryopreserved ethylenediamine tetra-acetic acid (EDTA)-plasma samples of the selected cases and controls were identified from the freezers and aliquoted for shipment to the IAVI Human Immunology Laboratory, London, UK, where the MSD assay was run.

The MSD platform allows for simultaneous analysis and quantification of many analytes in a single sample (multiplexing). This assay was conducted on a 96-well plate which contained 10 working electrodes denoted by 10 small spots in each well (Figure 8). Each spot represented a specific analyte to be assayed. The selected kit offered four multiplexing panels which target different analytes. The proinflammatory panel assays IFN-ϒ, IL-12 p70, IL-6, IL-1β, IL-8, IL-13, IL-2, IL-10, TNF-α and IL-4 (10 analytes). The cytokine panel assays GM-CSF, IL-12/23 p40, IL-17A, IL-1α, IL-15, TNF-β, IL-5, IL-16, VEGF-A, and IL-7 (10 analytes). The chemokine panel assays Eotaxin, IP-10, MCP-1, MIP-1β, MIP-1α, MDC, Eotaxin-3, IL-8, MCP-4 and TARC (10 analytes). The angiogenesis panel assays FGF, VEGF-A, PIGF, VEGF-C, Flt-1, Tie-2, and VEGF-D (7 analytes). The plates came with antibodies specific to the various analytes, each coated on a specific spot that was a working electrode surface. Twenty-five µl of calibrators and samples were added to the wells, in duplicates, sealed and incubated at room temperature for 2 hours with shaking. After incubation, the plates were washed 3 times with 300µl phosphate-buffered saline (PBS) and 0.05% Tween (PBS-T) to remove unbound compounds. Twenty-five µl of the detection reagent which contained the electrochemiluminescent tag (MSD-SULFO Tag)-conjugated antibody was added to each well, sealed and incubated for 1 hour with shaking to achieve the binding equilibrium (Figure 8). After incubation the plates were washed 3 times with 300 µl PBS-T. One hundred and fifty µl of the MSD read buffer was then added, so as to create a conducive environment for electroluminescence to occur. The plates were inserted into an MSD imager, where a voltage was applied to the plates, and this activated the MSD-SULFO tag, triggering a cascade of redox reactions resulting into emission of light by the tag, which was detected and quantified by the imager using a standard curve.

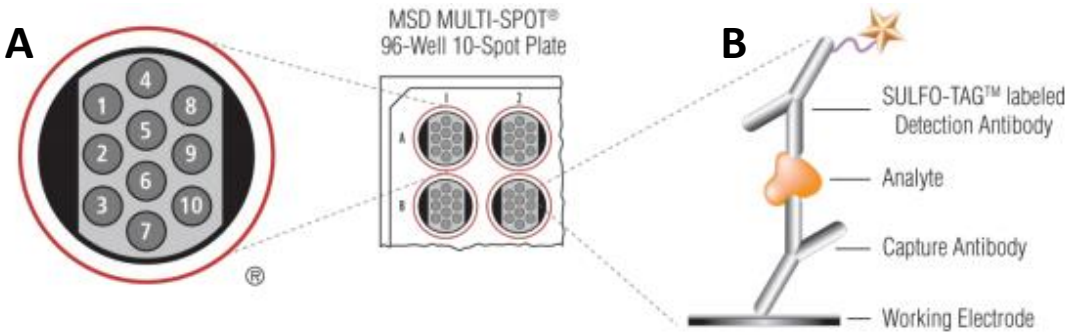


Figure 8: The set-up of the MSD assay**.** (MesoScale Discovery, LLC) A) The 10 MSD working electrode spots on which capture antibodies are coated. B) The resulting sandwich immunoassay system following addition of the sample containing the analyte and the detection antibody.

3.4.2 Preparation of schizont extract for coating ELISA plates to determine malaria exposure

To test for malaria exposure, a malaria parasite schizont extract ELISA was used. Schizont responses have been widely used as a marker of malaria exposure (Perraut et al., 2002). Measuring malaria plasma IgG antibodies as overall exposure as opposed to a current acute infection (IgM antibodies) provides more accurate information on the overall immunological insult caused by *Plasmodium falciparum* infection.

The schizont extract was prepared following malaria parasite culture, trophozoite harvesting and maturation. To ensure that the parasite cultures were not contaminated, mycoplasma testing was routinely performed. Each of these processes are elaborated below.

Parasite culture

A vial of 1 ml frozen malaria parasites was retrieved from the liquid nitrogen tank and thawed by rubbing the vial between gloved hands. In a biosafety cabinet, the vial was opened and the parasites transferred to a 15 ml Falcon tube, where 200 µl of 12% NaCl

was added to the parasites dropwise while shaking after every drop, followed by a 5-minute incubation. The same process was repeated, using 1.8% NaCl and subsequently 10 ml of 0.9% NaCl. This step-wise addition of decreasing concentrations of NaCl is to prevent the parasitized red blood cells (RBCs) from lysis due to rapid change in osmotic pressure and consequent influx of water into the cells (Blomqvist, 2008). The revived parasites were then centrifuged at 440xg for 5 minutes to separate the NaCl solution from the RBCs. The supernatant was aspirated out and the pellet resuspended in 10 ml incomplete media. Incomplete media comprised of 1X Roswell Park Memorial Institute Medium (RPMI) 1640 medium, 1M (4-(2-hydroxyethyl)-1-piperrazineethanesulfonic acid (HEPES), 10mg/ml gentamicin, 20% D [+] glucose, and 120 µM sodium hypoxanthine. RPMI is an artificial medium which contains vitamins, amino acids, glucose, salts and a pH indicator (SigmaAldrich). HEPES is a buffer which maintains physiological pH (Baicu & Taylor, 2002). Gentamicin is a prophylactic antibiotic which inhibits bacterial growth in the culture media (Fischer, 1975). Glucose is the source of carbon for the parasites and sodium hypoxanthine is the source of hypoxanthine required for parasite nucleic acid biosynthesis (WWARN, 2012). The resuspended parasites were then centrifuged at 440 x g for 5 minutes to wash the culture and get rid of any remaining NaCl solution. After centrifugation, the supernatant was aspirated, and the culture was resuspended in 10 ml of complete media comprising of incomplete media and 10% fetal calf serum (FCS) which offers more protein sources for parasite growth such as in bovine serum albumin (van der Valk et al., 2010). The culture was transferred to a 25 ml culture flask. The culture was then subjected to a gas mixture of 3% O_2, 5% CO_2, and 92% N_2 for 30 seconds and incubated at 37°C for 48 hours.

The culture was retrieved from the incubator and a 200 µl aliquot of the culture was transferred to an Eppendorf tube and centrifuged on pulse for 10 seconds to pellet the RBCs. The supernatant was discarded and 3 µl of the pellet was dispensed on a

microscope slide to make a thin blood smear. The slide was blow-dried, fixed with 3 ml of methanol for 30 seconds to fix the cells onto the slide, and blow-dried again. The dry slide was then flooded with 20% Giemsa in working phosphate buffer and allowed to stain the cells for 10 minutes. Giemsa is a differential stain composed of eosin which is an acidic dye and binds the basic components of the cell, and methylene blue which is a basic dye that binds the acidic components of the cell (Horobin & Walter, 1987). The Giemsa was washed off under slow running water and the slide blow-dried. The slide was then viewed under a light microscope using the X100 oil emulsion objective lens. Uninfected RBCs stained pink because of the alkaline cytosol and infected RBCs picked up methylene blue which binds acidic DNA. It is worth noting that uninfected RBCs do not possess any nucleic acid, but upon infection, the parasitic DNA can be spotted on the slide within the pink RBC. The parasitemia was calculated as the number of infected RBCs as a percentage of the total RBCs in 10 randomly-selected fields of view. The culture was maintained at 4% parasitemia to prevent the culture from crashing. If parasitemia rose above 4%, blood group O rhesus-positive RBCs collected from malaria-negative individuals, were added to the culture to provide the merozoites with adequate fresh RBCs to infect, following schizont maturation, RBC lysis and merozoite egression. To determine the volume of fresh RBCs to add to the culture, the formula $C_1V_1=C_2V_2$ was used.

Trophozoite harvesting

When the culture had attained 1500 µl of the RBC pellet and 8% parasitemia, with majority of the parasites at the trophozoite stage, the trophozoites were ready to be harvested using magnet-assisted cell sorting (MACS). The culture was centrifuged at 440 x g for 5 minutes to separate the culture media from the RBCs. The supernatant was aspirated and the pelleted RBCs were resuspended in 20 ml incomplete media and incubated at 37°C awaiting MACS.

MACS separation columns were fitted into the vario MACS magnetic support®. Each column was washed with 2 ml of incomplete media to equilibrate the column. The flow-through was discarded. The resuspended parasites were then added to the column, and flowed through at a slow rate, to allow the trophozoites to attach to the magnet. The MACS technique takes advantage of hemozoin production by parasites. The parasites utilized hemoglobin within the RBC for nutrition. Hemoglobin is a diamagnetic Fe (II) complex which is degraded by malaria parasites into paramagnetic hemozoin, an Fe (III) complex (Ribaut et al., 2008). This allows for the magnetic attraction and separation of trophozoites and schizonts which adhere to the magnetic column, whereas the 'younger' parasites in the ring stage and uninfected RBCs flow through the column. Three ml of incomplete media was layered over the culture, to elute any ring-stage parasite or uninfected RBCs which had adhered to the column. After all the incomplete media had passed through, 3 ml of incomplete media was added to the column and the magnet was detached and column inserted into a 15 ml Falcon tube to elute the attached trophozoites and schizonts into the tube. The eluted parasites were centrifuged at 440 x g for 5 minutes. The supernatant was discarded and the pellet was used to make a thin blood smear, and the parasitemia was calculated to be 97%.

The parasites were resuspended in 10 ml complete media, transferred to a 250 ml culture flask ~~a~~nd incubated at 37°C for 6 hours to allow the trophozoites to mature and segment. A thin blood smear was made and when majority of the trophozoites had segmented, 400 µl of 10 µM protease inhibitor trans-Epoxysuccinyl-L-leucylamido (4-guanidino) butane (E64) was added to the culture and continued to incubate at 37°C overnight to allow the schizonts to mature without rupturing. E64 maintains RBC membrane integrity, thus inhibiting merozoite egression from mature schizonts (Salmon et al., 2001).

Schizont lysate preparation

The next morning, the culture was centrifuged at 2000 x g for 5 minutes and the supernatant was aspirated. The schizont extract was resuspended in 50 µl of incomplete media and transferred into a 1 ml vial. To lyse the schizont extract, a freeze-thaw cycle was repeated twice by placing the vial at -80°C for 30 minutes to freeze, then removed to thaw. The lysate was further sonicated for 30 minutes to ensure that all the schizonts had lysed. The lysate was then stored at -80°C.

Mycoplasma testing for contamination

Mycoplasmas are prokaryotic micro-organisms which lack a cell wall, therefore can go undetected in a Giemsa-stained thin blood smear. Mycoplasmas are stubborn contaminants of parasite cultures which impair growth, inhibit enzyme activity and possess superantigen activity (Rowe et al., 1998). Therefore, it was crucial to maintain a malaria parasite culture free of Mycoplasma. The culture was checked for contamination using polymerase chain reaction (PCR), to test for the presence of Mycoplasma DNA, using the QIAamp Blood Mini Kit (QIAGEN).

DNA extraction

An aliquot of 200 µl of the culture was picked and transferred into an Eppendorf tube. Two µl QIAGEN protease was added to the sample and vortexed for 10 seconds to mix. Protease breaks down membrane proteins on the RBC membrane and histone proteins on which DNA is wrapped around within the nucleus. Two hundred µl of Buffer AL which was the lysis buffer required to disintegrate the phospholipid bilayer of the cell and nuclear membrane, was added to the sample, and the mixture was incubated at 56°C for 10 minutes, to create optimal temperature for the protease to act. Following incubation, 200 µl of 99% ethanol was added and vortexed for 15 minutes to precipitate DNA, because aqueous DNA segregates from ethanol, an organic solvent, which does not

dissolve DNA. The mixture was added to a QIAamp mini spin column containing a silica membrane, which binds DNA. The column was centrifuged at 2900 x g for 1 minute to force the mixture to flow down the column. The DNA was held back by the membrane, while the cell debris flows through to the collection tube. The collection tube containing the filtrate was discarded and a new 2 ml tube was fitted to the spin column. Five hundred μl of Buffer AW1 was added to the column and centrifuged at 2900 x g for 1 minute. The collection tube with the filtrate was discarded and replaced with a new one. The same was repeated using 500 μl of Buffer AW2 and centrifugation at 2900 x g for 3 minutes. The collection tube was discarded and replaced with a new one. The column was dry-centrifuged at 2900 x g for 1 minute to allow for the evaporation of any residual ethanol. The collection tube was discarded and replaced with a 1.5 ml microcentrifuge Eppendorf tube. Thirty μl of Buffer AE was added to the spin column and incubated at room temperature for 5 minutes, to elute the DNA (Gupta, 2019), after which the column was span at 2900 x g for 1 minute. The spin column was discarded and the eluted DNA carried forward to PCR.

