Impact of inflammation-induced oxidative stress on the integrity of immuno-haematopoietic cells and potential ameliorating interventions in an *in vitro* HIV model

By
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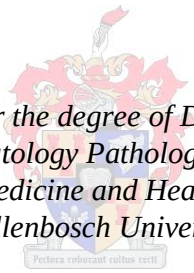

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DECLARATION

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ABSTRACT

Chronic inflammation and immune activation are hallmarks of HIV infection, resulting in chronic oxidative stress with over-utilization of antioxidant defences, which may contribute to the loss of immune cells and faster disease progression. Low levels of antioxidants in HIV-infected individuals have been associated with frequent opportunistic infections and an increased risk of mortality. HIV infection is also associated with on-going and aberrant activation of both the innate and adaptive immune systems. An important aspect of innate immune stimulation is derived from the leakage of lipopolysaccharide (LPS) across the damaged mucosal lining of the gut in early HIV infection. The impact of this innate immune stimulation on the adaptive arm of the immune system, as represented in this study by levels of CD4+ T-cell activation and death, have not been explored previously in untreated HIV infection. Using an integrated approach of immune activation, inflammation, oxidative stress and ameliorating antioxidant intervention for the first time, this thesis reports the impact of inflammatory induced-oxidative stress on CD4+ T-cells in an *in vitro* HIV model.

In a preliminary study, baseline levels of neutrophil respiratory burst as an *in vitro* indication of immune stimulation were investigated. The relationships between baseline total antioxidant status (TAS), Red blood cell (RBC) antioxidant enzyme activities (catalase, superoxide dismutase & glutathione peroxidase), lipid peroxidation and glutathione redox ratio and other markers of disease in asymptomatic, untreated HIV infection were also explored. The design and optimization of an assay that could determine the effects of LPS-induced oxidative stress on CD4+ T-cells, was a critical part of this study. The development of this assay enabled the measurement of the effects of selected antioxidant interventions N-acetyl cysteine (NAC) and vitamin C, on LPS-induced CD4+ T-cell activation and death. The results were also correlated with CD4 count, viral load and markers of inflammation (fibrinogen & D-dimers) in HIV-infected and uninfected groups.

Neutrophils from HIV-infected persons at rest showed a “primed” response to low stimulating agent, bacterial N-formyl peptides (fMLP), which was significantly ($P = 0.04$) higher than uninfected individuals. There was increased oxidative stress as evidenced by increased catalase activity, malondialdehyde (MDA) and conjugated dienes (CDs) with a corresponding decrease in antioxidant capacity in HIV-infected individuals with lower CD4 count. NAC in combination with vitamin C, significantly ($P = 0.0018$) reduced activation of CD4+ T-cells to a greater degree than with either antioxidant alone. NAC and vitamin C individually and in combination significantly ($P = 0.05$, $P = 0.012$ and $P<0.0001$) decreased the expression of the markers of apoptosis, Annexin V and 7-amino-actinomycin (7-AAD). Importantly, the antioxidant combination decreased MDA values and significantly ($P = 0.01$) increased the glutathione redox ratio in the HIV-infected group.

Based on these results, the respiratory burst and LPS-induced activation may be important contributing factors in inflammatory-associated oxidative stress in HIV infection and contribute to the depletion of CD4+ T-cells in the asymptomatic stage of HIV infection. These results also indicate the potential inhibitory effects of NAC and vitamin C in combination as agents that may limit immune activation and inflammation-induced oxidative stress. Importantly, the study showed that at this asymptomatic stage, CD4+ T-cells of the HIV-infected group responded similarly to stimulation as the HIV negative group, indicating that antioxidant defences were still functional and that appropriate early intervention at this stage may be protective against oxidative damage to the immune cells.

To the best of our knowledge, this study is the first to use an integrated approach involving not only plasma levels of antioxidant status, but also RBC antioxidant enzyme activities, oxidative damage (lipid peroxidation), inflammation, cellular levels of immune activation and potential ameliorating interventions in evaluating the problem of inflammation-induced oxidative stress in HIV infection.

Based on the results of this study, it is envisaged that an insight into the immune activation, inflammatory and oxidative stress status of patients will enable long-term profiling of each patient with a view to individualized therapy. This approach may have a direct impact on patient care in resource-limited settings such as sub-Saharan Africa.

OPSOMMING

Chroniese inflammasie en immuunaktivering is kenmerke van MIV-infeksie. Dié twee prosesse lei tot chroniese oksidatiewe stres en oorbenutting van antioksidant verdedigingstelsels, wat lei tot die verlies van die immuun selle en vinniger siektevordering. Lae vlakke van antioksidante in MIV-positiewe individue stem ooreen met gereelde opportunistiese infeksies en 'n verhoogde risiko van mortaliteit. MIV-infeksie word ook geassosieer met langdurige en abnormale aktivering van beide die ingebore en aanpasbare immuunstelsels. 'n Belangrike aspek van ingebore immuun stimulasie in die raamwerk van vroeë MIV-infeksie, is die lekkasie van LPS oor die beskadigde slymvlies voering van die dunderm. Die impak van die ingebore immuun stimulasie op die aanpasbare arm van die immuunstelsel, soos aangetoon in hierdie studie deur die vlakke van CD4 T-sel aktivering en apoptose, is nog nie voorheen ondersoek in onbehandelde MIV-infeksie nie. Met behulp van 'n oorspronklike, geïntegreerde benadering van immuun aktivering, inflammasie, oksidatiewe stres en ‘n lae vlak van antioksidant intervensie, lewer hierdie tesis verslag oor ‘n *in vitro* model van inflammasie-geïnduseerde oksidatiewe stres op CD4 T-selle.

In 'n voorlopige studie, is basislyn vlakke van die neutrofiel respiratoriese uitbarsting as 'n *in vitro* aanduiding van immuunstimulasie ondersoek. Die verhoudinge tussen basislyn totale antioksidant status (TAS), rooi bloed sel (RBC) antioksidant ensiemaktiwiteit (katalase, superoksied dismutase, en glutatioon peroksidase), lipied peroksidasie en glutatioon redoks-verhouding, asook ander merkers van siektevordering in asimptomatiese, onbehandelde MIV-infeksie is ook ondersoek. Die ontwerp en optimisering van 'n toets wat die effek van LPS-geïnduseerde oksidatiewe stres op CD4 T-selle kan bepaal, was 'n kritieke deel van hierdie studie. Die ontwikkeling van hierdie toets het ook die meting van die effek van toevoeging van twee geselekteerde anti-oksidante, N-asetiel sisteïen (NAC) en vitamien C, op LPS-geïnduseerde CD4 T-sel aktivering en apoptose ondersoek. Die resultate is ook gekorreleer met CD4-telling, virale lading en merkers van inflammasie (fibrinogeen en D-dimere) in groepe met en sonder MIV-infeksie.

Neutrofiele van asimptomatiese persone met MIV infeksie, het 'n 'voorbereide' reaksie gehad teen ‘n lae stimulerende agent, bakteriële N-formiel peptied (fMLP), wat beduidend ($P = 0,04$) hoër was as in individue sonder MIV infeksie. Daar was verhoogde oksidatiewe stres soos bewys deur verhoogde katalase aktiwiteit, malondialdehied (MDA) en gekonjugeerde diëne (CDs), saam met 'n ooreenstemmende afname in anti-oksidant kapasiteit in MIV-positiewe individue met laer CD4-tellings. NAC in kombinasie met vitamien C, het die aktivering van CD4 T-selle beduidend verminder ($P = 0,0018$), ‘n effek wat groter was in vergelyking met elke antioksidant alleen. NAC en vitamien C alleen, en in kombinasie het beduidend die

uitdrukking van die merkers van apoptose, Annexin V en 7-AAD verminder (P = 0,05, P = 0.012 en P<0,0001). Wat belangrik is, is dat die afname in MDA waardes as gevolg van antioksidante in kombinasie, ‘n beduidende styging in die glutatioon redoks verhouding in die MIV-positiewe groep tot gevolg gehad het.

Hierdie resultate het aangetoon dat die respiratoriese uitbarsting en LPS-geïnduseerde aktivering belangrike bydraende faktore mag wees in inflammasie-verwante oksidatiewe stres in MIV-infeksie, wat kan bydra tot die uitputting van CD4 T-selle in die asimptomatiese stadium van MIV-infeksie. Hierdie resultate dui ook aan dat die moontlike inhiberende effekte van NAC en vitamien C in kombinasie, immuun aktivering en geïnduseerde oksidatiewe stres kan beperk. Van belang is die feit dat hierdie studie bewys het dat in die asimptomatiese stadium van MIV-infeksie, CD4 T-selle weens stimulasie dieselfde gereageer het as dié van mense sonder MIV infeksie. Dit het aangedui dat antioksidant verdediging in hierdie stadium nog funksioneel was, en dat ‘n toepaslike vroeë intervensie op hierdie stadium beskermend teen immuun-sel oksidatiewe skade kan wees.

Tot die beste van ons kennis, is hierdie studie die eerste om 'n geïntegreerde benadering te gebruik, waar plasma vlakke van antioksidant status saam met RBC antioksidant ensiemaktiwiteit, oksidatiewe skade (lipied peroksiidasie), inflammasie, sellulêre vlakke van immuunaktivering, en potensiële beskermende ingrypings ondersoek is in die evaluering van die probleem van oksidatiewe stres in MIV-infeksie wat tot inflammasie lei.

Gebaseer op dié resultate, word dit in die vooruitsig gestel dat 'n insig in die status van immuunaktivering, inflammasie, en oksidatiewe stress van pasiënte, dit moontlik sal maak vir langtermyn- beplanning om vir elke pasiënt individuele terapie voor te skryf. Hierdie benadering kan 'n direkte impak op die sorg van pasiënte in hulpbron-beperkte gebiede soos sub-Sahara Afrika hê.

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DEDICATION

To the almighty God for His mercies, grace, direction, guidance, providence and protection. Thanks, praises and glory be to the almighty God through whom all this was possible.

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PREFACE

This thesis is organized in a format of five articles (written according to specific journal guidelines) with plans to submit them for publication in peer reviewed journals sooner rather than later and with an opening introduction, literature review and concluding chapters. The first chapter is a general overview of the research framework, which includes a brief description of the research problem, justification, aims and objectives of the research. The second chapter is a review of what other studies have reported in the field of inflammation-induced oxidative stress and how this has contributed to the development of the research topic. The research gaps in previous studies that form the basis of present study have also been discussed. The third chapter reports on a preliminary study that was conducted to investigate neutrophil respiratory/oxidative burst response in asymptomatic untreated HIV infection as an *in vitro* indication of a response to immune stimulation. The fourth chapter reports on how baseline antioxidant status and oxidative stress indicators relate to markers of disease and inflammation in untreated asymptomatic HIV infection. The fifth chapter reports on optimization of an assay that could determine the effects of LPS-induced oxidative stress on CD4+ T-cells, which enabled the measurement of the effects of selected antioxidant interventions N- acetyl cysteine (NAC) and vitamin C, on LPS-induced CD4+ T-cell activation and death. The sixth chapter is an article on modulation of LPS-induced CD4+ T-cell activation and apoptosis by antioxidants in untreated HIV infection. The seventh chapter is an article on modulation of LPS-induced immune activation and oxidative stress by vitamin C and *N*-acetyl cysteine in untreated asymptomatic HIV individuals

The eighth chapter is the conclusions, summary and recommendations. The developed HIV model for intervention is also discussed in this chapter.

GLOSSARY

Adaptive Immunity:	Arm of the immune system composed of T and B-cells with antigen-specific receptors that upon activation use multiple effector mechanisms to respond to antigen challenge.
Antioxidant:	A chemical when present in small amounts reduce or prevent oxidation of other molecules by being oxidized.
Apoptosis:	Programmed cell death or a genetically controlled mechanism of cell suicide involved in regulation of tissue homeostasis.
Asymptomatic:	A stage of disease in which there are no symptoms of the disease.
Biomarker:	A measurable biochemical indicator of a biological state, such as severity of presence of disease.
CD4+T-cells:	Mature T-helper cells expressing surface protein CD4.
Chemokine:	Low molecular weight cytokine capable of inducing chemotaxis/movement in leukocytes.
Chronic disease:	A long term disease that can be controlled but not cured.
Correlation:	Any broad class of statistical relationship involving dependence.
Cytochrome p-450:	A group of hemoproteins, which form multi-component electron transfer chains, useful in metabolism of endogenous and exogenous molecules e.g. drugs and toxins in humans.
Cytokine:	Is a cell signaling molecule made by cells, which modulate immune response such as inflammation cell growth & proliferation, differentiation and apoptosis.
Dismutation:	A reaction involving two identical molecules in which one gain what the other loses e.g. oxidation/reduction or phosphorylation/dephosphorylation.
Ex vivo:	An experiment conducted outside of the organism.
Fenton Reaction:	A non-enzymatic reaction of hydrogen peroxide with transition metals such as Fe^{3+} and Cu^{2+} to produce hydroxyl radicals and ferric iron.
Flow cytometry:	Process of measuring or counting cells or a technique of quantitative single cell analysis.
Flow-check Fluorosphere:	Suspension of fluorescent microspheres used for daily verification of flow cytometers optical (laser) alignment and fluidic system.
Flow-set Fluorospheres:	Suspension of fluorospheres (fluorescent micro beads) used in optimizing a flow cytometer for qualitative analysis of cells:

Free radical:	A highly reactive molecule with one or more unpaired electrons in its outer orbit.
Full matrix color Compensation:	Procedure used in flow cytometry to ensure and improve inter-instrument agreement in display and light sources measurements.
GRADE:	A type of systematic review, which separates the rating of the quality of evidence (by classifying it as high, moderate, low or very low) from the rating of the strength of recommendation.
HIV transcription:	Process by which single-stranded RNA with a base sequence complementary to one strand of double-strand DNA is synthesized via DNA-dependent RNA polymerase.
Immune activation:	State of immune stimulation characterized by high expression of CD38 and HLA-DR.
Immunophenotyping:	Analysis of antigens expressed by cells as by flow cytometry or immunohistochemistry.
In vitro:	An experiment or an occurrence on isolated cell components e.g. in a test tube.
In vivo:	An experiment or an occurrence in or on intact or whole organism.
Inflammation:	A protective tissue response to harmful stimuli such as pathogens, damaged cells or irritants.
Innate Immunity:	Arm of immune system composed of phagocytes (dendritic cells, macrophages & neutrophils), natural killer cells with germ line-encoded receptors specific for markers of perturbations (infection, damage or loss of exposure of self-molecules), complement and antimicrobial peptides.
Interleukins:	Cytokines produced by cells (WBCs) to modulate immune responses such as inflammation, growth, proliferation or apoptosis.
Kinase:	An enzyme that transfers phosphate groups from a high energy donor molecule e.g. ATP to specific substrates (phosphorylation).
Lentivirus:	A genus of virus of the retroviridae family characterized by a long incubation period
Lipid peroxidation:	Oxidative degradation of lipids by free radicals.
Metalloenzymes:	Enzymes containing a tightly bound metal e.g. zinc, cobalt, iron or magnesium as an integral part of its structure.
Monoclonal Antibodies:	A single clone of antibodies derived from a single cell.
Neutrophils:	Types of white blood cells characterized by the ability to mediate immune responses against infectious microorganisms.

