The relationship between Cytomegalovirus-specific cellular immune response and $CD4^+$ T cell count in HIV-positive individuals in a South African setting

by
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Declaration

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Abstract

Introduction: Reactivation of human cytomegalovirus (HCMV) infection in individuals infected with human immunodeficiency virus (HIV) may lead to life-threatening end-organ diseases (EOD). The EOD becomes clinically apparent when a critical number of cells in the affected organs become damaged as a consequence of HCMV-infection. Treatment of the HCMV-associated disease at this point may not be effective. Therefore, early detection of HCMV reactivation may be useful to guide pre-emptive therapy.

Aim: The aim of this study was to determine whether there is a point at which the HCMV-specific cellular immune response breaks down, as determined by the interferon-gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assay, and HCMV reactivation occurs in HIV-positive, antiretroviral therapy (ART)-naïve individuals in a South African setting. This was done in relation to the $CD4^+$ T cell count and the HCMV viral load as determined by real-time polymerase chain reaction (qPCR).

Materials and methods: Fifty-two (52) HIV-infected, ART-naïve subjects were recruited from primary healthcare centres that they attended for the management of their HIV infection. Heparinised blood samples were collected to quantify the HCMV-specific cellular immune response using the IFN-γ-ELISPOT assay and to determine the HCMV IgG serostatus. Ethylenediaminetetraacetic acid (EDTA) blood samples were collected for the determination of the $CD4^+$ T cell counts and the HCMV viral loads.

Results: All 52 subjects recruited were confirmed to be HIV-HCMV co-infected based on their HCMV IgG serostatus. The results of 34 subjects with completed data sets were analysed. The $CD4^+$ T cell counts of these subjects ranged from 10 to 784 cells/µl. Twenty-two (22) (65%) subjects had positive HCMV IFN-γ-ELISPOT results with 94% having no detectable HCMV viral loads. All subjects (28) with a $CD4^+$ T cell count above 100 cells/µl had undetectable HCMV viral loads. Two of the six subjects with $CD4^+$ T cell counts <100 cells/µl had detectable HCMV viral loads. There was no

statistically significant association between the $CD4^+$ T cell counts and the HCMV IFN-γ-ELISPOT results.

Conclusion: No specific point could be determined when there is loss of integrity of the HCMV-specific cellular immune response in HIV-positive individuals. Low $CD4^+$ T cell counts did not correlate with HCMV IFN-γ-ELISPOT results suggesting that the HCMV-specific cellular immunity did not necessarily break down at low $CD4^+$ T cell counts. Nevertheless, a $CD4^+$ T cell count above 100 cells/µl appeared to be protective against viraemia as determined by the HCMV viral load qPCR. The IFN-γ-ELISPOT assay was employed as a tool to determine the integrity of the HCMV-specific cellular immune response in HIV-positive individuals. However, the IFN-γ-ELISPOT assay should be used in conjunction with the $CD4^+$ T cell count and the HCMV viral load qPCR to determine when there is loss of integrity of the HCMV-specific cellular immune response and HCMV reactivation occurs. This may assist clinicians in their choice of management and appropriate pre-emptive treatment in the HIV-HCMV co-infected individual at a risk for HCMV reactivation.

Opsomming

Inleiding: Heraktivering van menslike sitomegaalvirus (MSMV) in menslike immuniteitsgebreksvirus (MIV)-MSMV ko-geïnfekteerde individue kan lei tot dodelike end-orgaan siektes (EOS). Die EOS word klinies duidelik wanneer 'n kritieke aantal selle in die organe beskadig raak as gevolg van die MSMV-infeksie. Behandeling van die MSMV-verwante siekte op hierdie punt mag moontlik nie meer effektief wees nie. Daarom kan die vroeë opsporing van MSMV heraktivering nuttig wees in die gebruik van voorkomende terapie.

Doel: Die doel van hierdie studie is om die punt te bepaal wanneer die MSMV-spesifieke sellulêre immuun reaksie afgebreek word met behulp van die interferon gamma (IFN-γ) ensiem-gekoppelde immunospot (ELISPOT) toets en MSMV heraktivering voorkom in MIV-positiewe, antiretrovirale terapie (ART)-naïewe individue in' n Suid-Afrikaanse instelling. Dit word gedoen in verhouding met die $CD4^+$ T sel telling en die MSMV virale lading.

Materiale en metodes: Twee-en-vyftig (52) MIV-geïnfekteerde, ART-naïewe pasiënte is vanaf primêre gesondheidsentrums, wat hul bywoon vir die behandeling van hul MIV infeksie, genader. Gehepariniseerde bloedmonsters is gebruik om die MSMV-spesifieke sellulêre immuun reaksie met behulp van die IFN-γ-ELISPOT toets en die MSMV IgG serostatus te bepaal. Etileendiamientetra-asynsuur (EDTA) bloed monsters is versamel vir die bepaling van hul $CD4^+$ T sel telling en hul MSMV virale lading met behulp van die ~~"r~~eal-time" polimerase kettingreaksie (qPKR) waardes.

Resultate: Al 52 pasiënte is bevestigde MIV-MSMV ko-infeksies, gebasseer op hul serologiese status. Die resultate van 34 pasiënte met voltooide data is ontleed. Die $CD4^+$ T sel tellings van hierdie pasiënte het gewissel 10-784 selle/µl. Twee-en-twintig (22) (65%) pasiënte het positiewe MSMV IFN-γ-ELISPOT resultate met 94% wat n negatiewe qPKR resultate. Alle pasiënte (28) met 'n $CD4^+$ T-seltelling bo 100 selle/µl het' n negatiewe qPKR resultate. Twee van die ses pasiënte met 'n $CD4^+$ T-seltelling <100 selle/µl het waarneembare MSMV virale ladings oor die qPKR. Daar was geen statisties

beduidende assosiasie tussen die $CD4^+$ T sel tellings en die MSMV IFN-γ-ELISPOT resultate nie.

Gevolgtrekking: Geen spesifieke punt wanneer die MSMV-spesifieke sellulêre immuun reaksie afgebreek word kon in MIV-positiewe individue bepaal word nie. Lae $CD4^+$ T sel tellings het nie ooreengestem met die MSMV IFN-γ-ELISPOT resultate nie en dui daarop dat die MSMV-spesifieke sellulêre immuniteit nie noodwendig afgebreek word teen 'n lae $CD4^+$ T sel tellings nie. Tog blyk 'n $CD4^+$ T-seltelling bo 100 selle/µl om beskerming teen viremie te bied. Die meriete van die gebruik van die IFN-γ-ELISPOT toets die integriteit van die MSMV-spesifieke sellulêre immuun response in MIV-positiewe individue te bepaal, is waargeneem in die opgehoopte data. Tog kan die gebruik van die IFN-γ-ELISPOT toets in samewerking met die $CD4^+$ T sel telling en die MSMV virale lading meer voordelig in die bepaling van 'n punt wanneer die MSMV-spesifieke sellulêre immuun reaksie afbreek en herstel plaasvind. Dit kan help om klinici in hul keuse van bestuur en gepaste voorkomende behandeling in die MIV-MSMV mede-geïnfekteerde individu op 'n risiko vir herstel.

"A rock pile ceases to be a rock pile the moment a single man contemplates it, bearing within him the image of a cathedral." Antoine de Saint-Exupéry

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List of Abbreviations

ºC	Degree Celsius
©	Copyright
®	Registered
µg	Microgram
µl	Microlitre
A	Absorbance
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
aU	arbitrary units
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
CPE	Cytopathic effect
cpm	Counts per minute
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
dNTP	deoxyribonuleoside triphosphates
EDTA	Etileendiamientetra-asynsuur
EDTA	Ethylene diamine tetra acetic acid
ELFA	Enzyme-linked fluorescent assay
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immune spot
EOD	End organ disease
EOS	End organ siekte
FCS	Foetal calf serum
g	Glycoprotein
g	Gravity
gag	Group antigen gene
3H-thymidine	tritiated thymidine
HAART	Highly active antiretroviral therapy

H and E	Haematoxylin and eosin
HCMV	HumanCytomegalovirus
HHV-5	Human herpes virus-5
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horse-radish perioxidase
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IE-1	Immediate early-1
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRIS	Immune reconstitution inflammatory syndrome
IRU	immune recovery uveitis
IRV	Immune recovery vitreitis
KLRG1	Killer cell lectin-like receptor G1
l	Litre
LPA	Lymphocyte proliferation assay
MCMV	Murine Cytomegalovirus
MgCl2	Magnésium chloride
MHC	Major histocompatibility complex
MIV	Menslike immuniteitsgebreksvirus
min	minutes
MIP-1β	Macrophage inflammatory protein-1 beta
ml	Millilitre
mM	Millimolar
MSMV	Menslike sitomegaalovirus
ng	Nanogram
NHLS	National Health Laboratory Services
NK	Natural killer
nm	Nanometer

NO	Nitric oxide
NTCs	Non-template controls
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween 20
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
pp	Phosphoprotein
PVDF	Polyvinylidene fluoride
qPCR	Real-time polymerase chain reaction
qPKR	Real-time polymerase ketting reaksie
RNA	Ribose nucleic acid
s	Second
SCT	Stem cell transplant
SFU	Spot forming units
SI	Stimulation index
SOT	Solid organ transplant
Taq	Thermus aquaticus
TB	Tuberculosis
T cell	Thymus cell
Th	T helper
™	Trade mark
tt	Tetanus toxoid
TNF-α	Tumour necrosis factor-alpha
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation

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Chapter 1

1. Introduction and Literature Review

1.1. Introduction

South Africa is a developing country with a high human immunodeficiency virus (HIV) burden (Department of Health, 2008). Although highly active antiretroviral therapy (HAART) has significantly reduced the progression to acquired immunodeficiency syndrome (AIDS) in HIV-positive individuals in developed countries (Mocroft *et al*., 2000; Palella *et al*., 1998), only a small percentage of HIV-positive individuals in developing countries receive this treatment (Manosuthi *et al*,2007). These patients typically seek treatment when their immune systems are profoundly compromised and their $CD4^+$ T cell counts are often below 100 cells/µl (Murdoch *et al*., 2008). At such a low $CD4^+$ T cell count the risk of opportunistic infections is increased (Erice *et al*., 2003; Holmes *et al*., 2006; Sacre *et al*., 2005; Salmon-Ceâron *et al*., 2000).

Human cytomegalovirus (HCMV) retinitis is regarded as an opportunistic AIDS-defining infection by the World Health Organisation (WHO) (WHO, 2005). The seroprevalence of HCMV in many developing countries approaches 100% so that most HIV-infected individuals will also have HCMV co-infection (Ben-Smith *et al*., 2008; Rabenau *et al*., 2010). Primary infection with HCMV is most often asymptomatic or subclinical in immunocompetent individuals (Lautenschlager *et al*., 2009). Active HCMV infection is associated with the shedding of infectious virus particles that may be asymptomatic or symptomatic. The symptoms of HCMV infection may include fever, malaise and diarrhoea and the laboratory findings include leukopenia and transaminitis. In contrast, HCMV disease is associated with the dysfunction of specific end-organs such as the eyes, lungs, liver, colon and brain (Bronke *et al*., 2005; Engelmann *et al*., 2008; Heiden *et al*., 2007). This dysfunction occurs when a critical number of cells in the organs are infected and damaged (Griffiths, 2009). Progressive organ damage may lead to life-threatening disease which presents as fulminant hepatitis, colitis, pneumonitis and encephalitis. In most individuals, following primary infection, the virus enters a state of latency.

Compromise of the host‘s immune system may lead to reactivation of HCMV and eventually to life-threatening disease (Griffiths, 2009).

In HIV-HCMV co-infected, antiretroviral therapy (ART)-naïve individuals, immunosuppression due to the HIV infection increases the risk of HCMV reactivation and the progression to HCMV end-organ disease (EOD) (Heiden *et al*., 2007; Ohnishi *et al*., 2005). Sabin (2000) reported that HIV-HCMV co-infected individuals may progress more rapidly to AIDS than those that are HCMV-negative. In the pre-HAART era, the incidence of HCMV diseases were associated with an increase in the risk of mortality in HIV/AIDS patients. Although the incidence of HCMV diseases has being significantly reduced by the initiation of HAART in developed countries (Mocroft *et al*., 2000; Palella *et al*., 1998), it is still problematic in developing countries where only a few HIV-positive individuals receive HAART (Manosuthi *et al*., 2007). Therefore, early identification of HCMV reactivation and prompt appropriate antiviral therapy, especially in resource-limited areas, may help to reduce morbidity and mortality associated with HCMV EOD in HIV-positive individuals.

Biomarkers that have been evaluated as possible predictors of HCMV reactivation include the HCMV viral load, HIV viral load and the $CD4^+$ T cell count. No consensus has been reached as to which biomarker is the most appropriate predictor of HCMV reactivation (Erice *et al*., 2003; Fernandez *et al*., 2006; Jacobson *et al*., 2008; Salmon-Ceâron *et al*., 2000; Singh *et al*., 2007; Weinberg *et al*., 2006; Wohl *et al*., 2009). A high $CD4^+$T cell count appears to be protective against HCMV reactivation (Weinberg *et al*., 2006). However, the $CD4^+$ T cells are not exclusive in their action to suppress HCMV reactivation. The $CD8^+$ T cells and natural killer (NK) cells act in concert with the $CD4^+$ T cells to effectively suppress HCMV reactivation (Gamadia *et al*., 2004; Gratama *et al*., 2008; Sacre *et al*., 2005; Tay-Kearney *et al*., 1997).

A host of cytokines including interferon gamma (IFN-γ) are secreted by $CD8^+$ T cells, NK cells and $CD4^+$ T cells. IFN-γ secretion is induced during primary infection and maintained during latency (Cheeran *et al*., 2008; van de Berg *et al*., 2010). The secretion

of IFN-γ may be measured by the IFN-γ-ELISPOT assay which does this by measuring the frequency of T cells that secrete IFN-γ. In this way the frequency of IFN-γ secretion in response to HCMV antigens as measured with the IFN-γ-ELISPOT assay is purported to be an effective measure of the HCMV-specific cellular immune response (Macatangay *et al*., 2010). An inadequate cell-specific immunity as may occur in an immunocompromised state is characterised by a low frequency of T cells that secrete IFN-γ. In this regard, we hypothesise that an individual with a compromised cell-specific immunity is at risk of HCMV reactivation.

The aim of this study was to determine whether there is a point at which there is loss of integrity of the HCMV-specific cellular immune response using the IFN-γ-ELISPOT assay and HCMV reactivation occurs in HIV-positive, ART-naïve individuals in a South African setting. HCMV reactivation is determined by the presence of detectable viral particles using quantitative real-time polymerase chain reaction (qPCR) in blood. The $CD4^+$T cell count was measured to determine the severity of the HIV-infection and used as a general measure of immune compromise.

This research project had several objectives that investigated the purpose of the study and allowed for a suitable conclusion.
These included:

1. the establishment of a cohort of HIV-HCMV co-infected individuals from the HIV-positive, ART-naïve individuals recruited from selected primary healthcare facilities in Cape Town, South Africa and surrounding areas;
2. the quantification of the $CD4^+$ T cell count at the time of recruitment and sample collection in the HIV-HCMV co-infected cohort;
3. the determination of the HCMV-specific cellular immune response of this cohort using the IFN-γ-ELISPOT assay and
4. the quantification of the HCMV viral load with the use of qPCR.

1.2. Literature Review

1.2.1. Structure and Taxonomy of HCMV

HCMV, also known as Human Herpesvirus5 (HHV-5), belongs to the *Betaherpesvirinae* subfamily of the *Herpesviridae*. This double-stranded deoxyribose nucleic acid (DNA) virus was first isolated in 1957 (Hassan and Connell, 2007). It is the largest member of the herpesvirus family with a diameter of 260 nm and a core diameter of 64nm. The core is surrounded by the icosahedral capsid consisting of 162 capsomeres (refer to Figure 1.1). The integument surrounded by the envelope encloses the capsid. The synthesis of the virus takes place over three phases, the immediate early, the early and the late phases. The proteins important for viral production are the envelope proteins, glycoprotein (g) B, gH, gL and gM. No fewer than 25 virion-encoded proteins and glycoproteins make up the envelope enclosing the virion. The matrix proteins are composed of the phosphoprotein (pp) 65, pp150, pp71 and pp88 (Harari *et al*., 2004).

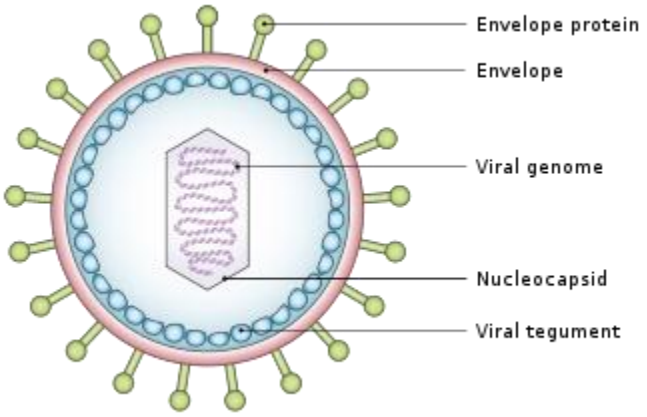

Figure 1.1: Illustration of the human cytomegalovirus (http://mbio.asm.org**)**

Both wild type strains and laboratory adapted strains exist. The wild type strains are recent isolates from humans and the laboratory strains such as AD169 and Towne are strains that have been repeatedly cultured in the laboratory over a number of years (Novak *et al*., 2009).

In addition to the laboratory strains murine CMV (MCMV) is also used as a model of human infection for pathogenesis, tissue tropism, and latency studies in the laboratory mouse (Aiba-Masago *et al*., 1999).

1.2.2. Transmission of HCMV

Humans are the only reservoirs for the very species-specific HCMV. It is a highly successful pathogen that is easily transmitted with normally little or no ill-effect on its immunocompetent host (van der Sande *et al*., 2007).The virus can be transmitted vertically or horizontally (de la Hoz *et al*., 2002).

Vertical transmission or trans-placental transmission is the transfer of the virus across the placenta from the HCMV-infected mother to the developing foetus. In a study in The Gambia, it was determined that vertical transmission was responsible for 5% live-born new-borns being infected with HCMV (van der Sande *et al*., 2007). Trans-placental transmission can occur at any stage of pregnancy. However, a pregnant woman in the third trimester with a primary infection or a reactivation of the virus is more likely to transmit the virus than at other stage of the pregnancy. Of note, a primary infection in a pregnant woman is a greater risk to the foetus than the reactivation or re-infection of the virus in the pregnancy (Griffiths, 2009; Munro *et al*., 2005).

Breast milk has been found to be the main source of postnatal, horizontal HCMV transmission in infancy. In an early study Friis (1982) purported that freezing milk and pasteurising it destroyed any viable virus. Maschmann (2006), however, provides evidence that freezing does not completely reduce HCMV infectivity and caution must be taken to prevent postnatal HCMV transmission. Day-care centres have been implicated in HCMV transmission due to the high rate of viral shedding in urine and saliva by young children. The risk of transmission is higher in children since, in most cases the children do not display any clinical signs of the HCMV infection and may unsuspectingly shed the virus over a long period of time. Hygiene, thus, plays a pivotal role in the reduction of the rate of transmission (Busse *et al*., 2008; de la Hoz *et al*., 2002; Joseph *et al*., 2006).

Other methods of horizontal transmission of HCMV documented in a review by Kano and Shiohara (2000) include sexual intercourse, blood transfusions, bone marrow transplantations and solid organ transplantations (SOT).

1.2.3. Epidemiology of HCMV

HCMV is an almost ubiquitous virus (Fletcher *et al*., 2005). At least 1.6% HIV-positive individuals between the ages of 12 and 49 years in the United States seroconverted to a positive HCMV status annually (Colugnati *et al*., 2007). In addition, 92.6% of pregnant Cuban women are reported to be HCMV seropositive (Correa *et al*., 2010). At least 2.4% of these women developed active HCMV infection during pregnancy. The risk of congenital HCMV is great in mothers with active HCMV. This is illustrated by the prevalence of HCMV disease in neonates in a multicentre study in France. Of these 23% had clinically apparent HCMV disease in those co-infected with HIV at birth (Guibert *et al*., 2009). Similarly, 85% of infants in The Gambia were found to be HCMV seropositive by the end of the first year of life. A further 70% of the HCMV seronegative infants at the end of the first year of life seroconverted in their second year of life. Consequently, the prevalence of HCMV seropositivity at the end of the second year of life was 96% (Miles *et al*., 2008). This emphasises the rate of HCMV infection early in life which may occur as a result of maternal transmission or postnatal infection. This has further catastrophic consequences should there be HCMV reactivation as a result of HIV co-infection.