Nested Polymerase Chain Reaction (PCR)

PCR is a technique which allows for the amplification of double-stranded DNA strands over a cycle of changes in temperature (Green & Sambrook, 2018). The master mix is a mixture of all the reagents required for DNA amplification, added to a PCR tube. The master mix contained 1.25 μl deoxyribonucleotide triphosphate (dNTPs), 1.12 μl primer mix, 1.5 μl $MgCl_2$, 5 μl NH_4 buffer, 38 μl PCR water and 1 μl Taq polymerase which is added to the master mix last because it is affected by changes in temperature, therefore only retrieved when needed. Nested PCR involves two sequential amplification reactions thus increases the sensitivity and specificity of PCR (Gupta, 2019). The different reactions use two different master mix solutions (master mix 1 and 2) which use a different set of primers; however, the rest of the reagents are similar. The reagents were

thawed by rubbing against two hands. Primer mix contains two sets of primers: forward primers which anneal to the template strand and reverse primers which anneal to the complementary strand of the denatured double-stranded DNA. The primers flank the locus of interest. Primers for the first round of PCR: Forward primer [5'-ACACCATGGGAG(C/T)TGGTAAT-3'], Reverse primer [5'-CTTC(A/T)TCGACTT(C/T)CAGACCCAAGGCAT-3']. Primers for the second round of PCR: Forward primer: [5'-GTC(C/G)GG(A/C)TGGATCACCTCCT-3'], Reverse primer: [5'-GCATCCACCA(A/T)A(A/T)AC(C/T)CTT-3'] (ThermoFischer). Taq polymerase is the enzyme which binds to the primer and catalyzes the synthesis of DNA from dNTPs, the building blocks of DNA. $MgCl_2$ provides $Mg2^+$ which is a cofactor enhancing the catalytic nature of Taq polymerase. NH_4 buffer provides the alkaline environment required for Taq polymerase activity. PCR water is a DNase, RNase and free of all contaminants which offers a medium for the reaction to occur (Lorenz, 2012).

Two µl of the sample was added to the master mix in the PCR tube and mixed by pipetting up and down. The tube was transferred to the thermocycler and DNA amplified under the following cycling conditions: Hot start temperature of 94°C for 3 minutes, denaturation temperature of 94°C for 30 seconds, 30 cycles of 50°C for 30 seconds each for hybridization, extension temperature of 72°C for 1 minute and a final extension temperature of 72°C for 10 minutes.

The same process was repeated with master mix 2, and the PCR product of reaction 1 as the sample for reaction 2 of the nested PCR.

Gel electrophoresis

To prepare a 1.5% agarose gel, 1.5 g of agarose powder was added to 100 ml of 0.5X TBE buffer in a 250 ml conical flask, and heated in a microwave to boil, for 1 minute. The flask was then left to cool, and 3.5 µl of RedSafe nucleic acid staining solution was

added. RedSafe binds and stains DNA enabling DNA visualization in the gel (Haines et al., 1989). The gel setting tank was assembled and a gel electrophoresis comb was fitted to create wells in the gel. The gel was gently poured into the assembled casting tray and allowed to set. After solidifying, the casting tray was put into the electrophoresis tank and 0.5XTBE buffer was poured into the tank, enough to submerge the gel. Two µl of loading dye was dispensed on a sheet of parafilm, and 10 µl of the sample was mixed with the dye, and loaded into the designated wells in the gel. The same was repeated for the control and the DNA hyperladder. Electrophoresis was run at 80V, 400A for 45 minutes. The DNA bands were visualized using the Bio-rad ChemiDoc XRS imaging system.

Schizont ELISA Optimization

In order to determine the optimum concentration at which to use the schizont extract to run the ELISA, the extract was titrated. Using 96-deep-well plates, schizont extract was serially diluted 2-fold in coating buffer, beginning with a dilution of 1:8. The different dilutions were then transferred to a 96-well plate in duplicates, at a volume of 50 µl and incubated overnight. Pooled hyper immune sera (PHIS) was used as the positive sample. PHIS is pooled from individuals residing in a malaria-endemic area and previously confirmed to have high antibody responses to malaria. The negative serum used for optimization was obtained from an individual residing in the United Kingdom who had no prior exposure to malaria and previously confirmed to be malaria antibody negative. The following morning, the plates were washed 4 times with 200 µl PBS-Tween to remove the unbound compounds, and banged to remove any residual PBS-Tween. A hundred µl blocking buffer was added to prevent non-specific binding and incubated at room temperature for 5 hours. The plates were then washed 4 times with 200 µl PBS-Tween and 100 µl of PHIS and the malaria-negative serum were added at 1:1000 dilution using blocking buffer. The plates were then incubated overnight at 4ºC. The following

day, the plates were washed 4 times with 200 µl PBS-Tween and 100 µl of a secondary antibody, horse radish peroxidase (HRP)-conjugated rabbit anti-human IgG added to each well and incubated for 3 hours. The o-Phenylenediamine dihydrochloride (OPD) substrate (100 µl) was added and reacted with the HRP enzyme to give a chromogenic yellow product and allowed to develop for 15 minutes. The reaction was then stopped by addition of 25 µl of 2 M H_2SO_4 and the optical density readings of the samples were read at 492 nm on a Synergy 4 (Bio Tek) plate reader. Figure 9 shows the results of the optimization process. The optimum schizont extract concentration to use for running the samples was 1 µl of extract in 1000 µl of coating buffer.

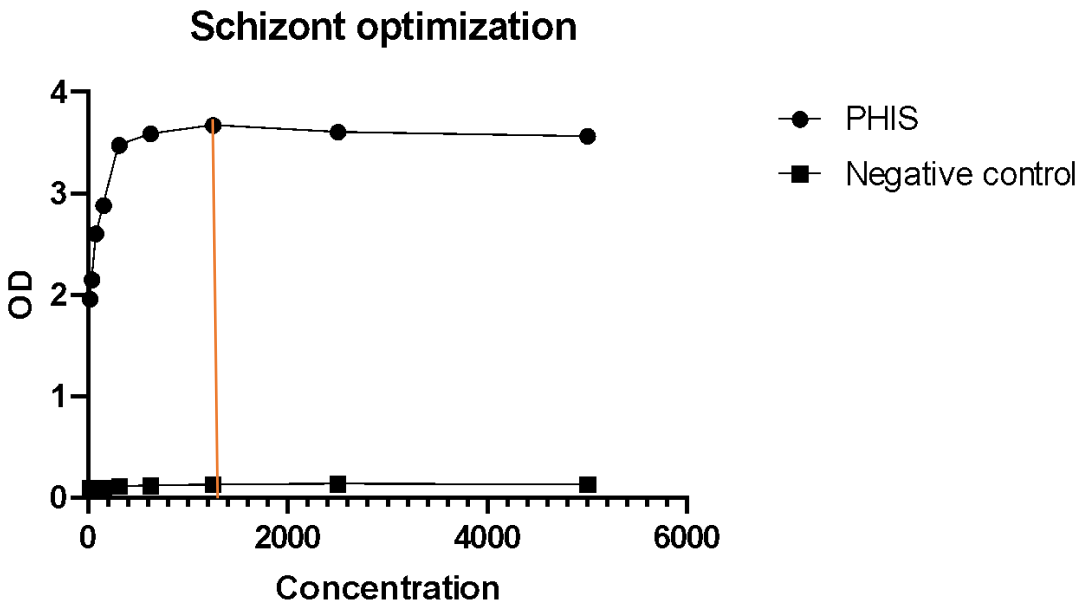


Figure 9: A dose-response curve for schizont lysate optimization**.** The curve with black circles represents titres of pooled hyper immune sera (PHIS) (positive control). The curve with black squares represents titres of malaria-naïve sera (negative control). The optimum concentration of schizont extract with the highest dynamic range was 1 µl of schizont extract in 1000 µl of coating buffer (1:1000). [OD-Optical Density].

Schizont ELISA

ELISA plates were coated with 50 µl of schizont extract in coating buffer and incubated overnight at 4ºC. The following day, the plates were washed 4 times with 200 µl PBS-Tween to remove the unbound compounds and banged on paper towel to remove residual PBS-Tween. A hundred µl of blocking buffer was added to prevent non-specific binding and incubated at room temperature for 5 hours. After incubation, the plates were washed 4 times with 200 µl PBS-Tween and 100 µl of the diluted standards, controls and samples (1:1000) were added to their respective wells on the plates, as per the plate map. The plates were then incubated overnight at 4ºC. The following day, the plates were washed 4 times with 200 µl PBS-Tween and 100 µl of a secondary antibody, horse radish peroxidase (HRP)-conjugated rabbit anti-human IgG added to each well and incubated for 3 hours. The o-Phenylenediamine dihydrochloride (OPD) substrate (100 µl) was added and reacted with the HRP enzyme to give a chromogenic yellow product and allowed to develop for 15 minutes. The reaction was then stopped by addition of 25 µl of 2 M H_2SO_4 and the optical density readings of the samples were read at 492 nm on a Synergy 4 (Bio Tek) plate reader.

3.4.3 Anti-CMV ELISA

Presence of IgG antibodies against CMV has also been used as a correlate of CMV exposure (Staras et al., 2006). Measuring CMV plasma IgG antibodies as overall exposure as opposed to a current acute infection (IgM antibodies) provides more information on the overall immunological insult caused by CMV.

Commercial ELISA plates pre-coated with lysed MRC-5 cells infected with the CMV AD169 strain were used for this assay (EUROIMMUN), following the manufacturer's instructions. In brief, 100 µl of calibrators, controls and diluted samples were added to the plates as per the plate map and incubated for 30 minutes at room temperature.

Following incubation, the wells were washed 3 times with 300 µl of wash buffer and banged to remove any remaining wash buffer. A hundred µl of HRP-conjugated IgG was added to each well and incubated at room temperature for 30 minutes. The plates were washed as before, and 100 µl of substrate solution was added to each well and incubated for 15 minutes at room temperature, away from light. The reaction was stopped by addition of 100 µl of stop solution to each well. The optical density readings of the samples were read at 450 nm on a Synergy 4 (Bio Tek) plate reader. Anti-CMV antibody concentrations in each sample were quantified from a standard curve constructed using the calibrators.

3.5 Data analysis approach

3.5.1 Distribution of volunteers

A flow chart to showcase the selection of cases and controls was developed. Then, a table comparing distribution of socio-demographic and clinical characteristics between cases and controls was presented. Continuous data were presented using median (interquartile ranges, IQR) and comparison between cases and controls done using Wilcoxon rank sum test. Categorical data were presented using frequency (percentage) and comparison between cases and controls done using chi-square test.

3.5.2 Specific objective 1

Analyte/cytokine concentration data was generated in duplicate and the respective upper (ULOQ) and lower (LLOQ) limit of quantification was availed. Analyte concentrations below the LLOQ were assigned half the LLOQ concentration, whilst those above the ULOQ were assigned the ULOQ value. A mean of the duplicate observations was obtained and carried forward to the analysis. Correlation between the duplicate observations was assessed using correlation tests and visualized using scatter plots.

Log_{10} analyte transformations were done. Comparisons for all analytes between cases and controls were done using Wilcoxon rank sum tests and results presented in dot plots. Further, multivariable linear regression models were applied to identify analytes independently associated with HIV-1 acquisition after controlling for gender and age.

Cytokines that were significantly different between cases and controls, were carried forward to principal component analysis to delineate clustering patterns that helped explain underlying related immunological pathways. These cytokine clusters (or components) were then taken forward to multivariable linear regression models to assess their independent predictive value for HIV-1 acquisition.

3.5.3 Specific objective 2

Schizont extract antibody data were used to determine levels of exposure to malaria. Arbitrary units representative of malaria antibody titres within the samples were read from a PHIS-generated standard curve by interpolation. Prevalence of malaria was determined based on the number of volunteers who exhibited malaria antibody titres above the seropositivity threshold (negative control+2 standard deviations, SD). Comparison of antibody levels (continuous variable) between cases and controls was then done using Wilcoxon rank sum test. A stratification of malaria antibodies based on malaria seroprevalence (categorical variable) was done, and categories compared between cases and controls using Wilcoxon rank sum test.

For CMV, the same was repeated but with the standard curve being generated from a calibration curve and the positivity cut-off was set at 2,200 RU/ml, as per the manufacturer's instructions (EUROIMMUN). Prevalence was also calculated based on the volunteers who were found to be CMV seropositive. The CMV antibody titre data were compared between cases and controls using Wilcoxon rank sum test. Further, the CMV antibody titre data were stratified into two groups, volunteers with low and those

with high CMV antibody titres, based on the median as the cut-off. The stratified antibody data were then compared between cases and controls using Wilcoxon rank sum test. The data were stratified to determine whether the level of pathogen exposure impacts HIV-1 acquisition.

3.5.4 Specific objective 3

Malaria antibody data (continuous data) were assessed for association with cytokine concentration data using Pearson correlation. The results were presented using a correlation matrix. In addition, cytokine concentration data was compared between malaria seropositive and seronegative volunteers using Wilcoxon rank sum test. The results were presented using a radar plot.