NOX family: A group of white blood cells with NADPH oxidase complex, which is capable of generating ROS.

Optimization: Process or method of making something e.g. designs, system as fully perfect, functional or effective as possible.

Oxidation: The loss of electrons by a substance.

Oxidative stress: An imbalance between free radicals formation and antioxidant defense mechanisms.

Peroxímes: Cell organelles responsible for metabolism of fatty acids.

Peroxidation: Oxidation initiated by free radicals with the end products being hydroperoxides

Phagocytosis: Engulfing/endocytosis of particulate material or cell fragment by cells to be killed or digested.

Phospholipids: Any lipid containing phosphorous.

Phosphotidylserine (PS): A phospholipid predominantly occurring in the inner leaflet of eukaryotic cellular membranes

Plasma: Fluid part of blood in which the particulate components have been suspended.

Polymorphism: Ability to exist in several different forms.

Priming: Initial exposure to antigen of cells to facilitate the action of another cell.

Pro-inflammatory Cytokine: Cytokine capable of stimulating inflammation.

Pro-oxidant: A free radical of pathological importance or a toxic substance that can cause damage to biological molecules.

Protein Kinases: A kinase enzyme that modifies other proteins by chemically adding phosphate groups.

Pro viral: Precursor or latent form of a virus that is capable of being integrated into the genetic material of the host cell and being replicated with it.

P-value: Levels of significance within a statistical hypothesis test representing the probability of occurrence of a given event.

Redox status: Oxidation and reduction status.

Regimen: A regulated course of medical treatment designed to give a positive result.

Respiratory burst: Rapid release of reactive oxygen species (ROS) from different cells.

RNS:	Nitrogen containing molecules/species produced by incomplete reduction of oxygen and nitrogen.
ROS:	Oxygen containing molecules/species produced by incomplete reduction of oxygen.
Seronegative:	Absence of detectable specific antibodies to virus in the blood stream due to infection or immunization.
Seropositive:	Presence of detectable specific antibodies to virus in the blood stream due to infection or immunization.
Statistically Significant:	Statistical term that tells how reliable a difference or relationship exists.
T-cell hybridomas:	Somatic cells hybrid formed by fusion of normal T-cells and tumour cells resulting in cells that can produce same secretions as normal cells and able to proliferate indefinitely in culture medium
Toll-like receptors:	A group of pattern recognition receptors first discovered in Drosophila, which play a critical role in the innate immunity.
Total antioxidant Capacity:	The ability of antioxidants (both water-soluble and lipid-soluble) to clear/remove harmful free radicals in the blood or cells.
Total antioxidant Status:	The sum total of endogenous and diet/food/supplements derived antioxidants of the extracellular fluid of an individual.
Ubiquitination:	Process of addition of ubiquitin to proteins as one step of intracellular breakdown.
β-oxidation:	Oxidative degradation of saturated fatty acids in which two-carbon units are sequentially removed from the molecule with each turn of the cycle.

LIST OF SELECTED ABREVIATIONS

°C:	Degrees Celsius
7-AAD:	7- Aminoactinomycin D
AAPH:	2,2'-Azobis (2-amidinopropane) hydrochloride
ABTS:	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic) diammonium salt
AIDS:	Acquired immunodeficiency syndrome
APC:	Allophycocyanin
ATF-2:	Activating transcription factor 2
ATP:	Adenosine triphosphate
ARV's:	Antiretroviral therapies
AUC:	Area under the curve
BCL-2:	B-cell lymphoma-2
BHT:	Butylated hydroxytoluene
BMK1:	Big mitogen activated kinase 1
CD:	Cluster differentiation
CDs:	Conjugated dienes
CPUT:	Cape Peninsula University of Technology
DHR 123:	Dihydrorhodamine 123
DMACA:	4-dimethylaminocinnamaldehyde
DNA:	Deoxyribonucleic acid
DPPH:	2,2-diphenyl-1-picrylhydrazyl
DTNB:	5-5'-dithiobis [2-nitrobenzoic acid]
ERK:	Extracellular signal-regulated kinases
EtOH:	Ethanol
FACS:	Fluorescein activated cell sorting
FBS:	Foetal bovine Saline
Fe:	Iron
FITC:	Fluorescein isothiocyanate
FL:	Fluorochlome
fMLP:	Bacterial N-formyl peptides
FRAP:	Ferric reducing antioxidant power/potential
GCLC:	Glutamate cysteine ligase catalytic unit
GPx:	Glutathione peroxidase
GR:	Glutathione reductase

GRADE:	Grading of recommendations assessment, development and evaluation
GSH:	Reduced Glutathione
GSSG:	Oxidised Glutathione
HAART:	Highly active antiretroviral therapy
H_2O:	Water
H_2O_2:	Hydrogen peroxide
HCl:	Hydrochloric acid
HLA-DR:	Human leukocyte antigen D related
HO-:	Hydroxyl radical
ICAM:	Intracellular adhesion molecule- 1
IKK:	Inhibitor of kappa B kinase
JAK:	Janus kinase
JNK:	c-JUN N-terminal kinases
LDL:	Low density lipoproteins
LPS:	Lipopolysaccharides
MAPK:	Mitogen activated protein kinase
M2VP:	1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate
MBH:	Membrane bound haemoglobin
MCP-1:	Monocyte chemotactic protein-1
MDA:	Malondialdehyde
MeOH:	Methanol
MIP-1:	Macrophage inflammatory protein- 1
MPA:	Metaphosphoric acid
mRNA:	Messenger ribonucleic acid
Na_2CO_3:	Sodium carbonate
NaCl:	Sodium chloride
NADPH:	Reduced nicotinamide dinucleotide phosphate
NaOH:	Sodium hydroxide
NF-kB:	Nuclear factor kappa-light chain enhancer of activated B-cells
Nrf2:	Nuclear factor (erythroid-derived) like 2
O2.-:	Superoxide radical
ORAC:	Oxygen radical absorbance capacity
PE:	Phycoerythrine
PerCP:	Peridinin chlorophyll protein

CHAPTER 1

PI3:	Phosphoinositide 3 kinase
PMA:	Phorbol myristate acetate
PUFA'S:	Polyunsaturated fatty acids
r:	Spearman's correlation coefficient
R 123:	Rhodamine 123
rRNA:	Ribosomal ribonucleic acid
RBC:	Red blood cells
ROS:	Reactive oxygen species
SAPK:	Stress associated protein kinase
SOD:	Superoxide dismutase
TAC:	Total antioxidant capacity
TBA:	Thiobarbituric acid
TBARS:	Thiobarbituric acid reactive substances
TB:	Tuberculosis
TE:	Trolox equivalents
TEAC:	Trolox equivalent antioxidant capacity
TLR:	Toll- like receptor
TNB:	5-thionitrobenzoic acid
TNF:	Tumour necrosis factor
TPTZ:	2,4,6-Tri [2-pyridyl]-s-triazine
TRAF:	Tumour necrosis factor receptor associated factor
TRAIL:	Tumour necrosis factor-related apoptosis inducing ligand
Trolox:	6-Hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid
µM:	Micromole
UNAIDS:	United nation programme of acquired immune deficiency syndrome
USA:	United States of America
V-CAM:	Vascular cell adhesion molecules
WHO:	World health organisation
ΔA:	Absorbance difference

CHAPTER 1

Introduction

CHAPTER 1

1.1 Introduction

Although the HIV pandemic has globally stabilised since 2008 due to the introduction of the highly active antiretroviral therapy (HAART) in 1996 (Gil *et al.,* 2013; UNAIDS, 2011), it still remains a serious health threat. This pandemic continues to devastate resource-poor countries, especially in sub-Saharan Africa, which bears the highest burden (WHO, 2009; UNAIDS, 2012). Moreover, the inability of HAART to completely eradicate HIV, in association with the on-going immune activation and inflammation, has resulted in inflammation-associated complications and non-AIDS-associated deaths, such as myocardial infarction (El-Sadr *et al.,* 2006; Lau *et al.,* 2006; Kuller *et al.,* 2008). A major characteristic of HIV infection is the continuous systemic immune activation and inflammation (Brenchley *et al.,* 2006; Haynes, 2006; Nixon, 2010) which may result in oxidative stress, weakened immune responses, pro-inflammatory cytokine production, inflammation-associated complications and uncontrolled viral replication, in activated CD4+ T-cells (Haynes, 2006; Morris *et al.,* 2012). Excessive immune activation, inflammation and oxidative stress are associated with loss of immune cells, faster disease progression and increased risk of mortality (Pace & Leaf, 1995; Hazenberg *et al.,* 2003, Tan *et al.,* 2008; Tien *et al.,* 2010). Inflammation which is an important non-specific protective response of a tissue to harmful stimuli such as pathogens, damaged cells or irritants (Prescott *et al.,* 2002), is associated with increased production of reactive oxygen species (ROS) which are associated with apoptosis of CD4+ T-cells in HIV infection (Hockenbery *et al.,* 1993; Kotler, 1998; Gil *et al.,* 2003). Oxidative stress which has been defined as the cytopathological consequence of an imbalance between free radical production and the antioxidant status of the cell (Franco & Panayiotadis, 2009), is thought to add significantly to the depletion of CD4 + T-lymphocytes in HIV infection, thereby contributing to the progression to AIDS (Gil *et al.,* 2003; Pasupathi *et al.,* 2009; Wanchu *et al.,* 2009).

Mitochondria are a major source of ROS in the cells through electron leakage from the mitochondrial respiratory chain. The leaking electrons react with molecular oxygen to form superoxide and other ROS (Andreyev *et al.,* 2005). Other major endogenous sources of ROS include neutrophil respiratory burst, whereby some phagocytic cells in an oxidative burst, produce ROS through NADPH oxidase catalysed reaction, intentionally to destroy cells infected with bacteria or virus. The activated neutrophils and macrophages, during inflammation produce hydrogen peroxide (H_2O_2) and other oxidants (Kotler, 1998) during respiratory/oxidative burst due to increased oxygen use and ATP production, which further promotes tissue injury and inflammation (Israel & Israel, 2002; Prescott *et al.,* 2002). As a result, neutrophil respiratory burst is thought to contribute to the overall oxidative stress, which is implicated in the massive

depletion of CD4+ T-cells in early HIV infection (Reeves, *et al.,* 2002). Interestingly, neutrophil respiratory burst levels have not been described previously in the context of asymptomatic untreated HIV infection nor have the effects of this oxidative stress been studied in the CD4+ T-cell group.

Since the human biological system constitutively produces free radicals and other oxygen-derived radical species, cells have evolved complex antioxidant defence systems to combat this (Halliwell & Cross, 1994; Evans & Halliwell, 2001; Leonarduzzi *et al.,* 2010). The antioxidant defence system consists of primary or preventive antioxidants, which limit the initial formation of oxygen-centred radicals of organic compounds. The first primary defence mechanism consists of antioxidant enzymes such as catalase (CAT), found in peroximes in most tissues, which catalyse the two stage change of H_2O_2 to water and oxygen; glutathione peroxidase (GPx) and glutathione reductase (GR), which catalyse oxidation of glutathione at the cost of H_2O_2 in the cytosol and mitochondria and superoxide dismutase (SOD) which catalyse dismutation of superoxide to H_2O_2, which can then be removed. Secondary scavenging or chain breaking antioxidants are present to trap intermediate ROS and thus interrupt the chain reaction. The third line of defence consists of repair systems for damaged nucleic acids, proteins and lipids (Halliwell, 1994).

The, cells of the immune system are remarkably sensitive to oxidative stress, since their plasma membranes contain high levels of polyunsaturated acyl lipids, which are vulnerable to peroxidation. Consequently, excessive ROS causes damage to biomolecules such as DNA, carbohydrates, proteins and uric acids (Prior & Cao, 1999; Devasagayam *et al.,* 2004). More importantly, this oxidative damage is particularly marked in the phospholipids, which constitute the cell membrane (Block *et al.,* 2002; Roberts *et al.,* 2010), therefore making lipid peroxidation a convenient marker of oxidative stress in living systems (Nalsen, 2006). Furthermore, peroxidation of the polyunsaturated acyl chain in the cell membranes leads to loss of membrane integrity and altered membrane fluidity, consequently leading to impairment of intracellular signalling and the overall cell function (Chew & Park, 2009). As a consequence, immune cells in HIV infection are in constantly challenged by oxidative stress (Willcox *et al.,* 2004; Guerra *et al.,* 2011; Morris *et al.,* 2012), which further damages the already compromised immune system and contributes to disease progression (Haynes, 2006). Therefore, maintenance of a proper functioning antioxidant defence system in HIV-infected patients is vital, as it protects the cells against oxidative damage, hence promoting their survival (Kotler, 1998; Wanchu *et al.,* 2009; Morris *et al.,* 2013).

Several studies have linked a weakened antioxidant defence system to the increased oxidative stress found in HIV infection (Dobmeyer *et al.,* 1997; Pasupathi *et al.,* 2009; Suresh *et al.,* 2009). Pasupathi and co-workers (2009). observed a markedly decreased activity of SOD, CAT, GPx, GR, glutathione S-transferase (GST), reduced glutathione (GSH), in HIV/AIDS patients as compared to healthy controls (Pasupathi *et al.,* 2009). In this study, significantly decreased levels of plasma GSH, vitamin A (β-carotene), vitamin C, vitamin E, serum uric acid, albumin, selenium and zinc in AIDS patients compared to HIV positive patients and controls were reported. In addition, elevated markers of lipid peroxidation such as malondialehyde/ thiobarbituric acid reactive substances and conjugated dienes have been reported in previous studies (Allard *et al.,* 1998; Pasupathi *et al.,* 2009; Suresh *et al.,* 2009). Decreased levels of serum vitamins and minerals have been linked to a higher risk of disease progression and mortality in HIV infection (Pace & Leaf, 1995; Hazenberg *et al.,* 2003,). Supplementation with micronutrients was shown to delay HIV progression and reduce mortality in HIV-infected individuals not receiving HAART (Jaruga, *et al.,* 2002; Drain *et al.,* 2007). Therefore, maintenance of antioxidant defences in HIV infections may be important as it prolongs the survival of the patient by delaying the accumulation of oxidative stress-induced tissue damage.