Studies suggest that developing countries have a higher HCMV seroprevalence than developed countries. A 100% seroprevalence was reported in a Malawian HIV-negative group of adolescents which contrasted significantly with the 36% in a matched UK HIV-negative group (Ben-Smith *et al*., 2008). In developing countries such as Ghana and Lesotho the seropositivity rate of HCMV amongst HIV-infected individuals was reported as 77.6% and 100%, respectively (Adjei *et al*., 2008; Rabenau *et al.,* 2010). Early literature reported a seropositivity rate of 17.7% and 5% in HIV-HCMV co-infected homosexual males and healthy male blood donors, respectively in Washington DC and New York City (Nerurkar *et al*., 1987).

Other factors that may have influenced HCMV seropositivity rate include ethnicity and socio-economic settings (Colugnati *et al*., 2007; Griffiths, 2009). Individuals from a low socio-economic environment appeared to be more prone to HCMV infection. It is suggested that HCMV infection is increased in densely populated areas that are unhygienic and may be the reason for HCMV infection being more prevalent in low socio-economic environments (Colugnati *et al*., 2007; Griffiths, 2009).

1.2.4. Pathogenesis and HCMV diseases

Once HCMV has been transmitted to the relevant host, an asymptomatic or symptomatic primary infection occurs. This involves the shedding of viral particles. In healthy individuals the primary infection is suppressed and is usually subclinical. This is due to an intact and controlled cell-mediated immunity, cytotoxic T cells and NK cells [*refer to section 1.2.6*] (Lautenschlager *et al*., 2009). The virus then enters a state of lifelong latency in healthy individuals. Latency is accomplished by the virus seeking refuge in circulating monocytes, epithelial cells, hepatocytes, smooth muscle cells, fibroblasts, bone marrow progenitors and endothelial cells where it is able to evade the immune surveillance system [*refer to section 1.2.8*] (Hahn *et al*., 1998; Jarvis *et al.,* 2007; Kondo *et al*., 1996;Lautenschlager *et al*., 2009).

Reactivations of the same strain or re-infections of a different strain of the virus may occur periodically (Griffiths, 2009; Novak *et al*., 2009). Reactivation is usually asymptomatic in immunocompetent individuals. However, in some immunocompetent individuals, HCMV mononucleosis may occur but is generally self-limiting (Rodríguez-Baпб *et al*., 2004).

The virus, however, has a greater clinical significance in immunocompromised individuals such as in HIV/AIDS patients [*refer to section 1.2.10*] and in recipients of organ transplants receiving immunosuppressive drugs. In these individuals the virus may reactivate and cause life-threatening diseases such as HCMV colitis and rarely HCMV pneumonitis and HCMV encephalitis. Furthermore, HCMV retinitis is purported to be responsible for 90% of cases of HIV-related blindness (Augustine *et al*., 2007; Heiden *et*

al., 2007). Cellular immunity is shown to be essential for limiting the expression of HCMV infection (Ohnishi *et al*., 2005). Therefore, the cell-mediated immune response of these individuals is by implication severely suppressed (Augustine *et al*., 2007).

Notably, although they may not be immunocompromised, the primary infection, re-infection of a different HCMV strain or reactivation of HCMV in pregnant women, may have dire consequences for the developing foetus. Congenital HCMV is asymptomatic in 90% of infants who acquired the virus *in-utero.* Hearing loss is most prevalent in the sypmtomatic group. The prognosis for symptomatic congenital HCMV is, however, extremely poor. sensorineural hearing loss, microcephaly, motor defects, mental retardation, chorioretinitis and dental defects are some of the clinical manifestations of these infants (Cheeran *et al*., 2009; Nassetta *et al*., 2009).

1.2.5. Humoral immune response to HCMV infection

Primary HCMV infection results in the differentiation of the B-lymphocytes in response to various HCMV peptides that include HCMV pp65, immediate early-1(IE-1) and gB. It is purported that production of anti-HCMV antibodies during primary infection reduces the degree of the dissemination of the HCMV virus (Alberola *et al*., 2000). The immunogenic viral envelope glycoprotein, gB, of HCMV elicits a strong neutralizing antibody response (Alberola *et al*., 2000, Macagno *et al*., 2010). Anti-HCMV antibodies produced in pregnant women following primary HCMV infection reduces the risk of congenital HCMV (Jackson *et al*., 2010).

In response to pathogens B-lymphocytes differentiate into plasma cells producing specific antibodies. HCMV-specific antibodies, however, do not appear to play a role in maintaining the latent state of HCMV infection. Rather they appear to be markers of past exposure (Griffiths, 2009). HCMV immunoglobulin (Ig) M is usually an indication of a primary, an acute or recent infection whereas HCMV IgG antibodies are an indication of a past or persistent latent infection (Lazzarotto *et al*., 2008; van der Giessen *et al*., 1990).

1.2.6. Cell-mediated immune response to HCMV infection

Krause (1997) reported that in order to successfully respond to the HCMV antigens and suppress HCMV reactivation optimum T cell function must be maintained. Since HCMV is an intracellular pathogen the viral peptides such as HCMV pp65and IE-1 proteins are usually processed via a cytosolic pathway. These peptides are then presented by the major histocompatibility (MHC) class I molecules to the $CD8^+$ T cells. The $CD8^+$ T cells have a cytotoxic function and can lyse virally infected cells. They also release pro-inflammatory cytokines such IFN-γ, macrophage inflammatory protein (MIP)-1β and tumour necrosis factor (TNF)-α in response to these virally-infected cells (Sinclair *et al*., 2004; Stone *et al*., 2006).

Naïve $CD8^+$ T cells are $CD45RA^+$, $CD28^+$ and express $CCR7^+$ and CD62L. Soon after a primary HCMV infection clonal expansion of the $CD8^+$ T cells occur that express perforin and granzyme. These $CD8^+$ T effector cells express CD45RO but have lost the expression of CCR7 and CD62L (Gamadia et al., 2003). During an acute infection, $CD8^+$ HCMV-specific T cells show a $CCR7^-CD27^+CD45RA^-CD45R0^+$ phenotype. The late differentiated phenotype of the $CD8^+$ effector T cells express CD57. The $CD8^+$ memory T cells formed after suppression of the HCMV infection are selected from the initial responding $CD8^+$ T cell population (Waller *et al*., 2008). These $CD8^+$ memory T cells express CD45RA and lack the expression of CD28.

In asymptomatic patients, HCMV-specific $CD4^+$ T cells emerge in the peripheral blood compartment preceding both CMV-specific antibodies and $CD8^+$ T cells (Gamadia *et al*., 2003). HCMV-specific $CD8^+$ T cells, however, are predominant over the $CD4^+$ T cells in individuals with symptomatic HCMV infection. Although various studies have demonstrated the importance of $CD8^+$ T cells in suppressing the virus (Sacre *et al*., 2005; Tay-Kearney *et al*., 1997), $CD4^+$ T cells also play a vital role in suppression of viral replication (Gamadia *et al*., 2004; Gratama *et al*., 2008).

Numerous studies provide evidence that the Th1 arm of the $CD4^+$ T cell response to HCMV peptides is dominant during a primary or active HCMV infection. This was

demonstrated by van de Berg (2010) in renal transplant recipients. Increased levels of the type 1 cytokines, IFN-γ, IL-12 and IL-18 were detected. Similar results of elevated levels of IFN-γ as well as TNF-α and IL-2 were obtained by Villacres (2004) in healthy HCMV-positive individuals. The latter study also demonstrated decreased levels of IL-4, a signature cytokine of the Th2 $CD4^+$ T cell response. Cervera (2007) showed an increase in TNF-α in response to HCMV in solid organ transplant recipients demonstrating a bias towards a Th1 $CD4^+$T cell response. Contrary to these studies, there is also evidence of a dominant Th2 $CD4^+$ T cell response to HCMV peptides. In kidney transplant recipients increased levels of IL-10 indicative of a higher Th2 response to certain HCMV peptides was observed (Essa *et al*., 2009). Decreased levels of IFN-γ and TNF-α were simultaneously observed suggesting a lower Th1 response. Sadeghi (2008) also provides evidence of a dominant Th2 $CD4^+$ T cell response to HCMV in renal transplant recipients. Decreased levels of IFN-γ and increased levels of IL-10 and IL-6 suggest a dominant Th2 response.

The $CD8^+$ T cells play a major role during HCMV reactivation. There is evidence that $CD4^+$ T cells are essential for the sustained antiviral response of the $CD8^+$ T cells (Komanduri *et al*., 2001; Lilleri *et al*., 2006; Sacre *et al*., 2005). $CD4^+$ T cells secreting IFN- γ decrease during the year prior to the onset of HCMV EOD (Bronke *et al*., 2005). Although the $CD8^+$ T cell concentration remains high in progressive HIV infection, IFN-γ producing $CD8^+$ T cells decrease in those progressing to AIDS. This is possibly due to insufficient $CD4^+$ Th1 cells while proportions of $CD8^+$ T cells that secrete perforin and granzyme increase compared to those that secrete INF-γ (Bronke *et al*., 2005).

A study comparing the differences between naïve and memory T cells in developing and developed countries such as Malawi and UK has shown that the Malawian adolescent has fewer naïve T cells and higher $CD28^-$ memory T cells than the UK adolescent. This has been hypothesised to be due to exposure at a younger age to specific antigens in the Malawian population. These memory cells have reduced ability to proliferate and may adversely affect the immunological response to HCMV antigens (Ben-Smith *et al*.,

2008).It is not yet clear whether HCMV exposure at a young age does in fact increase the risk of HCMV reactivation during a state of immune compromise.

$CD8^+$ T cells that lack expression of CD28 and express CD57 are associated with impaired immune responses as is in the case of a chronic HCMV infection and in the elderly (Khan *et al*., 2002).Many HCMV-specific $CD8^+$ T cells expressing CD45RA and lacking the expression of CD28 express inhibitory and activating NK cell receptors such as KLRG1 (killer cell lectin-like receptor G1) and low levels of IL-7Rα suggesting that these cells are terminally differentiated. These cells have a reduced proliferative capacity affecting their response to continued stimulation by HCMV antigens (Joshi *et al*., 2007). However, with the proper combination of antigen and cytokines these cells can expand (van de Berg *et al*., 2008).

In a HIV-HCMV co-infected population the HIV-specific $CD8^+$T cells of ART naïve individuals have a surface phenotype $CD45RA^-/CCR7^-$ termed ―pre-terminally differentiated". This differs from the HCMV-specific $CD8^+$T cells which predominantly express a $CD45RA^+/CCR7^-$ phenotype (Jagannathan *et al*., 2009). It has been reported that IL-2 promotes the number of cells proliferating as well as the number of divisions of the HCMV-specific $CD8^+$ T cells, whereas the proliferative capacity of the HIV-specific $CD8^+$ T cells is reduced (Jagannathan *et al*., 2009).

1.2.7. Components of HCMV that elicit a cellular immune response

Once the virus has entered the host, an immune response is mounted against the HCMV antigens. The HCMV genome codes for more than 200 immunogenic proteins that include the major IE-1 protein, gB and the matrix protein, pp65 (Griffiths, 2009). These are targeted by the $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ T cells (Gilbert *et al*., 1993; Bao *et al*., 2008).

Data have indicated that the response of the $CD4^+$ T cells and the $CD8^+$ CTL to the same HCMV antigens, differ (Gratama *et al*., 2008; Sinclair *et al*., 2004). The $CD4^+$ T cell response to the HCMV antigens in healthy individuals is more robust than the $CD8^+$ T

cell response. In contrast, in the HIV-HCMV co-infected individuals, the $CD8^+$ T cell response is greater than the $CD4^+$ T cell response (Sinclair *et al*., 2004). Besides a difference in response of the T cells to the same antigens, reports have indicated a difference in the response of the T cells to different antigens. The $CD8^+$ CTL are purported to mount a more robust immune response to the pp65 antigen than the IE-1 antigen (Gilbert *et al*., 1993; Bao *et al*., 2008). Sacre (2005), however, has demonstrated a similar degree of response of the HCMV-specific $CD8^+$ CTL cells to the HCMV pp65 protein and the HCMV IE-1 protein in healthy individuals.

Genetic variations of the human leukocyte antigen (HLA) genes (Valluri *et al*., 2005) may result in changes in the amino acid sequences of genes coding for different proteins (Mazzarino *et al*., 2005). Specifically, changes in the MHC-class I molecules [*refer to section 1.2.6*] will result in variations in the presentation of peptides such as HCMV pp65 or HCMV IE-1 to $CD8^+$ CTL (Mazzarino *et al*., 2005). Thus, this may lead to variations in responses to HCMV peptides by different individuals.

1.2.8. Immune evasion by HCMV

Once the virus has established itself in the human host it can successfully evade the immune system. Various mechanisms to evade the immune system have been described. One is that the expression of the MHC class I molecules that present viral infected cells to the $CD8^+$ CTL are inhibited by the HCMV infection (Mocarski, 2004). Normally the $CD8^+$ CTL lyse these virally infected cells. By inhibting the MHC class 1 expression HCMV may evade the antiviral mechanism. A second theory is that the HCMV adversely affects IFN-γ mediated MHC class II-dependent antigen presentation by macrophages to T cells (Cheung *et al*., 2009; Kano and Shiohara, 2000; Mocarski, 2004). The HCMV UL111A gene has been identified as a contributory factor to the latter mechanism and thereby contributing to the latent state of the virus (Cheung *et al*., 2009). Interleukin (IL) -10 is a human cytokine that inhibits the inflammatory and cell-mediated immune response by suppressing the production of pro-inflammatory cytokines and the expression of MHC class II and costimulatory molecules. It has been demonstrated that the virus uses the human IL-10/IL-10R1 signalling pathway to evade the immune system. One of

the HCMV genescodes for the human IL-10 homolog called cmvIL-10.The homolog cmvIL-10 can bind to IL-10R1, utilising the IL-10R complex and inhibiting the inflammatory and cell-mediated immune response, thereby preventing its clearance from the host (Sezgin *et al*., 2010).

1.2.9. Interferon gamma and HCMV

The invasion of cells by HCMV promotes the release of various cytokines by immune cells. In the current study IFN-γ is the cytokine [*refer to section 2.4.4 in Chapter 2*] which was investigated. It is secreted by $CD8^+$ T cells, Th1 $CD4^+$ T cells and NK cells in response to cells infected by pathogens. It contributes to the host‘s defence in a number of ways. Once secreted by the $CD8^+$ T cells, IFN-γ up regulates MHC class I molecule expression in cells infected with HCMV. In this way it facilitates the recognition of infected cells by the $CD8^+$ CTL (Cheeran *et al*., 2008). IFN-γ induces the products of cellular molecules such as nitric oxide (NO) that directly interferes with viral replication (Harris *et al*., 1995; Whitman *et al*., 2008). The IFN-γ stimulates macrophages to up regulate inducible nitric oxide synthase (iNOS) which catalyses the formation of nitric oxide (NO) (Henke *et al*., 2003).

1.2.10. HIV-HCMV co-infected individuals

HIV is a retrovirus whose infection rates have reached pandemic proportions in Sub-Saharan Africa (Connolly *et al*., 2004). It targets and specifically depletes $CD4^+$ T cells leading to impairment of the immune system. The relevance of studying HCMV seropositive subjects who subsequently acquire HIV is that the HCMV can promote HIV replication accelerating progression to HIV/AIDS as reviewed by Griffiths (2009). Sabin (2000) reported that haemophilic, HIV-positive individuals co-infected with HCMV progresses more rapidly to AIDS than haemophilic, HIV-positive individuals who are HCMV-negative. This is consistent with the hypothesis that a heightened pro-inflammatory state is induced by HCMV infection (van de Berg *et al*., 2010) and may accelerate progression to chronic diseases.

Before the HAART era, HCMV-related diseases were associated with an increased risk in morbidity and mortality in HIV/AIDS patients. The HCMV-associated diseases were usually under-diagnosed and often mistakenly not attributed to mortality in HIV/AIDS (Heiden *et al*., 2007). HAART has significantly reduced the incidence of HCMV-associated diseases in developing countries (Mocroft *et al*., 2000; Palella *et al*., 1998). However, only a small percentage of HIV-positive individuals receive HAART in developing countries (Manosuthi *et al.,* 2007).

Among the HIV-seropositive subjects, CMV-specific $CD8^+$ T cell responses were proportionately lower during recent infection, higher during chronic untreated infection and higher still during long-term antiretroviral treated infection.

With regards the effect of HIV-infection on HCMV reactivation, the $CD4^+$ T cells and $CD8^+$ T cells secrete cytokines that are able to efficiently suppress viral infections in immunocompetent individuals but in HIV-positive individuals the latent HCMV may reactivate (Augustine *et al*., 2007). In HIV-positive individuals, levels of HCMV-specific $CD8^+$ T cells were lower during a recent infection, higher during chronic untreated infection and higher still during long-term antiretroviral treated infection (Naeger *et al*., 2010). Studies have been done to determine the number of HIV-positive individuals who have HCMV viraemia and subsequently develop HCMV EOD such as HCMV retinitis and HCMV colitis. Wohl (2009) found that 14% of HCMV viraemic patients compared to only 1% of aviraemic patients developed HCMV EOD. Casado (1999) found a 12 month incidence rate of 38% of HCMV EOD and only a 2 % rate amongst aviraemic patients. A much lower rate of 6% with EOD was reported by Erice (2003) in 85% of viraemic patients receiving HAART. The lower incidence rate in the study of Erice (2003) was due to the initiation of HAART. However, despite the availability of HAART, HCMV EOD is still possible in profoundly immunocompromised HIV-positive individuals. This warrants the need for monitoring HCMV reactivation in HIV-positive individuals.

Another factor that warrants the further investigation of HCMV in the HAART era is ART resistance. HIV/AIDS patients who do receive antiretroviral drugs may become resistant to them due either to a genetic predisposition or due to non-compliance. The risk of HCMV reactivation is therefore significant (Kakehasi *et al*., 2007; Rodes *et al*., 2005; Steegen *et al*., 2009).

An additional complication that may occur in HIV-HCMV co-infected individuals is the appearance of the immune reconstitution inflammatory syndrome (IRIS) which exhibits clinical scenarios such as HCMV immune recovery vitritis (IRV) or HCMV immune recovery uveitis (IRU). IRV and IRU are forms of inflammation of the eye which causes blurred vision (Bonham *et al*., 2008; Smith *et al*., 2009; Springer and Weinberg, 2004). IRIS is the exaggerated inflammatory response to HCMV antigens after immune reconstitution. The response could activate a previously treated disease or unmask a newly identified disease. Various factors such as advanced HIV/AIDS, a low $CD4^+$ T cell count and opportunistic infections at the time of HAART initiation increases the risk of IRIS (Murdoch *et al*., 2008; Smith *et al*., 2009). HIV/AIDS persons in resource-limited regions are at a greater risk of developing IRIS because they often have advanced HIV/AIDS, low $CD4^+$ T cell counts and more opportunistic infections at the time of initiation of HAART (Bonham *et al*., 2008; Smith *et al*., 2009).

1.2.11. Markers to detect HCMV reactivation in HIV-positive individuals

Various parameters have been investigated as possible markers predicting HCMV reactivation in HIV-positive individuals before the introduction of HAART and during the HAART era. These include $CD4^+$ T cell count, HIV viral load and HCMV viral load (Erice *et al*., 2003; Salmon-Ceâron *et al*., 2000). Establishing the most appropriate marker for HCMV reactivation in HIV-positive individuals resulted in numerous investigations with varying and conflicting results. Different assays were also employed in the search for a suitable marker. As yet no consensus has been reached regarding the appropriate marker or assay predicting HCMV reactivation in HIV-positive individuals.

1.2.11.1. HCMV viral load

Primary infection or HCMV reactivation is characterised by the presence of the virus in blood (Bronke *et al.,* 2005). HCMV viraemia is a risk factor for HCMV EOD in immunocompromised individuals (Boeckh and Boivin, 1998; Ruell *et al*., 2007; Salmon-Ceâron *et al*., 2000). The HCMV pp65 antigenemia assay [*refer to section 1.2.12.2*] is routinely used to detect HCMV in the blood. However, the qPCR [refer to section *1.2.12.3*] has proven more sensitive than the pp65 antigenemia to detect HCMV viraemia (Allice *et al*., 2006; Boeckh *et al*., 2004).

It is purported that the qPCR is not 100% sensitive for HCMV viraemia. In a study of stem cell transplant recipients, the qPCR was not able to detect a HCMV viraemia in 50% of individuals who developed HCMV EOD. Of these individuals, 75% were aviraemic throughout the disease. However, it should be noted that a cut-off of 500 copies/ml was used to detect viral loads and it is likely that it may not have been sensitive enough to detect lower limit viral loads (Ruell *et al*., 2007).