Similarly, for CMV, antibody titre data were assessed for correlation with cytokine concentration data using Pearson correlation. The results illustrated using a correlation matrix. In addition, cytokine data was compared between volunteers with low CMV antibody titres and those with high CMV antibody titres using Wilcoxon rank sum test. The results were illustrated using a radar plot.

Statistical analyses were done using Stata/IC version 17 (StataCorp LP, California). Outputs were generated using GraphPad Prism version 7.0 (GraphPad Software, California).

CHAPTER FOUR: RESULTS

4.1 Study population baseline characteristics

Overall, 1,977 volunteers were enrolled and followed up under protocol B. Of these, 142 tested HIV-1 positive during follow up and 124 had a sample available at 3(+/-1) months pre-infection. Out of these, 97 volunteers had 2 or more eligible matched controls available. Of the selected samples, 89 cases and 190 controls' locations were found on LIMS. These samples were then searched for in the freezer. As a result, 80 cases and 160 controls were traced. However, only 47 of the 80 cases had 2 or more matched controls available in the freezer. Therefore, following the rigorous selection process, 47 cases and 94 controls met the selection criteria and were traced, hence carried forward to the analysis (Figure 10).

There was no significant difference between cases and controls based on comparison by gender (male, 78.2% vs. 78.2%, p=0.999), median age (23.9 vs. 23.7 years, p=0.910), risk group (MSM-W, 66.0% vs. 66.0%, p=0.827), median follow-up time (4.4 vs. 3.8 months, p=0.999), marital status (single, 83.0% vs. 84.0%, p=0.425), education level (None, 6.4% vs. 3.2%, p=0.741) and circumcision status (amongst men only: yes, 86.5% vs. 86.5%, p=0.741) respectively (Table 3), implying that matching of the cases and controls was achieved.

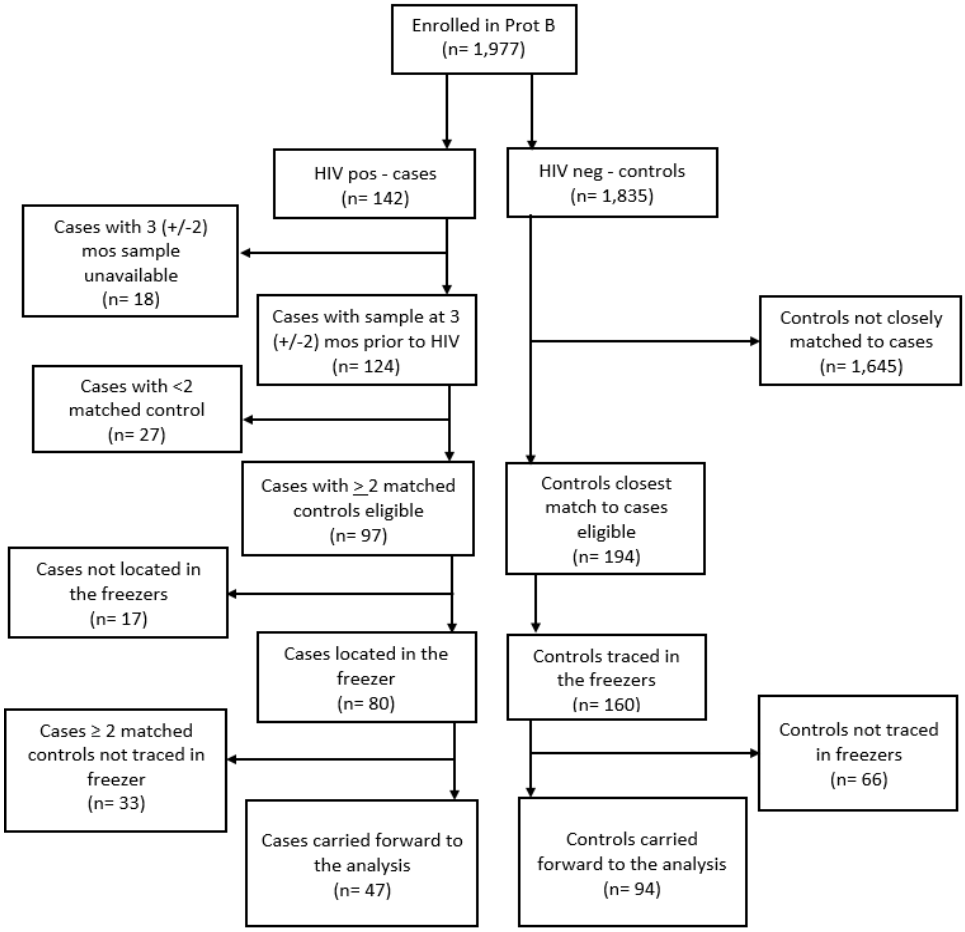


Figure 10: Flow chart of volunteers enrolled into Protocol B, followed up over time and included in the analysis based on sample availability. [*Prot B (Protocol B); HIV pos (HIV-1 positive); HIV neg (HIV-1 negative)*]

Table 3: Baseline characteristics. Description of social, demographic and clinical characteristics of volunteers at high risk of HIV-1 acquisition from Coastal Kenya (n=141). Comparison between cases and controls matched by gender, age, risk group and follow-up time. *Risk group categories: [MSM-W (men who have sex with men and women), MSM-E (men who have sex with men exclusively), MSW-E (men who have sex with women exclusively/heterosexual males), WSM-E (women who have sex with men exclusively/heterosexual females)]; IQR (Interquartile range)].*

**From enrolment into protocol B to date of sampling*

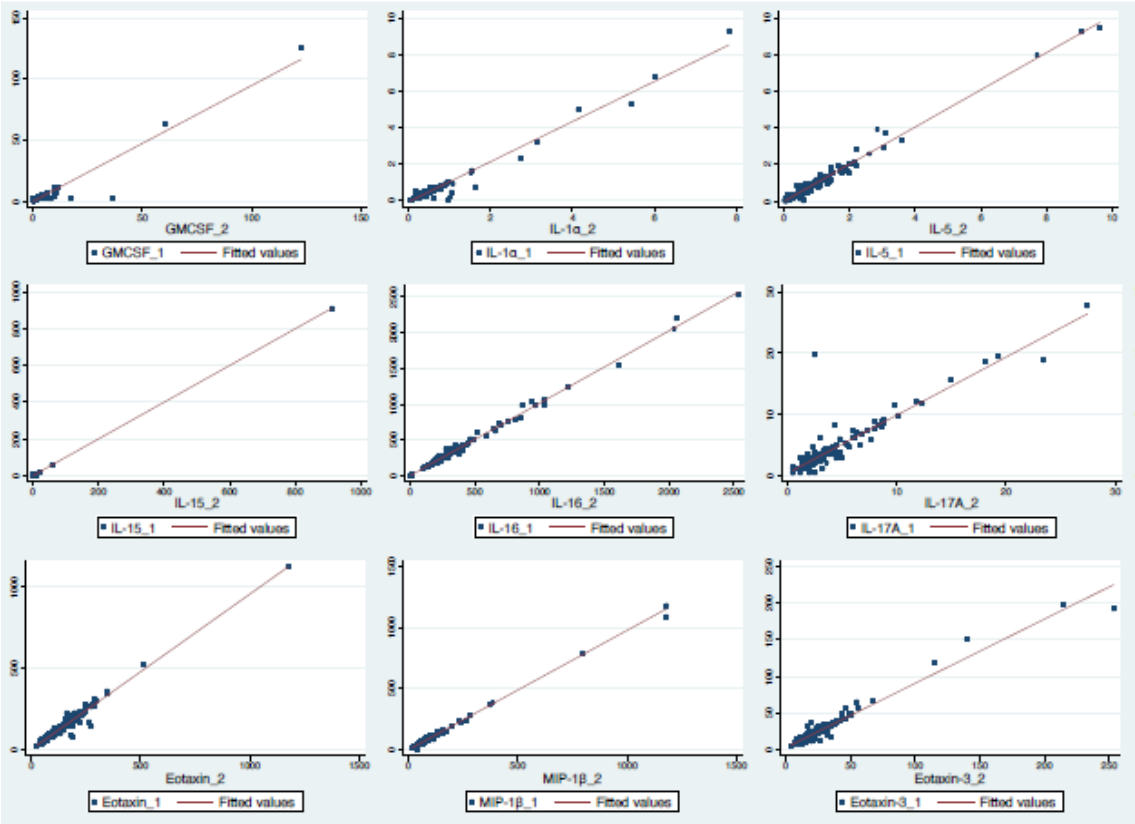
***Applies for male volunteers only*

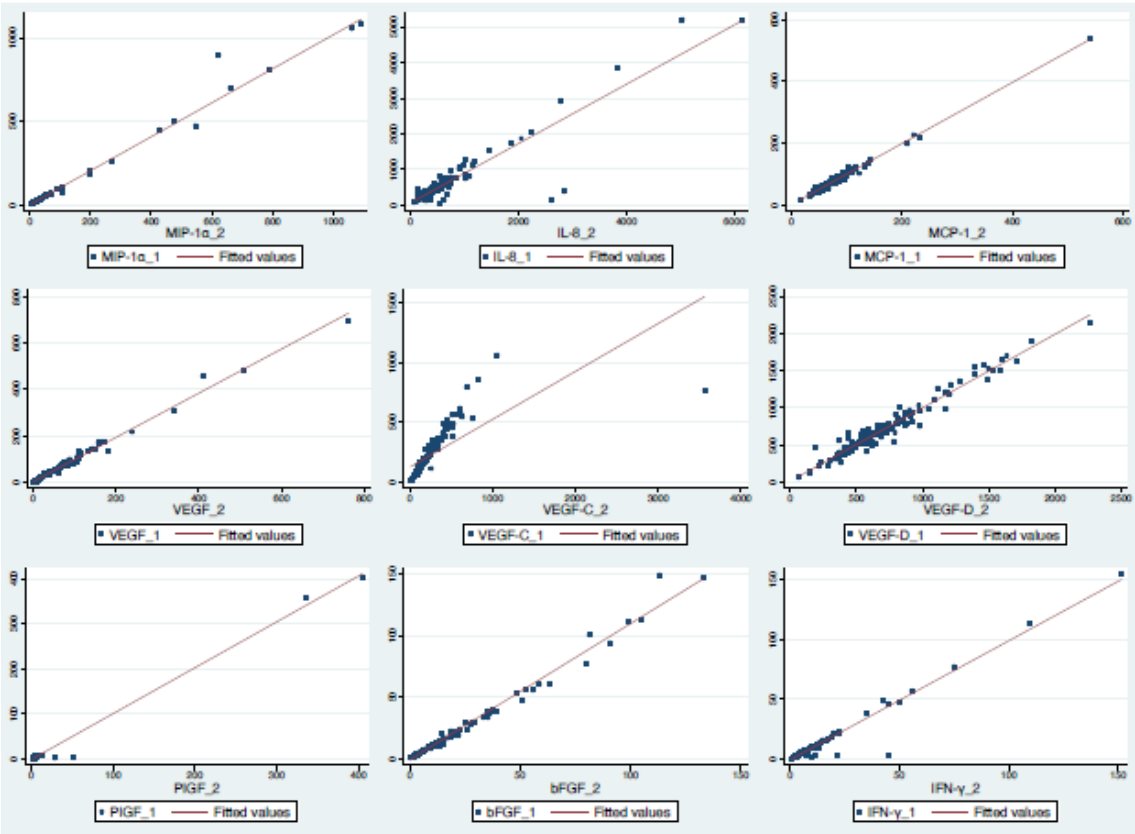
Characteristic	**Grouping**	**Cases (n=47)**	**Controls (n=94)**	**P-value**
Gender	Male Female	37 (78.72) 10 (21.28)	74 (78.72) 20 (21.28)	0.999
Age in years	Median (IQR)	23.86 (21.25 - 26.69)	23.74 (21.34 - 26.55)	0.910
Age group in years	18.0 – 24.9 25.0+	28 (59.57) 19 (40.43)	56 (59.57) 38 (40.43)	0.999
Risk group	MSM-W MSM-E MSW-E WSM-E	31 (65.96) 3 (6.38) 3 (6.38) 10 (21.28)	62 (65.96) 6 (6.38) 6 (6.38) 20 (21.28)	0.827
Follow-up time in months*	Median (IQR)	4.40 (0 - 13.57)	3.83 (0 - 12.85)	0.999
Follow-up time in months (group)*	0 – 5.9 6.0+	27 (57.45) 20 (42.55)	54 (57.45) 40 (42.55)	0.886
Marital status	Single Married Widowed	39 (82.98) 4 (8.51) 4 (8.51)	79 (84.04) 6 (6.38) 9 (9.57)	0.425