The role of immune activation and inflammation, as stronger indicators of HIV pathogenesis than CD4 count and viral load, has become an important subject of HIV research (Giorgi *et al.,* 1993; Fahey *et al.,* 1998; Liu *et al.,* 1998; Brenchley *et al.,* 2004; Cassol *et al.,* 2010). The role played by LPS translocation and other microbial products was a key finding in understanding that other factors besides the viral load, may be contributing to ongoing to immune activation (Brenchley *et al.,* 2006). The translocation of microbial products from the gastrointestinal tract (GIT) into the systemic circulation as a result of breakdown of the gut mucosal integrity (“leaky gut” phenomenon) in the acute phase of the infection induces the activation of innate immune cells such as neutrophils, monocytes and dendritic cells. This is likely to result in increased oxidative stress, depletion of antioxidant defence mechanisms and increased susceptibility to apoptosis (Brenchley *et al.,* 2006; Cassol *et al.,* 2010). Therefore, “the leaky gut phenomenon” is regarded as an important source of on-going immune stimulation and consequently immune activation persists as a characteristic of HIV infection (Hazenberg *et al.,* 2003; Cassol, 2010). Immune activation is associated with the depletion of CD4+ T-cells and increased risk of disease progression (Pace & Leaf, 1995; Hazenberg *et al.,* 2003). This occurs during the early phases of HIV infection, and more specifically, stage II of HIV infection has been associated with higher free radical production (Bundres *et al.,* 1993; Favier *et al.,* 1994; Jarstrand & Akerlund, 1994; Pugliese *et al.,* 2005). Some viral proteins (such as vpr) are capable of binding to mitochondria

of infected CD4+ T-cells, directly inducing apoptosis (Jacotot *et al.,* 2000). Figure 0.1 below summarizes the link between HIV, immune activation, inflammation, oxidative stress and CD4+ T-cell depletion. Allard *et al.* showed increased oxidative stress in HIV-positive patients when compared to seronegative control subjects and decreased plasma concentrations of various antioxidant vitamins and selenium (Allard *et al.,* 1998). Previous studies have reported clinical improvement in AIDS patients who willingly consumed high doses (500 mg, 800 mg and 1 800 mg) of ascorbic acid (Allard *et al.,* 1998; Fawzi *et al.,* 2002; Kaiser *et al.,* 2006) and the potential of vitamin C to inhibit apoptosis, albeit in erythrocytes has been described (Cathcart, 1984; Mahmud *et al.,* 2010). Previous studies involving LPS-induced activation of cells have utilized varying concentrations of LPS (100 ng/ml - 5 µg/ml), N-acetyl cysteine (NAC) (5 - 50 µM), and vitamin C (25 - 50 nM) therefore the current study required considerable optimization of conditions.(Dobmeyer *et al.,* 1996; Shang *et al.,* 2003; Yamanda *et al.,* 2006).

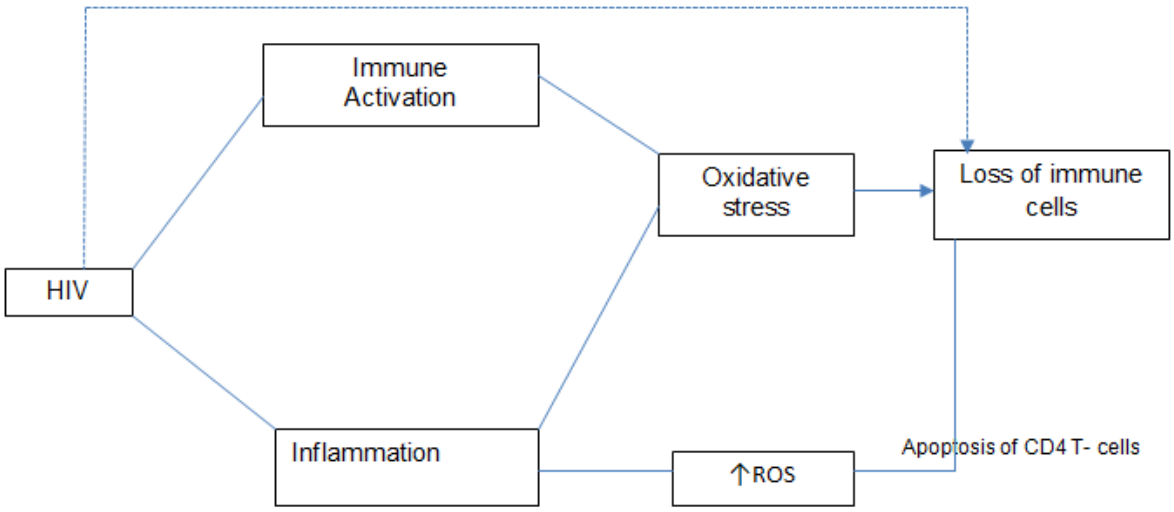

Figure 0:1: Proposed link between HIV immune activation and CD4+ T-cells depletion

1.2 Statement of the problem

Various studies have confirmed that HIV-infected patients are under chronic oxidative stress and that oxidative stress plays a key role in HIV pathogenesis (Pace & Leaf, 1995; Jaruga *et al.,* 2002; Gil *et al.,* 2003; Wanchu *et al.,* 2009; Morris *et al.,* 2012). More importantly, all these studies on oxidative stress and HIV have consistently implicated changes in the antioxidant defence system and increased ROS production during infection. They have also concluded that micronutrients and dietary antioxidants, could offer a cost-effective supplementary therapy to

HIV patients, which may prolong and improve their survival rate (Wanchu *et al.,* 2009; Morris *et al.,* 2013). However, despite the numerous studies done on HIV and oxidative stress, several gaps remain in the understanding of the link between oxidative stress and immune deficiency. Since oxidative stress is implicated in inflammation, HIV replication and apoptosis of immune cells and therefore the progression of HIV to AIDS (Dobmeyer *et al.,* 1997; Kotler, 1998; Papasuthi *et al.,* 2009), reducing the impact of oxidative stress in immune cells may present an additional strategy for slowing down HIV progression and thereby increasing the survival rate of infected persons. Hence, it becomes necessary to find ways of ameliorating oxidative stress during early HIV infection before the oxidative damage and excessive immune depletion of immune cells starts in order to delay the progression to AIDS. The impact of direct stimulation of the innate immune system on CD4+ T-cell death has not been well documented in the literature to date. Studies on oxidative stress levels in asymptomatic untreated HIIV infection in conjunction with effects of LPS-induced stimulation on CD4+ T-cell activation and death; and potential of antioxidants to ameliorate these effects, are still scarce and limited especially in the South African context. ART remains the best treatment option for HIV/AIDS. While not advocating antioxidant therapy in general; but rather specific, scientifically determined combinations with validated *in vitro* effects on the immune system, which might offer a supplementary therapeutic strategy that aims to minimize immune activation, inflammation and oxidative stress responses in HIV infection.

1.3 Aims and objectives

The general objective of this study was to investigate the impact of inflammation-induced oxidative stress on the integrity of CD4+ T-cells in asymptomatic untreated HIV infection and potential ameliorating interventions. This was based on two hypotheses; 1) that a dysfunctional enzymatic antioxidant defense system (SOD, CAT, GPx) will lead to an overall increase in H_2O_2 levels which has been implicated in apoptosis of cells from HIV-infected individuals as H_2O_2 is membrane soluble and can easily enter the cells and cause apoptosis (Dobmeyer, *et al.,* 1996) and 2) that antioxidant intervention ameliorates HIV inflammation-induced oxidative stress and delays apoptosis of the cells from HIV-infected individuals, thereby promoting their survival.

Results from the current study will make an important contribution to the current knowledge in the field of HIV and oxidative stress, by addressing one of the underlying mechanisms by which dietary antioxidants (in combination) can modulate oxidative stress in specific immune cells (CD4+ T-cells) from HIV-infected individuals. Through data from this study, possible management strategies can be devised for HIV patients.

Ethics approval was obtained from both the clinical site: University of Cape Town: **REC: REF: 417/2006** and University of Stellenbosch (laboratory site): **N07/09/197**. This sub-study was added as an addendum to the already approved original project, **N07/09/197.** The current study was divided into four phases, each addressing different aspects as mentioned below.

1.3.1 A preliminary study of the neutrophil respiratory burst in asymptomatic untreated HIV individuals as an *in vitro* indication of response to immune stimulation

HIV is characterized by persistent inflammation and immune activation resulting in chronic oxidative stress with over-utilization of endogenous antioxidant defences. The aim of this preliminary study was to determine the capacity of innate immune cells to produce a respiratory burst, which may impact on the integrity of adaptive immune cells such as the CD4+ T-cells in HIV infection. Specifically, the neutrophil respiratory burst response in asymptomatic untreated HIV-infected individuals was determined and compared with uninfected controls as an *in vitro* indication of response to immune stimulation. In this preliminary study, the neutrophil respiratory burst response of 14 HIV-infected and 12 uninfected controls as well as TAS, measured as oxygen radical absorbance capacity (ORAC), were determined. Results of the phagoburst test were correlated with CD4 count, viral load and other markers of immune system activation and inflammation in these individuals and control group.

1.3.2 Baseline antioxidant status and oxidative stress in asymptomatic untreated HIV infection

Scientific evidence suggests that HIV-infected patients are under chronic oxidative stress (Pace & Leaf, 1995) and that a weakened antioxidant defence system is associated with the increased oxidative stress found in HIV infection (Dobmeyer *et al.,* 1998; Pasupathi *et al.,* 2009; Suresh *et al.,* 2009). In this phase of the study, the aim was to determine the baseline antioxidant status and oxidative stress status in untreated asymptomatic HIV-infected individuals and its relationship (if any) to markers of disease and inflammation. Therefore, the total antioxidant status, lipid peroxidation markers, activity of antioxidant enzymes and the glutathione (GSH) redox status, were determined and correlated with other markers of the disease. In total 20 HIV-infected asymptomatic untreated participants and 20 uninfected controls were used in this preliminary study.

1.3.3 Effects of temperature, time and concentration on LPS-induced whole blood activation and antioxidant intervention in asymptomatic untreated HIV infection: An optimization study

Previous studies involving LPS-induced activation of cells have utilized varying concentrations of LPS (100 ng/ml - 5 µg/ml), NAC (5 - 50 µM), and vitamin C (25 - 50 nM) and therefore it was

critical to optimize the conditions for the current study (Dobmeyer *et al.,* 1996; Shang *et al.,* 2003; Yamanda *et al.,* 2006). The LPS stimulation and Annexin V/7-AAD [apoptosis] assays were optimized before undertaking the current study. The aims of this phase of the study were to explore and optimize the *in vitro* effects of time, temperature and concentration on LPS-induced immune activation and apoptosis in asymptomatic, untreated HIV-infected individuals. The effect of varying doses of selected antioxidants (vitamin C & NAC) on LPS-induced activation was also explored. For the optimization phase, blood samples from 20 HIV-infected individuals and 20 uninfected controls were utilized.

1.3.4 LPS-induced activation, oxidative stress and modulation with antioxidants

The general objective of this phase was to study the effects of selected antioxidants such as vitamin C and a glutathione precursor (NAC) on the oxidative stress status and apoptosis of CD4+T-cells from HIV-infected individuals. Specific aims of the study were to first develop a flow cytometry assay for the assessment of levels of CD4+ T-cell activation in HIV-infected individuals and secondly, to measure immune activation before and after stimulation with LPS and incubation with the selected antioxidants. The ability of selected antioxidants to modulate LPS-induced immune activation was also investigated in this phase. A total 20 HIV-infected asymptomatic untreated participants and 20 uninfected controls were analysed in this phase of the study.

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CHAPTER 2

Literature review

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Literature review

2.1 Introduction

UNAIDS estimates that there were 34.5 million people living with human immunodeficiency virus (HIV) at the end of 2011, the majority of whom (about 23.5 million or about 70% of total HIV burden) were from sub-Saharan Africa (UNAIDS, 2012). As the pandemic continues to devastate these resource-poor countries, sub-Saharan Africa bears two thirds of the total global HIV infections. Over 7500 people become infected with HIV daily and over 5600 people die daily from the disease globally, according to the 2009 joint United Nations programme on HIV/AIDS (UNAIDS, 2009). In 2009 alone there were 1.3 million AIDS-related deaths among adults and children in sub-Saharan-Africa (UNAIDS, 2010). To date, the discovery of a cure for the HIV pandemic remains elusive and the factors precipitating the progression of HIV infection to acquired immunodeficiency syndrome (AIDS) still remains unclear (Appay & Sauce, 2008). In addition to reviewing and analyzing the previous studies on oxidative stress and its role in the progression and pathogenesis of the HIV infection, this review discusses the interrelationship between inflammation, immune activation, oxidative stress and apoptosis and its impact on CD4+ T-cells in HIV infection. Potential ways to ameliorate oxidative stress using selected antioxidants, which could delay the progression and thereby increase the survival rate of infected persons have also been discussed. A brief overview of some of the immunohaematopoietic cells targeted by HIV has also been discussed. The review was conducted by searching PUBMED, MEDLINE, SCOPUS; GOOGLE SCHOLAR, SCIENCEDIRECT databases using the following search words; HIV, inflammation, oxidative stress, antioxidant defense systems, immune activation and apoptosis of CD4+ T-lymphocytes in asymptomatic HIV infection. The search was not limited by date, treatment or stage of disease, but only to HIV disease.

2.2 HIV disease: Current and future perspectives

The HIV infection is perhaps the most widely studied disease since its discovery in 1983 (Papadopulos-Eleopulos, *et al.,* 1992; Appay & Sauce, 2009). Successful vaccines and treatments for this small virus comprising of 9 genes, continues to elude scientists for decades without a remedy in sight in the near future. How this virus is capable of rapid mutation and evasion of the host's immune system still remains a mystery to scientists. However, a great body of information regarding HIV pathogenesis, such as depletion of both infected and "innocent bystander" CD4+ T-cells through apoptosis, has been gathered over the last two decades (Appay & Sauce, 2008; Hunt, 2010). An interesting phenomenon in HIV pathogenesis has been

the role of chronic immune activation and inflammation in disease progression (Brenchley *et al.,* 2004).

The introduction of HAART regimens in the mid-1990s has resulted in a 50% decline in AIDS related deaths (UNAIDS, 2010), although, this is mostly in developed countries. In developing countries however, the access to antiretroviral therapy (ARVs) is still low due to economic reasons, therefore a large number of infected persons continue to die before they can benefit from the ARV's. The role out of ARV programmes has been slow. Although more than 6.2 million people in sub-Saharan Africa are currently receiving ARV's, more than 10 million people living with HIV and qualifying for treatment, according to the 2010 WHO guidelines, are still on a waiting list (UNAID, 2012). According to WHO, individuals with symptomatic HIV disease and those with lower CD4 count are like to benefit more for initiating ARVs. Based on increasing evidence supporting earlier initiation of ARVs, WHO in 2010 recommended commencement of ARVs for HIV-infected individuals with CD4 count of ≤350 cells/mm^3 regardless of clinical stage and for those with clinical stages 3 or 4 regardless of CD4 count (WHO, 2012). Evidence gathered from systematic review of previous observational studies and randomized control trials using grading of recommendations, assessments, development and evaluation (GRADE) evidence profiles; all indicated benefits of early ARVs initiation, such as reduced risk of progression to AIDS and/or death, TB, development of a non-AIDS-defining illness and increased likelihood of immune recovery. Consequently, WHO currently recommends initiation of ARVs to all individuals with confirmed HIV infection with a CD4 count of ≤500 cell/mm^3, prioritizing those with advanced clinical stages (3 or 4) or a CD4 count of ≤350 cells/mm3 (WHO, 2013). This may be difficult to implement in resource-poor countries, since significant numbers of patients with CD4 count of less than 200 cells/mm^3 have not yet had access to ARV's.

The depletion of CD4+ T-lymphocytes is a characteristic of HIV infection (Brenchley *et al.,* 2004; Brenchley *et al.,* 2006; Appay & Sauce, 2009), which is complemented by the HIV's ability to mutate rapidly, thereby capable of evading its host's immune system and persistently causing serious damage (Hahn *et al.,* 1986; Hunt, 2010). Another major characteristic of HIV infection, is the intense generation of pro-inflammatory cytokines (Morris *et al.,* 2010) with a sustained loss of immune competent and experienced cells, resulting in aberrations in the host's immune integrity (Haynes, 2006; Pasupathi *et al.,* 2009). Scientific evidence suggests that this is strongly associated with increased oxidative stress at cellular levels within the lymphatic and systemic tissues (Pace & Leaf, 1995; Dobmeyer, 1998; Pasupathi *et al.,* 2009). More importantly, recent studies in developed countries have indicated that ongoing immune activation and inflammation, despite adequate control of viral load, increases the risk of thrombotic and inflammatory-

associated complications such as myocardial infarctions and certain cancers (Nakhla & Rumble, 2010). As a result, the role of immune activation and inflammation as stronger indicators of HIV pathogenesis rather than CD4 count and viral load has become a common subject of study (Fahey, *et al.,* 1998; Carbone *et al.,* 2000; Brenchley *et al.,* 2004).