There is no consensus as to what is the most appropriate HCMV viral load value to distinguish between HCMV latency and reactivation. The lack of consensus may well be the reason for the discrepancy in results of studies that have tried to distinguish between latency and reactivation (Engelmann *et al*., 2008; Peres *et al*., 2010; Sanghavi *et al.,* 2008).

A study conducted by Wohl (2009) investigated quantitatively the HCMV DNA and the HIV ribose nucleic acid (RNA) by PCR and the $CD4^+$ T cell count of HIV-positive individuals. Wohl (2009) did not investigate the HCMV-specific immune response of individuals. The authors demonstrated that the PCR is more effective in determining survival rate of HIV-positive individuals than $CD4^+$ T cell count and HIV RNA load.

Various investigations have been undertaken to establish which analyte would be more suitable in predicting HCMV viraemia in qPCR (Koidl *et al*., 2008; Peres *et al*., 2010; Preiser *et al*., 2003a; Razonable *et al.*, 2002). It is important to note that during latency

the virus is cell bound. Active HCMV infection sees the release of the virus into the blood plasma. Thus, it would appear that plasma would be a more suitable predictor of viraemia (Preiser *et al*., 2003a). Despite this no consensus has been reached regarding the most suitable blood analyte for quantifying HCMV DNA. Technical differences between the various commercial and in-house PCR kits that are utilised have been implicated as contributing to the lack of consensus. A comparison of data from different laboratories proves difficult since qPCR methods are not standardized. The use of different target sequences, primer sets, and extraction and detection methods results in varying results (Koidl *et al*., 2008; Peres *et al*., 2010; Preiser *et al*., 2003a).

1.2.11.2. HIV viral load

HIV viral load has also been investigated as a possible marker to determine when the HCMV-specific cellular immune response breaks down and HCMV reactivation occurs. In a univariate analysis Salmon-Ceâron (2000) showed that plasma HIV viral load may be considered a possible marker. An HIV viral load greater than 100 000 copies/ml was predictive for HCMV reactivation. However, in a multivariate analysis in the same study, plasma HIV viral load did not prove significant. In the multivariate analysis only the plasma HCMV DNA, pp65 antigenemia >100 nuclei/200 000 cells and a $CD4^+$ T cell count, 75 x 10cells/litre (*l*) were associated with progression to EOD.

1.2.11.3. $CD4^+$ T cell count

Studies have found that immunocompromised individuals with a low $CD4^+$ T cell count (less than 50 cells/µl) are at risk of developing HCMV disease while a high $CD4^+$ T cell count is protective against HCMV reactivation and progression to HCMV disease (Holmes *et al*., 2006; Sacre *et al*., 2005; Salmon-Ceâron *et al*., 2000). In addition, Erice (2003) has demonstrated that despite receiving HAART, a high percentage of individuals with a low $CD4^+$ T cell count are at risk of HCMV reactivation.

However, the relationship between $CD4^+$ T cell count and HCMV related EOD is not that straightforward as is discussed in the following two paragraphs. In some instances HIV-positive individuals with a high $CD4^+$ T cell count or those with an increase in $CD4^+$ T

cell count after treatment with HAART are still at risk (Keane *et al*., 2004). The authors of the latter study hypothesised that this observation may be due to profound immunosuppression before initiation of HAART (Keane *et al*., 2004) or may be due to the impairment of $CD8^+$ T cells necessary for the suppression of viral replication (Singh *et al*., 2007).

Wohl (2009) showed that a small percentage of HIV-positive individuals with a low $CD4^+$ T cell count, despite being on HAART, progressed to HCMV EOD. This was in agreement with the findings of Song (2002) who reported that individuals resistant to HAART with a low $CD4^+$ T cell count and not on prophylactic antiviral treatment did not develop HCMV retinitis. Song (2002), further reported, no predictive pattern between patients with reactivated HCMV retinitis and those who did not develop HCMV retinitis. Their study suggests utilising retinal examinations to determine whether reactivation of HCMV retinitis is imminent rather than using a specific $CD4^+$ T cell count which may appear unreliable.

Weinberg (2006) found that a robust response was usually associated with a higher $CD4^+$ T cell count. Similarly, a weak HCMV-specific cellular immune response was associated with a low $CD4^+$ T cell count. This suggests a close relationship between $CD4^+$ T cell count and HCMV-specific cellular immune response.

However, Fernandez (2006) demonstrated that the HCMV-specific cellular immune response was independent of the $CD4^+$ T cell recovery in HIV-positive individuals initiated on HAART. This confounds the relationship between HCMV-specific cellular immune response and $CD4^+$T cell count. The relationship between HCMV-specific cellular immune response and $CD4^+$ T cell count requires clarification.

1.2.12. Laboratory methods of diagnosing HCMV infection and disease

The rapid detection of active HCMV infection in immunocompromised individuals plays a pivotal role in pre-emptive treatment strategies. Direct detection of the virus can be achieved by virus isolation on cell cultures, detection of viral antigens and the detection

of viral nucleic acids (Preiser *et al*., 2001). Indirect techniques include detection of anti-HCMV antibodies (Preiser *et al*., 2001) and the cellular antiviral immune response to HCMV in the host.

Blood and tissue from end-organs such as the kidney or liver (Storch *et al*., 1994) as well as cerebrospinal fluid (Stanojevic *et al*., 2002) can be obtained and analysed using various assays to detect HCMV disease entities discussed below. In addition specimens such as urine, faeces, bone marrow tissue and respiratory tissue can also be utilised to determine the presence of HCMV (Engelmann *et al*., 2008).

1.2.12.1. Viral culture

Human fibroblasts are used to isolate HCMV. Conventional viral culture takes up to 14 days to obtain results, while rapid shell vial cultures provide results within 24hours to 48hours. A distinctive cytopathic effect (CPE) with a confirmatory fluorescent antibody stain is visualised (de la Hoz *et al*., 2002; Storch *et al*., 1994). Tissue samples required for viral culture includes urine, throat swabs and saliva (Hassan and Connell, 2007).

1.2.12.2. HCMV pp65 antigenemia assay

The HCMV pp65 antigenemia assay allows for the direct detection of HCMV pp65 antigen in peripheral blood leukocytes. In the HCMV pp65 antigenemia assay the circulating cells are stained with monoclonal antibodies that target the HCMVpp65 antigen. HCMV infection is evident as intranuclear inclusion bodies called "owl's eye" inclusion bodies that stain dark pink on haematoxylin and eosin (H and E) staining (Boeckh *et al*., 2004). Erice (1992) presented evidence that the antigenemia assay was more sensitive than the shell vial culture in detecting HCMV. In contrast, Storch (1994) has demonstrated that the pp65 antigenemia assay and the shell vial culture are equally sensitive in detecting HCMV in solid organ transplant (SOT) recipients. However, the advantage of the pp65 antigenemia assay is that fewer cells are required. A further advantage is that the sensitive process of cell culture is omitted.

1.2.12.3. Polymerase chain reaction

The polymerase chain reaction (PCR) can be used to detect viral DNA in ethylenediaminetetraacetic acid (EDTA) whole blood or EDTA plasma, peripheral blood mononuclear cells (PBMC) or bronchoalveolar lavage. This method was developed by Kary B. Mullis in 1985 (Mullis and Faloona, 1987). During PCR, a specific viral DNA sequence is amplified from its initial concentration to a level at which it can be accurately analysed either quantitatively or qualitatively. The PCR cycle consists of consecutive steps of DNA heat denaturation, primer annealing and DNA extension that are repeated several times. In order for this to occur two oligonucleotide primers specific to the target sequence and heat stable DNA polymerase enzymes are required. High temperatures allow the DNA to unwind. The temperature is lowered and the primers anneal to the target sequence. Finally an optimal temperature is set allowing for the heat stable DNA polymerase enzymes such as *Thermus aquaticus* (*Taq*) DNA polymerase to extend the primers by the addition of deoxyribonucleoside triphosphates (dNTPs) (Kubista *et al*., 2006).

A number of different PCR techniques have been developed on the blue print of that described by Mullis (1987), such as qPCR. A positive qPCR indicates an increase in the viral load and possible reactivation of the virus. An advantage of the qPCR over the conventional PCR is that the qPCR provides both qualitative and quantitative detection of the product formed during the cycle. This is in contrast to the conventional PCR that provides a qualitative result at the end of its cycle. In the qPCR a fluorescent reporter is required which binds to the newly formed DNA and fluoresces allowing for early detection (Kubista *et al*., 2006). Studies have reported a greater sensitivity in qPCR detecting HCMV than the pp65 antigenemia assay (Boeckh *et al*., 2004; Preiser *et al*., 2003a).

1.2.12.4. Antibody detection

Antibodies, however, may not always be formed. In a report by van der Giessen (1990), neither IgM nor IgG antibodies to HCMV were detected in certain HCMV-infected individuals using the ELISA. Novak (2009) describes a novel enzyme-linked

immunosorbent assay (ELISA) technique that is able to detect the presence of antibodies against different strains of HCMV. This provides opportunities for the investigation of HCMV re-infections and its effect on individuals. However, this technique was unable to detect antibodies in some individuals who had been exposed to HCMV suggesting the possibility of the presence of other epitopes of HCMV not detected by the ELISA.

The presence of IgG HCMV-specific antibodies in immunocompromised individuals suggests past exposure to the virus. Knowledge of past exposure is particularly important in individuals with advanced HIV/AIDS since an immunocompromised state may trigger HCMV reactivation.

ELISA is commonly used to detect HCMV antibodies (Priya *et al*., 2002; van der Giessen *et al*., 1990). An alternate assay is Western blotting but this is not commercially available (Hassan and Connell, 2007). Commercial IgG ELISA is simple to use. In the course of the current study the commercially available VIDAS®HCMV IgG test is used to determine the HCMV IgG serostatus of the subjects. A drawback, however, is that they use arbitrary units that vary between assays (Mendelson *et al*., 2006).In the instance that IgG and IgM antibodies are both positive on a sample, an IgG avidity assay is then required to determine the time of infection. This is essential in pregnant women as a primary HCMV infection carries a higher risk of vertical transmission than a reactivated one (Lazzarotto *et al*., 2008). A low avidity index indicates a recent primary infection and prenatal diagnosis should follow. In contrast, a high avidity index indicates neither recent nor a primary infection (Lazzarotto *et al*., 2008; Mendelson *et al*., 2006).

1.2.12.5. Immunological assays

The cell-mediated immune response to HCMV has been investigated in immunocompromised individuals such as those living with HIV/AIDS, recipients of immunosuppressant drugs after organ or SCT. The cell-mediated immune response [*refer to section 1.2.6*] is essential for suppressing HCMV and monitoring the activity of T cells in response to specific antigens may be a suitable marker to assess the integrity of the immune system (Ohnishi *et al*., 2005). Failure of the immune system as measured by

reduced HCMV-specific activity of the T cells may be an indicator for pre-emptive therapy against HCMV. To assess the active immunity against HCMV various immunological assays can be employed such as the lymphocyte proliferation assay (LPA) (Augustine *et al*., 2007; Gehrz and Knorr, 1979), the IFN-γ-ELISPOT assay (Macatangay *et al*., 2010; Stone *et al*., 2006), intracellular cytokine staining (ICS) (Kern *et. al*., 1999) and tetramer staining (Hernandez-Fuentes *et al*., 2003). A variety of assays are commercially available (QuantiFERON®-CMV, Cellestis Limited, Melbourne, Australia; IFN-γ ELISPOT assay®, Mabtech AB, Sweden) to determine the cell-mediated immune response of an individual to HCMV infections. However, these assays are not routinely used in a diagnostic setting.

In order to measure the magnitude of the response of the T cells, different antigens or mitogens are used to stimulate them. To determine an individual‘s response to the HCMV, different antigens such as the whole HCMV lysate or overlapping peptide pools (pp65 or IE-1 proteins) are utilised. Mitogens such as phytohemagglutinin (PHA) are usually used as a positive control in immunological assays (Augustine *et al*., 2007).

The lymphocyte proliferation assay *(*LPA) measures the response of total T-lymphocytes to antigens such as HCMV (Augustine *et al*., 2007). Numerous research efforts have been made using the LPA to study the immunological function of peripheral blood mononuclear cells (PBMC). It was suggested that lymphocyte proliferation involved the intricate interaction of multiple mononuclear cell subpopulations as antigen presenting cells (Gehrz and Knorr, 1979). Various mitogens such as phytohemagglutinin (PHA), concanavalin A, and pokeweed have been utilized in the LPA to elicit a cell-mediated immune response (Augustine *et al*., 2007). Only PHA was utilised in the current study to stimulate the isolated PBMC of subjects. The LPA requires heparinised blood and the isolation of PBMC from whole blood by density gradient centrifugation. They are cultured with a mitogen and/or an antigen with varying incubation periods (4 to 7 days) (Macatangay *et al*., 2010). Proliferation of lymphocytes can be measured by the integration of a radioactive material, tritiated thymidine (^{3}H-thymidine), into the DNA of

dividing cells. Results are expressed as counts per minute (cpm) and/or as a stimulation index (SI) (Augustine *et al*., 2007; Gotch *et al*., 2005; Macatangay *et al*., 2010).

The labelling of lymphocytes with carboxyfluorescein succinimidyl ester (CFSE) and its combination with ICS is considered a useful tool in monitoring T-cell proliferative responses. The carboxy-fluorescein diacetate succinimydyl ester (CFDA–SE) is a lipophilic molecule with a low fluorescence. It enters PBMC and an acetyl group is cleaved resulting in the fluorescent CFSE. The intensity of the fluorescence is halved each time the cell divides. The cells are harvested and stained with various antibodies to delineate different T cells. Flow cytometry is used to analyse lymphocyte proliferation. Fluorescent labelled antibodies are used to isolate specific T cells. The simultaneous determination of the proliferation, cytokine release and the phenotypic characterization of the stimulated cells is a major advantage of this assay (Gotch *et al*., 2005; Luzyanina *et al*., 2007). Tetramer staining consists of four MHC–peptide complexes that are covalently linked to a fluorochrome. It is a powerful tool to detect T cells but requires matching of tetramers to the MHC of the donors (Hernandez-Fuentes *et al*., 2003).

The ELISPOT assay was first introduced to measure the frequency of B-lymphocytes that secrete antibodies (Czerkinsky *et al*., 1983). Subsequently, it became a valuable assay to measure the frequency of cells that secretes various cytokines such as IFN-γ, IL-4, IL-5 and 1L-10 (Hauer *et al*., 1997) in response to various pathogens such as the influenza virus (Jia *et al*., 2009), *Mycobacterium tuberculosis* (Karam *et al*., 2008; Lawn *et al*., 2007) and HIV (Gray *et al*., 2009). The granzyme B ELISPOT may be utilised to determine cell-mediated immune response (Rininsland *et al.,* 2000). Nonetheless, the IFN-γ-ELISPOT assay is still the most widely used and generally accepted read-out of the response to HCMV (Barron *et al*., 2009, Gray *et al*., 2009; Macatangay *et al*., 2010, Weinberg *et al*., 2006, Zhang *et al*., 2009) It is easier to undertake than a dual-cytokine ELISPOT assay which includes IL-2 (Boulet *et al*., 2007).Unlike an ELISA it does not measure the concentration of the secreted cytokine. Further roles for the ELISPOT assay were described recently for the development of vaccines against a variety of infections (Pass *et al*., 2009) and in cancer research (Schmittel *et al*., 2000).

The ELISPOT assay requires the isolation of PBMCs from heparinised blood as described in the material and methods in Chapter 2. Typically the cytokine secreted by the T cells is captured by anti-cytokine antibodies. Capturing of the cytokine is detected by a second antibody. A spot representing a single cytokine secreting T cell is formed. The frequency of cells secreting IFN-γ is given as spot forming units (SFU)/10^6 cells, measured with an automated reader (Gotch *et al*., 2005; Hobeika *et al.*, 2005; Macatangay *et al*., 2010; Mashishi and Gray, 2002).

To obtain a positive result for the IFN-γ-ELISPOT assay certain criteria must be met. These usually include a low background control and a high positive control which need to meet defined criteria. These criteria vary within the different laboratories utilising the IFN-γ-ELISPOT assay. The spot parameters used by most automated ELISPOT readers, are diameter, contrast, hue, saturation, shape and slope (Hobeika *et al*., 2005; Macatangay *et al*., 2010; Mashishi and Gray, 2002). This assay is performed over three days.

1.2.13. IFN-γ-ELISPOT assay and HCMV-specific cellular immunity

In many studies the IFN-γ-ELISPOT assay has been demonstrated to be effective at measuring the HCMV-specific cellular immune response. Weinberg and Pott (2003) demonstrated that the IFN-γ-ELISPOT assay was more sensitive than either of the LPA or flow cytometry to detect changes in HCMV-specific cellular immunity in HIV-positive children on HAART.

In one study the cellular immune response of 20 HIV-infected individuals was measured with the IFN-γ-ELISPOT assay and cytokine flow cytometry (Karlsson *et al*., 2003). This was done to compare the sensitivities of the two assays. The antigens used in the study were HCMVpp65 antigens and HIV-gag peptide pools. While the two assays were concordant, the IFN-γ-ELISPOT assay, however, was better able to detect low–level responses than cytokine flow cytometry. In contrast, Sun (2003) demonstrated that the IFN-γ-ELISPOT assay and the cytokine flow cytometry showed similar frequencies of HIV-specific $CD8^+$ T cells responses. A further discrepancy between the two assays (Karlsson *et al*., 2003) was the difference in magnitude of the cellular immune response

to HCMV antigens. The response to the HCMV pp65 peptide pool was greater when using flow cytometry in this study.

A prospective study by Weinberg (2006) demonstrated that the IFN-γ-ELISPOT assay was more sensitive than the $CD4^+$ and $CD8^+$ intracellular IFN-γ assays in determining a link between HCMV-specific cellular immunity, HCMV viraemia and HCMV-EOD in HIV-positive individuals. In their study, a positive IFN-γ-ELISPOT assay result indicated an effective HCMV-specific cellular immune response which delayed the onset of HCMV viraemia and HCMV EOD.

The IFN-γ-ELISPOT assay was compared with the proliferation assay to detect the immune response to various antigens (Goodell *et al*., 2007). The proliferation assay accurately detected low (antigens to HER-2/neu), moderate (antigens of tetanus toxoid (tt)) and robust T cell responses (antigens to HCMV). The INF-γ ELISPOT assay was able to accurately detect the robust T cell immune response (antigens to HCMV). However, when the sensitivities of the two assays in response to HCMV antigens were compared, the sensitivity of the HCMV IFN-γ-ELISPOT assay was 92% versus 88% for the HCMV proliferation assay.

Barron (2009) used the IFN-γ-ELISPOT assay, flow cytometry and LPA to determine possible markers for HCMV reactivation in stem cell transplant recipients. The flow cytometry and IFN-γ-ELISPOT results of aviraemic and viraemic individuals were similar. However, a strong HCMV-specific cellular immune response using the LPA was associated with a lower risk of HCMV viraemia. LPA, although considered the gold standard for detection of HCMV-specific cellular immune response by Augustine *(*2007), often involves the use of a radioactive material which requires special containment facilities. These are not required for the IFN-γ-ELISPOT assay. The LPA is more time consuming than the IFN-γ-ELISPOT assay. Stimulation of the PBMC with different antigens takes 7 days in the LPA, whereas the IFN-γ-ELISPOT assay usually requires three days for its completion (Macatangay *et al*., 2010).

Although the IFN-γ-ELISPOT assay does not allow for the identification of the exact phenotype of T cells stimulated as in the flow cytometry, it requires far less PBMC than the ICS technique for analysis and may be a better choice in a diagnostic setting (Goodell *et al*., 2007). Tetramer binding looks at the binding of HLA-peptide tetramers to specific T cells that requires flow cytometry to be detected. In a previous study in our laboratory, we established that the IFN-γ-ELISPOT assay was more sensitive than LPA in determining the HCMV-specific cellular immune responses in HIV-positive individuals. The evidence described in the above studies supports the use of the IFN-γ-ELISPOT assay in our research project and as a routine diagnostic tool in determining the HCMV-specific cellular immune response in HIV-positive individuals.

1.2.14. Treatment of HCMV infections

At present five main drugs are available for treatment of HCMV disease in immunocompromised patients. These include ganciclovir, foscarnet, cidofovir, acyclovir and fomivirsen (Kalil *et al*., 2005; Paya *et al*., 2004). The drugs improve the prognosis of the disease but factors such as high toxicity, development of drug resistant strains and cost of treatment are problematic (Godard *et al*., 2004). Recipients of SOT and stem cell transplants (SCT) on immunosuppressive drugs at risk of developing HCMV reactivation may be administered pre-emptive or prophylactic treatment. Pre-emptive treatment entails the regular monitoring, using PCR or the pp65 antigenemia assay of individuals at risk of HCMV reactivation (Ruell *et al*., 2007). When the virus is detected and HCMV reactivation is diagnosed, prophylactic treatment is provided. In contrast, prophylactic treatment provides all individuals at risk of developing HCMV disease with drug therapy irrespective of their HCMV viraemic status. It is thus essential to determine a means by which only the individuals at risk of reactivation progressing to EOD receive pre-emptive treatment.