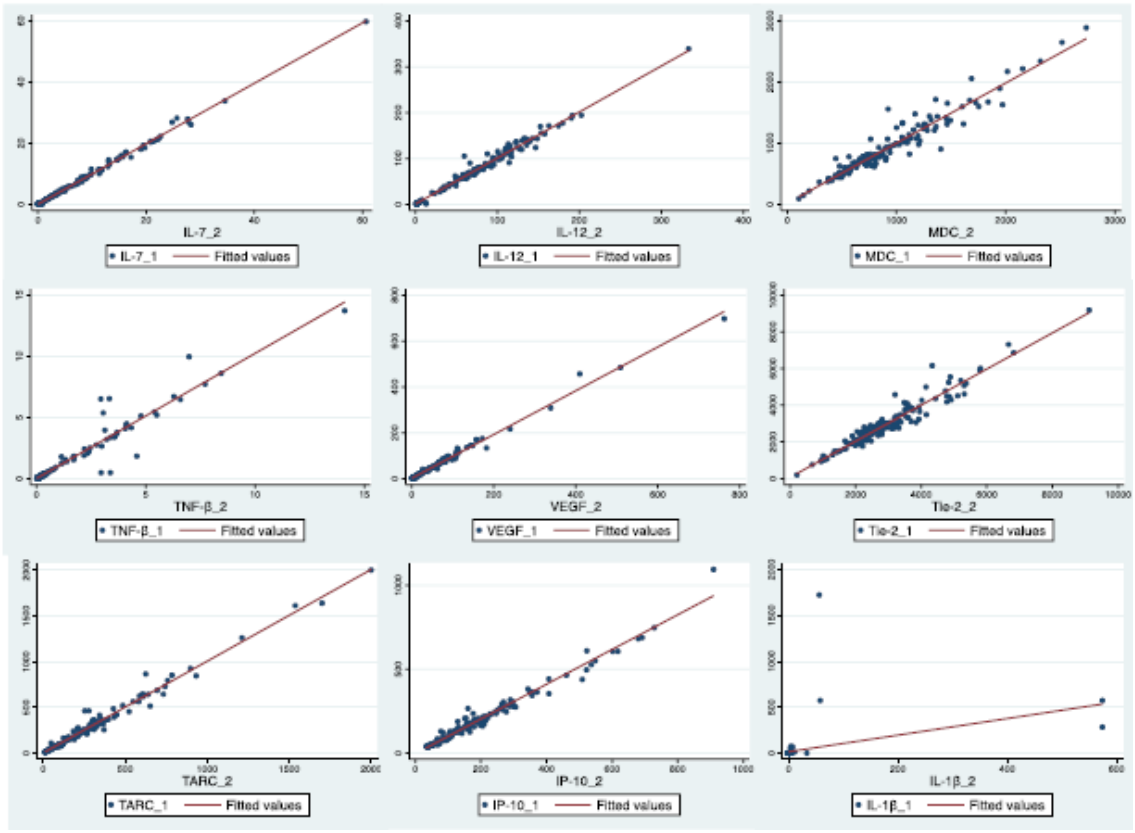
Characteristic	Grouping	Cases (n=47)	Controls (n=94)	P-value
Education level	None Primary Secondary Higher	3 (6.38) 17 (36.17) 23 (48.94) 4 (8.51)	3 (3.19) 47 (50.00) 37 (39.36) 7 (7.45)	0.741
Circumcision status**	No Yes Missing	4 (10.81) 32 (86.49) 1 (2.70)	6 (8.11) 64 (86.49) 4 (5.41)	0.741

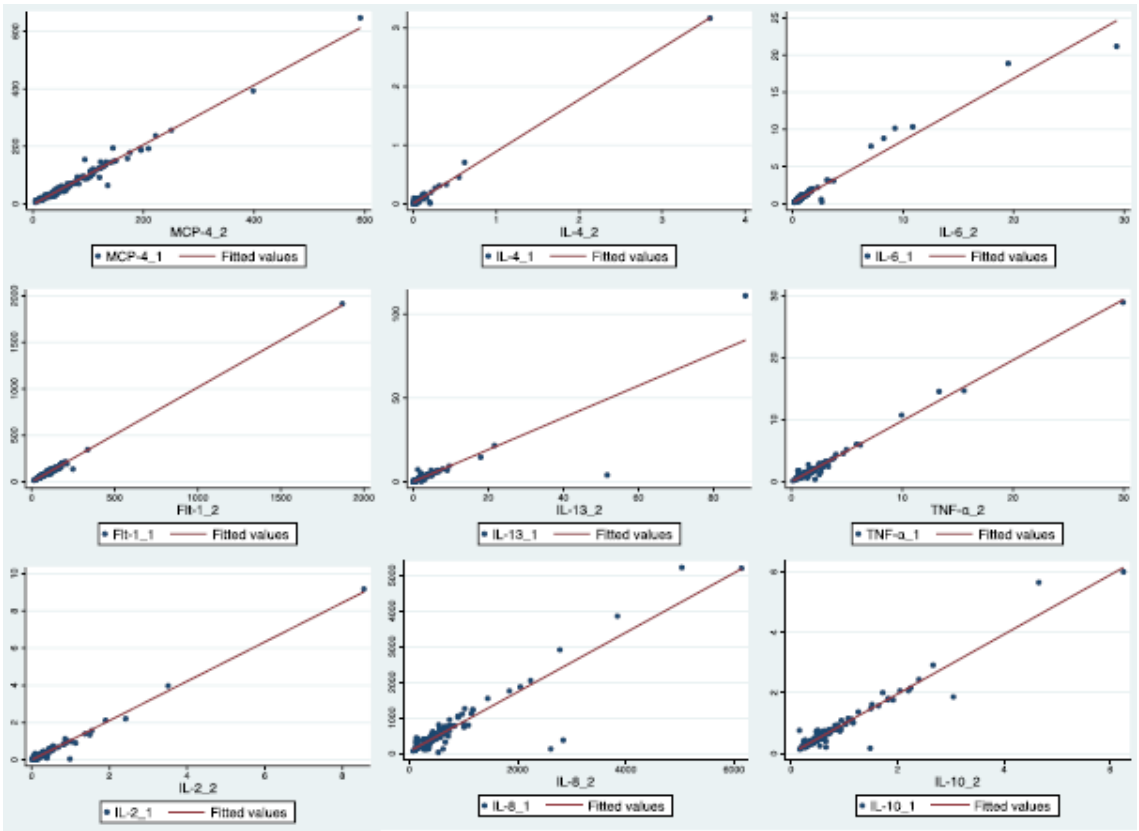
4.2 Measurement of cytokine levels

Following the MSD assay described in chapter 3 section 3.4, data were cleaned and quality checked. To do this, correlation between the duplicate pairs of each cytokine was assessed and a threshold of acceptability was set at correlation coefficient (rho) $\geq$ 0.9 (Figure 11). Only 3 analytes, VEGF-C (rho=0.72), IL-1β (rho=0.38) and IL-13 (rho=0.88) reported rho < 0.9 between the duplicate assays, suggesting excellent duplicity in the data and justifying the move to aggregate the duplicate into a mean value for downstream analysis. Outlier measurements from the specific scatter plots were also assessed (n = 7) and were excluded from the analysis to avoid biased downstream effects on the data output such as wide variance.


GMCSF_2
GMCSF_1 Fitted values
IL-1α_2
IL-1α_1 Fitted values
IL-5_2
IL-5_1 Fitted values
IL-15_2
IL-15_1 Fitted values
IL-16_2
IL-16_1 Fitted values
IL-17A_2
IL-17A_1 Fitted values
Eotaxin_2
Eotaxin_1 Fitted values
MIP-1β_2
MIP-1β_1 Fitted values
Eotaxin-3_2
Eotaxin-3_1 Fitted values


MIP-1α_2
MIP-1α_1 Fitted values
IL-8_2
IL-8_1 Fitted values
MCP-1_2
MCP-1_1 Fitted values
VEGF_2
VEGF_1 Fitted values
VEGF-C_2
VEGF-C_1 Fitted values
VEGF-D_2
VEGF-D_1 Fitted values
PlGF_2
PlGF_1 Fitted values
bFGF_2
bFGF_1 Fitted values
IFN-γ_2
IFN-γ_1 Fitted values


IL-7_2
IL-7_1 Fitted values
IL-12_2
IL-12_1 Fitted values
MDC_2
MDC_1 Fitted values
TNF-β_2
TNF-β_1 Fitted values
VEGF_2
VEGF_1 Fitted values
Tie-2_2
Tie-2_1 Fitted values
TARC_2
TARC_1 Fitted values
IP-10_2
IP-10_1 Fitted values
IL-1β_2
IL-1β_1 Fitted values


MCP-4_2
MCP-4_1 Fitted values
IL-4_2
IL-4_1 Fitted values
IL-6_2
IL-6_1 Fitted values
Flt-1_2
Flt-1_1 Fitted values
IL-13_2
IL-13_1 Fitted values
TNF-α_2
TNF-α_1 Fitted values
IL-2_2
IL-2_1 Fitted values
IL-8_2
IL-8_1 Fitted values
IL-10_2
IL-10_1 Fitted values

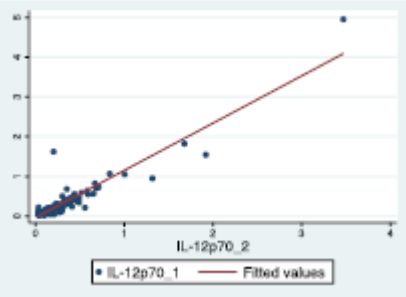
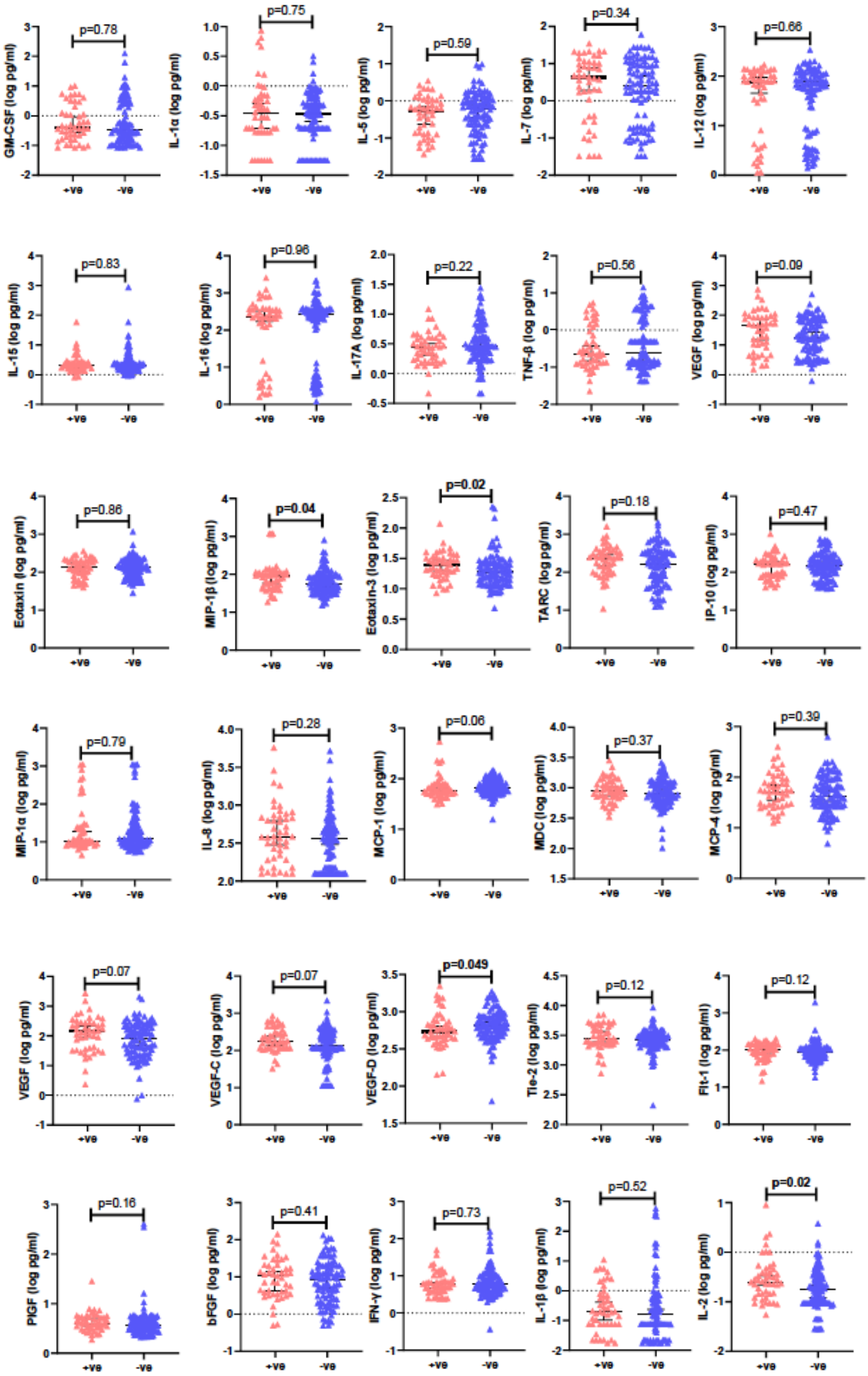


Figure 11: Scatter plots illustrating correlation between the two analyte duplicates of the MSD assay. The first duplicate (y axis), the second duplicate (x axis). Red line represents the line of best fit. Each dot represents the concentration of the first replicate (y axis) and the second replicate (x axis) of the analyte in the particular sample. The data points which are separated from the majority, are outliers.