2.2.1 WHO clinical and immunological staging of HIV infection

In its effort to assist in clinical management and to maximize ARV therapy, World Health Organization (WHO) is continuously revising its clinical and immunological classification of HIV guidelines (WHO, 2007; WHO, 2009). The clinical staging of HIV is used only when HIV infection has been confirmed, which enables the assessment at baseline (first diagnosis of HIV infection) or for long term HIV care and in the follow up of patients in care and treatment programmes (Mellors *et al.,* 1997; Bonnet *et al.,* 2005; WHO, 2007). Clinical staging is also useful in decision making, such as when to commence HIV interventions (WHO, 2007). More importantly, clinical stages of HIV have been shown to correlate with survival, prognosis and progression of clinical disease without ARVs in adults and children (Malamba *et al.,* 1999; Dilys *et al.,* 2002; Dunn, 2003). WHO uses clinical (HIV-associated symptoms) and immunological classification to categorize HIV infection. Using HIV-associated symptoms, WHO classifies HIV infection into four clinical stages, depending on severity of symptoms; 1) clinical stage 1, the asymptomatic stage, in which there is no symptoms or there can be a persistent generalized lymphadenopathy 2) Clinical stage 2 in which there are mild symptoms, such as moderate unexplained weight loss, recurrent respiratory infection, Herpes Zoster, recurrent oral ulceration and popular pruritic eruptions among other symptoms; 3 clinical stage 3, which is an advanced-symptoms stage, with unexplained severe weight loss, unexplained chronic diarrhea (>1 month), unexplained persistent fever (>1 month), persistent oral candidiasis, pulmonary tuberculosis, unexplained anaemia, neutropenia etc. and 4) clinical stage 4, characterized by severe symptoms such as the HIV wasting syndrome, *Pneumocystis* pneumonia, extra pulmonary tuberculosis, Kaposi sarcoma, lymphomas, invasive cervical carcinoma among others.

Since the pathogenesis of HIV infection is associated with depletion of CD4+ T-cells (Brenchley *et al.,* 2006; Appay & Sauce, 2009), using this information, WHO has provided guidelines for categorizing HIV infection using immunological classification (WHO, 2007). Based on this and the normal CD4 count which is 500 - 1500 cells/mm^3 of blood, WHO and CDC have classified HIV infection into four stages depending on immune deficiencies as shown in Table 2.1. As CD4 count may vary within an individual, assessing the CD4 count over time is necessary. The response to therapy will also depend on the immune stage at which it is started. Better outcomes

and response to ART at higher CD4 counts (CD4>200 – 350) have been reported (Bennett *et al.,* 2002; Grabar *et al.,* 2005; WHO, 2007).

Table 2:1: WHO immunological classification of established HIV infection (WHO, 2007)

	Age-related CD4 values			
HIV associated immunodeficiency	<11 Months (%CD4+)	12-35 months (%CD4+)	36-59 months (%CD4+)	>5 years (absolute number per mm^3 or CD4+)
None or not significant	>35	>30	>25	>500
Mild	30-35	25-30	20-25	350-499
Advanced	25-29	20-24	15-19	200-349
Severe	<25	<20	<15	<200 or <15%

2.2.2 Current ARV treatment regimens

According to WHO and UNAIDS, at least 15 million people needed ARV treatment by 2011 and over 8 million had access to ART in low and middle income countries (UNAIDS, 2012; WHO, 2012). WHO in 2010 recommended the commencement of ARV therapy for adults at CD4 count of ≤350 cells/mm^3. However, recent studies in addition to improved understanding of the chronic inflammation caused by HIV, all point to clinical benefits of starting ART at CD4 count above 350 cells/mm^3. Accordingly, the new revised WHO guidelines on use of ARVs for treating and preventing HIV infection, recommends beginning of treatment for all individuals with confirmed HIV infection with a CD4 count of ≤500 cells/mm^3 (WHO, 2013). Unfortunately, changing the threshold from 350 to 500 cells/mm^3 would further increase the pool of people eligible for ART to 25 million, despite most of low and middle income countries yet to achieve a "universal access" to ART for the majority of their eligible people (WHO, 2012).

2.2.3 Regimen composition

The current ARV regimens recommended for HIV treatment include two nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) as the backbone and either a non-nucleoside reverse transcriptase inhibitors (NNRTIs) or a protease inhibitor (PIs) as the base (WHO, 2013). The main reason for combining different anti-HIV drugs in the current treatment regimens of HIV/AIDS is to increase synergy, reduce toxicity and prevent development of drug resistance (De Clercq, 2009; Gil *et al.,* 2013; UNAIDS, 2011). Currently there are more than 20 ARV medications available, classified into six major classes (CDC report, 2009; De Clercq, 2009; UNAIDS, 2011);

- Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) such as Abacavir, Stavudine, Tenoforvir etc.
- Non-Nucleoside reverse transcriptase inhibitors (NNRTIs) that includes Nevirapine Efavirenz etc.
- Protease inhibitors (PIs) such as Amprenavir, Atazanavir, Darunavir etc.

- Fusion inhibitors such as Enfuvirtide
- Integrase inhibitors such as Elvitegravir
- CCR5 or CXCR4 antagonists such as Maraviroc

Fixed dose combinations are also available such as Zidovudine + Lamivudione or Zidovudine + Lamivudine + Abacavir etc. (Lubinski *et al.,* 2009). However, due to availability of new medications, formulations and dosage recommendations, ARV treatment is continuously being updated (De Clercq, 2009). Although adverse effects have been reported with use of all ARVs, the use of newer regimens has reduced these effects. Numerous factors such as gender, concomitant use of medications, comorbidities, drug-drug interactions and genetic factors influence the development of adverse effects. Women have a higher susceptibility to develop Steven-Johnson syndrome, rashes and hepatotoxicity from NNRTIs such as Nevirapine. However, all said and done, the overall benefits of ARVs outweigh the risks (El-Sadr *et al.,* 2006; Lichtenstein *et al.,* 2008).

2.3 HIV, immunobiology and pathogenesis

2.3.1 The HIV

The HIV belongs to the genus Lentivirus, a group of enveloped ribonucleic acid (RNA) viruses under the family Retroviridae. This group of viruses produces typically slow, progressive infections and their replication depends on the occurrence of an active reverse transcriptase, which transforms the viral RNA genome into a proviral deoxyribonucleic acid (DNA) copy that integrates into the host cell chromosome. The proviral DNA is finally transcribed into messenger RNA (mRNAs) that encodes the viral proteins and progeny genomic RNA (Gao *et al.,* 1999).

The HIV is sub-divided into two groups; HIV-1, which is more virulent and a less virulent, less transmissible, HIV-2. HIV-1 is further sub-divided into three sub-groups; M (“major”), O (“outlier”) and N (“non M/non O”). Group M, which has evolved in humans to form at least 10 genetic subtypes (designated by letters A to K), is responsible for the majority of global AIDS pandemic (Tatt *et al.,* 2001). The majority of infections in sub-Saharan Africa, including Southern and Eastern Africa, are of sub type C. In Central Africa, sub-types A and D predominate (McCutchan, 2001). In geographical areas, where more than one HIV-1 genetically distinct subtypes exist, recombination of subtypes currently referred to as circulating recombinant forms (CRFs) have been identified (McCutchan, 2000; Filho *et al.,* 2006). This usually occurs during replication in order to generate diversity. Several CRFs such as CRF 12_BF in South America, CRF-06 in West Africa and CRF16 in Kenya, just to mention a few are now recognized as important sub-types. Most of the CRFs have been discovered in sub-Saharan Africa, which also bears the greatest genetic diversity of HIV (Dowling *et al.,* 2002; Montavon *et al.,* 2002).

A mature virion is made up of a cone-shaped protein capsid enclosing two strands of genomic RNA, replication enzymes and cellular proteins as shown below in Fig. 2.1, (Goldstein, 1996). A lipid envelop, which comprises of the viral glycoproteins and cellular proteins, encloses the capsid. The envelop glycoprotein consists of two sub-units, external gp120, which binds to the CD4 receptor and CCR-5 or CXCR5 co-receptors on the surface of target cells and gp41, trans membrane subunit, anchoring the spikes in the viral envelope to maintain their trimeric organization and help in fusion of the virus and the host cell membrane (Brelot & Alizon, 2001).

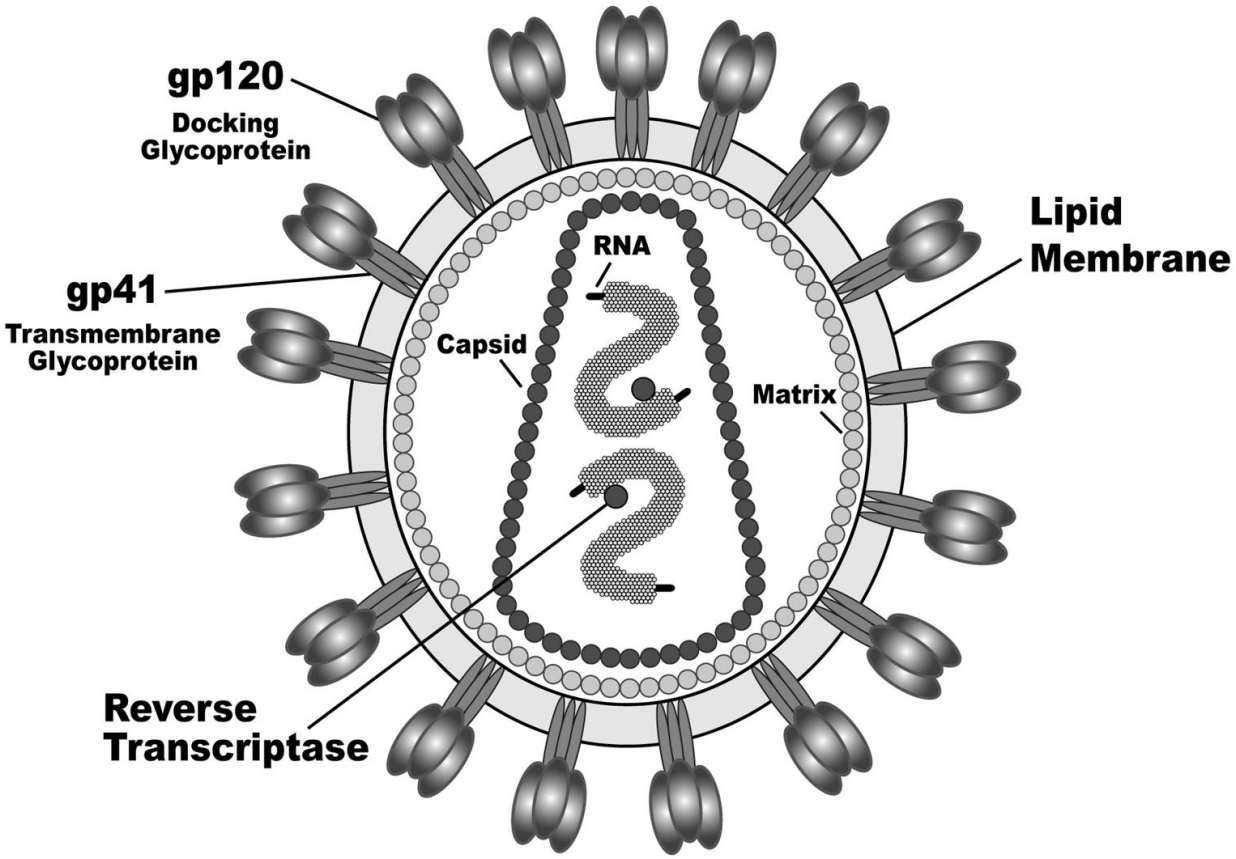


Figure 2:1: A mature virion showing capsid with genomic RNA, lipid 'envelop', with viral glycoproteins gp120 and gp41. (Source: http://upload.wikimedia.org/wikipedia/commons/2/2b/HIV_Virion-en-2.png).

2.3.2 HIV entry into the cells

Entry of HIV into the host cell is via CD4-gp120 interaction and gp120-chemokine receptor (CCR5 and CXCR4) interactions (Dimitrov, 1997; Brelot & Alison, 2001). The gp120 binds to CD4 and a co-receptor, either CCR5 or CXCR4, which are present on the surface of susceptible cells such as T-lymphocytes or macrophages. Immunohaematopoietic cells

expressing these receptors and co-receptors of HIV are targets for infection by the virus and are therefore discussed briefly below. Consequent upon the binding of the virus with CD4 and gp120, gp41 undergoes a conformational change that facilitates fusion of the viral membrane and the target cell membrane (Chan & Kim, 1998). The virus loses its protein capsid coat and is transcribed and integrated into the hosts DNA by reverse transcriptase and integrase respectively. Further transcription and translation produces new viral particles, which are assembled at the cell membrane to make a new virion through a budding process. In addition, cell to cell infection or the release of viral particles during budding propagates the virus to other cells (Sierra *et al.,* 2005).

2.3.3 Immunohaematopoietic cells infected by HIV

HIV requires its target cells to express two molecules i.e. CD4, which is the primary receptor for HIV and a second co-receptor either CCR5 or CXCR4 in order to infect the cell (Appay & Sauce, 2009). A number of cell populations occurring in various mucous membranes, such as the genital tract, express the required receptors for HIV infection (Hunt, 2010). These include CD4+ T-lymphocytes, Langerhans cells, dendritic cells and macrophages (Siliciano & Siliciano, 2000; Hunt, 2010).

Most of the infected cells die quickly by cytopathic effects of the virus, apoptosis or by hosts' antiviral immune response, while a fraction of them represents a stable pool of long-lived memory T-cells. These memory T-cells carry the proviral DNA, but do not transcribe the viral genes and thus evades the hosts HIV specific cellular effectors killing (Ho *et al.,* 1995: Hunt, 2010).

2.3.3.1 CD4+ T helper cells

The hallmark of HIV infection is the massive depletion of CD4+ T-helper cells and the subsequent inability to fight opportunistic infections (Appay & Sauce, 2009). CD4+ T-lymphocytes express CD4 receptors and both CCR5 and CXCR4 co-receptors making them perfect targets for HIV (Hunt, 2010). Thus, it is not surprising that they are the major type of cells infected by the virus. Furthermore, HIV directly depletes these cells through; its cytopathic effects, killing by activated CD8+ cytotoxic cells, or indirectly through apoptosis, eventually causing immunosuppression, the hall mark of HIV/AIDS (Gandhi *et al.,* 1998; Cummins & Badley, 2010). Interestingly, infected CD4+ T-helper cells become targets of CD8+ T-cells, but can also die by other mechanisms. During acute phase of HIV infection, CD4+ T-helper cells mostly in the GIT mucosal are depleted. However, during chronic phase they generally proliferate and die due to the persistent immune activation and other reasons (Brenchley *et al.,* 2004; Haynes, 2006). After activation by specific HIV antigens, the CD4+ T-helper cells either die or become non-proliferating memory cells, which are rapidly activated after re-encounter with a similar antigen (Hunt, 2010).