1.3. Problem Statement

HCMVEOD is life-threatening in HIV-positive individuals. Establishing a biomarker that can predict possible HCMV reactivation and EOD is most desirable. Various biomarkers have been considered such as HCMV viral load, $CD4^+$ T cell count and HIV viral load. Alternative tests such as the IFN-γ-ELISPOT assay have shown promise in identifying HCMV reactivation. However, no consensus has been reached as to which is the most suitable biomarker. (Erice *et al*., 2003; Fernandez *et al*., 2006; Jacobson *et al*., 2008; Singh *et al*., 2007; Weinberg *et al.*, 2006; Wohl *et al.,* 2009).

The aim of the study was thus to determine whether there is a point at which the HCMV-specific cellular immune integrity was lost by using the HCMV IFN-γ-ELISPOT assay as a tool in HIV-positive, ART-naïve individuals in a South African setting. This was done in relation to the HCMV viral load, as determined by the HCMV viral load qPCR and the $CD4^+$ T cell count.

Chapter 2

2. Materials and Methods

2.1. Introduction

A cross-sectional observational study was undertaken during the period January 2009 to August 2010. Fifty-two HIV-positive, ART-naïve, adult subjects were recruited for the study. The HCMV IgG test, the $CD4^+$ T cell counts, an IFN-γ-ELISPOT assay and the HCMV viral load qPCR, utilising the subjects' blood samples were conducted in the laboratory of the Division of Medical Virology, Stellenbosch University, Tygerberg Campus, South Africa. The HCMV-specific cellular immune response of each subject was ascertained by using the IFN-γ-ELISPOT assay. The qPCR provided the HCMV viral load of the subjects at the time of blood sample collection. The HCMV IgG serostatus of each subject determined whether they had previously been exposed to the virus. The $CD4^+$ T cell count of each subject was also determined at the time of recruitment. The purpose of the assays was to determine whether there is a point at which the HCMV-specific cellular immune response breaks down in relation to the $CD4^+$ T cell count and HCMV viral load. In this chapter a detailed description of the cohort sample and the research assays used in this study are presented.

2.2. Ethics Approval

This research project was approved by the Ethics Committee of Human Research, Division of Research, Development and Support, Stellenbosch University, Tygerberg Campus, Cape Town, South Africa (Appendix A). The ethics reference number for the study was: N09/01/007. The approval dates for the study was from the 14 April 2009 to the 15 April 2010. The dates were extended from the 15 May 2010 to the 15 May 2011 (Appendix B) to allow for further sample collection. Permission was also granted by the Department of Health to allow for sample collection at the various primary healthcare facilities in the greater Cape Town area (Appendix C).

2.3. Cohort Sample

2.3.1. Demographics of cohort sample

Fifty-two human subjects were recruited from the Idas Valley clinic in Stellenbosch, the Kraaifontein HIV clinic and the Durbanville HIV clinic in Cape Town, South Africa. All the subjects provided written informed consent for participation in the study. (Appendix D). The cohort of 52 subjects consisted of 34 females and 18 males with an average age of 34.9 (± 8.1) years. Subject volunteers routinely attended the clinics for regular health checks and counselling related to their HIV status.

2.3.2. Inclusion criteria of study

All subjects recruited were HIV-positive, ART-naïve adults (≥18 years).

2.3.3. Exclusion criteria of study

Subjects excluded from the study were those individuals who were receiving ART, TB patients, pregnant women and children (<18 years). To avoid any potential conflicts between HCMV and TB, HCMV subjects co-infected with TB were excluded from the current study.

2.3.4. Blood samples of recruited subjects

Approximately 20 ml heparinised blood and 10 ml of EDTA blood was taken from each subject. For the validation of the HCMV IFN-γ-ELISPOT assay two 20 ml heparinised blood samples from four HIV-negative individuals were obtained.

2.4. Methods

2.4.1. Sample Preparation

The 20 ml heparinised blood obtained from subjects was used for two different assays. These included the IFN-γ-ELISPOT assay and the HCMV IgG serology test. The heparinised blood was centrifuged at 275 x gravity (g) for 10 minutes (min) at 20ºC. A volume of 500 µl plasma from the heparinised blood was removed and stored at -20ºC for the later determination of the HCMV IgG serostatus of each subject. The remaining blood was used to isolate PBMC by density gradient centrifugation for the determination of the

HCMV-specific cellular immune response when stimulated with HCMV pp65 antigens, PHA-P and anti-CD3.

The 10 ml volume of EDTA blood provided whole blood and plasma for the determination of the HCMV viral load using qPCR. A volume of 500 µl of the EDTA whole blood was removed for qPCR and stored in the -20ºC refrigerator. A further 150 µl of EDTA whole blood was removed for the determination of the $CD4^+$ T cell count and stored in -20ºC refrigerator. The rest of the EDTA blood was then centrifuged at 800 x g for 10 min. A volume of 500 µl plasma was removed for HCMV viral load qPCR and stored in the -20ºC refrigerator for later analysis. HCMV DNA was extracted from the 200 µl of frozen plasma and whole blood using the QIAamp® DNA Blood Mini kit (Qiagen, Hilden, Germany). Thereafter, HCMV DNA was amplified as per an in-house HCMV viral load qPCR. The $CD4^+$ T cell count was determined within 24 hours of sample collection using the FACSCalibur® flow cytometer (Becton, Dickson Co., United States of America (USA). [*See details below*]

2.4.2. The VIDAS® HCMV IgG test

Approximately 500 µl of plasma from the 20 ml heparinised blood was used to determine the HCMV IgG serostatus of the subjects. The HCMV IgG test was performed by members of the National Health Laboratory Services (NHLS) in the Division of Virology at Stellenbosch University, Tygerberg Campus, Cape Town. The VIDAS® HCMV IgG test (Biomerieux Corporate, France) amended protocol was utilised by the NHLS, Division of Virology, Stellenbosch University. It is an automated quantitative measurement of HCMV IgG in human heparinised plasma or serum using the enzyme-linked fluorescent assay (ELFA). A VIDAS® HCMV IgG kit was used that included washing solution, conjugate enzymes and a positive control and a negative control that validated quality control.

The test utilised a 2-step enzyme immunoassay ‗sandwich' method. The sandwich method required two antibodies that bound to different epitopes on the anti-HCMV IgG antibodies in the subject plasma. A purified, inactivated HCMV viral antigen was bound

to a solid phase receptacle. Once the plasma sample was added the following steps proceeded automatically. Anti-HCMV IgG antibodies in the subject plasma bound to the purified antigens on the solid phase receptacle. Unbound substances were removed during the washing process. A labelled second antibody called the detection antibody was added. These antibodies bound to different epitopes of the anti-HCMV IgG antibodies. The anti-HCMV IgG antibodies were enclosed by the two antibodies. Thus a _sandwich' method was achieved. The anti-HCMV IgG antibodies and the two antibodies were hydrolysed by a conjugate enzyme, alkaline phosphatase that was added. This resulted in a product that fluoresced that was measured at 450 nm. The intensity of the fluorescence was proportional to the concentration of the anti-HCMV IgG antibodies. All results were analysed automatically by the instrument. To ensure the validity of the results obtained a positive and negative control was run each time a new lot number was opened. Table 2.1 provides information on the interpretation of the HCMV IgG results. Concentrations are expressed in arbitrary units per millilitre (aU/ml).

Table 2.1: HCMV IgG values and interpretation

Value (*aU/ml)	**Interpretation**
** <4	Negative
From*** ≥ 4 to < 6	Equivocal
≥ 6	Positive

* aU/ml- arbitrary units per millilitre

2.4.3. The $CD4^+$ T cell count assay

The $CD4^+$ T cell count of each subject was determined within 24 hours of receiving the EDTA blood sample. A volume of 20 µl of MultiTEST CD3/ CD8/ CD45/ CD4 reagent (BD Bioscience, USA) was added to the TruCount tube (BD Bioscience, USA) above the metal plate. A volume of 50 µl of well-mixed whole EDTA blood was added. The tube was closed and gently vortexed. The tube and contents were incubated in a dark cupboard for 15 min. Thereafter, a volume of 450 µl of FACS lysing solution (BD Bioscience, USA) was added and the contents gently vortexed. The tube was incubated for 15 min in a dark cupboard. The FACSCalibur® flow cytometer (Becton, Dickson Co., USA) was set up as per the manufacturer‘s protocol. The sample number and date was added to the program. The processed EDTA sample was inserted into the FACSCalibur® flow cytometer and results were generated automatically.

Validation of $CD4^+$ T cell count assay: To ensure that the $CD4^+$ T cell counts obtained with the FACSCalibur® flow cytometer were valid a LymphoSure flow cytometry standard (Synexa Life Sciences, South Africa) was run simultaneously with the blood samples of the subjects once a week. This standard was prepared from human blood that was stabilised and screened non-reactive for syphilis, hepatitis B, HIV-1 and HIV-2. The expected values of the different T cell subsets were provided by the manufacturers and these remained constant. The LymphoSure flow cytometry standard was used as a reference control in the assay that determined the $CD4^+$ T cell count of each subject. If the $CD4^+$ T cell count of the LymphoSure flow cytometry standard fell within the expected range as provided by the manufacturers then the $CD4^+$ T cell counts of the subjects were deemed valid.

2.4.4. The IFN-γ-ELISPOT assay

An IFN-γ-ELISPOT assay was used to measure the frequency of T cells that produced the cytokine, IFN-γ, when stimulated with a HCMV pp65 peptide pool (BD Pharmingen™, BD Bioscience, USA). The positive controls used in the assay were PHA-P (Sigma-Aldrich Inc., USA) and anti-CD3 (Mabtech AB, Sweden). The assay was performed over three days. The assay protocol was followed as described by Mashishi and Gray (2002) as detailed below. Cryopreservation has been known to result in a decrease in IFN-γ release as detected by IFN-γ-ELISPOT assay (Macatangay *et al*., 2010). Thus, fresh PBMC were used in this study.

2.4.4.1. Preparation of reagents for IFN-γ-ELISPOT assay

Preparation of medium

AR-medium:

50% of RPMI (Gibco™, Invitrogen Corporation, UK) and 50% AIMV (Gibco™, Invitrogen Corporation, UK) were mixed together. A volume of 1 mM sodium-pyruvate (Gibco™, Invitrogen Corporation, UK) and a 50 microgram (µg)/ml of gentamicin (Gibco™, Invitrogen Corporation, UK) were added to this. The AR-medium was stored at 4 ºC.

AR-10/AB plasma medium:

10% volume AB plasma (Sigma-Aldrich Inc., USA) was added to the AR-medium.

Preparation of wash solutions

Fetal calf serum-phosphate buffered saline–Tween 20 (FCS-PBST):

Five phosphate buffered saline (PBS) capsules (Sigma-Aldrich Inc., USA) were dissolved in one litre of milli-Q water prepared in the MilliQ™ Water System (Millipore, Ireland) in the Division of Virology. Thereafter, a 1/2000 dilution of Tween-20 (Sigma-Aldrich Inc., USA) in PBS was prepared, obtaining phosphate

buffered saline -Tween 20 (PBST). PBST was supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Life Technologies, Germany).

Preparation of antigens and mitogens

HCMV pp65 peptide pool:(BD Pharmingen™, Becton, Dickson and Co., USA)

The HCMV pp65 peptide pool consisted of 138 peptides, 15mrs with 11 aa overlaps of the HCMV strainAD169 (Kern *et al*., 2000). A stock solution of the HCMV peptide pool was prepared (as per manufactures instructions). A volume of 15 µl of dimethyl sulphoxide (DMSO, BDH Laboratory Supplies, UK) was added to lyophilised HCMV pp65 peptide pool (BD Pharmingen™, Becton, Dickson and Co., USA).

*Anti-CD3 (*Mabtech AB, Sweden):

The stock solution of the anti-CD3 was 100 µg/ml. A dilution factor of 500 was used. The final concentration in the assay was 0.1 µg/ml.

PHA-P (Sigma-Aldrich, USA):

The stock solution of PHA-P was 5 milligram (mg)/ml. A dilution factor of 100 was used. Thus the final concentration of PHA-P used in the assay was 25 µg/ml.

Preparation of the monoclonal antibodies

Capturing antibody 1-DIK (Mabtech AB, Sweden):

The stock solution of the capturing antibody was 1 mg/ml. A dilution factor of 200 was used. The final concentration used in the assay was 2.5 µg/ml.

Detection antibody-biotinylated monoclonal antibody 7-B6-1(Mabtech AB, Sweden):

The stock solution of the detection antibody was 1 mg/ml. A dilution factor of 500 was used. Thus the final concentration in the assay was 1 µg/ml.

Preparation of horse-radish peroxidase (HRP)-substrate (detection reagent):

A Vector NovaRED kit (VECTOR, Burlingame, USA) was used to prepare the HRP-substrate. Three drops of reagent 1 and two drops each of reagent 2, 3 and 4 were added to 15 ml of milli-Q water in a 50 ml centrifuge tube. The contents of the centrifuge tube were mixed after each drop. This was done in the dark since the detection agent is sensitive to light.

Preparation of Turks solution

0.02% crystal violet (Sigma-Aldrich Inc., USA) and 7% glacial acetic acid (Sigma-Aldrich Inc., USA) were added to water.

2.4.4.2. Procedure of the IFN-γ-ELISPOT assay

Day 1:

Coating of plates: A 5 µg/ml (1/200) dilution of primary or capturing antibody 1-DIK (Mabtech AB, Sweden) in sterile PBS (Sigma-Aldrich Inc., USA) was prepared. Each well of the 96-well polyvinylidene difluoride (PVDF) backed plates (Millipore, Billerica, USA) were coated with 100 µl diluted capturing antibody. This antibody bound with the cytokine produced by the T cell. Plates were sealed with an adhesive ELISA plate sealer (Costar) and stored at 4˚C overnight.

Day 2:

Incubation of plates: Plates were washed 3 times with sterile PBS (Sigma-Aldrich Inc., USA) (200 µl per well). The contents were discarded and the plate was tapped dry on absorbent paper. This removed any capturing antibody. Plates were blocked with AR-10 medium (10% AB plasma, Sigma-Aldrich Inc., USA). This saturated the binding sites and prevented the binding of any other proteins. The plates were then incubated for 2 hours at room temperature.

Isolation of fresh PBMC: The isolation of the PBMC was undertaken in a biosafety II cabinet. Approximately 20 ml of heparinised blood was obtained from patient volunteers

for the IFN-γ-ELISPOT assay. While the plates were incubating, the PBMC isolation was undertaken. The blood was decanted into 15 ml centrifuge tubes. It was centrifuged at 275 x g for 10 min at 20˚C with the brake off. To establish the HCMV IgG serostatus of each donor, 500 µl of plasma was first removed after centrifugation, and frozen in a -20˚C refrigerator for later analysis. Thereafter, a 4 ml volume of buffy layer consisting of mononuclear cells was removed and mixed with 4 ml of sterile PBS (Sigma-Aldrich Inc., USA). The solution was then layered onto a 4 ml volume of histopaque 1077 (Sigma-Aldrich Inc., USA). The histopaque 1077 was at room temperature. The PBMC were separated by ficoll gradient centrifugation at 800 x g for 30 min at 20°C. The red blood cells and granulocytes that were denser than the histopaque 1077 aggregated towards the bottom of the centrifuge tube below the histopaque 1077. The PBMC consisting of lymphocytes and monocytes were less dense than the histopaque 1077 and were found above it. A volume of not more than 2 ml PBMC were removed and suspended in up to 12 ml of sterile PBS. Cells were then washed once at 325 x g for 10 min at 20°C and once at 275 x g for 10 min at 20°C using a Jouan GR412 centrifuge unit (Jouan S.A., France) in order to remove any histopaque 1077. The cells were suspended in 1 ml AR-10 medium (10% AB plasma, Sigma-Aldrich Inc., USA) and counted.

Cell count: After the final centrifugation a PBMC aliquot was counted using a 1:4 dilution of Turks solution (Gibco^{TM}, Invitrogen Corporation, UK) with a Nikon Microscope Eclipse E200 (Nikon Instruments Inc., USA).

Calculating the number of PBMC using a haemocytometer:

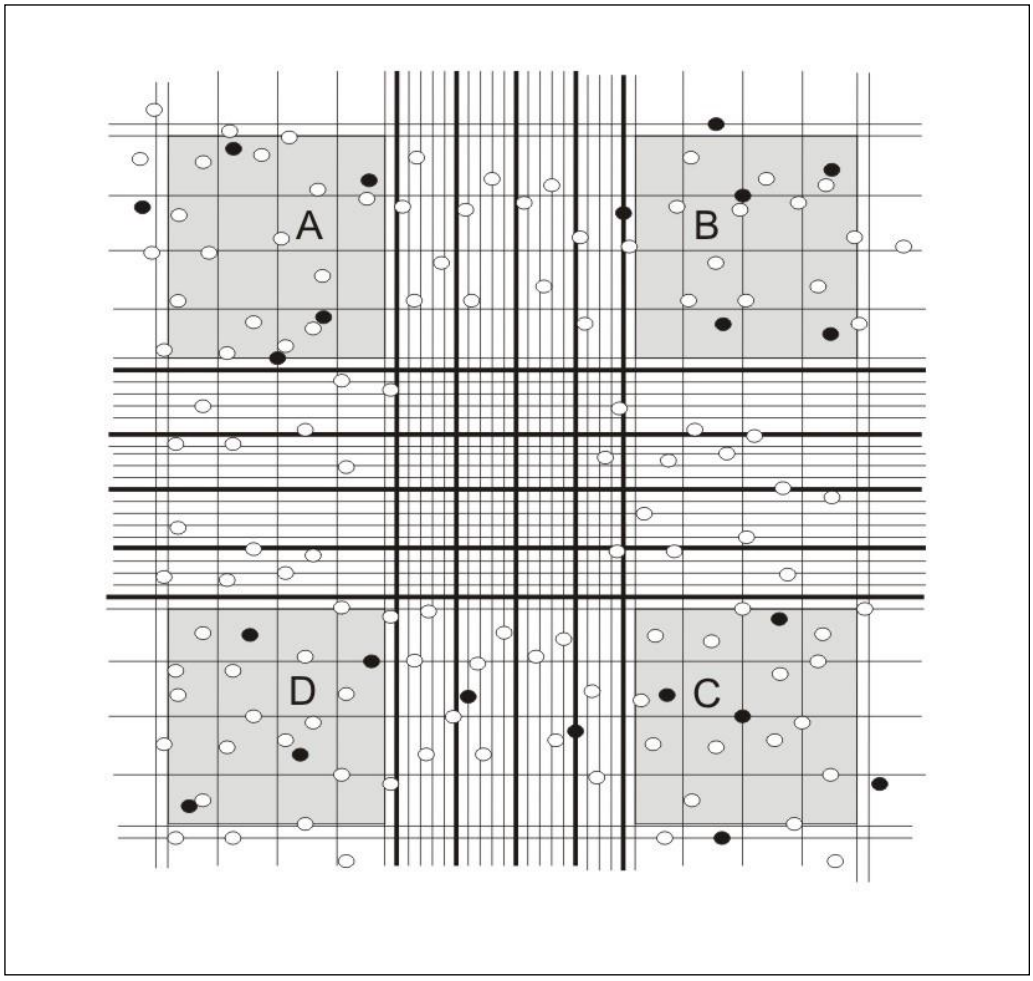

Figure 2.1: The haemocytometer used in the counting of isolated PBMC diluted in Turks solution. Blocks A, B, C and D are the blocks in which the PBMC were counted.

The number of PBMC was determined as follows using the haemocytometer (refer to Figure 2.1):

Cells in 4 large blocks (A, B, C and D)

Depth of the counting area = 0.1mm

Length of the counting area =1mm

Volume = length x breadth x height

= 1 x 1 x 0.1

= 0.1mm^3

The volume was converted to 1 mm^3 (x10)

To convert 1mm^3 to cm^3 the length, breadth and depth must be multiplied by 10.