Mean cytokine concentrations for all the analytes were then compared between cases and controls (Wilcoxon rank sum test, $p<0.05$ considered significant) (Figure 12). Of the 37 measured cytokines, MIP-1β ($p=0.04$), Eotaxin-3 ($p=0.02$), IL-12p70 ($p=0.04$), IL-2 ($p=0.02$), IL-4 ($p=0.008$), were significantly elevated in cases compared to controls, while VEGF-D ($p=0.049$) was significantly lower in the cases compared to the controls (Figure 13).


GM-CSF (log pg/ml)
p=0.78
IL-1α (log pg/ml)
p=0.75
IL-5 (log pg/ml)
p=0.59
IL-7 (log pg/ml)
p=0.34
IL-12 (log pg/ml)
p=0.66
IL-15 (log pg/ml)
p=0.83
IL-16 (log pg/ml)
p=0.96
IL-17A (log pg/ml)
p=0.22
TNF-β (log pg/ml)
p=0.56
VEGF (log pg/ml)
p=0.09
Eotaxin (log pg/ml)
p=0.86
MIP-1β (log pg/ml)
p=0.04
Eotaxin-3 (log pg/ml)
p=0.02
TARC (log pg/ml)
p=0.18
IP-10 (log pg/ml)
p=0.47
MIP-1α (log pg/ml)
p=0.79
IL-8 (log pg/ml)
p=0.28
MCP-1 (log pg/ml)
p=0.06
MDC (log pg/ml)
p=0.37
MCP-4 (log pg/ml)
p=0.39
VEGF (log pg/ml)
p=0.07
VEGF-C (log pg/ml)
p=0.07
VEGF-D (log pg/ml)
p=0.049
Tie-2 (log pg/ml)
p=0.12
Flt-1 (log pg/ml)
p=0.12
PlGF (log pg/ml)
p=0.16
bFGF (log pg/ml)
p=0.41
IFN-γ (log pg/ml)
p=0.73
IL-1β (log pg/ml)
p=0.52
IL-2 (log pg/ml)
p=0.02
+ve
-ve

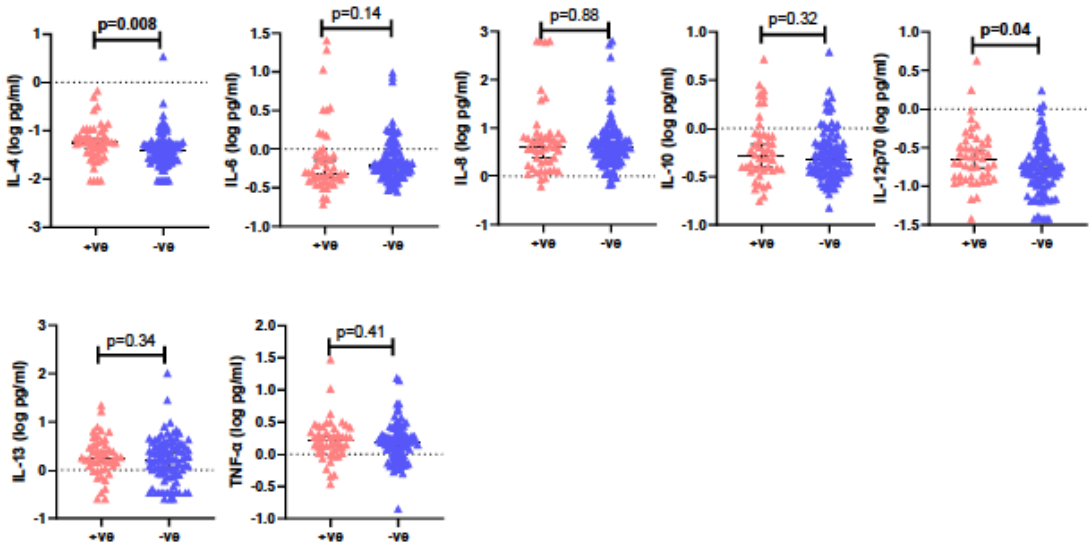


Figure 12: Dot plots showing comparison of cytokine concentrations between cases and controls. *+ve (orange): volunteers who contracted HIV-1 during follow up (cases), -ve (blue): volunteers who remained HIV-1 negative (controls).* (Wilcoxon rank-sum test, two-tailed, $p<0.05$ considered significant, median log pg/ml (95% confidence interval), n=141).

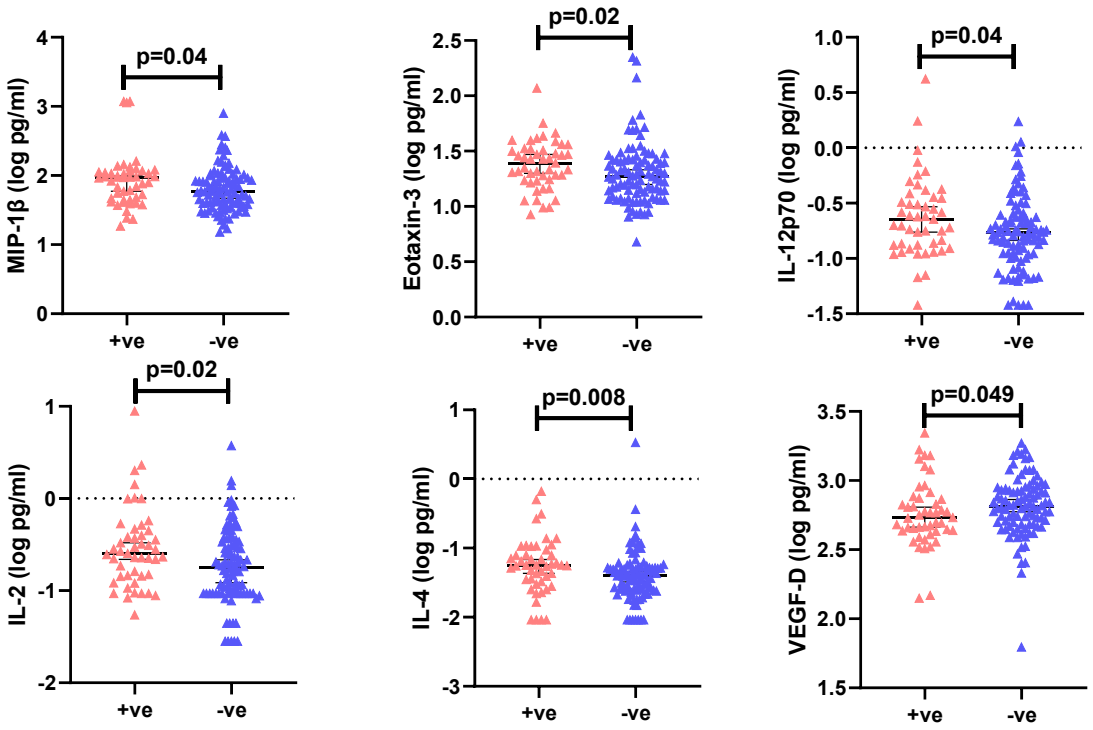


Figure 13: Dot plots showing cytokine concentrations found to be significantly different between cases and controls. *+ve (orange): volunteers who contracted HIV-1 during follow up (cases), -ve (blue): volunteers who remained HIV-1 negative (controls).* (Wilcoxon rank-sum test, two-tailed, $p<0.05$ considered significant, median log pg/ml (95% confidence interval), n=141).

4.2.1 Association between elevated cytokines and HIV-1 acquisition

To determine whether the six cytokines that were significantly different in the cases and controls were associated with HIV-1 acquisition, linear regression was used (Table 4). While the Wilcoxon rank sum test compares cases and controls to identify significant differences between cases and controls, linear regression allows us to further determine whether the observed difference is associated with HIV-1 acquisition. In the bivariate analysis, IL-2 (p=0.01), IL-4 (p=0.04) and IL-12p70 (p=0.03) showed significant association with HIV-1 acquisition, however in the multivariate analysis, none of the

cytokines showed significant association with HIV-1 acquisition ($p<0.05$ considered significant) (Table 4).

Table 4: Linear regression analysis showing the association between significantly elevated cytokines and HIV-1 acquisition (n=141). [*95% CI (95% Confidence interval); Crude coefficient: Bivariate analysis; Adjusted coefficient: Multivariate analysis*]. *Adjusted for gender and age.*

Cytokine	**Crude coefficient (95% CI)**	**P-value**	**Adjusted coefficient (95% CI)**	**P-value**
MIP-1β	1.00 (-0.04 - 2.04)	0.06	**…**	**…**
Eotaxin-3	1.09 (-0.25 - 2.44)	0.11	**…**	**…**
IL-2	1.11 (0.24 – 1.99)	0.01	0.84 (-0.13 – 1.81)	0.09
IL-4	0.99 (0.04 – 1.95)	0.04	0.40 (-0.90 – 1.69)	0.55
IL-12p70	1.12 (0.09 – 2.15)	0.03	0.38 (-1.09 – 1.86)	0.61
VEGF-D	-1.05 (-2.63 – 0.52)	0.19	**…**	**…**

4.2.2 Principal component analysis of cytokines

As cytokines tend to work interdependently and/or synergistically, I sought to delineate clustering patterns that would elucidate potential immunological pathways that may be involved in immune activation. To do this, measured cytokines levels were included in a principal component analysis (PCA). Only cytokines that showed significant differences ($p<0.1$ considered significant) between cases and controls were included in the PCA.

Using a threshold of $p < 0.1$, cytokines were selected for building the structural PCA. Ten cytokines, VEGF (p=0.09), MIP-1β (p=0.04), Eotaxin 3 (p=0.02), MCP-1 (p=0.06), VEGF (p=0.07), VEGF-C (p=0.07), VEGF-D (p=0.049), IL-2 (p=0.02), IL-4 (p=0.008),

IL-12p70 (p=0.02) met the criterion and were carried forward to PCA. A Kaiser-Meyer-Olkin (KMO) value of 0.69 suggested sufficient variation in the ten analytes to justify the use of PCA for these data. Using a scree plot to determine the number of principal components to be retained for the PCA, 4 components had eigenvalues above 1 (Figure 14). Eigenvalues quantify how much variance each principal component explains. Therefore, a 4-component PCA was selected using the eigenvalue >1 rule.

Overall, PCA clustered the 10 analytes into four components comprising PC1 (comprising VEGF, MIP-1β, VEGF-C and IL-4), PC2 (comprising MCP-1β, IL-2 and IL-12p70), PC3 (comprising VEGF-D) and PC4 (comprising Eotaxin-3).

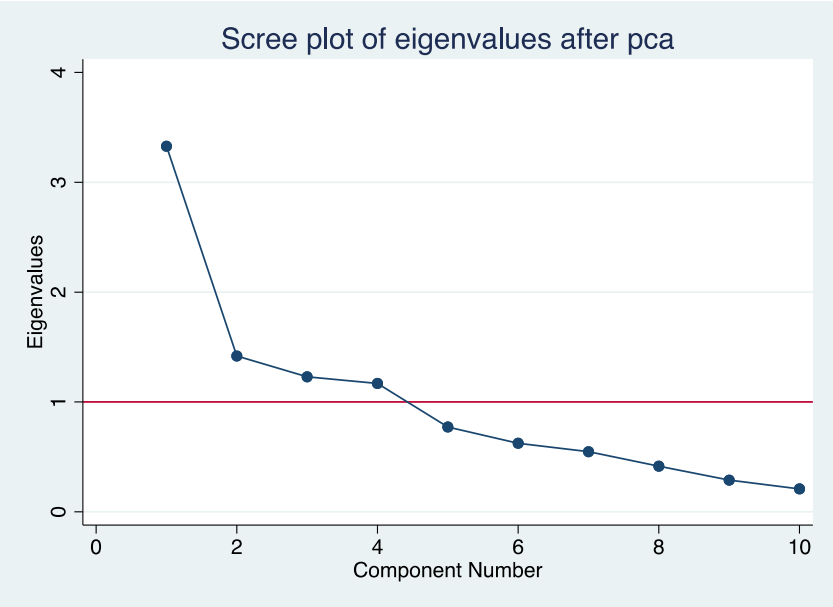


Figure 14: A scree plot showing eigenvalues from factor analysis. Ten principal components (x axis) plotted against their corresponding eigenvalues (y axis). Four principal components above the red line were retained for PCA.

The 4 principal components (PCs) were carried forward to linear regression. PC1 was found to be significantly associated with HIV-1 acquisition after controlling for age and gender (adjusted coefficient, (95% CI), p-value: 0.06 [0.02 – 0.11], p=0.004) (Table 5). Cytokines comprising PC1 include VEGF, MIP-1β, VEGF-C and IL-4 (Figure 15).

Therefore, a unit increase in cytokines with the highest component loading in PC1 corresponds to a 0.06 increased risk for HIV-1 acquisition. PC3 comprising VEGF-D was also found to be significantly associated with HIV-1 acquisition after controlling for age and gender (adjusted coefficient, (95% CI), p-value: -0.07, [-0.14 – -0.002], p=0.040, Table 5). Only VEGF-D had the highest component loading in PC3. Therefore, for every unit increase in VEGF-D, HIV-1 acquisition decreases by 0.07. On the other hand, PCs 2 and 4 were not significantly associated with HIV-1 acquisition.