2.3.3.2 CD 8+ T lymphocytes

These cells express low levels of CD4 antigens when activated and are only infected by HIV in small numbers during the later stages of the disease. Naïve CD8+ T-cells do not express CD4 antigen, therefore do not become infected by HIV, although they express the co-receptors CCR5 and CXC4 (Hunt, 2010).

2.3.3.3 Macrophages/monocytes

Macrophages/monocytes express CD4 antigens, although in low amounts, and CCR5 co-receptor and are, therefore infected by HIV in the later stages of the disease (Kedzierska *et al.,* 2003; Alfano & Poli, 2005). Although only a few of them are infected by HIV, monocytes activation contributes significantly to pathogenesis of HIV disease by inducing ROS production (Elbim *et al.,* 1999; Bailey *et al.,* 2006; Brown *et al.,* 2008). They are also capable of phagocytosing opsonized virus particles by anti-HIV antibodies via Fc or complement receptors e.g. C5b (Dimitrov, 1997; Hunt, 2010). In addition, macrophages provide an important reservoir for the virus within the hosts' body. They bind HIV gp120 proteins via syndecan and CD91 consequently becoming infectious to other cells. Non-proliferating mature macrophages can sustain HIV production for long periods without being killed by the virus (Hunt, 2010).

Activated macrophages lead to aberrant production of pro-inflammatory cytokines in HIV infection, leading to a vicious cycle of secondary effects like wasting, inflammation and oxidative stress (Brown *et al.,* 2008). Macrophages are also involved in the removal of CD4+ T-helper cells expressing phosphatidylserine (PS) on their surface, which is one of the specific signals for recognition and removal of apoptotic cells (Fadok *et al.,* 1992; Verhoven *et al.,* 1995).

2.3.3.4 Natural killer cells

Natural killer cells express CD4 antigens and interact with antigen presenting cells like dendritic cells. They also express the co-receptor CCR5 and thus are infected by CCR5 specific HIV strains (Hunt, 2010).

2.3.3.5 Dendritic cells

Dendritic cells are antigen-processing cells (APCs) that recognize antigens and present them to T-cells. Although they are not promptly infected by HIV, these cells express low levels of HIV receptors CD4 antigen and co-receptors CCR5 and CXCR4, which make them candidates for infection (Alfano & Poli, 2005; Hunt, 2010). The dendritic cells trap HIV on their surfaces through a surface lectin, called dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) at the site of infection, migrate to lymph nodes and transmit the trapped virus to CD4+T-cells (Bailey *et al.,* 2006; Gil *et al.,* 2013). This

ensures that no fusion of the cell membrane with the virus occurs and hence dendritic cells cannot get infected via this route (Israel & Israel, 2002; Hunt, 2010). However, they bind the virus when it enters through the mucosal route and migrates to the lymph nodes where they present it to the T-cells, infecting them in the process (Bailey *et al.,* 2006; Hunt, 2010; Gil *et al.,* 2013).

2.3.3.6 Cells of the nervous system

HIV infects oligodendrocytes, astrocytes, neurones, glial cells and brain macrophages which express low levels of CD4 antigens (Del la Monte *et al.,* 1987; Ranki *et al.,* 1995; Kruman *et al.,* 1998; Clements *et al.,* 2002). Infection of these cells causes disease of the central nervous system through the viral protein *Tat*, which binds to neural cells through CD91 antigen (Westendorp *et al.,* 1995; Jaworowski *et al.,* 1999 Hunt, 2010). Although, all the cells discussed previously are infected by HIV, however, untreated asymptomatic HIV infection is characterized by massive depletion of CD4+T-cells.

2.3.4 HIV mediated depletion of CD4+ T-cells

The HIV is unique and different from other viruses in that it targets CD4+ T-cell pool and to a small extent macrophages and dendritic cells, which are key players in the immunity of the host (Kedzierska *et al.,* 2003; Alfano & Poli, 2005; Hunt, 2010). Acute HIV infection is characterized by a temporary decrease in peripheral blood CD4+ T-cell count (Figure 2.2). Since the majority of CD4+ T-cells reside in the mucosal-associated lymphoid tissues (MALT), particularly GIT, the peripheral blood count is not representative of the total body CD4+ T-cell count. The CCR5 expressing mucosal CD4+ T-cells consist mostly of memory CD4+ T-cells, hence are the primary targets for the HIV (Brenchley *et al.,* 2004; Harnes, 2006; Hunt, 2010).

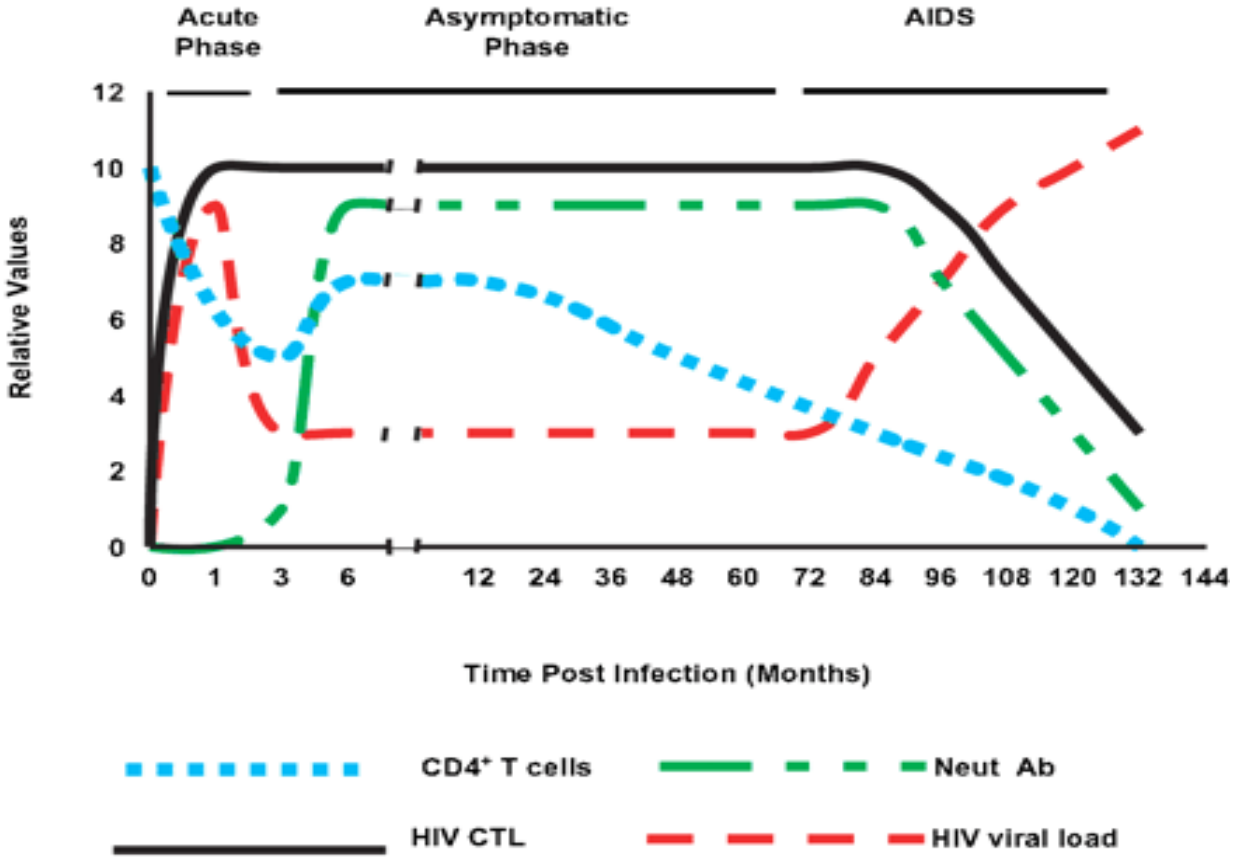


Figure 2:2: The phase of HIV infection showing course of HIV infection in terms of the levels of CD4+ T cells, HIV viral load, neutralizing antibodies, and cytotoxic T lymphocyte levels in different phases of HIV infection (Source: Appay & Sauce, 2009).

Studies done on primates, infected with Simian equivalent of HIV (SIV) and on humans, infected with HIV, indicated that most of the depletion of the CD4+T-cells occurs in the mucosal tissues during acute phase of the infection either as direct targets of the cytopathic effects of the virus or indirectly by apoptosis (Veazey *et al.,* 2003; Brenchley *et al.,* 2004; Haynes, 2006). Chronic HIV infection is characterized by a gradual decline of peripheral blood CD4+ T-cell counts and gradual increase in viral load as shown in Figure 2.2. Consequently, the low circulating CD4 count correlates well with the onset of AIDS, as minimum levels of circulating CD4+ T-cells are important for maintaining immune integrity (Pantaleo, 1993).

The number of HIV-infected circulating CD4+ T-cells in peripheral blood (1 in 10 000 in acute phase and 1 in 40 in chronic phase) is too low to account for their depletion (Douek *et al.,* 2002; Lassen *et al.,* 2004; Duoek, 2007). Therefore, the loss of uninfected CD4+ T-cells (bystander cells) suggests other mechanisms through which these cells are depleted. Several theories explaining the cause of CD4+ T-cell depletion in HIV have been proposed.

These theories include direct effect of virion synthesis in the cell and budding from the cells, thus puncturing the membranes initiating their destruction (Carbonari *et al.,* 1995; Dandekar, 2007; Cummins & Badley, 2010; Hunt, 2010). This theory falls short, as the cells need to be infected and it has been confirmed that only a few of the cells are infected. The second theory proposed is the fusion of the HIV with the cell membrane. This fusion is thought to result in the formation of syncytia that can lead to the spread of the virus to other uninfected cells, though this is not common (Hunt, 2010).

It is also proposed that infected cells expressing viral proteins in association with MHC I molecules on their surface, might become targets of cytotoxic T-cells destruction (Gil *et al.,* 2003 Hunt, 2010). However, this phenomenon does not explain the death of the "innocent bystander" CD4+ T-cells. Another hypothesis about the depletion is that viral proteins such as gp120, *Nef* and *Tat* shed from the infected cells or from the virus particles, may bind to the uninfected cells through CD4 antigens, thus appearing as infected cells and are thereby "cleared" by the immune system (Westendorp *et al.,* 1995; Schindler *et al.,* 2006; Appay & Sauce, 2008; Hunt, 2010). Some viral proteins (such as vpr) are capable of binding to mitochondria of infected CD4+ T-cells, directly inducing apoptosis (Jacotot *et al.,* 2000). It has also been suggested that HIV-related cytotoxic antibodies are produced in infected individuals that may target specific antigens on the surface of activated uninfected CD4+ T-cells (Hunt, 2010). Autoimmune component targeting gp120 via anti-gp120, which is capable of binding to CD4+ T- cells, has also been proposed (Betts *et al.,* 2001; Alimonti *et al.,* 2003). HIV proteins have also been shown to alter the T-cell function (Hunt, 2010).

Though not exclusive, the above theories try to elucidate why, despite only a few of CD4+ T-cells being infected, massive depletion of these cells occurs as the disease progresses. The prospects of HIV-induced apoptosis of CD4+ and CD8+ T-cells as the main cause of the depletion of these cells has become a common theme of many studies (Gougeon, & Gougen & Montagnier, 1993; Dobmeyer *et al.,* 1998; Hunt, 2010). Consequently, apoptosis of the cells initiated via various pathways is thought to be a major contributing factor in the depletion of CD4+ T-cells and the eventual progression to AIDS (Cummins & Badley, 2010).

Immune activation, inflammation and inflammation-induced oxidative stress is thought to contribute significantly to the depletion of peripheral CD4+ T-cells in HIV infection and have become a popular research subjects (Dobmeyer *et al.,* 1998; Brenchley *et al.,* 2004; Marques *et al.,* 2009; Plaeger *et al.,* 2012).

2.3.5 Inflammation and Immune activation in HIV

Chronic inflammation and immune activation also characterises HIV infection (Haynes, 2006; Villinger & Ansari, 2010), which results in weakened immune responses, increased pro-inflammatory cytokines production and uncontrolled viral replication in the activated CD4+ T-

cells (Cassol *et al.,* 2010; Morris *et al.,* 2012). The significant depletion of memory CD4+ T-cells lining the GIT mucosa in early HIV infection results in loss of mucosal integrity and ongoing translocation of microbial products such as LPS across the epithelial surface (Brenchley *et al.,* 2004). Consequently, LPS induces the activation of innate immune cells such as neutrophils, monocytes and dendritic cells resulting in increased oxidative stress, depletion of antioxidant defence mechanisms and an increased susceptibility to apoptosis (Bailey *et al.,* 2006; Gendron *et al.,* 2011).

As a consequence, neutrophil respiratory burst contributes significantly to oxidative stress, inflammatory and immune activation states in living systems (Fuchs *et al.,* 2007; Rada & Leto, 2008; Gonzalez-Dosal *et al.,* 2011), which is thought to contribute to the depletion of CD4+T-cells in HIV infection (Aquaro *et al.,* 2008). Although the process of recruitment and activation of neutrophils and macrophages at the site of infection is tightly regulated, if in excess or prolonged, it can lead to inflammation and damage to the surrounding tissues and cells (Crocker *et al.,* 2012; Drescher & Bai, 2013). As previously mentioned, depletion of CD4+T-cells is characteristic of HIV infection and recent studies have linked the loss with chronic immune activation and inflammation (Brenchley *et al.,* 2004; Haynes, 2006; Desail & Lindayl, 2010).

As a common routine, the pathogenesis of HIV infection is linked to CD4+ T-cells and the immune status of an HIV-infected individual is assessed by measuring the absolute number per mm^3 or percentage CD4+ T-cells as the golden standard of immune deficiency (MacDonnell *et al.,* 1998; Vlahov *et al.,* 1998; Vajpayee *et al.,* 2005; WHO, 2007). However, peripheral blood CD4 count is not representative of the total body CD4+ T-cell count as discussed in section 2.3.4, the role of immune activation and inflammation as stronger indicators of HIV pathogenesis has become more prominent (Fahey *et al,* 1998; Brenchley *et al.,* 2004). Inflammation is an important non-specific protective response of a tissue to harmful stimuli e.g. a pathogen, damaged cells or irritants (Prescott *et al.,* 2002). Activated neutrophils and macrophages during inflammation produce H_2O_2 and other oxidants (Kotler, 1998). This occurs during respiratory burst due to increased oxygen use and ATP production, which further promotes tissue injury and inflammation (Prescott *et al.,* 2002). Importantly, inflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), are known to stimulate HIV transcription and replication and thus may be important factors in the progression of HIV to AIDS (Aquaro *et al.,* 2008). Elevated markers of immune activation, inflammation and apoptosis such as CD 38/8, CD 95, pro-inflammatory cytokines e.g. TNF α, IL-1β, IL-6 in both plasma and lymph nodes, as well as chemokine such as macrophage inflammatory protein 1 (MIP-1 α, MIP 1β and RANTES) have been reported in HIV-infected patients (Giorgi *et al.,* 1999; Gil *et al.,* 2003; Deeks *et al.,* 2004). Giorgi *et al.* reported that T-cell activation levels as expressed by the activation

marker CD38 on CD8+ T-cells, predicted a poor prognosis for the infected patients which have been confirmed by other investigators (Giogi *et al.,* 1999; Hazenberg *et al.,* 2003; Deeks *et al.,* 2004). More specifically, HIV gene products, such as *Tat*, *Nef*, *Vpr, Raf* and *Env* have been shown to induce immune activation of macrophages and lymphocytes directly, however they cannot solely account for the complete immune activation (Kedzierska *et al.,* 2003; Brown *et al.,* 2008; Hunt, 2010). In addition, HIV is capable of causing immune activation and inflammation indirectly through antigenic stimulation (Hunt, 2010).