The cells were diluted 25% with Turks solution thus multiply by 4 to get the count of 100% cells

The number of cells is given as cells/ml

Preparation of IFN-γ-ELISPOT plate: The fresh PBMC were diluted with media to obtain a working concentration of 4 x 10^6 cells/ml. A negative control or background control that consisted of 50 µl of PBMC of each subject and 50 µl of AR-10 medium was added in triplicate to the IFN-γ-ELISPOT plate. A volume of either 50 µl of HCMV pp65 peptide pool, 50 µl PHA-P or 50 µl anti-CD3 monoclonal antibody was also added in triplicate to the IFN-γ-ELISPOT plate. The PHA-P and the anti-CD3-stimulated wells were used as positive controls. The positive controls confirmed cell viability and appropriate assay procedure. The positive and negative controls together confirm the validity of the results produced by the assay. Each stimulant was assayed in triplicate to minimise the chance of technical error. Aliquots of 50 µl of PBMC of each subject were

added to the wells containing the stimulants. The plates were incubated for 20 hours at 37˚C, 95% humidity and 5% CO_2 (Nuaire^{TM}, Plymouth, Minnesota, USA).

Day 3:

Development of plates: The development of the plates was undertaken in a biosafety II cabinet. The plates were washed 3 times with the prepared non-sterile PBS (200 µl per well) and 3 times with FCS-PBST (200 µl per well). A volume of 100 µl detection antibody-biotinylated monoclonal antibody 7-B6-1 at 1 mg/ml in PBS and azide (Mabtech AB, Sweden) diluted to 2 µg/ml, was added to each well and the plates were incubated in a dark cupboard for 3 hours. During the incubation period this antibody reacted with a specific epitope of the IFN-γ and bound to it forming an antibody-antigen complex. Plates were then washed 6 times with FCS-PBST (200 µl per well) to remove any unbound biotinylated antibodies. A volume of 100 µl of a 1/100 dilution Streptavidin-Horseradish Peroxidase (Mabtech AB, Sweden) was added. The plates were incubated for 1 hour at room temperature in a dark cupboard allowing the Streptavidin bound enzyme to produce a substrate which was detected by the detection substrate. Thereafter, plates were washed 6 times with FCS-PBST (200 µl per well) to remove debris. A volume of 100 µl/well of Vector NovaRED substrate was added and the plates were incubated for 20 minutes. This allowed the substrate to be converted to a coloured product. When a colour was visible the reaction was stopped by washing the plates thoroughly under cold water. Plates were then air-dried overnight in a dark cupboard.

Day 4:

Once dry, the plates were transferred to an adhesive ELISA plate sealer (Costar). An Eli-puncher kit (Zell Net, Germany) was used to punch out the PVDF membranes onto an adhesive plate sealer. The wells were covered with the plastic cover provided with the plate sealer. An automated ELISPOT reader (KS ELISPOT 4.5, Carl Zeiss Limited, Germany) was used to read the plate. The number of spot forming units (SFU) was detected using the automated reader. The five parameters that were necessary for determining a spot were diameter, contrast, hue, saturation, shape and slope. The parameters were recorded and not changed within a plate. The mean number spots of the

background were obtained. This mean value was then subtracted from the SFU obtained in each well. The assay used approximately 100 000 to 200 000 cells/well. To express these cells in SFU/million cells, the values in each well was multiplied by a factor of five. The mean values of the different stimulants were then obtained. To obtain a positive result for the IFN-γ-ELISPOT assay certain criteria had to be met. These included a background control less than 50 million SFU, a PHA-P more than 100x background control and/or an anti-CD3 greater than 50x background control.

2.4.4.3. Validation of the IFN-γ-ELISPOT assay

To determine the validity of the IFN-γ-ELISPOT assay its repeatability was tested. For the validation four HIV-negative volunteers were recruited from the Division of Virology, Stellenbosch University, Cape Town, South Africa. A volume of 20 ml of heparinised blood was obtained from each volunteer for each ELISPOT assay. An inter-assay comparison of the PBMC of the four volunteers was completed on two different days. An intra-assay comparison was also undertaken within every experiment using the HIV-HCMV co-infected samples as well as the HIV-negative volunteer samples. In this regard the PBMC of each volunteer and HIV-HCMV co-infected subject was also stimulated in triplicate and the IFN-γ-ELISPOT assays of two of the volunteers were also completed in duplicate in one assay. The PBMC of the HIV-negative volunteers were stimulated with a HCMV pp65 peptide pool (138 peptides, 15mrs with 11 aa overlaps, pp65 sequences for HCMV strain AD169) (Kern *et al*., 2000), PHA-P and anti-CD3 as described in section 2.4.4.2.

2.4.5. The HCMV viral load real-time PCR

A volume of approximately 10 ml of EDTA blood was used to determine the HCMV DNA load in each plasma and whole blood sample. Both analytes were utilised in the study in order to determine which of the two is more predictive of HCMV viraemia. The HCMV viral load qPCR entailed HCMV DNA extraction and amplification and analysis of data.

2.4.5.1. Preparation of standards and reagents used in the HCMV viral load qPCR

Preparation of the standards

Aliquots of 207 µl of plasma from HCMV-negative volunteer donors were added to five 1.5 ml centrifuge tubes. Serial dilutions of cell culture supernatant containing a known concentration of HCMV strain AD 169 (Division of Virology, Stellenbosch University) were prepared. The tubes contained the following HCMV copies/ml namely, 300000, 30000, 3000, 300.

Preparation of the AL buffer mix

7 µg of carrier DNA, a poly A 18mer (Integrated DNA Technologies, Germany) was added to 200 µl of lysis buffer (Qiagen buffer, kit buffer) per sample extraction. The carrier DNA was added to improve the yield of the DNA extracted, especially in the low copy number target samples. Also added to the buffer mix were 200 copies of internal control per sample extraction.

2.4.5.2. DNA extraction from samples

Quantification standards, negative controls, and samples were extracted under the same conditions prior to each HCMV viral load qPCR run. For each of the extractions the spin protocol for purification from blood or body fluids was undertaken as described in the QIAamp® DNA Blood Mini kit handbook (Qiagen, Hilden, Germany).

DNA was extracted from 200 µl of plasma and 200 µl of whole blood using the DNA Blood Mini Kit (Qiagen, Hilden, Germany). This volume of plasma or whole blood was added to 20 µl of protease K (Qiagen kit) and 200 µl of Buffer AL (kit buffer) mix. The

contents were vortexed for 15 s and thereafter, incubated at 56ºC for 10 min. Contents were briefly centrifuged. Thereafter, 200 µl ethanol (Sigma-Aldrich Inc., USA) was added with a further 15 s vortexing and 10 s centrifuging. The contents were transferred to a QIAamp mini spin column (Qiagen, Hilden, Germany), capped and centrifuged at 6000 x g for 1 min. The spin column was then placed in a clean 2 ml collection tube (Qiagen kit). The tube containing the filtrate was discarded. A volume of 500 µl Buffer AW1 (kit buffer) was added, the tube capped and centrifuged at 6000 x g for 1 minute. The spin column was again placed in a mini spin column and the filtrate was discarded. A volume of 500 µl of Buffer AW2 (kit buffer) was added, the tube capped and centrifuged at 20 000 x g for 3 min. The spin column was placed in a 2 ml collection tube to remove all fluid in the final centrifugation. The filtrate was discarded. The tube was centrifuged for 1 min at 6000 x g. The spin column was placed in a clean 1.5 ml centrifuge tube and the collection tube containing the filtrate was discarded. Finally, instead of using 200 µl of the elution buffer, Buffer AE (kit buffer), as prescribed by the QIAamp® DNA Blood Mini kit handbook, 65 µl Buffer AE was added. This modification of the protocol allowed for the increase in yield of the extracted DNA. The contents were then incubated for 1 min at room temperature and centrifuged at 6000 x g for 1 minute. This elution buffer allowed for the release of the DNA from the Qiagen silica membrane. Thereafter, the DNA that was obtained was stored in a -80 ºC refrigerator for later amplification.

2.4.5.3. Determination of the DNA concentration of subjects sample extracted

The concentration of DNA extracted was determined by using the Nanodrop™ ND-1000 system (Nanodrop Technologies Inc.; Delaware, USA). A volume of 1 µl of the DNA sample extracted was required. The method was considered fast, accurate and reproducible and did not require any concentration dilutions (Ausubel *et al*., 2003; Sambrook *et al*., 1989). The DNA concentration of the eluate was determined by measuring the absorbance (A) of the sample at 260 nm. The purity of the sample was measured by calculating the absorbance at 260 nm divided by the absorbance at 280 nm. A value between 1.7 and 2.0 ng/µl indicated the presence of pure DNA without any proteins or other contaminants. However, a value between 1.5 and 1.7 ng/µl was also considered acceptable (Ausubel *et al*., 2003; Sambrook *et al*., 1989).

The following equations are applicable:

$$\text{DNA concentration} = \frac{A260}{20} \text{ x dilution factor}$$

$$\text{DNA purity} = \frac{A260}{A280}$$

2.4.5.4. Amplification and analysis of DNA sample

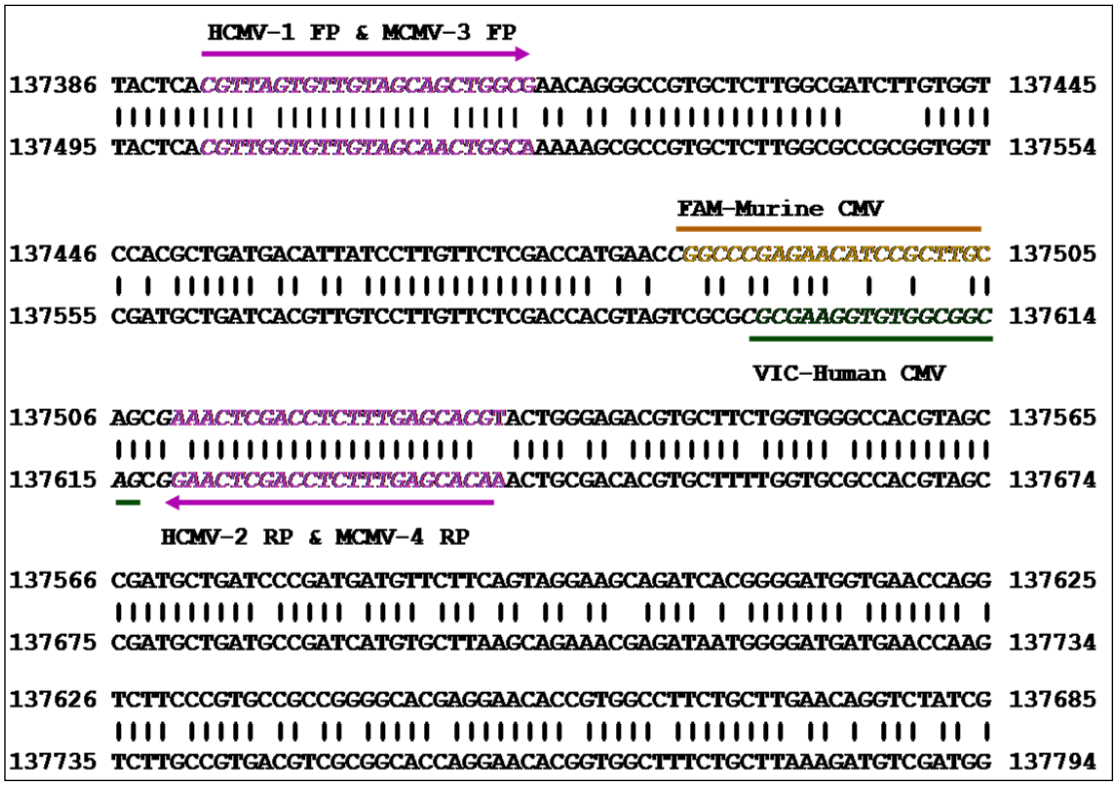

Figure 2.2: **Sequence homology between the target HCMV sequence and the internal (MCMV) control.** The bottom sequence is HCMV and the top sequence is the MCMV. The sequences highlighted in pink are the targets for the forward and reverse primers. The yellow sequence is the target for internal control probe and the green sequence is the target for the HCMV [Modified version of Figure.1 in Preiser *et al.*, 2003b].

The eluate contained an unknown concentration of HCMV DNA. The amplification of the unknown sample of HCMV DNA in the eluate together with specific primers and probes allowed for the quantitation of the extracted HCMV DNA from subject samples. An internal MCMV control illustrated in Figure 2.3. was added to the sample, co-amplified with the sample but detected with a MCMV specific probe. It ensured a high reliability for the specific test especially in cases of negative and low positive results. The ideal internal control is one that is closely related to the target and non-pathogenic in humans (Preiser *et al*, 2003b; Whiley & Sloots, 2006).

Each HCMV viral load qPCR run also included two non-template controls (NTCs) and one negative control. Nuclease-free water replaced the template in the NTC which was used as a negative control. A second negative control was used. This included a template from the eluate, extracted from a HCMV-negative individual. The negative controls were important to detect the presence of contaminating DNA. These inclusions provided valid HCMV viral load qPCR results.

The standards, prepared in duplicate,had known concentrations of HCMV DNA. The standards, internal controls, NTC‘s, negative controls and the extracted HCMV DNA were amplified simultaneously.The Rotor-Geen 6000 cycler (Corbett Life Science, Australia) generated standard curves. The unknown viral copies that were initially present in the subjects‘ whole blood and plasma were determined by interpolating the values of the amplified products into the standard curves. Standard curves allowed for the quantitation of an unknown concentration of HCMV. The HCMV viral load qPCR was determined in the exponential phase of the reaction with a set number of cycles. This produced valid results.

CGTTAGTGTTGTAGCAGCTGGCGAACAGGGCCGTGCTCTTGGCGATC
TTGTGGTCCACGCTGATGACATTATCCTTGTTCTCGACCATGAACCGG
CCCGAGAACATCCGCTTGCAGCGAAACTCGACCTCTTTGAGCACG

Figure 2.3: An MCMV 140bp sequence served as internal control for the qPCR

In the current study the HCMV and MCMV forward and reverse primers for gene region UL89 were used as well as dually labelled virus-specific probed for HCMV and murine CMV during amplification (Preiser *et al.*, 2003b). The forward and reverse HCMV primers were 5‘-CGTTGGTGTTGTAGCAACTGG-3‘ and 5‘-TGTGCTCAAAGAGGTCGAGTTCC-3‘, respectively (refer to Figure 2.2). The TaqMan® probe selected between both primers was fluorescence labelled with 6-carboxyfluorescein at the 5' end as the reporter dye and 6-carboxytetramethylrhodamine at the 3' end as the quencher (VIC 5‘ CGCGAAGGTGTGGCGGCAG 3‘ TAMRA) as illustrated in Figure 2.2 (Preiser *et al.*, 2003b).

Each HCMV viral load qPCR was performed in a 25 µl reaction volume. It consisted of a Sensi Mix™ Probe kit (Bioloine, UK), reverse and forward primers and probes. The kit contained a chemically modified *Taq* DNA polymerase, a hot-start enzyme which remained inactive at room temperature. The *Taq* DNA polymerase required activation at 95°C for 15 min, 0.2 mM dNTPs and 1.5 mM magnesium chloride ($MgCl_2$), 1.5 µl forward primer, 1.5 µl of reverse primer and a volume of nuclease free water allowing for 25 µl reaction volume

Using an in-house qPCR the following cycling conditions were employed in the amplification process:two hold steps at 50˚C for 2 min and at 95˚C for 10 min, 40 repeat cycles at 95˚C for 10 s (denaturation/strand separation) and 61˚C for 45 s (primer annealing and extension). Data was acquired at 61˚C and both the green and yellow channels were selected for target detection. The green channel was used to detect the fluorescent dye for the internal control (FAM). The yellow channel detected the dye for

the target sequence, HCMV (VIC). The target and the internal control were co-amplified but detected separately.

The HCMV viral load qPCR was run on the Rotor-Gene™ 6000 rotary real-time analyzer (Corbett Life Science, Australia). Data was provided automatically. A value exceeding 100 copies/ml was deemed as a postive response. Valid results were also based on the efficiency of the qPCR which needed be as close to 100%. The total run time on the Rotor-Gene™ 6000 depended on the number of samples in the run ranging between 70 and 90 min. The software has an auto-find threshold option which determined the threshold of detection. The threshold was set in the exponential phase, taking both the correlation coefficient (R^2-value) and reaction efficiency in to consideration. A correlation coefficient value between 0.99 and 1.00 indicates a good standard curve. Reaction efficiency above 0.90 is sufficient for use in the interpolation of the viral load in the unknown samples.

2.5. Statistical analysis

A statistician, Professor Martin Kidd, from the Stellenbosch University was consulted to determine the correlation between $CD4^+$ T cell counts and IFN-γ-ELISPOT result as well as correlations between IFN-γ-ELISPOT result and $CD8^+$ T cell counts and IFN-γ-ELISPOT result and HCMV IgG values. Spearman‘s correlation coefficient was used for these comparisons.

Chapter 3

3. Results

3.1. Subjects

3.1.1. Subjects recruited for the study

Fifty-two (52) human, adult subjects were recruited for the purpose of this study from primary healthcare facilities that specifically managed the health of persons infected with HIV. The subjects provided written informed consent for the perusal of their medical records and collection of blood samples. Review of the subjects‘ medical records confirmed that they were all infected with HIV and naïve to ART. The exact test used to determine the HIV status of each subject was not available at the time of recruitment. In addition, none of the subjects displayed any clinical features of HCMV-associated diseases as assessed and documented by the medical staff at the primary healthcare facilities. A HCMV IgG test conducted in the Division of Virology, Stellenbosch University revealed that all subjects were HCMV IgG positive.

3.1.2. Subject data excluded from the final analysis of the study

Eighteen (18) subjects were excluded from the final analysis of data in the current study as illustrated in Figure 3.1. Subjects were excluded for the following reasons. Eight of the 18 subjects provided an insufficient quantity of heparinised blood to allow for the completion of the IFN-γ-ELISPOT assay. In addition, in the IFN-γ-ELISPOT assays of nine of the subjects the PHA-P and the anti-CD3 responses were too low. The data of these subjects were thus excluded. One subject provided a sufficient volume of heparinised blood and displayed a positive HCMV IFN-γ-ELISPOT result. However, an insufficient volume of EDTA blood was drawn from the same subject. Thus, no HCMV viral load could be determined using the HCMV viral load qPCR. As a result this subject‘s data was excluded from the final analysis. The demographics of the 34 subjects that had valid HCMV IFN-γ-ELISPOT and HCMV viral load qPCR results are discussed below.

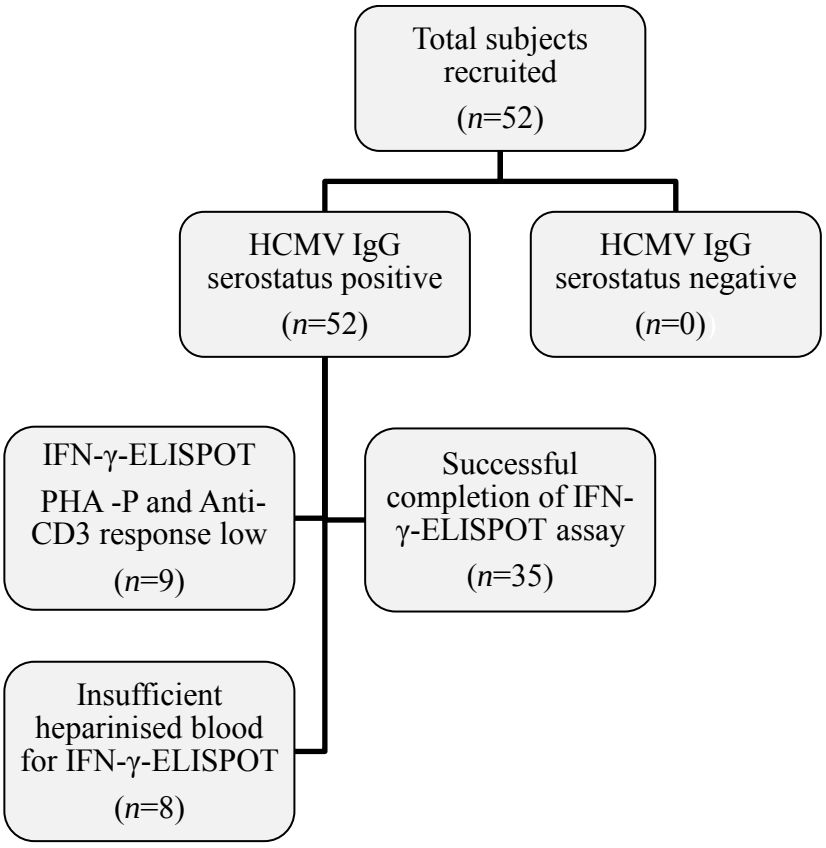

Figure 3.1: **An illustration of the process by which 18 subjects were excluded from the initial cohort of 52 HIV-positive subjects.** Eight subjects provided an insufficient quantity of heparinised blood for the IFN-γ-ELISPOT assay. Nine subjects had inappropriate responses to the positive control, PHA-P and anti-CD3. One subject was excluded due to an insufficient volume of EDTA blood sample drawn.