Table 5: Linear regression analysis representing the association between the 2 clusters of the selected 14 cytokines and HIV-1 acquisition (n=141). [*PC (Principal component); 95% CI (95% Confidence interval); Crude coefficient: Bivariate analysis; Adjusted coefficient: Multivariate analysis]. Adjusted for gender and age.*

Cytokine	**Crude coefficient (95% CI)**	**P-value**	**Adjusted coefficient (95% CI)**	**P-value**
PC1	0.06 (0.02 – 0.11)	0.003	0.06 (0.02 – 0.11)	0.004
PC2	-0.008 (-0.07 – 0.06)	0.808	**…**	**…**
PC3	-0.07 (-0.14 – (-0.001))	0.048	-0.07 (-0.14 – (-0.002))	0.040
PC4	0.015 (-0.05 – 0.09)	0.680	**…**	**…**

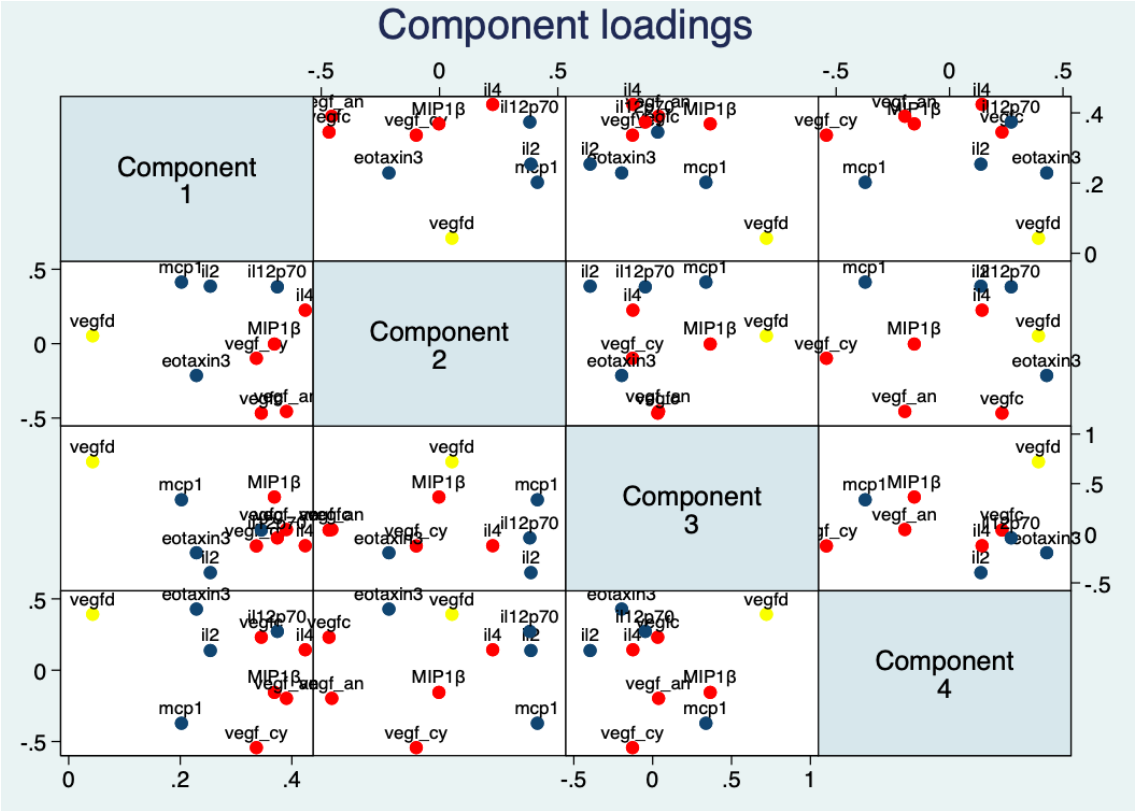


Figure 15: PCA loading multi-plot showing the clusters associated with HIV-1 acquisition. Bottom axis from the left: PC1, PC2, PC3 scores. Left axis from the top: PC2, PC3, PC4 scores. Top axis from the left: PC2, PC3, PC4 scores. Right axis from the top: PC1, PC2, PC3 scores. The plot illustrates how each of the four components relate to each other. The four cytokines coloured red in each plot, VEGF, MIP-1β, VEGF-C and IL-4, represent PC1 cytokines. The cytokine coloured yellow in each plot represents PC3 cytokine, VEGF-D. Both PC1 and PC3 have shown significant association with HIV-1 acquisition, as clusters ((adjusted coefficient, 0.06, (confidence interval, 0.02 – 0.11), p=0.004) and (adjusted coefficient, -0.07, (confidence interval, -0.14 – (-0.002)), p=0.040) respectively) [vegf_cy: VEGF assayed in the cytokine panel; vegf_an: VEGF assayed in the angiogenesis panel].

4.3 IgG antibodies specific to schizont extract, a marker for malaria exposure.

4.3.1 Malaria exposure

Overall, samples from 39 cases and 78 controls were assayed for antibodies against malaria. Seropositivity was determined based on a seropositivity threshold (negative control+2 standard deviations, SD). Of the 117 samples assayed, 81 (69.2%) were positive for schizont-specific IgG antibodies.

4.3.2 Association between malaria exposure and HIV-1 acquisition

To determine whether exposure to malaria has a direct contribution to eventual HIV-1 acquisition, malaria antibody levels were compared between cases and controls. When antibody levels were compared between cases and controls, both groups had similar levels (p=0.68) implying that they had similar exposure to malaria (Figure 16).

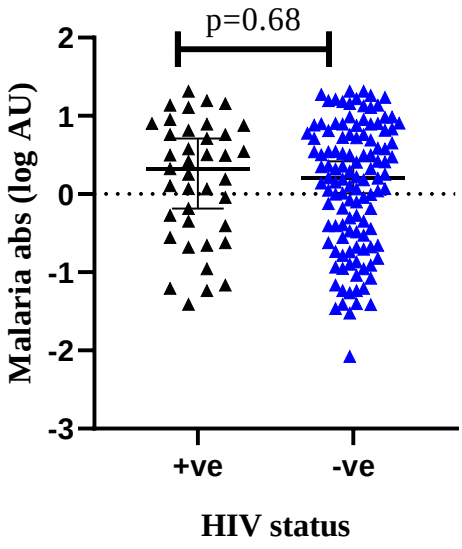


Figure 16: A graph illustrating differences between median $\log_{10}$ concentrations of malaria antibody titres between cases and controls (p=0.68). Black triangles (+ve) represent volunteers who contracted HIV-1 during follow up (cases). Blue triangles (-ve) represent volunteers who remained HIV-1 negative (controls). [*AU (Arbitrary units),*

Malaria abs (Malaria antibodies)]. (Wilcoxon rank sum test, p<0.05 considered significant, n=117).

To determine whether considering the antibody titres would give a better indication of past level of pathogen exposure and impact that these differing levels of exposure would have on HIV-1 acquisition, volunteers were grouped into those who had detectable levels of antibodies against schizont extract and those who did not. No significant difference was observed between the cases and the controls (Figure 17).

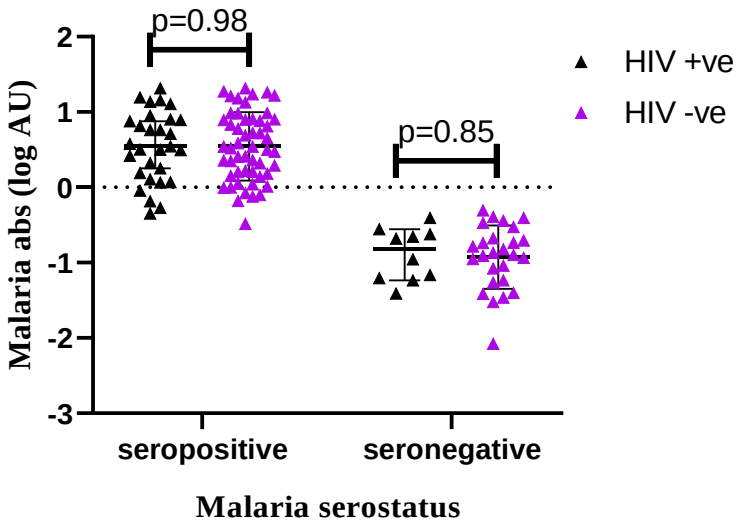


Figure 17: A graph illustrating differences between median $\log_{10}$ concentrations of malaria antibody titres of cases and controls in reference to malaria serostatus. Volunteers were grouped into malaria seropositive and malaria seronegative groups. Black triangles (HIV +ve) represent volunteers who contracted HIV-1 during follow up. Purple triangles (HIV -ve) represent volunteers who remained HIV-1 negative. [*AU (Arbitrary unit), Malaria abs (Malaria antibodies)*]. (Wilcoxon rank sum test, p<0.05 considered significant, n=117).

4.3.3 Association between malaria exposure and immune activation

To determine whether exposure to malaria was the driver of the immune activation profiles observed, antibody titres against malaria were correlated to the cytokines measured. There was no significant correlation between malaria antibodies and any of the assayed cytokines. ($p<0.05$ considered significant) (Figure 18). Implying that additional parameters assisted in driving the cytokine profiles observed.

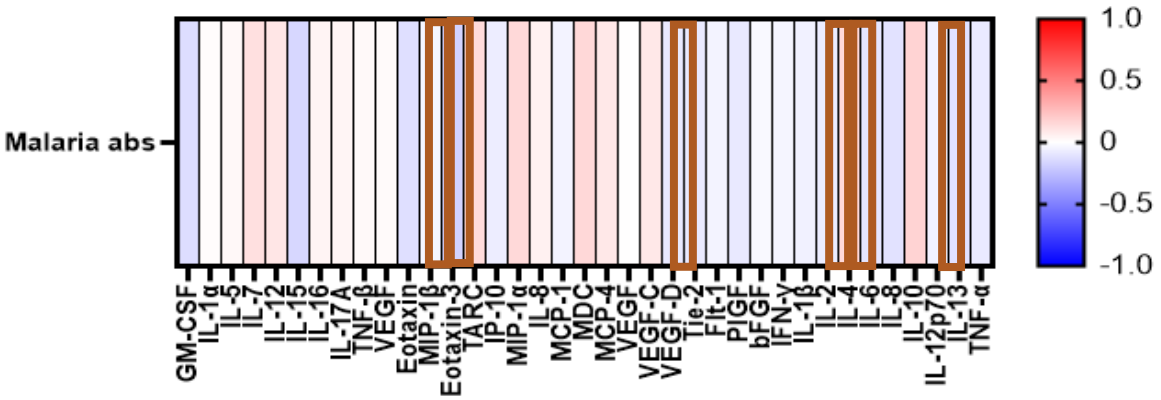


Figure 18: A heat map displaying correlation between detectable malaria antibody titres and assayed cytokines**.** Cytokines within the red borders highlight cytokines significantly different between cases and controls ($p<0.05$ considered significant). Rho=1.0 (in red) represents a strong positive correlation; Rho= -1.0 (in blue) represents a strong negative correlation; Rho=0 (in white) represents no correlation. No significant association between malaria antibody titres and any of the assayed cytokines. [*Malaria abs (Malaria antibodies)*].

To establish whether volunteers with higher antibody levels had a different cytokine profile, volunteers were stratified into malaria seropositive and malaria seronegative groups as previously described in section 4.3.2. Cytokine concentrations were then compared between the two groups. Three cytokines, IL-1α ($p=0.03$), IL-5 ($p=0.02$), IL-10 ($p=0.02$), were observed to be significantly higher in malaria seropositive than seronegative volunteers (Figure 19).

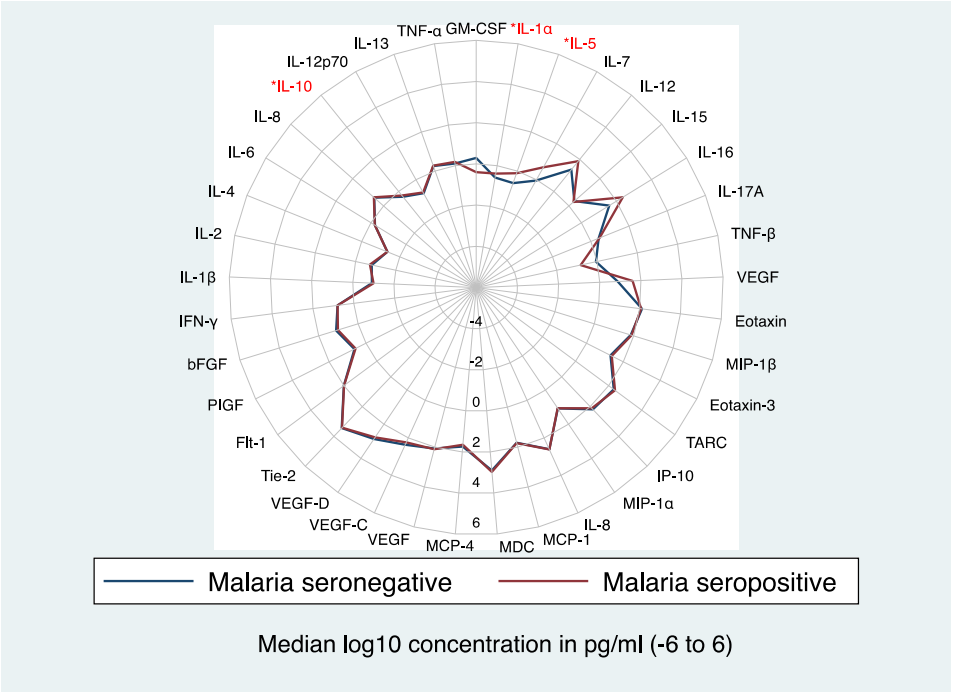


Figure 19: A radar plot illustrating differences in cytokines assayed between malaria seronegative and seropositive volunteers. Blue line represents malaria seronegative samples; Red line represents malaria seropositive samples. IL-1α (p=0.03), IL-5 (p=0.02), IL-10 (p=0.02) were significant higher in malaria seropositive than seronegative volunteers. (Wilcoxon rank sum test, $p<0.05$ considered significant, n=117) ($p<0.05$[*], $p<0.01$[**], $p<0.001$[***]).