Brenchley *et al.* recently proposed another potential mechanism of immune activation that involves the innate immune system (Brenchley *et al.,* 2006). The study suggested that the massive depletion of CD4+ T-cells by HIV in the MALT lining the gut, compromises the integrity of the mucosal barrier which usually prevents translocation of the intestinal tract flora, restricting them to the lamina propia and the mesenteric lymph nodes. Consequently, the loss of integrity of the mucosal lining results in microbial translocation from the gut to the systemic immune system. As a result, the translocation of microbial products such as LPS leads to an intense activation of the innate immune response. LPS, flagellin and CpG DNA, which are toll-like receptor (TLR) ligands, are capable of stimulating macrophages and dendritic cells to produce a range of pro-inflammatory cytokines (TNF-α, IL-6 & IL-1β) and ROS. As a result, increased oxidative stress, depletion of antioxidant defence mechanisms and increased susceptibility of the cells to apoptosis, may occur (Merrill *et al.,* 1989: Cheung *et al.,* 2008; Morris *et al.,* 2012). The ultimate effect of this phenomenon is activation of "innocent bystander cells", differentiation of lymphocytes, monocytes and an establishment of a pro-inflammatory condition which characterizes HIV infection. Hence, immune activation has been shown to be a stronger predictor of disease progression to AIDS than CD4 count and viral load (Carbone *et al.,* 2000; Giorgi *et al.,* 1993; Fahey *et al.,* 1998; Liu *et al.,* 1998; Brenchley *et al.,* 2004).

HIV infection has been shown to affect levels of antioxidant enzymes such as glutathione peroxidase (Stephensen, 2007). In addition, HIV-*tat* gene has been shown to induce H_2O_2 signaling, resulting in enhanced T-cell activation and death signal expression (Campbell, 2005). Therefore, it is likely that both the depletion of endogenous antioxidants such as glutathione and the increased production of H_2O_2 as a result of chronic inflammatory processes, contribute to the deaths of CD4+ T-lymphocytes in HIV infection (Gil *et al.,* 2003; Wanchu *et al.,* 2009; Morris *et al.,* 2012).

2.3.6 The natural course of HIV infection

Although, the clinical manifestation of full blown AIDS from the time of HIV infection takes about 8-10 years (Hunt 2010), this may not always be the case, as sometimes it can be less than two years depending on factors such as the hosts' immune system and dietary factors

(Lederman & Valdez, 2000; Hunt, 2010). The acute phase of the infection lasts for 6-12 weeks (Figure 2.3) before anti-HIV antibodies are detectable in the blood. Initially, the virus produces a mild disease which is self-limiting or asymptomatic.

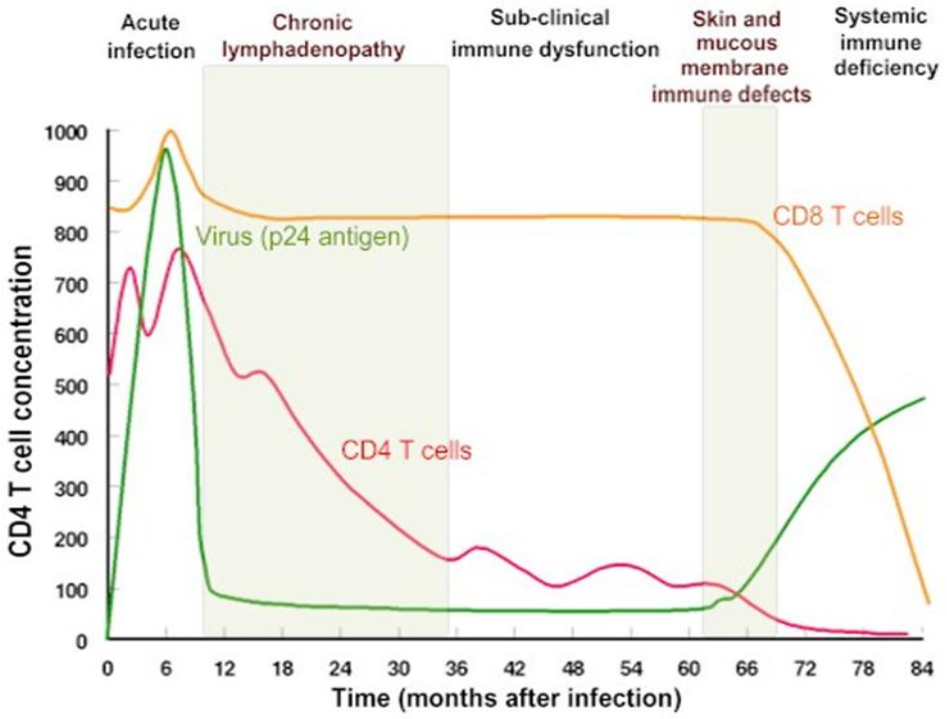


Figure 2:3: The CD4 count and viral load in HIV infection, showing the natural course of CD4 count viral load CD8 in different phases of HIV infection (source: Hunt, 2010).

Furthermore, during early infection, an initial fall in CD4+ T-cells and a rise in CD8+ T-cells occur, which quickly return to normal as seen in figure 2.4 below. The virus titer levels at this time are very high (figure 2.4). The first two weeks of infection are marked by a massive loss of CD4+ T-cells of the MALT, resulting from immune activation due to translocation of microbes from the damaged gut mucosa and local immunodeficiency (Lifson *et al.,* 1997; Brenchley *et al.,* 2004; Hunt, 2010).

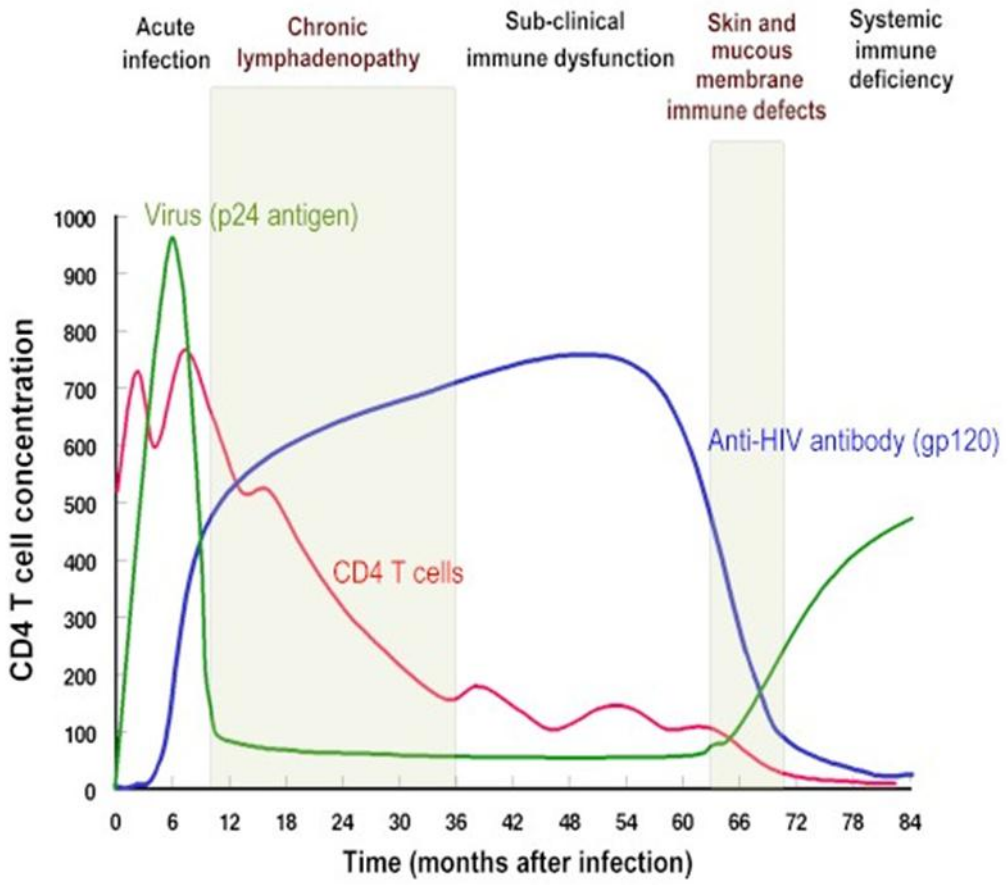


Figure 2:4: The natural course of HIV infection in terms of the CD4 count, HIV antibodies, and viral load (source: Hunt, 2010).

As a result, the cytotoxic B and T-lymphocytes intensify their defense and the virus disappears from the peripheral circulation as seen in figure 2.3. In addition, the cell-mediated immune response will also be accompanied by increased humoral antibodies (anti-HIV antibodies (figure 2.2 & 2.4). More specifically, high levels of virus are produced daily (10 billion particles) but are cleared rapidly by the immune system (Hunt, 2010). Although, the infected cells producing the virus are destroyed by the immune system or by the virus itself, however, a small fraction survives as resting memory cells and make up a reservoir that is crucial in drug-based therapy (Martinez-Maza *et al.,* 1987; Giorgi *et al.,* 1999; Hunt, 2010; Zeng *et al.,* 2011). Immune activation is also associated with up regulation of CCR5, which along with CD4 receptor allows HIV entry into more cells enhancing the virus replication and infection (El-Far *et al.,* 2008)

The strong immune response reduces the viral particles in the blood stream, although the particles persist elsewhere and continue to replicate and infect more cells. The patient enters a latent phase as seen in figure 2.3. This period vary from as little as 1 year to more than 15 years (Pantaleo, 1993; Appay & sauce, 2009). Activated CD4+ T-cells, which are susceptible to apoptosis, are continuously and permanently depleted from the beginning of the infection,

unless ARV treatment commences. Ultimately, the virus cannot be controlled, as the CD4+ T-cells becomes depleted and falls below 200 cell/mm^3, the virus titers rise, while immune response falls dramatically (Fig. 2.3).

The loss of immune competence driven by immune activation, inflammation, oxidative stress apoptosis-induced depletion of CD4+T-cells, marks the onset of opportunistic infections and HIV-associated cancers characterizing AIDS (Lederman *et al.,* 1993; Pace & Leaf, 1995; Allard *et al.,* 1998; Hazenberg *et al.,* 2003, Hunt, 2010; Tien *et al.,* 2010). The specific roles of some of the common pro-inflammatory cytokines in the inflammatory process are discussed below.

2.3.7 Pro-inflammatory cytokines, Inflammation, oxidative stress and apoptosis

The persistent systemic immune activation that characterizes HIV infection, results in enhanced pro-inflammatory cytokine production such as TNF-α, which have been implicated in the activation of extrinsic pathway of apoptosis (Ashkenazi & Dixit, 1999; Haynes, 2006). Pro-inflammatory cytokines refers to a group of inducible cytokines with properties that promote inflammatory reaction. These include; IL-1, IL-2, IL-6, TNF family (CD40 ligand, Fas ligand, CD27 ligand, CD30 ligand etc.) and interferon's (IFN's) (Ware *et al.,* 1998). Although these cytokines stimulate increased production of ROS, thereby inducing oxidative stress, they assist in antigen presentation by dendritic cells in addition to promoting phagocytosis (Ware *et al.,* 1998; Luiz, 2005). The pro-inflammatory cytokines drive the many processes underlying the inflammatory response, cell apoptosis, activation and differentiation (Tracey *et al.,* 1993; Luiz, 2005). In the LPS-induced activation of innate immunity, the LPS bind to CD14 on macrophages or monocyte surface. This binding is enhanced by a plasma LPS-binding protein (LPB). The LPS/CD14 complex formed activates toll like proteins 2 (TLR2) and 4 (TLR4) which consequently activates the nuclear factor of Kappa B (NF-kB) resulting in production of pro-inflammatory cytokines and ROS (Ware *et al.,* 1998; Liu, 2005).

More specifically, TNF which is the primary mediator of immune regulation plays a crucial role in various cellular processes including cell proliferation, differentiation, necrosis and apoptosis. TNF exerts cytotoxic effects on a wide range of tumour cells and other target cells (Liu, 2005). As a result, TNF is involved in both intracellular inflammatory signalling pathways and apoptotic signalling through its death domain of the p55 tumour necrosis factor receptor (TNFR). The IL-1 and TNF up-regulate the E-selectin, ICAM-1, and V-CAM adhesion molecules on vascular endothelial cells. Furthermore, they also stimulate production of chemokines such as IL-8 and MCP-1, which helps to attract and trap neutrophils to the inflammatory sites (Ware *et al.,* 1998). These two cytokines (IL-1 & TNF) also produce small mediators, such as cyclooxygenase 2 which induces production of prostaglandin E2 or prostacyclin from endothelium, causing vasodilation and feeling of pain during inflammation

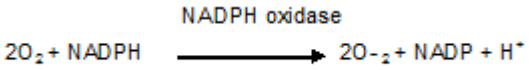
(Israels & Israels, 2002). In concert with IFN-γ, they induce production of nitric oxide, a vasodilator from NO synthase. They also cause expression of tissue factor and platelet activating factor, which boosts the clotting mechanisms. IL-6, a product of IL-1 and TNF-stimulated cells, causes production of acute phase proteins and also aids in promoting fever during inflammatory process. Many of the TNF super families e.g. Fas, TNFR and death receptor 3 are known to induce apoptosis in HIV infection (Lichtner *et al.,* 2004; Herbein & Khan, 2008). The TNF-TNFR complex is known to enhance the NADPH oxidase activity that increases oxidative stress leading to necrotic cell death (Feldmann & Saklatvala, 2000). Due to its cytotoxic effects, the biosynthesis of TNF is tightly controlled and therefore produced in very small quantities in quiescent cells, but secreted in large amounts in activated cells (Liu, 2005).

Elevated markers of TNF, IL-1 & 6, in plasma and lymph nodes have been observed in HIV infection from the early stages of the disease (Molina *et al.,* 1989; Weiss, 1989; Emilie, 1990; Lafeuillade, 1991; Wanchu, *et al.,* 2009; Morris *et al.,* 2012). Importantly, in HIV infection, activated macrophages are a major source of cytokines such as TNFα, IL-1, IL-6, IL-12, IL-15, IL-18 and chemokines such as IL-8, MIP-1, MIP-1, MCP-1, interferons, etc. (Alfano & Poli, 2005; Brown *et al.,* 2008). An association between elevated pro-inflammatory markers sTNFR-1, sCD27, sCD40L and CD8 T-cell activation, with disease progression in patients whose CD4+ T-cell counts were less than 200 cells/cm^3 and who had suppressed viral replication but who either died, developed an AIDS-defining malignancy or other complications has been described previously (Kalayjian *et al.,* 2010). As a result, these pro-inflammatory markers may highlight important cellular pathways in pathogenesis of HIV independent of on-going viral replication which could be targeted for immune-based therapies in the future (Kalayjian *et al.,* 2010). As previously mentioned, macrophages/monocytes and neutrophils are major targets and source of the pro-inflammatory cytokines and ROS (Ware *et al.,* 1998; Liu, 2005; Lam *et al.,* 2010), and hence neutrophil respiratory burst, which is a major source of oxidative stress is an example of pathways that could be ideal targets in the future.