3.2. Demographics of subjects recruited

The data of 34 subjects were analysed in the study to determine the point at which the HCMV-specific cellular immune response breaks down in relation to the HCMV viral load and $CD4^+$ T cell count. The majority of the subjects were female and the mean age of the 34 subjects was 33.9 (± 6.8) years with a range of 25 to 48 years.

Table 3.1: Demographics of HIV-HCMV co-infected subjects (*n*= 34)

Demographics	**Completed data**
Gender	
Male	16 (47.1%)
Female	18 (52.9%)
Age, mean (± SD) years	33.9 (± 6.8)

3.3. The $CD4^+$ T cell counts of subjects recruited

The $CD4^+$ T cell counts ranged from 10 to 784 cells/µl (refer to Table 3.2). The most frequent $CD4^+$ T cell count was in the 100 to 199 range. There was an equal frequency of subjects in the $CD4^+$ T cell ranges of 0 to 99 and 200 to 299 cells/µl. The mean value of the $CD4^+$ T cell counts was 237 (± 168.3) cells/µl.

Table 3.2: The $CD4^+$ T cell counts (cells/µl) of the subjects at the time of recruitment (*n*= 34)

Subject Number	$CD4^+$ T cell counts (cells/µl)
1	10
2	50
3	53
4	62
5	73
6	85
7	116
8	121
9	133
10	133
11	141
12	152
13	163
14	167
15	171
16	183
17	185
18	190
19	194
20	202
21	214
22	217
23	243
24	265
25	298
26	355
27	377
28	412
29	413
30	444
31	465
32	465
33	545
34	784

3.4. HCMV IFN-γ-ELISPOT assay results for HIV-HCMV co-infected subjects

A representative image of the wells of the IFN-γ-ELISPOT plates as read by the ELISPOT reader (KS ELISPOT 4.5, Carl Zeiss Limited, Germany) used in the assay is illustrated in Figure 3.2. With a strong response the number of SFU is high, resulting in confluence of spots in positive control wells. This limits the counting of the data (Zhang *et al*., 2009).

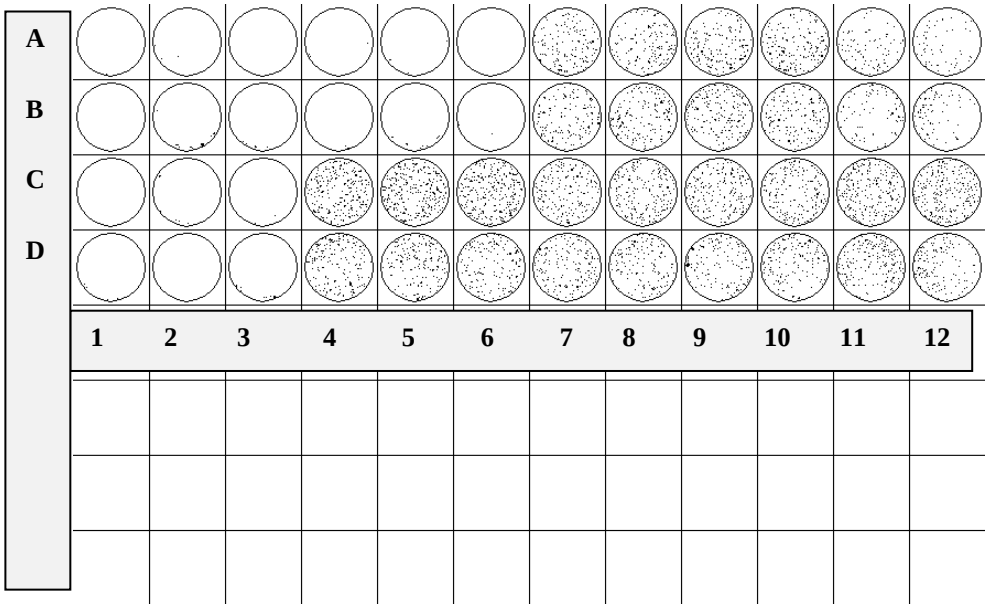

Figure 3.2: A representative image of the 96-well ELISPOT plates used in the IFN-γ-ELISPOT assay and the spot forming units counted. At higher numbers, confluence of spots interferes with the countability of the data. The PMBC of four subjects (A, B, C and D) were utilised in the IFN-γ-ELISPOT assay. The first three wells (1, 2 and 3) represented the unstimulated cells (negative control). A few spots were observed in all four subjects for this control. Wells numbered 4, 5 and 6 represented the SFU of cells stimulated with HCMV pp65 peptide pool. Subjects A and B had few spots in these wells which indicated a negative response to the HCMV pp65 peptide pool. Numerous SFU were observed in wells numbered 4, 5 and 6 for subjects C and D. This indicates that a strong HCMV-specific cellular immune response was generated by these subjects. The wells numbered 7, 8 and 9 represented the SFU of cells stimulated with a mitogen, PHA-P that produces a confluence of SFU. A similar result is obtained in wells 10, 11 and 12 in which cells were stimulated with a positive control, anti-CD3.

As described in section 2.4.4.2 in Chapter 2, subjects with positive HCMV IFN-γ-ELISPOT results were defined as those with more than 50 SFU/million cells. In contrast, subjects with negative HCMVIFN-γ-ELISPOT results were defined as those with less than 50 SFU/million cells. Sixty five percent (65%) of the subjects had a positive HCMV IFN-γ-ELISPOT result with valid background controls and positive controls (refer to Table 3.3). Twelve (12) of the 34 subjects (35%) had a valid negative HCMV IFN-γ-ELISPOT result with less than 50 SFU/million cells. Two subjects (11 and 34) (6%)had high HCMV IFN-γ-ELISPOT results with a value of more than 1000 SFU/million cells. This high HCMV IFN-γ-ELISPOT result is indicative of a strong HCMV-specific cellular immune response.

In the current study 18 females and 16 males were evaluated regarding their IFN-γ-ELISPOT response to the HCMV peptides. Sixty-one percent (61%) of the females had a positive IFN-γ-ELISPOT response. In contrast, 69% of the males had a positive response.

In order for the HCMV IFN-γ-ELISPOT result to be deemed valid, certain criteria had to be met. The background control that consisted of unstimulated cells in AR-10 medium only had to have a value of less than 50 SFU/million cells. The background control values ranged from 0 to 15 SFU/million cells for all subjects and thereby met the criteria for valid HCMV IFN-γ-ELISPOT results. The PHA-P and the anti-CD3 were the positive controls used in the assay. Either one or both of these controls had to be positive according to our definition in order for the HCMV IFN-γ-ELISPOT result to be valid. Eighteen (18) subjects had a positive response to the PHA-P and 27 subjects had a positive response to the anti-CD3 stimulant (refer to Table 3.3). Eleven subjects had a positive response to both PHA-P and the anti-CD3. A positive PHA-P value had to exceed a value of 100 x the SFU/million cells of the background control. A positive anti-CD3 value had to exceed a value of 50 x the SFU/million cells of the background control (Mashishi and Gray, 2002).

Table 3.3: HCMV IFN-γ-ELISPOT results of subjects co-infected with HIV-HCMV and their relevant background and positive controls (*n*= 34)

Subject Number	Gender	Background control SFU/10^6 cells	CMV pp65 peptide pool SFU/10^6 cells	PHA-P SFU/10^6 cells	Anti-CD3 SFU/10^6 cells
1	F	4	353	-	+
2	M	0	32	+	+
3	M	0	8	-	+
4	M	0	210	+	-
5	M	2	285	+	+
6	F	0	43	+	+
7	F	0	52	-	+
8	F	4	295	-	+
9	M	0	975	+	-
10	F	2	315	+	+
11	M	2	1200	+	-
12	F	15	183	-	+
13	M	0	25	+	+
14	F	0	293	+	+
15	F	2	23	-	+
16	M	0	122	-	+
17	M	0	57	-	+
18	F	2	228	-	+
19	F	0	47	-	+
20	M	0	3	-	+
21	F	5	420	+	+
22	M	5	3	+	+
23	M	0	262	+	+
24	F	0	53	+	-
25	F	0	0	-	+
26	M	10	605	+	-
27	M	10	415	-	+
28	F	0	43	-	+
29	M	0	63	-	+
30	F	0	20	-	+
31	F	5	90	+	-
32	F	5	143	+	-
33	F	0	25	+	+
34	M	4	2260	+	+

The PHA-P and the anti-CD3 are denoted as positive or negative depending on the following:

+ Positive PHA-P value [>100x background]

- Negative PHA-P value [<100x background]

+ Positive anti-Cd3 value [> 50x background]

- Negative anti-CD3 value [< 50x background]

Validation of the ELISPOT assay

Two IFN-γ-ELISPOT assays were run on two different days to assess inter-assay repeatability. Four HIV-negative volunteers were recruited. Table 3.4 illustrates this validation of the inter-assay comparison. The samples numbered 1 and 2 represented subject one. The sample numbered 3 represented subject two. The sample numbered 4 represented subject three and samples numbered 5 and 6 represented subject four. Samples 1, 2 and 3 had low HCMV IFN-γ-ELISPOT results. In contrast, samples 4, 5 and 6 had high HCMV IFN-γ-ELISPOT results. Similar responses were garnered by the subjects between the two assays on different days.

Table 3.4: Inter-assay comparison of the HCMV IFN-γ-ELISPOT results

Sample Number	ELISPOT assay 1 HCMV pp65 peptide pool (SFU/10^6 cells)*	ELISPOT assay 2 HCMV pp65 peptide pool (SFU/10^6 cells)
1	2	0
2	3	0
3	0	2
4	238	172
5	1445	2118
6	2210	2217

*Average of triplicate wells

Two IFN-γ-ELISPOT assays were also run simultaneously in the same IFN-γ-ELISPOT plate for two of the subjects. In each assay, PBMC of each subject were stimulated in triplicate. Similar HCMV IFN-γ-ELISPOT results of each well of cells were obtained for each subject.

3.5. The HCMV viral load qPCR results

The purity of the HCMV DNA extracted as described in section 2.4.5.1 of Chapter 2 all fell within the acceptable range that is 1.5 to 2.0 ng/µl. This is the minimum amount of HCMV DNA required for qualifying and quantifying the HCMV DNA using the HCMV viral load qPCR (Ausubel *et al*., 2003).

The HCMV viral load qPCR results of the 34 HIV-HCMV co-infected subjects as determined from analysis of the EDTA samples as described in section 2.4.5.3 of Chapter 2 are tabulated below (refer to Table 3.5). A value exceeding 100 copies/ml of the virus was interpreted as being a positive HCMV viral load qPCR. Two subjects (subjects 1 and 4) (6%) had positive HCMV viral load qPCR result. Subject one (female) had HCMV viral loads greater than 100 copies/ml in whole blood. Subject four (male) had HCMV viral loads greater than 100 copies/ml in both plasma and whole blood. The remaining 31 subjects did not have any detectable HCMV viral copies as determined by the HCMV viral load qPCR and were considered aviraemic.

Table 3.5: The HCMV DNA concentrations (copies/ml) of plasma and whole blood as determined by HCMV viral load qPCR (*n*= 34)

Subject Number	Plasma CMV DNA concentration (copies/ml)	Whole blood CMV DNA concentration (copies/ml)
1	*LDL	**713**
2	LDL	LDL
3	LDL	LDL
4	**207**	**1087**
5	LDL	LDL
6	LDL	LDL
7	LDL	LDL
8	LDL	LDL
9	LDL	LDL
10	LDL	LDL
11	LDL	LDL
12	LDL	LDL
13	LDL	LDL
14	LDL	LDL
15	LDL	LDL
16	LDL	LDL
17	LDL	LDL
18	LDL	LDL
19	LDL	LDL
20	LDL	LDL
21	LDL	LDL
22	LDL	LDL
23	LDL	LDL
24	LDL	LDL
25	LDL	LDL
26	LDL	LDL
27	LDL	LDL
28	LDL	LDL
29	LDL	LDL
30	LDL	LDL
31	LDL	LDL
32	LDL	LDL
33	LDL	LDL
34	LDL	LDL

LDL : Less than detectable levels [100 copies/ml]

3.6. Comparison of the $CD4^+$ T cell count, HCMV IFN-γ-ELISPOT results and HCMV viral load qPCR results

The $CD4^+$ T cell counts, HCMV IFN-γ-ELISPOT results and HCMV viral load qPCR results of the 34 HIV-HCMV co-infected subjects are compared in detail in Table 3.6 and summarised in Table 3.7. In the $CD4^+$ T cell count range 0 to 99 cells/µl (n=6), only 50% (n=3) of the subjects had a positive HCMV IFN-γ-ELISPOT result. In this $CD4^+$ T cell range two of the six subjects had detectable levels of HCMV in their whole blood as determined by HCMV viral load qPCR, despite a positive HCMV IFN-γ-ELISPOT result. One of the latter subjects also had a detectable HCMV viral load in plasma.

In the $CD4^+$T cell count range 100 to 199 cells/µl (n=13), 77% (n=10) of the subjects had positive HCMV IFN-γ-ELISPOT results. None of the subjects in this $CD4^+$ T cell range had detectable HCMV viral loads.

In the $CD4^+$T cell count range 200 to 299 cells/µl (n=6), 50% (n=3) of the subjects had negative HCMV IFN-γ-ELISPOT results. However, all subjects in this $CD4^+$ T cell range had negative HCMV viral load qPCR results.

In the $CD4^+$ T cell count range of more than 300 cells/µl (n=9), 67% (n=6) of the subjects had positive HCMV IFN-γ-ELISPOT results and all subjects in this $CD4^+$ T cell range had no detectable HCMV as per the HCMV viral load qPCR.

Table 3.6: Comparison of the $CD^{+}4$ T cell count, HCMV IFN-γ-ELISPOT results and HCMV viral load qPCR results (*n*= 34)

Subject Number	$CD4^{+}$ T cell count	$CMV\ IgG^{+}$ results	HCMV IFN-γ-ELISPOT results		HCMV viral load qPCR results	
		(arbitrary units)#	*Positive response SFU/10^6 cells	**Negative response SFU/10^6 cells	Plasma (copies/ml)	Whole blood (copies/ml)
1	10	155	353		LDL	713
2	50	238		32	LDL	LDL
3	53	137		8	LDL	LDL
4	62	165	210		207	1087
5	73	57	285		LDL	LDL
6	85	295		43	LDL	LDL
7	116	157	52		LDL	LDL
8	121	224	295		LDL	LDL
9	133	132	975		LDL	LDL
10	133	109	315		LDL	LDL
11	141	139	1200		LDL	LDL
12	152	57	183		LDL	LDL
13	163	124		25	LDL	LDL
14	167	102	293		LDL	LDL
15	171	173		23	LDL	LDL
16	183	166	122		LDL	LDL
17	185	132	57		LDL	LDL
18	190	96	228		LDL	LDL
19	194	202		47	LDL	LDL
20	202	217		3	LDL	LDL
21	214	57	420		LDL	LDL
22	217	80		3	LDL	LDL
23	243	71	262		LDL	LDL
24	265	218	53		LDL	LDL
25	298	93		0	LDL	LDL
26	355	166	605		LDL	LDL
27	377	82	415		LDL	LDL
28	412	84		43	LDL	LDL
29	413	80	63		LDL	LDL
30	444	155		20	LDL	LDL
31	465	68	90		LDL	LDL
32	465	163	143		LDL	LDL
33	545	79		25	LDL	LDL
34	784	16	2260		LDL	LDL

*a value more than and equal to 50 SFU/10^6 cells is positive
**a value less than 50 SFU/10^6 cells is negative
LDL: lower than detectable levels
a relative unit of measurement

Table 3.7: Summary of the $CD4^+$ T cell counts, HCMV IFN-γ-ELISPOT results and HCMV viral load qPCR results (*n*= 34)

$CD4^+$ T cell count distribution (cells/µl)	**CMV IgG^+ (arbitrary units)**	**HCMV IFN-γ-ELISPOT values *(SFU/10^6)**		**Real-time PCR (copies/ml)**			
				plasma		whole blood	
		Positive Response ≥50SFU/10^6	Negative response <50SFU/10^6	**LDL	positive viraemia	LDL	positive viraemia
0-99	6[100%]	3[50%]	3[50%]	5[83%]	1[27%]	4[67%]	2[33%]
100-199	13[100%]	10[77%]	3[23%]	13[100%]	0	13[100%]	0
200-299	6[100%]	3[50%]	3[50%]	6[100%]	0	6[100%]	0
≥300	9[100%]	6[67%]	3[23%]	9[100%]	0	9[100%]	0

*SFU - spot forming units

**LDL - lower than detectable levels of virus

There was no significant relationship between $CD4^+$ T cell count and HCMV IFN-γ-ELISPOT results of the whole group of subjects when analysed with Spearman's correlation co-efficient (refer to Figure 3.3). The $CD4^+$ T cell count of subjects with a negative HCMV IFN-γ-ELISPOT result (SFU/million cells <50) ranged from 50 to 545 cells/µl. The subjects with a positive HCMV IFN-γ-ELISPOT value (SFU/million cell >50) ranged from 10 to 784 cells/µl. The two subjects with viraemia as determined by the HCMV viral load qPCR were among the six subjects who had an HCMV IFN-γ-ELISPOT result< 400 SFU/million cells and a $CD4^+$ T cell count less than 100 cells/µl. One of the subjects with a detectable HCMV viral load and a positive HCMV IFN-γ-ELISPOT result was female and one was male.

No statistically significant results between IFN-γ-ELISPOT results and $CD4^+$ T cell counts and IFN-γ-ELISPOT results and $CD8^+$ T cell counts and IFN-γ-ELISPOT results and HCMV IgG results were established using Spearman's correlation. The SFU/10^6 cells below 50 of the ELISPOT results were excluded from the statistical analysis.

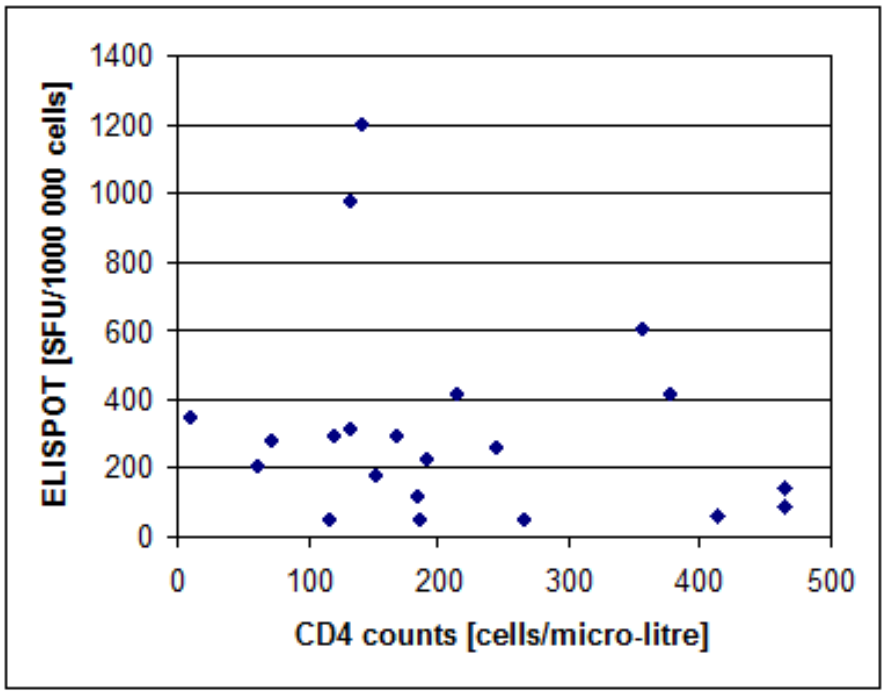

Figure 3.3: Relationship between $CD4^+$ T cell count and HCMV IFN-γ-ELISPOT values in HIV-HCMV co-infected subjects (*n*= 34)

Chapter 4

4. Discussion and conclusion

4.1. Introduction

HCMV is an ubiquitous virus (Fletcher *et al*., 2005). Primary infections and subsequent reactivations and re-infections in healthy individuals are usually asymptomatic (Lautenschlager *et al*., 2009). However, when an HCMV-positive individual is co-infected with HIV, the immune system is compromised. The HCMV-specific cellular immunity may be adversely affected and HCMV reactivation may occur. Life-threatening EOD such as retinitis, colitis and pneumonitis may ensue (Heiden *et al*., 2007; Ohnishi *et al*., 2005). The EOD in HCMV infection becomes clinically apparent when a threshold of cellular damage in the target organs is breached (Griffiths, 2009). Treatment of the HCMV-associated disease at this point may not be effective (Wohl *et al*., 2009), suggesting that prevention of the reactivation of the virus may be a more suitable treatment option. Determination of a point when the HCMV-specific cellular immune response breaks down and possible reactivation occurs in HIV-positive individuals may assist clinicians in providing appropriate pre-emptive treatment. A further advantage of the determination of this point would be the reduction in the use of prophylactic drugs against HCMV which increases the risk of HCMV resistance (Godard *et al*., 2004). Only individuals identified as high risk for HCMV disease would receive the appropriate preventative treatment (Godard *et al*., 2004; Len *et al*., 2008).