4.4 IgG antibodies specific to CMV, a marker for CMV exposure.

4.4.1 CMV exposure

IgG antibodies specific to the lysate of MRC-5 cells infected with the CMV AD169 strain were measured. A threshold of positivity was set at 2,200 RU/ml, as per the manufacturer's instructions (EUROIMMUN). Of the 117 samples tested, 115 (99.15%) had CMV antibody titres above the threshold.

4.4.2 Association between CMV exposure and HIV-1 acquisition

To determine whether exposure to CMV directly influences HIV-1 acquisition, CMV antibody titres were compared between cases and controls. There was no significant

difference in CMV antibody titres between volunteers who acquired HIV-1 and those who remained uninfected (p=0.47). Overall, volunteers had equal exposure to CMV (Figure 20).

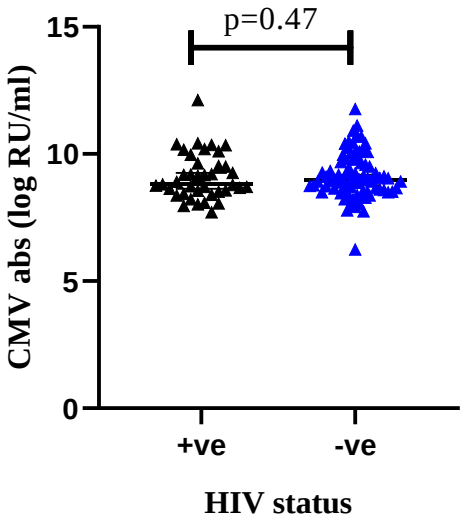


Figure 20: A graph illustrating differences between median $\log_{10}$ concentrations of CMV antibody titres between cases and controls. Black triangles (+ve) represent volunteers who contracted HIV-1 during follow up (cases). Blue triangles (-ve) represent volunteers who remained HIV-1 negative (controls). [*RU/ml (Relative units/ml), CMV abs (CMV antibodies)*]. (Wilcoxon rank sum test, p<0.05 considered significant, n=117).

To assess if considering the antibody titres would give a better indication of past level of CMV exposure and impact that these differing levels of exposure would have on HIV-1 acquisition, volunteers were categorized into those with low CMV antibody titres and those with high CMV antibody titres, using the median as a cut-off. This is because almost all volunteers were CMV IgG positive to the lysate of MRC-5 cells infected with the CMV AD169 strain 115 (99.15%), therefore dividing volunteers into seroconverters and non-seroconverters was not feasible. Neither of the two groups showed significant differences between volunteers who acquired or did not acquire HIV-1 (Figure 21).

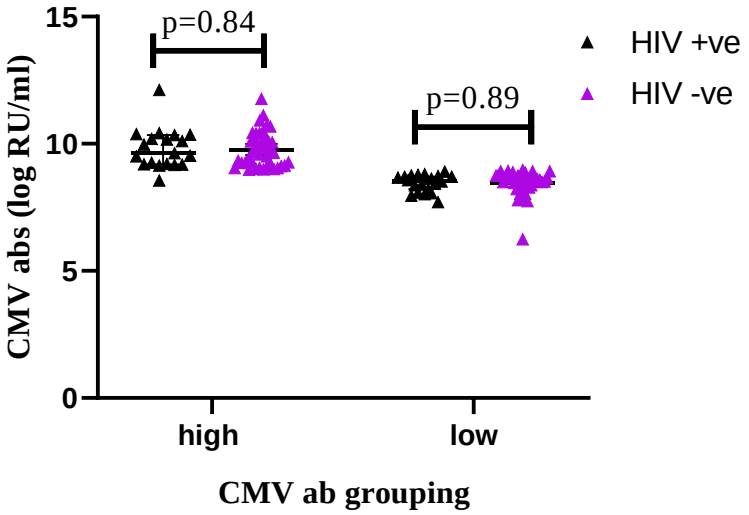


Figure 21: A graph illustrating differences between median log_{10} concentrations of CMV antibody titres of cases and controls based on CMV antibody grouping. Volunteers were grouped into low CMV antibody group and high CMV antibody group. Black triangles (HIV-1 +ve) represent volunteers who contracted HIV-1 during follow up. Purple triangles (HIV-1 -ve) represent volunteers who remained HIV-1 negative. [*RU/ml (Relative units/ml). CMV abs (CMV antibodies)*]. (Wilcoxon rank sum test, $p<0.05$ considered significant, n=117).

4.4.3 Association between CMV exposure and immune activation

To determine whether exposure to CMV was the driver of the immune activation profile observed, antibody titres against CMV were correlated to the cytokines measured. There was no significant correlation between CMV antibodies and any of the assayed cytokines. ($p>0.05$ considered significant) (Figure 22), suggesting that additional parameters assisted in driving the cytokine profiles observed.

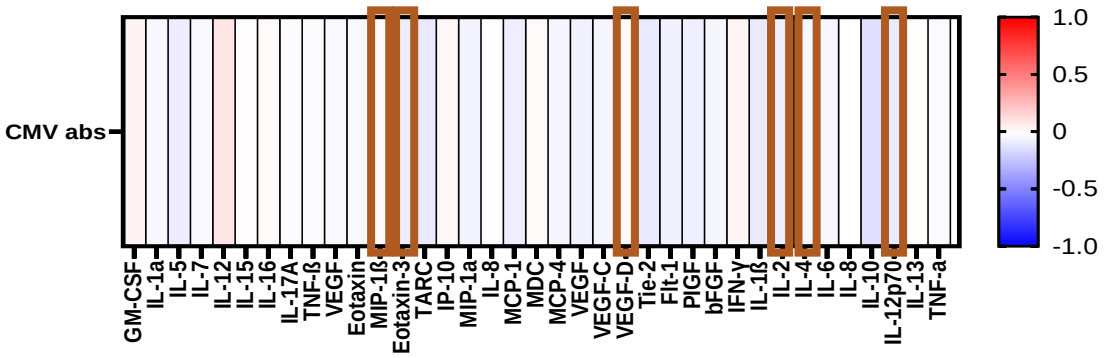


Figure 22: A heat map displaying correlation between detectable CMV antibody titres and assayed cytokines. Cytokines within the red borders highlight cytokines significantly different between cases and controls ($p<0.05$ considered significant). Rho=1.0 (in red) represents a strong positive correlation; Rho= -1.0 (in blue) represents a strong negative correlation; Rho=0 (in white) represents no correlation. No association between CMV antibody titres and any of the assayed cytokines.

To establish if volunteers with higher CMV antibody levels had a different cytokine profile, volunteers were stratified into low and high CMV antibody titres as previously described in section 4.4.2. No significant difference observed between the two groups with regard to cytokines assayed (Figure 23).

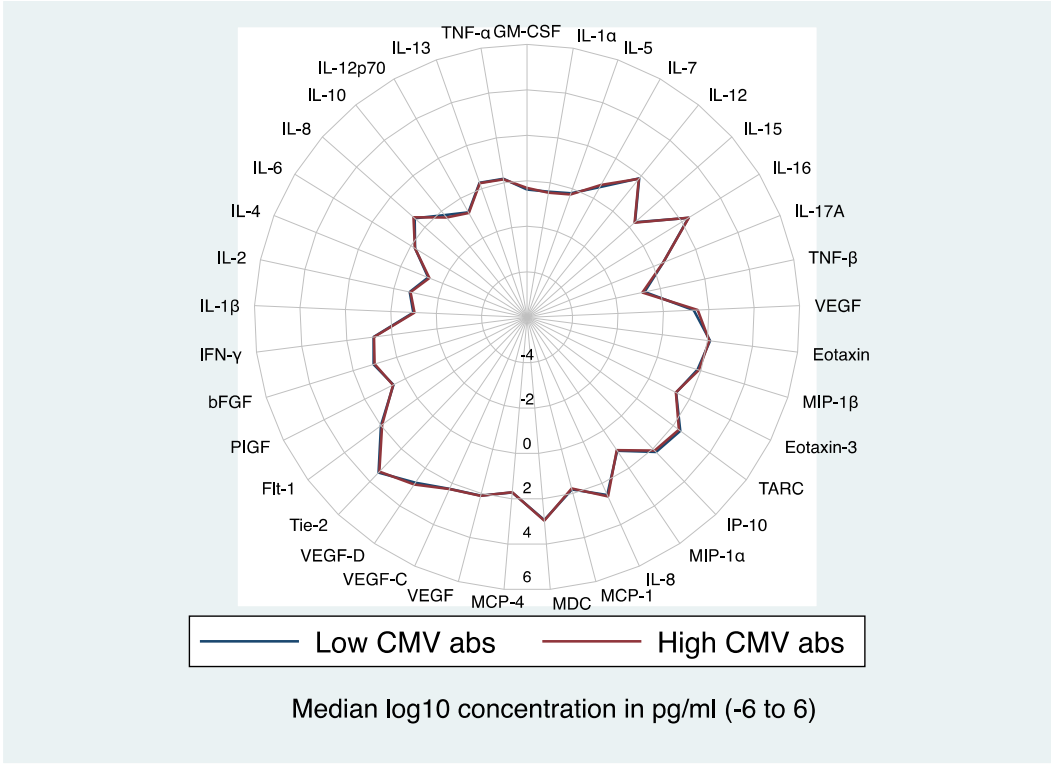


Figure 23: A radar plot demonstrating differences in cytokines assayed between volunteers who had low antibodies against CMV and those who had high CMV antibody titres. Red line represents volunteers with high CMV antibody titres; Blue line represents volunteers with low CMV antibody titres. The two lines overlay each other, therefore there is no significant difference between the two groups in regards to cytokines assayed. (Wilcoxon rank sum test, $p<0.05$ considered significant, n=117).

CHAPTER FIVE: DISCUSSION

There is a high pathogen endemicity in sub-Saharan Africa which may drive immune activation and alter immune responses to secondary infections and vaccine responses. This study suggests that an altered cytokine profile may contribute to HIV-1 acquisition. However, exposure to malaria and CMV infection, which are endemic in our setting, were neither associated with an altered immune response nor to HIV-1 acquisition. However, when volunteers were stratified into malaria seropositive and seronegative groups, IL-1β, IL-5 and IL-10 were significantly higher in malaria seropositive volunteers. It is likely that the drivers for immune activation are broader than the prevailing malaria and CMV infections common in this population.

In this study, MIP-1β, Eotaxin-3, IL-12p70, IL-2 and IL-4 were significantly elevated, while VEGF-D was significantly lower amongst volunteers that eventually acquired HIV-1 infection, compared to those that did not. However, none of these markers were significantly associated with HIV-1 acquisition in the multivariable models. When structural equation modelling approaches were applied, these analytes clustered into four groups suggestive of a Th2 profile (MIP-1β, VEGF, VEGF-C and IL-4), a Th1 profile (MCP-1, IL-2 and IL-12p70), a tissue repair role (VEGF-D) and an eosinophil chemotactic role (Eotaxin-3). Importantly, analytes suggestive of a Th2 profile and a tissue repair role stood out to be significantly associated with HIV-1 acquisition.