2.3.8 Neutrophil respiratory burst

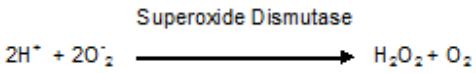
Activated neutrophils have been shown to produce large amounts of superoxide anion approximated to be 1 – 4 M/L in the vacuoles (Reeves *et al.,* 2002) therefore, identifying perturbations in cell redox triplets composed of mitochondrial potential, respiratory burst and intracellular thiol levels may help in understanding oxidative stress-related disease processes including HIV infection (Matteucci & Giampietro, 2008; Matteucci *et al.,* 2009). Polymorph nuclear neutrophils (PMNs) are a critical component of first line defense against pathogens (Salmen *et al.,* 2005; Crocker *et al.,* 2012; Drescher & Bai, 2013) and their enrolment and

activation at the location of infection or inflammation in order to eliminate pathogens is a key aspect of innate immunity (Greenberg & Grinstein, 2002; Choi & Dumler, 2003;). PMNs continuously traverse blood vessels scrutinizing for bacterial, viral infection and inflammation (Rada & Leto, 2008; Gonzalez-Dosal *et al.,* 2011) and when they encounter an infection they leave circulation, migrate to the site of infection where they phagocytose and kill the invading microbe by releasing packaged microbial systems (Prescot, 2002; Fuchs *et al.,* 2007; Papayannopoulus & Zychlinsky, 2009). At the site of infection, neutrophils kill the invading pathogens by a combination of two mechanisms; oxygen-independent and oxygen-dependent response or respiratory burst (Prescott *et al.,* 2002; Nathan, 2006; Fuchs *et al.,* 2007). The oxygen independent mechanism is through highly toxic cationic proteins and enzymes e.g. MPO, defensins, lactoferrin, lysozyme, gelatinase, etc. contained in azurophilic, gelatinase and specific granules (Engelich *et al.,* 2002; Segal, 2005). The oxygen-dependent mechanism or the respiratory or oxidative burst involves a sequential reduction of oxygen by an NADPH oxidase leading to production of toxic oxygen metabolites such as H_2O_2, hydroxyl radicals and singlet oxygen according to equation 2.1 - 2.7 (Parkin & Cohen, 2001; Pada & Leto, 2008).



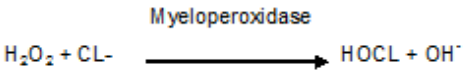
Equation 2.1

H_2O_2 is generated from the dismutation reaction of the superoxide radical catalysed by superoxide dismutase (Israels & Israels, 2002).



Equation 2.2

When the generated H_2O_2 reacts with a halide, hypochlorus acid is produced. This reaction is catalysed by myeloperoxidase enzyme. The hypochlorus acid is used to kill the ingested bacteria in the phagosome.



Equation 2.3

Catalase enzyme and reduced glutathione (GSH) protects the neutrophils from oxidative damage by these oxidants (Hunt *et al.,* 1998, Israels & Israels, 2002).

The NADPH oxidase system is inactive, but can be activated by chemokines, immune complexes, (activated complement 5) C5a, or N-formyl oligopeptides shed by microbes (Taylor *et al.,* 1993; Cruse & Lewis, 2010). The NADPH oxidase system is separated into two compartments in a resting cell: the membrane and the cytosol. The oxidase system is a complex put together from five proteins, two on the plasma membrane ($p22^{phox}$ and $gp91^{phox}$) and three in the cytoplasm ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$), but each group associated with monomeric GTP-binding (G) protein (Israel & Israel, 2002, Segal, 2005). When neutrophils are activated and the cytosolic protein ($p47^{phox}$) is phosphorylated, cytoplasmic protein complex are translocated to the membrane. The gathering of the cytoplasmic proteins with the membrane proteins activates the oxidase enzyme (NADPH oxidase) (Segal, 2005). The NADPH oxidase transports electrons across the plasma membrane from the cytosolic NADPH to oxygen to generate the superoxide anion radical ($O^{\cdot-}_2$) both in the phagosome and extracellular space (Israels & Israels, 2002; Fuscs *et al.,* 2007). H_2O_2 is generated from the dismutation reaction of the superoxide radical catalyzed by superoxide dismutase (Halliwell, 1994). When the generated H_2O_2 reacts with a halide, hypochlorus acid is produced, which is used to kill the ingested bacteria in the phagosome.

A rapid flow cytometric screening test for abnormalities of neutrophil function (Van Eeden *et al.,* 1999), whole blood PMNs oxidative burst (Alvarez-Larran *et al.,* 2005) and apoptosis in physiological and pathological situations has been described previously (Emmendorffer *et al.,* 1990; Bitzinger *et al.,* 2008; Brescia & Sarti, 2008). In addition, a burst test assay for PMNs oxidative burst which utilises unlabeled opsonized bacteria (*E. coli*), phorbol 12-myristate 13-acetate (PMA) and the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) as stimulants, dihydrorhodamine (DHR) 123 as a fluorogenic substrate and necessary reagents has been reported (Rothe *et al.,* 1988). The assay determines the percentage of phagocytic cells which produce ROS (conversion of DHR 123 to R 123) and their enzymatic activity. Based on the fact that, when stimulated, phagocytes produce ROS to destroy bacteria inside the phagosome during the oxidative burst, which can be monitored by the addition and oxidation of DHR 123 to R 123 and can be measured using flow cytometry.

Although, studies on neutrophil respiratory burst levels in HIV have been scarce and limited however, the few that exists have shown that pro-inflammatory cytokines such as TNF, GM-CSF and IL-8 strongly prime a sub-population of PMNs to generate H_2O_2 in response to fMLP stimulation (Elbim & Lizard, 2009). This results suggests that the priming effects of cytokines such as TNF is required for triggering the PMN oxidative burst and that it pre-activates resting PMNs, enabling them to produce a heightened and more powerful response

at the site of infection (Elbim *et al.,* 1999; Wittmann *et al.,* 2004). Additional evidence of pre-activation, priming and increased respiratory burst comes from a study by Bandres *et al.* who showed an enhanced respiratory burst and phagocytosis by neutrophils and monocytes of men with stage 1 HIV infection (Bandres *et al.,* 1998). In a study investigating priming by TNF, GM-CSF & IL-8, Elbim & Gougerot-Pocidalo, showed strong priming of polymorphonuclear neutrophils (PMN) by these cytokines in HIV (Elbin & Gougerot-Pocidalo, 1996). In this study, there was normal respiratory burst in both HIV positive and negative controls patients, however, there was enhanced oxidative burst of resting and fMLP stimulated neutrophils in HIV positive patients than in negative controls suggesting a preactivation or priming by the inflammatory cytokines (TNF, IL-1, 8). Dobmeyer *et al.* in another study showed a significant decrease in respiratory burst response of asymptomatic HIV infection patients in contrast to those with AIDS which correlated well with CD4 count (Dobmeyer *et al.,* 1995).

These studies and others discussed in later sections of this chapter, confirms the significant contribution of neutrophil respiratory burst to the overall oxidative stress indicated in HIV infection.

2.4 Oxidative stress

Zielch *et al.* have defined oxidative stress as “cellular state where ROS production exceeds the cells ability to metabolize them resulting in excessive accumulation of ROS that overwhelms cellular defenses” (Zielch *et al.,* 2011) whereas, Franco & Panayiotidis defined it as “the cytopathological consequence of an imbalance between free radical production and the ability of the cell to defend against them” (Franco & Panayiotidis, 2009). Oxidative stress may result from excessive production of ROS and the failure of the antioxidant defense system. Importantly, the human body produces ROS and reactive nitrogen species (RNS), constitutively through normal physiological and metabolic processes e.g. aerobic respiration. ROS are defined as oxygen-containing, reactive chemical species, while RNS refers to nitrogen containing species e.g. nitric oxide (NO), nitric dioxide (NO_2) and peroxynitrite. Furthermore, ROS have been categorised into two groups; the free radicals which contain one or more unpaired electron in their outer molecular orbitals e.g. superoxide, nitric oxide, and hydroxyl radicals and the non-radicals, which, although lacking unpaired electrons, they are chemically reactive and can be converted to free radical species e.g. (H_2O_2), ozone, peroxynitrate and hydroxide (Trachootham *et al.,* 2009). Mitochondria are a major source of ROS in the cells through electron leakage from the mitochondrial respiratory chain. The leaking electrons react with molecular oxygen to form superoxide and other ROS (Andreyev *et al.,* 2005). Other major endogenous sources of ROS include;

i. phagocytic cells and some cancer cells, which in an oxidative burst, produces ROS through an NADPH oxidase complexes catalysed reaction intentionally to destroy cells infected with bacteria or virus infected cells as discussed previously.

ii. β-oxidation of fatty acid reactions in the peroximes is a major source of ROS.

iii. Prostaglandin synthesis and detoxification reaction by cytochrome P-450 mixed function oxidase system (Kehrer & Smith, 1994; Abe & Berk, 1998).

It is a well-known fact that ROS plays a vital role in biological functions, as regulators of many signal transduction pathways, signalling cell growth and differentiation and regulation of certain enzymes (e.g. ribonucleotide reductase) (Trachootham *et al.,* 2009). In addition, they mediate inflammation by stimulating cytokine production. More importantly, ROS are also involved in elimination of pathogens and foreign particles, particularly in oxygen-dependent microbial killing of neutrophil respiratory burst (Israels & Israels, 2002). However, ROS being highly reactive molecules are capable of causing oxidative damage to biomolecules such as DNA, proteins and lipids, consequently altering their functions (Boonstra & Post, 2004; Devasagayama *et al.,* 2004). This may result in various pathological conditions and diseases (Prior & Cao, 1999). Excessive increase in ROS in cells may cause permanent oxidative damage which could lead to loss of immune function, inflammation and even death of the cell (Gil *et al.,* 2013).

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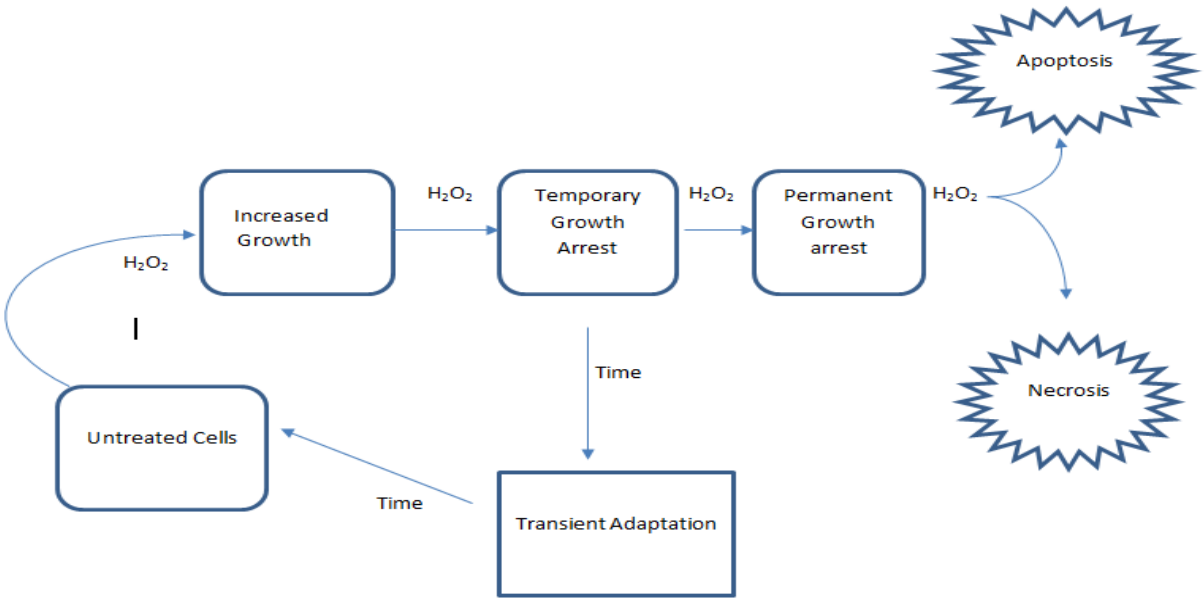


Figure 2:5: Responses to oxidative stress in proliferating cells. (Source: Davies, 2005).

2.4.1 Antioxidants defense system, oxidative stress and HIV

The human biological system constantly produce ROS as products of normal physiological processes to serve important functions such as electron transfer in mitochondrion and as signalling molecules (Devasagayam *et al.,* 2004; Valko *et al.,* 2007 Gil *et al.,* 2013). However, as mentioned previously, activated immune system such as activated phagocytes may generate excessive amounts of ROS both locally and in systemic circulation (Gil *et al.,* 2013), which might lead to oxidative modifications and damage to proteins, nucleic acids, carbohydrates and lipids (Block *et al.,* 2002; Kohen & Nyska, 2002). As a result, cells have evolved complex antioxidant defence systems to combat this (Halliwell & Cross, 1994; Evans & Halliwell, 2001; Leonarduzzi *et al.,* 2010). Antioxidants have been defined by Becker *et al.* as substances that at low concentrations prevent or retard oxidation of biomolecules such as lipids, proteins and DNA (Becker *et al.,* 2004). The antioxidant defence system consists of primary or preventive antioxidants, which limit the initial formation of oxygen-centred radicals of organic compounds. Secondary scavenging or chain breaking antioxidants are present to trap intermediate ROS and thus breaking the chain reaction. The third line of defence consists of repair systems for damaged nucleic acids, proteins and lipids (Halliwell, 1994; Masella *et al.,* 2005; Halliwell, 2006 Ratnam *et al.,* 2006).

More specifically, the first primary defence mechanism consists of antioxidant enzymes such as CAT, GPx, GR and SOD. Catalase is a ubiquitous antioxidant enzyme found in peroximes in most tissues, which catalyse the two stage change of H_2O_2 to water and oxygen. It is so efficient as an enzyme that it cannot be saturated by H_2O_2 at any concentration. Most important, catalase protects the cells from H_2O_2 generated within them therefore, playing a vital role in acquiring tolerance to oxidative stress in the adaptive response of cells (Hunt *et al.,* 1998). Catalase has a peroxidatic activity in which low molecular weight alcohols can serve as electron donors. Catalase is found in high levels in human liver, kidneys and erythrocytes, where it accounts for the bulk of its H_2O_2 decomposition, therefore essential in the protection of cells against severe oxidative stress (Yan & Harding, 1997). A significant decrease in activity of erythrocyte's catalase has been observed in AIDS patients when compared to HIV infection and healthy subjects (Pasupathi *et al.,* 2009)

GPx and GR catalyse oxidation of glutathione at the cost of H_2O_2 in the cytosol and mitochondria (Equation 2.4).