The association between the $CD4^+$ T cell count, the frequency of T cells secreting IFN-γ in response to HCMV antigens and HCMV viraemia has previously being reported in older, predominantly male populations (Erice *et al*., 2003; Jacobson *et al*., 2008; Weinberg *et al*., 2006). These studies were undertaken in developed countries. This association has not yet been reported in the HIV-infected population in South Africa which is younger and predominantly female (Connolly *et al*., 2004). To our knowledge the current study is the first to investigate if a point can be determined when the HCMV-

specific cellular immune response breaks down in HIV-positive, ART-naïve individuals in a South African setting using the IFN-γ-ELISPOT assay. The HCMV viral load of the subjects was determined using an in-house qPCR. The $CD4^+$ T cell count of each subject was also established with flow cytometry.

The main finding of the current study is that the IFN-γ-ELISPOT response cannot be used in isolation of the $CD4^+$T cell count to determine a specific point at which the HCMV-specific cellular immune response breaks down and HCMV reactivation occurs. In addition, we were unable to find a correlation between the numerical values of the IFN-γ-ELISPOT response and the $CD4^+$ T cell counts in HIV-HCMV co-infected individuals. However, what we did find was that HCMV viraemia as determined by HCMV viral load qPCR was more likely to occur at $CD4^+$ T cell counts less than 100 cells/µl. The IFN-γ-ELISPOT was positive in those individuals, contrary to what was anticipated. Nevertheless, we believe that the IFN-γ-ELISPOT assay does have role in evaluating the HCMV-specific cellular immune response in the individual HIV-positive patient. In this chapter we make a compelling argument as to why the IFN-γ-ELISPOT assay is useful at quantifying the integrity of the HCMV-specific cellular immune response in HIV-positive individuals and together with the $CD4^+$ T cell counts and HCMV viral load may prove useful in the clinical applications for pre-emptive therapy in patients at risk of HCMV reactivation.

4.2. Demographics of recruited subjects

4.2.1. Cohort size

The cohort sample size of studies investigating HIV-HCMV co-infected individuals in the available literature has varied widely. Jacobson (2008) investigated 11 case patients and 12 control subjects, Sacre (2005) studied 30 HIV-HCMV co-infected subjects, Weinberg (2006) had 107 subjects and Wohl (2009) had 338 study subjects in prospective observational studies. The sample size of 34 HIV-HCMV co-infected subjects analysed in the current study is comparable with other reports in the literature.

4.2.2. Gender and age of subjects recruited

The HIV-HCMV subjects whose data were analysed in the current study were predominantly female. This is consistent with reports that in South Africa the HIV prevalence in women is greater in females (Connolly *et al*., 2004). There is currently no data to support a similar gender bias of HCMV infection in South Africa. Rather, Rabenau (2010) found the seroprevalence of HCMV to be the same in both genders. It is possible that the proportion of HIV-HCMV co-infected individuals in the current study is more likely to reflect an HIV gender bias than that of HCMV.

The mean age of the group in this study was 33.9 years with a range of 25 to 48 years. The population of this study is younger than those reported in other studies concerned with HIV-HCMV co-infection. Erice (2003), Jacobson (2008) and Weinberg (2006) looked at HCMV EOD in populations who were predominantly male (87%, 91%, and 94%, respectively) and older (median age 39, 42 and 39 years, respectively).

The implication of studying HCMV-specific cellular immune responses in an HIV-HCMV co-infected sample that is predominantly female is that women appear to have a stronger and more spontaneous release of the pro-inflammatory cytokines such as IFN-γ and IL-2 in response to HCMV (Villacres *et al*., 2004). However, we were unable to demonstrate a gender difference in the IFN-γ-ELISPOT response. This is particularly pertinent to the finding of a positive IFN-γ-ELISPOT result in the presence of HCMV viraemia at low $CD4^+$ T cell counts. One of the subjects with a positive IFN-γ-ELISPOT result at a $CD4^+$ T cell count <100 cells/μl and a detectable viral load was male and the other with a similar result was female. Reasons for the positive IFN-γ ELISPOT result and detectable HCMV viral load other than gender are discussed in section 4.4.1 of this chapter below.

4.2.3. HIV-HCMV co-infection seroprevalence

All 52 HIV-infected subjects recruited in the current study were also HCMV seropositive. They were recruited from an HIV clinic that served a low socio-economic population. This is consistent with other studies that have shown a high HCMV prevalence in

populations from poor socio-economic settings (Colugnati *et al*., 2007). Rabenau (2010) documented a 100% seroprevalence of HCMV in HIV-positive individuals in the Southern African country, Lesotho. Adjei (2008) reported the seroprevalence of HCMV in 3275 HIV-seronegative, healthy, Ghanian blood donors and 250 HIV-infected individuals to be 77.6% and 59.2%, respectively. The seroprevalence in the Ghanaian population is less than that reported in Southern Africa but is still higher than that reported in developed countries. With regards developed countries, in an early study, Nerurkar (1987) reported a seroprevalence of 17.7% and 5% in HCMV-HIV co-infected homosexual males and healthy male blood donors, respectively in Washington DC and New York City. In a more recent study, Colugnati (2007) reported an incidence of new HCMV infections in a USA population of 1.6 per 100 persons per year with the infection rate higher in low socio-economic than middle- and high socio-economic groups.

4.3. The IFN-γ-ELISPOT assay and HCMV-specific cellular immune monitoring

There is no generally accepted management strategy for HCMV disease in HIV-positive patients who are not yet on ART and there is no consensus whether HCMV-specific cellular immunity is a marker of disease risk. In addition, there are also no routine diagnostic tests to quantify HCMV-specific cellular immune responses. The $CD4^+$ T cell count, HCMV viral load, and the magnitude of the HCMV-specific cellular immune response have been proposed as possible biomarkers for the breakdown of HCMV-specific cellular immunity in HIV/AIDS patients (Erice *et al*., 2003; Fernandez *et al*., 2006; Jacobson *et al*., 2008; Salmon-Ceâron *et al*., 2000; Singh *et al*., 2007; Weinberg *et al*., 2006; Wohl *et al*., 2009). However, no consensus has been reached as to the merits of these biomarkers.

T cells are essential in controlling HCMV and preventing reactivation (Gamadia *et al*., 2004; Gratama *et al*., 2008; Sacre *et al*., 2005; Tay-Kearney *et al*., 1997). Monitoring the activity of the T cells that suppress the virus requires the use of an efficient immunological assay. Different immunological assays are available to determine the HCMV-specific cellular immune response. These include the LPA, (Augustine *et al*., 2007; Gehrz and Knorr, 1979), the IFN-γ-ELISPOT assay (Macatangay *et al*., 2010;

Stone *et al*., 2006), intracellular cytokine staining (ICS) (Kern *et. al*., 1999) and tetramer staining (Hernandez-Fuentes *et al*., 2003).

The IFN-γ-ELISPOT assay has been demonstrated as an effective tool in measuring the HCMV-specific cellular immune response. Weinberg and Pott (2003) demonstrated that the IFN-γ-ELISPOT assay was more sensitive than either of the LPA or flow cytometry to detect changes in HCMV-specific cellular immunity in HIV-positive children on HAART. Karlsson (2003) found the IFN-γ-ELISPOT assay better able to detect low level-responses than the flow cytometry. Thus, in the current study the IFN-γ-ELISPOT assay was deemed appropriate at detecting any weak responses of the HIV-HCMV co-infected subjects. A prospective study by Weinberg (2006) demonstrated that the IFN-γ-ELISPOT assay was more sensitive than the $CD4^+$ and $CD8^+$ intracellular IFN-γ assays in determining a link between HCMV-specific cellular immunity, HCMV viraemia and HCMV-EOD in HIV-positive individuals. The IFN-γ-ELISPOT assay was more sensitive than the LPA in determining the immune response to HCMV antigens in HIV-positive individuals in a study undertaken by Goodell (2007). In an earlier, unpublished study we showed that the IFN-γ-ELISPOT assay proved more sensitive than the LPA in determining the HCMV-specific cellular immune response of healthy individuals. The LPA spanned seven days in contrast to the IFN-γ-ELISPOT which required three days. In addition, the LPA usually utilises radioactive material during the assay (Gotch *et al*., 2005) which also made it an unattractive option. Based on the above literature the IFN-γ-ELISPOT assay was favoured to determine the HCMV-specific cellular immune response of the HIV-positive individuals in the current study.

The IFN-γ-ELISPOT assay measures the frequency of the T cells that secrete IFN-γ (Barron *et al*., 2009, Gray *et al*., 2009; Macatangay *et al*., 2010, Weinberg *et al*., 2006, Zhang *et al*., 2009). However, one must bear in mind that IFN-γ is not the only cytokine expressed during the inflammatory process. Other cytokines and molecules include IL-2, MIP-1β, TNF-α and granzyme B. The granzyme B ELISPOT assay may be utilised to determine the cell-mediated immune response (Rininsland *et al.,* 2000). Nonetheless, the IFN-γ-ELISPOT assay is still the most widely used and generally accepted read-out of

the response to HCMV (Barron *et al*., 2009, Gray *et al*., 2009; Macatangay *et al*., 2010, Weinberg *et al*., 2006, Zhang *et al*., 2009). A dual- cytokine ELISPOT assay that utilises IFN-γ and IL-2 is also available. It has been reported that IL-2 increases the proliferation of the HCMV-specific $CD8^+$ T cells (Jagannathan *et al*., 2009). However, it requires extreme optimization to distinguish IFN-γ or IFN-γ + IL-2 or IL-2 only (Boulet *et al*., 2007). A simple diagnostic test which could be used routinely was our primary option. The frequency of T cells secreting IFN-γ was thus, used in the IFN-γ-ELISPOT assay in the current study as a surrogate marker for determining the magnitude of the HCMV-specific cellular immune responses of the HIV-infected subjects.

4.4. Comparative results

4.4.1. The HCMV IFN-γ-ELISPOT results and HCMV viral load qPCR results

Positive HCMV IFN-γ-ELISPOT results are associated with an absence of HCMV viraemia (Weinberg *et al*., 2006). Similar findings are reported in the current study. Of the 34 subjects studied, 23 subjects had a positive HCMV IFN-γ-ELISPOT result. Of these, 94% were aviraemic as determined by HCMV viral load qPCR. Moreover, none of the subjects displayed any clinical manifestations of HCMV disease at the time of recruitment for the study. This emphasises the possibility of the use of the IFN-γ-ELISPOT assay as a valuable tool to determine the integrity of the HCMV-specific cellular immune response in HIV-positive individuals which is in agreement with similar studies (Goodell *et al*., 2007; Karlsson *et al*., 2003; Weinberg and Pott, 2003).

Weinberg (2006) has, however, demonstrated that the IFN-γ-ELISPOT assay result can convert from positive to negative and from negative to positive in individuals with a low $CD4^+$ T cell count receiving HAART over a two year period. This suggests that the HCMV-specific cellular immune response is dynamic. The cross-sectional observational study design employed in the current study describes the status of the HCMV-specific cellular immune response at a single point in time. This may not be representative of the dynamic nature of the HCMV-specific cellular immune response which may fluctuate as demonstrated by Weinberg (2006). Therefore, the IFN-γ-ELISPOT assay may have more value in a longitudinal, diagnostic study.

Two subjects had positive HCMV IFN-γ-ELISPOT results with positive HCMV viral load qPCR results. The HCMV IFN-γ-ELISPOT result is indicative of the HCMV-specific cellular immune response of an individual (Cheeran *et al*., 2008; Whitman *et al*., 2008). This implies that HCMV reactivation was imminent in these subjects despite a positive HCMV-specific cellular immune response. Studies have shown that despite a positive IFN-γ-ELISPOT result an individual may still display viraemia as determined by the qPCR (Barron *et al*., 2009; Weinberg *et al*., 2006). Acute infections may periodically arise but are controlled by the HCMV-specific immune response. Reactivations may also occur in healthy individuals and are not necessarily an indication of a failing immune response. The latent HCMV may reactivate but is eventually controlled again. Since no clinical manifestation of the disease was apparent at the time of recruitment, this suggests that the HCMV-specific cellular immune response of the subjects was still intact and was able to control the viral replication to some extent and prevent the damage of a critical number of cells which would have resulted in HCMV EOD (Griffiths, 2009). Similar evidence of this was reported by Wohl (2009). Only 5.8% of the subjects in the study undertaken by Wohl (2009) who displayed viraemia were diagnosed with HCMV EOD. This suggests that an appropriate HCMV-specific cellular immune response was evident and prevented uncontrolled replication of the virus. Furthermore, Sacre (2005) stated that the strength of the HCMV-specific cellular immune response may not be a major factor in limiting HCMV replication and preventing the progression of HCMV-associated diseases. The antigenic repertoire, diversity and differentiation of the $CD8^+$ T cells may be more effective in controlling active HCMV disease (Sacre *et al*., 2005).

A positive HCMV IFN-γ-ELISPOT result with a positive HCMV viral load qPCR could also reflect the virus‘ ability to evade the immune system. The HCMV uses different mechanisms to limit the $CD4^+$ T cell recognition of latently infected cells and thus prevents its clearance from the host (Cheung *et al*., 2009; Kano and Shiohara, 2000; Mocarski, 2004; Sezgin *et al*., 2010). It is also possible that these mechanisms may have allowed the HCMV to reactivate and replicate to detectable levels of HCMV viraemia. In this scenario, the HCMV-specific cellular immune response appears intact as represented by the positive IFN-γ-ELISPOT result but is in fact ineffective at suppressing the HCMV

reactivation. However, as the subjects were not re-evaluated it is not possible to exclude that the HCMV viraemia resulted in clinically significant EOD. Therefore, in a clinical scenario, the IFN-γ-ELISPOT assay should be repeated if the first result is positive in the presence of detectable HCMV viral loads to exclude progression of the disease.

A negative HCMV IFN-γ-ELISPOT result was obtained for 11 of the 34 subjects in the current study which implies that they may be at risk of HCMV reactivation. The HCMV–specific cellular immune response in these subjects is assumed to be ineffective at suppressing the virus. This could lead to active HCMV infection which is associated with the shedding of infectious virus particles. However, none of them had a positive HCMV viral load qPCR value. An explanation for this observation may relate to the antigen we used in the IFN-γ-ELISPOT assay. The HCMV pp65 antigen was used to stimulate a cell-specific immune response. However, it is known that some subjects require HCMV antigens other than pp65 to mount a detectable HCMV-specific cellular immune response (Sinclair *et al*., 2004). The IE-1 peptide is one such antigen. In rare cases, some individuals do not respond to either IE-1 or HCMV pp65 antigens (Bao *et al*., 2008; Sacre *et al*., 2005). This would account for an aviraemic state in the light of an apparently deficient HCMV-specific cellular immune response. A combination the HCMV pp65 and the IE-1 peptide pool may be appropriate to elicit a HCMV-specific cellular immune response (Sinclair *et al*., 2004) and is recommended for future studies in this regard.

Genetic variations of the human leukocyte antigen (HLA) genes (Valluri *et al*., 2005) may result in changes in the amino acid sequences of genes coding for different proteins (Mazzarino *et al*., 2005). Specifically, changes in the major histocompatibility complex (MHC)-class I molecules [*refer to section 1.2.6*] will result in variations in the presentation of peptides such as HCMV pp65 or HCMV IE-1 to $CD8^+$ CTL (Mazzarino *et al*., 2005). Thus, this may lead to variations in responses to HCMV peptides by different individuals.

4.4.2. The $CD4^+$ T cell counts and the HCMV viral load qPCR results

Studies have found that immunocompromised individuals with a low $CD4^+$ T cell count (less than 50 cells/µl) are at risk of developing HCMV disease while a high $CD4^+$ T cell count is protective against HCMV reactivation and progression to HCMV disease (Holmes *et al*., 2006; Sacre *et al*., 2005; Salmon-Ceâron *et al*., 2000). The $CD4^+$ T cell counts of the 34 subjects in the current study ranged from 10 cells/µl to 784 cells/µl. All subjects who had $CD4^+$ T cell counts >100 cells/µl had undetectable viral loads as determined by the HCMV viral load qPCR. This is consistent with the literature that suggests that a $CD4^+$ T cell counts >100 cells/µl are protective against viraemia (Holmes *et al*., 2006; Sacre *et al*., 2005; Salmon-Ceâron *et al*., 2000).

Two subjects had detectable HCMV viral loads at $CD4^+$ T cell counts of 10 cells/µl and 62 cells/µl suggesting possible HCMV reactivation. However, two subjects with $CD4^+$ T cell counts of 50 cells/µl and 53 cells/µl, respectively did not have detectable HCMV viral loads. This implies that subjects with low $CD4^+$ T cell counts were still able to suppress the viral reaction. This is consistent with the findings of Wohl (2009), and Song (2002) who established that only a very small percentage of individuals develop detectable HCMV viral loads and progress to EOD when the $CD4^+$ T cell counts are low. The transient nature of the HCMV viraemia (Salmon-Ceâron *et al*., 2000) may account for the absence of HCMV viral loads at $CD4^+$ T cell counts at less than 100 cells/µl. We recommend that in the clinical scenario, patients with low $CD4^+$ T cell counts and negative HCMV viral loads have serial HCMV viral load qPCR to confidently exclude HCMV reactivation. The frequency of serial HCMV viral load qPCR assays to confidently exclude HCMV reactivation in a susceptible host has, however, not being evaluated in the current study.

4.4.3. The HCMV IFN-γ-ELISPOT results and the $CD4^+$ T cell counts

Of the 28 subjects with a $CD4^+$ T cell count above 100 cells/µl, 68% had positive HCMV IFN-γ-ELISPOT results. This is consistent with Weinberg (2006) who reported that a high $CD4^+$ T cell count is associated with a positive IFN-γ-ELISPOT result.

However, two of our subjects also displayed a positive IFN-γ-ELISPOT value at a $CD4^+$ T cell count below 100 cells/µl. This suggests that they were still able to mount a cellular immune response to HCMV despite the low $CD4^+$ T cell count. As a result the use of the $CD4^+$ T cell count to determine the point when the HCMV-specific cellular immunity breaks down is questionable. Our findings are consistent with the findings of other researchers. Fernandez (2006) found that after the initiation of ART the $CD4^+$ T cell-IFN-γ response to HCMV was similar irrespective of the value of the $CD4^+$ T cell count. Similarly, Song (2002) found that individuals with a $CD4^+$ T cell count below 50 cells/µl did not necessarily develop HCMV retinitis.

The observation that the HCMV IFN-γ-ELISPOT response was negative in nine of the 28 subjects with a $CD4^+$ T cell count more than 100 cells/µl may be explained on the basis of the antigenic stimulant used in the current study (Bao *et al*., 2008; Sacre *et al*., 2005; Sinclair *et al*., 2004). However, an alternative explanation may relate to inadequate T cell response. In this regard, T cell responses have been investigated by researchers such as Ben-Smith (2008) and Gupta (2004) in specific populations. In this regard Ben-Smith (2008) has reported a difference in the naïve T cells and memory T cells in Malawian and UK adolescents. The Malawians had fewer naïve T cells and an increase in the number of $CD28^-$ memory T cells. This was possibly due to an early and greater exposure of the individuals to HCMV in the Malawian group as is common in developing countries. A similar phenomenon was also observed in the elderly who were exposed to HCMV over a long period (Gupta *et al*., 2004). Gupta (2004) reported that in the elderly, the CD8 stable memory T cells and the CD8 central memory T cells most often did not express CD28. This impacted negatively on the proliferation and clonal expansion of T cells in the elderly which in turn adversely affected the immune response to antigenic stimulation. In a developing country such as South Africa early exposure of viruses such as HCMV is probable (Keane *et al*., 2004) as suggested by the 100% HCMV seroprevalence in the current study. Thus, early exposure to HCMV of the subjects in the current study may have resulted in fewer naïve T cells and an increase in memory T cells lacking $CD28^-$ similar to that observed in the Malawian group and the elderly. The implication of the high HCMV seroprevalence and possible early exposure to the HCMV antigens in the

population in the current study is not known but the possibility remains that it may have led to an adverse effect on the immune response to HCMV stimulation as reflected by a negative HCMV INF-γ- ELISPOT result despite a high $CD4^+$ T cell count.