PC1 cytokines play a role in Th2 response and trafficking of immune cells to the site of infection. MIP-1β is a chemokine produced by monocytes, activated T and B cells, and NK cells and acts to recruit leukocytes to sites of inflammation (Menten et al., 2002). Similar to this study, a recent study reported significantly higher MIP-1β in participants who eventually contracted HIV-1 compared to those who remained HIV-1 negative (McInally et al., 2021). Influx of activated T cells a key target cell of infection, may promote the establishment of an HIV-1 infection. Interestingly, MIP-1β is the natural

ligand for CCR5, a coreceptor for HIV-1 infection. An elevation in this chemokine may therefore compete with the R5 strains of HIV-1 for CCR5, thus reducing susceptibility to infection by M-tropic HIV-1 strain (Levy, 2007). Contrary to the known protective nature of MIP-1β, this study has reported MIP-1β to be a risk factor for HIV-1 acquisition, together with VEGF, VEGF-C and IL-4. VEGF and VEGF-C are potent inducers of lymphangiogenesis by binding to endothelial cells adjacent to the site of infection (Angelo & Kurzrock, 2007). Lymphangiogenesis facilitates the adequate trafficking of immune cells to the site of infection, including activated $CD4^+$ T cells. IL-4 is a Th2 cytokine which promotes differentiation of naïve T cells to Th2 cells, favours production of IgE-secreting B cells and activation of M2 macrophages (Junttila, 2018). Influx of Th2 cells avails these cells to HIV-1 infection. In addition, M2 macrophages are HIV-1 target cells as they express CD4 and CCR5, and are therefore infected by the R5 HIV-1 strain (Koppensteiner et al., 2012). It is possible that all PC1 cytokines, MIP-1β, VEGF, VEGF-C and IL-4, work synergistically contributing to increased HIV-1 acquisition. VEGF and VEGF-C induce lymphangiogenesis which stimulates the vascular generation of endothelial cells for leukocyte travel to sites of infection (Angelo & Kurzrock, 2007). VEGF also induces production of chemokines by endothelial cells (Reinders et al., 2003), among them MIP-1β (Shukaliak & Dorovini-Zis, 2000). MIP-1β attracts and activates monocytes, T cells and B cells. It has been reported that MIP-1β promotes IL-4 production in T cells ex vivo, which skews the cytokine profile towards a Th2 response, thus dampening the Th1 response required for anti-HIV-1 immunity. Therefore, upon exposure to HIV-1, the immune system may not be adequately equipped to prevent HIV-1 from establishing an infection, resulting in HIV-1 acquisition. A Th1 response is mainly induced by intracellular bacteria and viruses, and is characterized by the secretion of Th1 cytokines such as IFN-γ, IL-6 and IL-2 (Sompayrac, 2015). IFN-γ is particularly crucial for anti-viral immunity as it stimulates cells to upregulate anti-viral proteins, thus

preventing viral infection of target cells (Schroder et al., 2004). Therefore, this response is protective against establishment of HIV-1 infection. A Th2 response on the other hand is mainly induced by helminths, and is characterized by hyporesponsiveness and anergy (C. Chen et al., 2005). Th2 cytokines include IL-4, IL-5 and IL-10 which promote differentiation of naïve T cells to Th2 cells and activation of M2 macrophages, both of which are HIV-1 target cells (Miller & Shattock, 2003). Therefore, a Th2 response is a risk factor of HIV-1 acquisition, as observed in this study. Furthermore, IL-4 has been reported to increase expression of CXCR4, therefore promoting the infection of these cells by HIV-1 X4 strain. (Valentin et al., 1998).

PC3 cytokine, VEGF-D, is an angiogenic factor which has been associated with generation of blood and lymphatic vessels in disease. It has also been found to be responsible for wound healing in a mouse model (Stacker & Achen, 2018). In my study, VEGF-D was inversely associated with HIV-1 acquisition. Increased levels of VEGF-D in this HIV-1 high-risk population, who are also highly exposed to STIs, may have contributed to better wound healing which may be protective against HIV-1 entry across the epithelial layer.

Overall, inflammation has been found to be associated with HIV-1 acquisition. This may be attributed to the proliferation and activation of $CD4^+$ T cells which are the primary targets of HIV-1 as they express CD4, CXCR4 and CCR5 which are coreceptors required for entry of HIV-1 into the cell and subsequent establishment of infection. Furthermore, the altered cytokine profile may be an obstacle to mounting an appropriate and effective antiviral immune response against HIV-1, upon exposure. This dysregulation increases the risk of HIV-1 acquisition.

Exposure to malaria and CMV infection was quantified by measuring the IgG antibody titres in the plasma samples. Malaria and CMV threshold positivity were at 69.2% and

99.2% respectively, which verified that the infections were endemic within this cohort. No significant difference was observed between cases and controls with regard to both CMV and malaria antibody titres. This means that malaria and CMV may not be the only drivers of the observed cytokine profile. Two epidemiological studies in East Africa and Tanzania have reported an ecological overlap between malaria and HIV-1 prevalence and incidence, thus suggesting a possible interaction between the two infections (Cuadros et al., 2011; Nielsen et al., 2006). Conversely, a study conducted in West Africa did not find an association between malaria and HIV-1 (Cuadros et al., 2011). They attributed this to the lower HIV-1 prevalence in West Africa in comparison to East Africa. Despite its high seroprevalence of 55-97% in Africa (Bates & Brantsaeter, 2016), CMV is an understudied infection because of the false perception that the high prevalence does not pose a risk of congenital materno-fetal transmission of CMV. Taking into consideration that expectant mothers are presumed to have already acquired the infection during childhood (Foulon et al., 2008; Fowler et al., 1992; Ross et al., 2006). It appears that the drivers of HIV-1 acquisition in sub-Saharan Africa go beyond the high pathogen burden exposure that the population experiences.

There was no association between pathogen exposure and the six cytokines which were significantly different. Therefore, in this particular study, neither CMV nor malaria contributed to the observed immune activation. In addition, there was no association observed between malaria and cytokines that are key players in anti-malarial immunity such as TNF-α, IFN-ϒ, IL-6, IL-1β and IL-4 (Mshana et al., 1991). Likewise, no association was found between CMV and cytokines involved in anti-CMV immunity including IFN-ϒ, IL-6, IL-8 and TNF-α (Humar et al., 1999). Overall, there was no association between either of the infections and any of the measured cytokines. A recent study conducted on serodiscordant couples in Zambia and Rwanda reported an elevated cytokine profile before HIV-1 infection, which was neither associated with

schistosomiasis nor genital inflammation/ulceration in seronegative participants who contracted HIV-1 during follow up (McInally et al., 2021). There may be other factors responsible for the observed immune activation such as age (Mohr & Siegrist, 2016), host genetics (Boyd & Jackson, 2015; Haralambieva et al., 2013; Poland et., 2008), nutrition (Bhattacharjee & Hand, 2018; Hoest et al., 2014; Prendergast, 2015), socio-economic status (Pan et al., 2019), local health practices (Simpson et al., 2015), and circulating HIV-1 subtype (Wilkinson et al., 2015). Nevertheless, when pathogen exposure was stratified into categories, IL-5, IL-1α, and IL-10 were found to be significantly higher in malaria seropositive than malaria seronegative volunteers. This is consistent with previous findings which indicate that IL-5 levels increase during a malarial infection (Prakash et al., 2006). IL-5 activates eosinophils, which participate in parasite killing (Waters et al., 1987). IL-1α is usually released following liver inflammation and recruits leukocytes to the site of infection (de Menezes et al., 2019). This is expected, given that malaria parasites infect liver cells during the liver stage of malaria. IL-10 has also been reported to elevate in malaria-infected individuals (Peyron et al., 1994; Sarthou et al., 1997). IL-10 has a dual effect whereby it plays a critical role in controlling immunopathology by dampening inflammation resulting from the immune response against *Plasmodium falciparum* while conversely, down-regulating Th1 responses against malaria thus allowing infection to persist (Kumar et al., 2019). On the other hand, there was still no association between categorized CMV antibody titres and any of the cytokines. This suggests that there may be other factors that are driving the observed immune activation. For instance, STIs have been associated with an increased predisposition to HIV-1 acquisition (Cameron et al., 1989). Compounded with the fact that STIs are highly prevalent among people at high risk of HIV-1 acquisition (Boothe et al., 2020; Musyoki et al., 2015), STIs may be a potential driver of the observed cytokine profiles.

Several confounding factors need to be considered when determining key drivers of immune activation. Females have been reported to experience more robust immune responses as opposed to males (Klein & Flanagan, 2016). It is also possible that non-communicable diseases not often accounted for in our setting, may be drivers of varying cytokine profiles. Genetics and not just the magnitude of exposure may also play a major role as some individuals are innately predisposed to infections owing to genetic immune deficiency disorders (Damania & Münz, 2019). Therefore, gender and genetics are possible confounders for this study. Gender was controlled for in these data. In addition, cases and controls were selected from Coastal Kenya therefore were exposed to the same environmental exposures and were also the same gender.

This study had four main limitations. First, it was not possible to measure all pathogens that may have infected this population e.g. helminths which require a stool sample which was not available for this study. Also measurement would be biased towards already known pathogens, for which tests are available and completely ignore those for which tests are not available. Cytokine profile is therefore better reflective as it overcomes these limitations. Secondly, these data were generated from plasma which is a constituent of peripheral blood. Cytokines may be secreted in secondary lymph node organs and in small quantities that are targeted for specific functions and hence not easily captured in peripheral blood. Third, although analyzing cytokine concentrations before HIV-1 infection longitudinally would be more informative on the long-term dynamics of immune activation, this study analyzed samples cross-sectionally at 3 months before infection. This time point is close to the estimated date of infection; therefore, it is reflective of the immune environment right before infection. Also, it is possible that the cytokine milieu closer to the date of infection as opposed to this 3-month time point would have been better reflective. However, taking into consideration the challenges of longitudinally following up high-risk volunteers until point of infection, the ability to

accurately estimate the date of infection, and the high cost of the techniques, a 3-month time point prior to HIV-1 acquisition is practically close enough to the incidence. Lastly, taking into consideration that a Th2 response was observed to increase susceptibility to HIV-1 acquisition, quantifying exposure to helminths would have been beneficial to this study in retrospect. Followed by correlation tests to determine whether helminth infection was responsible or partially responsible for the observed cytokine profile in this population. Future studies allowing collection of stool samples can support such analysis. Underlying effects of continuous exposure over time were deliberately analyzed which would have a larger and more permanent insult/impact on long-term immunological responses as opposed to the detection of an acute infection, which would have been determined by pathogen detection, and may only be reflective of a short-term impact. This approach reflects better the continuous immunological insult from continuous ongoing exposure to pathogens as opposed to an active acute infection. Detecting acute infections may have resulted in larger immune activation alteration, but may not have been better reflective of the underlying continuous insult to the immune responses, which was the primary objective of this study.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

This study established that a cytokine cluster consisting of MIP-1β, VEGF, VEGF-C and IL-4, suggestive of a Th2 profile, is associated with HIV-1 acquisition. VEGF-D, suggestive of a tissue repair profile, was also found to be inversely associated with HIV-1 acquisition. A pre-existing Th2 response is therefore a predisposing risk factor for HIV-1 acquisition. Therefore, I recommend further study on the development of biological interventions, towards abrogating markers from PC1 which will moderate the Th2 response, thus consequently reduce the risk of HIV-1 acquisition. This may be particularly important towards preventing HIV-1 acquisition amongst high-risk volunteers and may play a role towards controlling the epidemic in this population. However, this should be done meticulously because cytokines play many important roles in immunity.

Secondly, although malaria and CMV infections were not found to be associated with the observed immune activation in this study, residents of sub-Saharan Africa are exposed to many infections. Additional studies should go into studying a wider panel of infections which may be responsible for the observed cytokine profiles, Also, treatment of local pathogens will be beneficial to reducing secondary infections and also in inducing optimal vaccine responses within the sub-Saharan African population.

Exposure to malaria and CMV was not found to be associated with HIV-1 acquisition. Therefore, there is need for the inclusion of a wide range of endemic infections in future studies within the sub-Saharan African setting. This will inform on whether treatment of endemic pathogens may reduce immune activation/modulation resulting from infection. Moreover, it would be beneficial to look into both infectious and non-infectious diseases, given that some non-communicable diseases such as sarcoidosis and systemic lupus erythematosus are associated with chronic inflammation (Ogoina & Onyemelukwe,

2020). Therefore, studies that aim to comprehensively analyze the entire immune activation profile, as that described in this thesis will be highly informative.

Furthermore, the impact of the elevated cytokines on disease progression would be a good step forward, to help inform strategies on slowing down disease progression after HIV-1 infection.

Malaria and CMV may not necessarily be the key drivers of immune activation observed in this particular study. Additional studies should investigate the possible inducers of immune activation within this population. It is worth acknowledging that, the high HIV-1 burden in Africa is not only due to exposure of the residents to multiple pathogens but may also be attributed to other factors such as age, host genetics, nutrition, socio-economic status, local health practices, and circulating HIV-1 subtype.

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APPENDICES

Pwani UNIVERSITY

ETHICS REVIEW COMMITTEE

ACCREDITED BY THE NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION (NACOSTI, KENYA)

CERTIFICATE OF ETHICAL APPROVAL

THIS IS TO CERTIFY THAT THE PROPOSAL SUBMITTED BY:

LYNN N. FWAMBAH

REFERENCE NO:
ERC/MSc/002/2021

ENTITLED:
Impact of immune activation on HIV acquisition and disease progression

TO BE UNDERTAKEN AT:
KILIFI COUNTY, KENYA

FOR THE PERIOD
FROM: 05/02/2021 TO: 04/02/2022

HAS BEEN **APPROVED** BY THE ETHICS REVIEW COMMITTEE

AT ITS SITTING HELD AT PWANI UNIVERSITY, KENYA

ON THE 4/02/2021

CHAIRMAN SECRETARY LAY MEMBER

Pwani UNIVERSITY

Ethics Review Committee

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The ERC: Giving Integrity to Research for Sustainable Development

Appendix 1: Ethical Approval

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Appendix 2: Funding