4.4.4. The qPCR and HCMV viral load

Real-time PCR provides a quantitative and qualitative measurement of viral load However, it does not tell us about the mechanism that has led to the presence of the virus. An interesting observation in the current study is the low HCMV viral load prevalence in the HIV-infected group who have a low $CD4^+$ T cell count. It has been reported in the literature that HIV-HCMV co-infected individuals with low $CD4^+$ T cell counts are at risk of reactivation as determined by HCMV viral load (Erice *et al*., 2003; Sachithanandham *et al*., 2009; Weinberg *et al*., 2006). However, only two of the six subjects with a $CD4^+$ T cell count less than 100 cells/µl in the current study displayed viraemia as determined by the HCMV viral load qPCR. This emphasises the transient nature of the HCMV viraemia (Salmon-Ceâron *et al*., 2000) and questions the sensitivity of the HCMV viral load qPCR to detect viral loads.

Conflicting evidence with regards the sensitivity of the qPCR exists in the literature. The lower limit of detection as a predictor for determining active HCMV disease has not yet been established. Various in-house qPCR are available with no standardisation in techniques and in the various primers and probes used to investigate the same genome regions (Peres *et al*., 2010; Preiser *et al*., 2003a). In a study undertaken by Ruell (2007) with stem cell transplant recipients, the qPCR was not able to detect a HCMV viraemia in 50% of individuals who developed HCMV EOD. Of these individuals, 75% were aviraemic as measured with qPCR throughout the disease. By implication a negative qPCR in symptomatic individuals does not exclude HCMV EOD. Boeckh (2004) reported a similar finding regarding qPCR. Some individuals in the latter study developed HCMV disease but did not display viraemia as measured with the qPCR. It has been suggested that the HCMV DNA load in plasma be used in conjunction with other biomarkers such as $CD4^+$ T cell count and the HCMV-specific cellular immune response to guide clinical management.

4.4.5. The use of a specific analyte for qPCR

The two analytes that were used in the current study to determine the HCMV viral load using qPCR were EDTA whole blood and EDTA plasma. There is controversy in the literature as to which analyte is more suitable for this purpose (Deback *et al*., 2007; Koidl *et al*., 2008; Michelin *et al*., 2008; Preiser *et al*., 2003a). Viraemia may be an indication of HCMV reactivation. However, the detection of HCMV DNA in whole blood may not necessarily indicate viraemia as the virus remains latent in the monocytes. Plasma is devoid of blood cells in the latent stage of the virus. Shedding of viral particles into the plasma is thus more likely to be representative of viraemia (Preiser *et al*., 2003a). In the current study, two subjects displayed viraemia in whole blood and only one subject displayed viraemia in plasma. However, only the subject displaying a plasma viral load of more than 100 copies/ml was deemed viraemic as determined by the HCMV viral load qPCR. Due to the low number of subjects with detectable viraemia, we were unable to determine which analyte, plasma or whole blood, would be more suitable for use in qPCR.

4.5. Clinical application of the IFN-γ-ELISPOT results and HCMV viral load

The results of our study suggest that the IFN-γ-ELISPOT assay could have relevant clinical applications. This is due to the HCMV IFN-γ-ELISPOT results in HIV-HCMV co-infected individuals being representative of the cellular immune mechanisms that suppress HCMV reactivation. However, the results of individual assays and tests may be misleading if interpreted in isolation. Thus, we suggest that the HCMV IFN-γ-ELISPOT results be interpreted in conjunction with the HCMV viral load and $CD4^+$ T cell counts. This may be useful to assist clinicians to determine the risk of reactivation and possible disease outcomes as illustrated in Table 4.1.

Table 4.1: HCMV IFN-γ-ELISPOT results and HCMV viral load results with possible outcomes in HIV-HCMV co-infected individuals

HCMV IFN-γ-ELISPOT result	HCMV qPCR result	Interpretation
Positive	Negative	Development of HCMV disease-unlikely
Positive	Positive	Development of HCMV disease-unlikely
		Development of HCMV disease-yes if viraemia persists
Negative	Positive	Development of HCMV disease-yes if viraemia persists
		Development of HCMV disease-unlikely
Negative	Negative	Risk of development of IRIS

In an HCMV IgG positive individual, a positive HCMV IFN-γ-ELISPOT result and a negative HCMV viral load as determined by qPCR suggests that the individual is able to suppress the replication of the virus and poses no risk of HCMV reactivation. This usually occurs in individuals with a high $CD4^+$ T cell count (Weinberg *et al*., 2006).

A positive HCMV IFN-γ-ELISPOT result and a positive HCMV viral load qPCR may be interpreted in two ways. The individual may develop HCMV disease if viraemia persists but this is usually a small proportion of subjects most often with a low $CD4^+$ T cell count (Song *et al*., 2002; Wohl *et al*., 2009). The other possibility is that this is simply a transient viraemia (Salmon-Ceâron*et al*., 2000) and the subjects may not develop HCMV disease (Song *et al*., 2002; Wohl *et al*., 2009). In this instance, the HCMV viral load qPCR should be repeated as the viraemia may be temporarily positive and not of clinical significance.

In subjects with a negative HCMV IFN-γ-ELISPOT result and a persistent positive HCMV viral load, the assumption can be made that there is reactivation of HCMV and that this is due to an inadequate suppression of the virus. The individual may be at risk of developing HCMV EOD and clinical judgement should ensue. Individuals with low $CD4^+$ T cell counts are at greater risk (Weinberg *et al*., 2006).

In an individual who is seropositive for HCMV but negative for IFN-γ-ELISPOT testing and without detectable viral load there is the risk of the development of HCMV-IRIS on initiation of HAART (Bonham *et al*., 2008; Rios *et al*., 2005). An exaggerated inflammatory response is mounted against latent HCMV and their antigens. This is relevant to the South African HIV-HCMV co-infected individuals as they may comprise a significant proportion of the 2.7 million people that are predicted to require HAART in 2012 (Walensky *et al*., 2008). Initiating HAART to these individuals with advanced HIV/AIDS and low $CD4^+$ T cell count increases the risk of IRIS (Murdoch *et al*., 2008; Smith *et al*., 2009). Thus, it may be important to ensure that HCMV disease be identified and treated before the initiation of HAART in the individuals with a negative HCMV IFN-γ-ELISPOT result and a negative HCMV viral load (Bonham *et al*., 2008; Murdoch *et al*., 2008).

Five main drugs are available for HCMV-associated diseases in HIV-positive patients and transplant recipients on immunosuppressive drugs. These include ganciclovir, foscarnet, cidofovir, acyclovir and fomivirsen (Kalil *et al*., 2005; Paya *et al*., 2004). The drugs improve the prognosis of the disease but factors such as high toxicity, development of drug resistant strains and cost of treatment are problematic (Godard *et al*., 2004). Thus, appropriate pre-emptive treatment should be administered to only those individuals at an increased risk of HCMV reactivation and progression to HCMV EOD.

4.6. Limitations and recommendations

The use of a single peptide (pp65) in this study may have led to failure to elicit an appropriate IFN-γ-ELISPOT response in some of the subjects. It is therefore, recommended that the use of an overlapping pool of peptides that includes both IE-1 and pp65 or the whole HCMV lysate may be useful as this may have greater success at eliciting an HCMV-specific cellular immune response (Sinclair *et al*., 2004). This would include those individuals who respond with a greater magnitude to the different epitopes.

The nature of the cross-sectional observational study is that it provides a snapshot of the state of a specific variable in the chosen population. The advantage of this design is the convenience with which it may be completed. The samples are collected from each subject with the minimum of investigator-subject contact, allowing for recruitment of greater subject numbers. Although, the HCMV IFN-γ-ELISPOT assay has proven to be efficient in measuring the magnitude of HCMV-specific immune response at a given time, it may have greater value at determining the fluctuations in cellular immune responses over a period of time. It may be suited to longitudinal study designs where the dynamics of the HCMV-specific cellular immune responses may be determined (Weinberg *et al*., 2006).

An in-house qPCR was utilised in the current study to determine the HCMV viral load in HIV-positive individuals. A value of at least 100 copies/ml was deemed a positive qPCR. However, no standardised cut-off for HCMV reactivation by qPCR has yet been determined (Peres *et al*., 2010). Thus, the positive HCMV viral load obtained may result in patients receiving treatment who would not necessarily progress to HCMV EOD (Godard *et al*., 2004). A standardised cut-off should therefore be established, clearly defining the point of HCMV reactivation and disease progression. More investigations are required in this regard. Hence, we suggest the clinical interpretation of the HCMV viral load testing be made in conjunction with IFN-γ-ELISPOT assay and $CD4^+$ T cell count as these may allow the clinician to make clearer clinical decisions in the treatment of HCMV reactivation in HIV-HCMV co-infected individuals.

4.7. Conclusion

The aim of the current study was to determine whether there is a point when there is loss of integrity of the HCMV-specific cellular immune response in a HIV-positive ART-naïve individual using the IFN-γ-ELISPOT assay. The aims and objectives of the study have been achieved.

The main finding of the current study is that the IFN-γ-ELISPOT response cannot be used in isolation of the $CD4^+$ T cell count to determine a specific point at which there is loss of integrity of the HCMV-specific cellular immune response and HCMV reactivation occurs. Furthermore, we were unable to find a correlation between the numerical values of the IFN-γ-ELISPOT response and the $CD4^+$ T cell counts in HIV-HCMV co-infected individuals. However, it was established that HCMV viraemia as determined by qPCR was more likely to occur at $CD4^+$ T cell counts less than 100 cells/µl. Surprisingly, the IFN-γ-ELISPOT was positive in those individuals and contrary to what was anticipated. Nevertheless, we believe that the IFN-γ-ELISPOT assay does have a role in evaluating the HCMV-specific cellular immune response in the individual HIV-positive patient.

The IFN-γ-ELISPOT used in conjunction with other biomarkers such as $CD4^+$ T cell count and HCMV viral load as determined by qPCR may be more effective in providing a true reflection of the state of the HCMV-specific cellular immune response of an HIV-positive individual. This may assist clinicians in providing appropriate pre-emptive therapy to HIV-positive individuals at risk of HCMV reactivation.

Chapter 5

5. References

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Appendix A: Ethics approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot · your knowledge partner

15 April 2009 **MAILED**

Dr W Liebrich & Mrs G. Arendse
Dept of Medical Virology
Stellenbosch University
Tygerberg
7505

Dear Dr Liebrich & Mrs G. Arendse

"The relationship between Cytomegalovirus-specific immunity and CD4 count in HIV-positive individuals."

ETHICS REFERENCE NO: N09/01/007

RE : PROVISIONAL APPROVAL

It is my pleasure to inform you that the abovementioned project has been provisionally approved on 14 April 2009 for a period of one year from this date. You may start with the project, but this approval will however be submitted at the next meeting of the Health Research Ethics Committee for ratification, after which we will contact you again.

Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary to make their final decision.

Please quote the abovementioned project number in all future correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Please note that in line with the recent changes to research ethics guidelines, including the Declaration of Helsinki, the CHR requires that all researchers specifically request and motivate for a "waiver of informed consent" for retrospective clinical audits.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

Verbind tot Optimale Gesondheid · Committed to Optimal Health
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Yours faithfully

DR MALCOLM DE ROUBAIX
RESEARCH DEVELOPMENT AND SUPPORT
Tel: +27 21 938 9207 / E-mail: mertrude@sun.ac.za
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Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

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Appendix B: Extension of Ethics approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
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21 May 2010 **MAILED**

Dr W Liebrich & Mrs G. Arendse
Dept of Medical Virology
Stellenbosch University
Tygerberg
7505

Dear Dr Liebrich & Mrs G. Arendse

"The relationship between Cytomegalovirus-specific immunity and CD4 count in HIV-positive individuals."

ETHICS REFERENCE NO: N09/01/007

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 19 May 2010, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 19 May 2010 Expiry Date:19 May 2011

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

21 May 2010 14:55 Page 1 of 1

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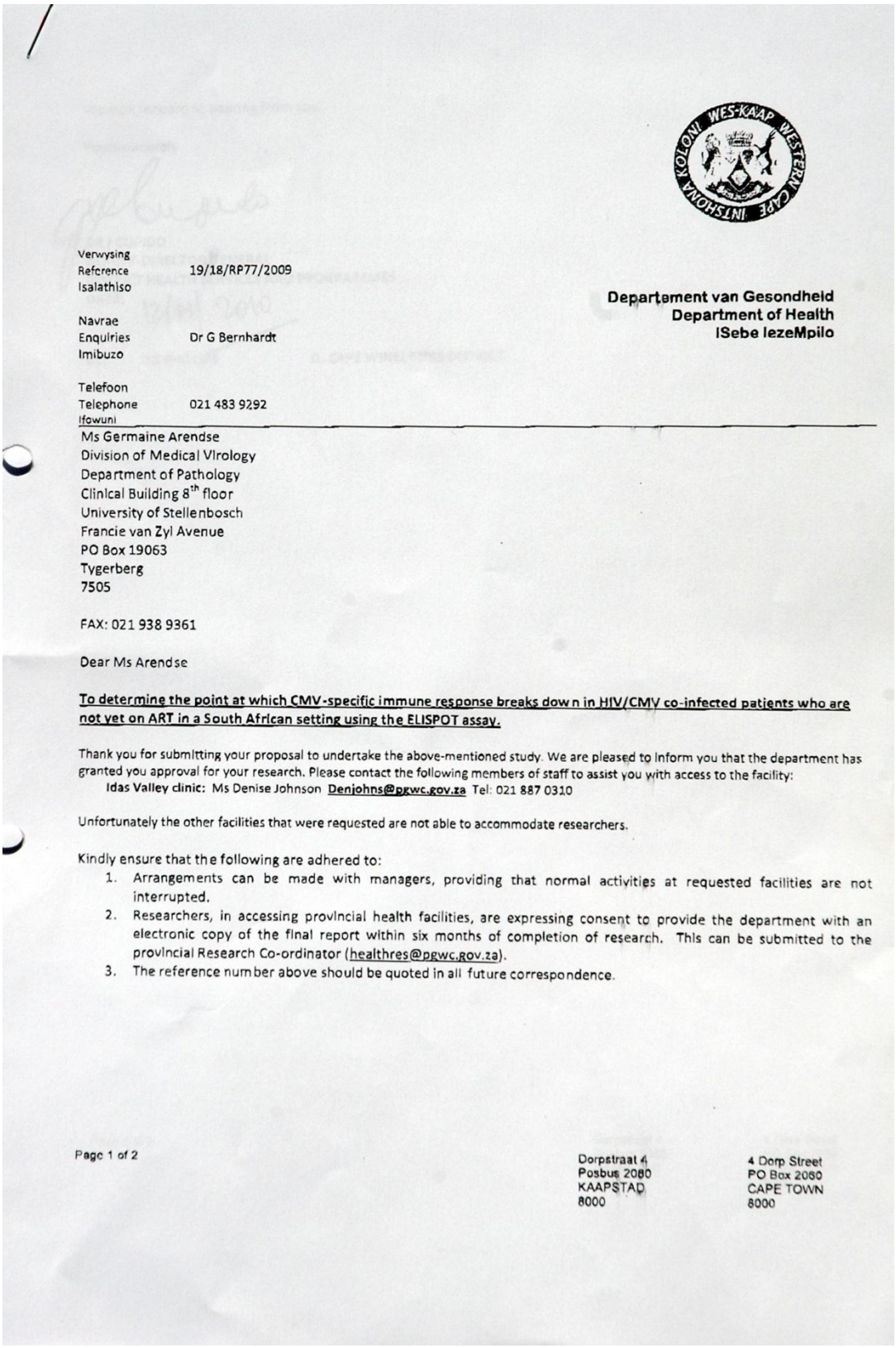
Appendix C: Department of Health approval



Verwysing
Reference 19/18/RP77/2009
Isalathiso

Navrae
Enquiries Dr G Bernhardt
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Ifowuni

Departement van Gesondheid
Department of Health
ISebe lezeMpilo

Ms Germaine Arendse
Division of Medical Virology
Department of Pathology
Clinical Building 8th floor
University of Stellenbosch
Francie van Zyl Avenue
PO Box 19063
Tygerberg
7505

FAX: 021 938 9361

Dear Ms Arendse

To determine the point at which CMV-specific immune response breaks down in HIV/CMV co-infected patients who are not yet on ART in a South African setting using the ELISPOT assay.

Thank you for submitting your proposal to undertake the above-mentioned study. We are pleased to inform you that the department has granted you approval for your research. Please contact the following members of staff to assist you with access to the facility:

Idas Valley clinic: Ms Denise Johnson Denjohns@pgwc.gov.za Tel: 021 887 0310

Unfortunately the other facilities that were requested are not able to accommodate researchers.

Kindly ensure that the following are adhered to:

1. Arrangements can be made with managers, providing that normal activities at requested facilities are not interrupted.
2. Researchers, in accessing provincial health facilities, are expressing consent to provide the department with an electronic copy of the final report within six months of completion of research. This can be submitted to the provincial Research Co-ordinator (healthres@pgwc.gov.za).
3. The reference number above should be quoted in all future correspondence.

Page 1 of 2

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Appendix D: Participant information leaflet and consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

The relationship between Cytomegalovirus-specific immunity and the CD4 count in HIV+ patients

REFERENCE NUMBER: N09/01/007

PRINCIPAL INVESTIGATOR:
Dr Walter Liebrich

ADDRESS:
Division of Medical Virology, Department of Pathology
Faculty of Health Sciences, University of Stellenbosch
Tygerberg Campus
Francie van Zijl Avenue, PO Box 19063
Tygerberg 7505

CONTACT NUMBER:
Tel:021 938 9054 / 9745

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not

affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

In this study we plan to do research in determining the relationship between the Cytomegalovirus-specific immunity (i.e. mechanisms the body users to protect us against CMV infection) and the CD4 T cell count (cells with specific markers that protect the body).The virus can enter the body through saliva, sexual contact, breast milk and blood transfusions. Once inside the body the virus multiplies causing CMV infection by interfering with the normal workings of the body making the person sick. CMV occurs in between 60% and 90% of humans with very few becoming sick because most have a strong immune system to protect them against the virus. If the immune system is weakened by illnesses such as HIV/AIDS then diseases caused by the virus may occur. The virus can affect the eyes, the lungs and the large intestine. We would like to see if the CD4 T cell count of the person can be used to determine when the immune system can no longer protect the body against CMV infection. The doctors can then use the CD4 T cell count as a way of deciding when patients can get treatment for CMV before it causes infection. (i.e. use of preventive treatment). Just before the CD4 T cell count becomes too low and CMV causes infection the patient can be treated and CMV diseases prevented.

Our aim is to invite a group of newly diagnosed HIV positive patients that are also suspected of being infected with CMV and who have different CD4 T cell counts ranging from above 200cells/µl to below 50cells/µl to take part in this project.

10-20ml (about 2 tablespoons) of blood will be taken for laboratory tests (virological, genetic and immunological). Volunteers may be recalled for testing. Test will be performed in the Division of Medical Virology, Stellenbosch University.

Why have you been invited to participate?

You have been invited to participate as an HIV-positive donor with suspected concurrent CMV infection or as a healthy donor for control purposes.

What will your responsibilities be?

You will be asked to donate 10-20ml (about two tablespoons) of blood for this study. You may be asked to donate a further 10ml (one tablespoon) of blood at a later stage.

Will you benefit from taking part in this research?

There are no direct benefits (financial or medical) at this stage for you. However, family members and future generations may benefit if researchers succeed in this particular field of research.

Are there in risks involved in your taking part in this research?

You may feel some pain associated with having blood withdrawn from a vein and may experience discomfort, bruising and/or slight bleeding at the site. There are no potential risks, other than those associated with normal blood taking.

If you do not agree to take part, what alternatives do you have?

Participation in the study is voluntary and if you wish you may withdraw at any time from the study without giving any explanation.

Who will have access to your medical records?

Your identity will be kept confidential throughout and any information collected will not be linked to your name. A code number will be used for research purposes and access will be limited to authorised personnel only. Your name will not be used in any

publication, lecture or report resulting from the study. No information will be shared with a third party such as insurance companies.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

Your participation in this study will not result in any injuries. Only minor risks are involved as associated with blood taking (see above).

Will you be paid to take part in this study and are there any costs involved?

No you will not be paid to take part in the study. No additional visits for the sole purpose of this study are required. There will be no costs involved for you, if you do take part.

Is there anything else that you should know or do?

There is no need to inform your family practitioner or usual doctor that you are taking part in a research study.

There is no need to inform you medical insurance company that you are participating in a research study.

You can make use of the contact numbers above if you have any further queries or encounter any problems.

You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled *(insert title of study)*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. On (*date*) ……...…….. 2009.

....

Signature of participant Signature of witness

Declaration by study staff

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....….....…….. On (*date*) ……...…….. 2009.

....

Signature of investigator Signature of witness

Declaration by interpreter (if applicable)

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....….....…….. On (*date*) ……...…….. 2009.

....

Signature of interpreter Signature of witness

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