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

Equation 2.4

GPx is a selenium containing peroxidase, which catalyse the reduction of various hydroperoxides at the expense of glutathione, thus protecting mammalian cells from

oxidative damage. At least five isoenzymes of GPx have been identified in mammals (De Haan *et al.,* 1998; Mates & Sanchez-Jimenez, 1999), which occur in the cytosol and mitochondria (e.g. cGPx or GPx1 which reduces fatty acid hydroperoxides and H_2O_2 at the expense of glutathione in mitochondria). GPx1 and phospholipid hydroperoxide peroxidase occurs in most tissues, while GPx4 occurs both in the cytosol and membranes. GPx1 occurs predominantly in erythrocytes, kidneys and liver. GPx4 occurs in high quantities in renal epithelial cells and testes with cytosolic GPx2 and extracellular GPx3, only detectable in gastrointestinal tract and kidneys. GPx contains one selenocystein residue, which is important for its enzymatic activity. The glutathione redox cycle, protects against low levels oxidative stress, while catalase is more significant in protecting against severe oxidative stress (De Haan *et al.,* 1998). GPx and GR catalyse oxidation of glutathione at the cost of H_2O_2 in the cytosol and mitochondria and thus protects the cell from oxidative damage. A reduction of erythrocyte GPx activity has been observed in HIV-infected individuals than in health individuals (Gil *et al.,* 2003; Pasupathi *et al.,* 2009).

SOD is a metalloenzyme that forms a fundamental component of the cellular antioxidant defense system. SOD catalyse the dismutation of superoxide anion to molecular oxygen and H_2O_2 as a vital part of cellular enzymatic antioxidant defence system as shown in equation 2.2 (Mates & Sanchez-Jimenez, 1999; Benov & Fridovich, 1998). Importantly, SOD also protects dehydratases (dihydroxy acid dehydratase, aconitase, 6-phosphogluconate and fumarase A and B) from inactivation by the superoxide radical (Benov & Fridovich, 1998). In humans, it is found in high concentration in brain, liver, kidneys, heart and erythrocytes. The amount of SOD present in the cellular and extracellular environments is important for the prevention of oxidative stress related damage.

In humans, three classes of SOD have been identified: the cytosolic copper superoxide dismutase (CU-SOD), zinc superoxide dismutase (zn-SOD), mitochondrial manganese superoxide dismutase (Mn-SOD) and extracellular superoxide dismutase (EC-SOD) which accounts for the majority of SOD in plasma, lymph and synovial fluid (Adachi & Wang, 1998). The mitochondrial Mn-SOD is a nuclear encoded primary antioxidant enzyme that functions to remove superoxide radicals in the mitochondria, where the respiratory chain serves as a major source of oxygen radicals. The expression of Mn-SOD is vital for the survival of aerobic life and cellular resistance to oxygen radical mediated toxicity. Copper and zinc-SOD, play a major role in the first line of antioxidant defense by catalyzing the dismutation of superoxide anion radicals, to form H_2O_2 and molecular oxygen. Extracellular-SOD occurs in the interstitial spaces of tissues and in extracellular fluids accounting for the majority of SOD activity of plasma, lymph and synovial fluid (Adachi & Wang, 1998). EC-SOD is not induced by its substrates as its regulation in mammalian tissues is coordinated by cytokines.

A significant decrease in the activity of erythrocyte SOD has been observed in AIDS patients when compared to HIV infection and healthy individuals (Pasupathi *et al.,* 2009; Suresh *et al.,* 2009).

As previously mentioned, the secondary defence system includes chain-breaking antioxidants, exogenous in source and derived from dietary intakes, are small molecules that prevent further interaction of free radicals with other molecules (this may generate secondary radicals). These chain-breaking antioxidants function by receiving electrons from a radical or by donating an electron to a radical to form a stable by-product (De Zwart *et al.*, 1999). These antioxidants are classified into water soluble and lipid soluble antioxidants. Lipid phase antioxidants, such as vitamin E and carotenoids, scavenge free radicals in membranes and lipoprotein particles, thus preventing lipid peroxidation. Vitamin E is a major lipid soluble antioxidant in body tissues, which provides the first line of protection against free radicals in cell membranes, by trapping peroxyl radicals (Lien *et al.,* 1999). The water soluble antioxidants such as vitamin C, uric acid, phenolic compounds and bilirubin scavenges free radicals present in the aqueous phase section of blood plasma (Evans & Halliwell, 2001). Vitamin C is an important water soluble antioxidant that is involved in the recycling of free radicals produced by oxidation of vitamin E. However, vitamin C under certain circumstances can function as pro-oxidant and therefore, damage the cells (Bayday *et al.,* 2007).

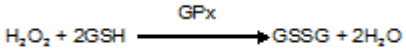
In addition to the primary and secondary antioxidant defence system, other endogenous non–enzymatic antioxidants such as reduced glutathione (GSH), bilirubin, ceruloplasmin, uric acid, nicotinamide adenine dinucleotide phosphate (NADPH), albumin, thiols, ubiquinol-10, melatonin, and metals and metal binding proteins such as, transferrin, manganese and copper exists. The mechanism of metal binding proteins (e.g. ferritin, transferrin and lactoferrin) involves capturing or seizing the iron and copper so that they are unavailable to form hydroxyl radicals, thus forming an essential constituent of the antioxidant defence system (Papas, 1999).

2.4.1.1 Glutathione redox status imbalance- reduced glutathione: oxidized glutathione ratio

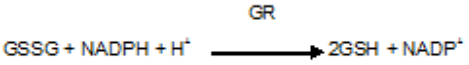
Glutathione (GSH), a free radical scavenger, is one of the major endogenous antioxidant, which plays key role in the cellular redox homeostasis and whose importance in redox balance and cell apoptosis is well established in various cell types (Circu *et al.,* 2008; Ushio, 2009). It is a tripeptide of glycine, glutamine (glutamic acid) and cysteine that is present in all eukaryotic cells, which exists in two forms; reduced GSH responsible for its antioxidant activity and GSSG which is a by-product of GSH free radical scavenging activity but lacking antioxidant activity (Zhang *et al.,* 2005; Heisterkamp *et al.,* 2008). Importantly, the cysteine residual in GSH provides an exposed sulphydryl group that is available for free radical attack,

conferring the strong antioxidant activity of this molecule. Although free radicals oxidises GSH, the oxidised form (GSSG) is recycled back to its reduced form (GSH) by GR and an electron acceptor NADPH (Masella *et al.,* 2005) (Equation 2.4).

As a consequence, the main form of GSH in the body is the reduced form, however an oxidised form (GSSG), although in much lower concentrations also exist. Glutathione reductase oxidises GSH to GSSG and then recycled back to GSH by NADPH in the GPx catalysed reduction of peroxides to water and alcohol:



Equation 2.6



Equation 2.7

Under normal conditions the ratio of GSH is about 90% to 10% GSSG in humans. A change in this ratio or decrease in GSH indicates an increase in GSSG, which in turn indicates lipid peroxidation and thus cellular oxidative stress (Sen, 1997; Schafer & Buettner, 2001).

2.4.1.2 Glutathione redox status and HIV

More specifically, glutathione being a major intercellular free radical scavenger is thought to inhibit activation of NF-kB which is involved in the transcription of HIV-1 (Walmsley*et al.,* 1997). In its inactive form NF-kB is bound to IkB in the cytoplasm, however factors like TNF-α and ROS can cause its release from IkB to the nucleus where it binds with DNA (Greenspan & Aruoma, 1994). Interestingly, intracellular GSH is known to control T-lymphocyte function. In addition, T-cells lack both the ability to synthesize cysteine, a primary component of glutathione and the ability to take up cysteine the form in which cysteine is present in circulation. As a result, intracellular glutathione levels depend on the presence and uptake of extracellular cysteine (Droge *et al.,* 1991; Droge *et al.,* 1992).

The measurement of GSH and GSSG has been used as an indicator of oxidative status and as an indicator of disease risk in humans (Pastore *et al.,* 2003; Rossi *et al.,* 2006). Recent studies have reported glutathione redox imbalance in HIV infection resulting from oxidative damage, viral replication, chronic inflammation and micronutrient deficiencies (Stephenson *et al.,* 2007; Awodele *et al.,* 2012; Morris *et al.,* 2012), which is implicated in apoptosis and

reduced immune proliferation (Morris *et al.,* 2012). In addition, deficiencies of intracellular GSH in red blood cells (RBCs) and in T-cells, natural killer cells and monocytes of HIV-infected individuals have been reported (Venketaraman *et al.,* 2006; Morris *et al,* 2012). Supporting this theory, Staal *et al.* observed significantly lower GSH levels in CD4+ and CD8+ T-cells from HIV-infected individuals than from uninfected controls, the greatest decrease occurring in the later stages of the infection (Staal *et al.,* 1992). These prior studies show that intracellular GSH levels could be a key aspect in HIV infection and progression to AIDS (Staal *et al.,* 1992). Morris *et al*. hypothesized that chronic HIV infection promotes excessive production of pro-inflammatory cytokines, leading to generation of ROS which leads to depletion of GSH as it scavenges for the free radicals. In addition, they suggested that elevated TGF-β block GCLC reducing production of GSH and that elevated levels of IL-1 lead to loss of intracellular cysteine, further inhibiting production of new GSH molecules (Morris *et al.,* 2009). In addition, *N*-acetyl cysteine (NAC), a cysteine analogue that replenishes intracellular GSH and glutathione peroxidase, antioxidants that scavenge peroxides have been shown to inhibit apoptosis (Hockenbery *et al.,* 1993). NAC acts directly as a free radical scavenger by reducing hypochlorus acid produced by neutrophils to kill target pathogens and cells. Although, this direct antioxidant activity of NAC could inhibit the natural mechanisms of neutrophils cytolysis, however, cell studies have indicated that NAC essentially boosts intracellular killing of bacteria by protecting the neutrophils and macrophages from free radical generated during phagocytosis (Oddera *et al.,* 1994).

Various studies support the hypothesis that oxidative stress is implicated in the progression of HIV disease (Dobmeyer *et al.,* 1997; Kotler, 1998; Pasupathi *et al.,* 2009). Disturbances in glutathione and antioxidant defence system in HIV-infected persons, decreased levels of other antioxidants e.g. ascorbic acid, tocopherols, carotenoids and selenium, altered activities of enzymes of the oxidant defence system and higher levels of serum markers of oxidative stress including malondialdehyde and hydroperoxides and higher oxygen consumption rates supports the conclusion that HIV-infected persons are exposed to chronic oxidative stress (Pace & Leaf, 1995; Kotler, 1998). The discovery that NAC or glutathione can protect T-cell hybridomas from activation-induced apoptosis, imply that HIV-linked CD4+T-cell activated apoptosis may be prevented by either replacement of intracellular reduced glutathione or by addition of other internal antioxidants (Sandstrom *et al.,* 1994).

The above antioxidant defence mechanisms, however, are not entirely efficient and thus repair enzymes also exist intracellularly to destroy proteins damaged by free radicals, remove oxidised fatty acids from membranes and repair free radical-damaged DNA (Halliwell, 1994; Masella *et al.,* 2005). In addition other extracellular antioxidant defence mechanisms exist, which includes; transferrin (the plasma iron transport protein), the iron binding protein lactoferrin, ceruloplasmin (transport form of copper), hemopexin and

haptoglobin (which binds free heme and heme proteins to decrease their capability to catalyse free radical damage), albumin and urate (end products of purine metabolism which scavenges several free radicals) (Morrissey & O'Brien, 1998; Halliwell, 1989).

Several studies have linked a weakened antioxidant defence system to the increased oxidative stress found in HIV infection (Dobmeyer, 1998; Pasupathi *et al.,* 2009; Suresh *et al.,* 2009). This could be as a result of increased utilization of the antioxidant defense system or reduced intakes of antioxidant micronutrients (Allard *et al.,* 1998; Pasupathi *et al.,* 2009). A depleted TAS leading to increased oxidative stress in HIV infection, has been previously reported (Mates *et al.,* 2000;Gil *et al.,* 2005; Pasupathi *et al.,* 2009). Pasupathi, *et al.* reported a markedly decreased activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase, and reduced levels of GSH, in HIV/AIDS patients as compared to healthy controls (Pasupathi *et al.,* 2009). They also observed significantly decreased levels of plasma GSH, vitamin A (β-carotene), vitamin C, vitamin E, serum uric acid, albumin, selenium and zinc in AIDS patients than in HIV positive patients and controls. Elevated markers of lipid peroxidation such as malondialdehyde/ thiobarbituric acid reactive substances (MDA/TBARS) and conjugated dienes have been reported in previous HIV studies (Allard *et al.,* 2009; Pasupathi *et al.,* 2009; Suresh *et al.,* 2009). Therefore, maintenance of the antioxidant defence system in HIV infections is critical as it prolongs the survival of the patient. This is due to the fact that accumulation of tissue damage due to oxidative stress takes long to manifest (Kotler, 1998).

2.4.2 Oxidative stress and disease development

All eukaryotic cells with the exception of red blood cells (RBC's) contain mitochondria, the principal organelle that generates ROS (Shanoun *et al.,* 1997: Roberts *et al.,* 2010), which is not only important for the normal physiological processes such as differentiation and cell signalling, but also in pathological processes (Halliwell, 2006; Kondo *et al.,* 2009; Gil *et al.,* 2013). Cells are capable of dealing with mild oxidative stress via their antioxidant defence mechanisms, however, severe oxidative stress causes damage to biomolecules, cells and tissues. Prevention of oxidative stress is therefore paramount for good health and modulation of disease (Morrissey & O'Brien, 1998; Young & Woodside, 2001). In healthy tissue, ROS and RNS generation involves a well and tightly regulated mechanism that helps to maintain redox homeostasis at cellular level (Yoshikawa *et al.,* 2000). However, excessive ROS causes oxidation of nucleic acids, chromosome breaks, and peroxidation of unsaturated fatty acids in the cell membrane (Devasagayam *et al.,* 2004), causing a loss of cell function, which might lead to apoptosis and in the process promote pathology of diseases (Kotler, 1998; Roberts *et al.,* 2010). Furthermore, extensive DNA damage induced by oxidative stress not only promotes apoptosis of the affected cell, but also results in cell necrosis, which releases

lysosomal enzymes, further producing ROS (Greenspan & Aruoma, 1994; Devasagayama *et al.,* 2004).

As previously mentioned, the body has developed a repair mechanism which includes both non-enzymatic antioxidants and enzymatic antioxidants to deal with the slight imbalance (Morrissey & O'Brien, 1998; Young & Woodside, 2001; Leonarduzzi *et al.,* 2010). The body can derive antioxidants such vitamin C, E, carotenoids, polyphenols and micronutrients such as zinc, magnesium and selenium from dietary intakes (Podsedec, 2007; Valko *et al.,* 2007). Consequently, the body also uses a type of negative feedback regulation mechanism in a process referred to as redox homeostasis which produces endogenous antioxidants to correct this imbalance (Valko *et al.,* 2007). The interrelationships between the effect of the imbalance in ROS, oxidative stress and human disease and their consequences on cell growth and differentiation and function are illustrated in figure 2.6. Several studies indicate that oxidative stress may be implicated in the progression of numerous disorders, which include HIV/AIDS, atherosclerosis, diabetes, inflammatory conditions, certain cancers and the aging process (Lipinsky, 2001; Barja, 2004; Devasagayam *et al.,* 2004; Kondo *et al.,* 2009). However, according to other studies, oxidative stress may be a secondary phenomenon and not the primary cause of the disease (Gutteridge, 1993).

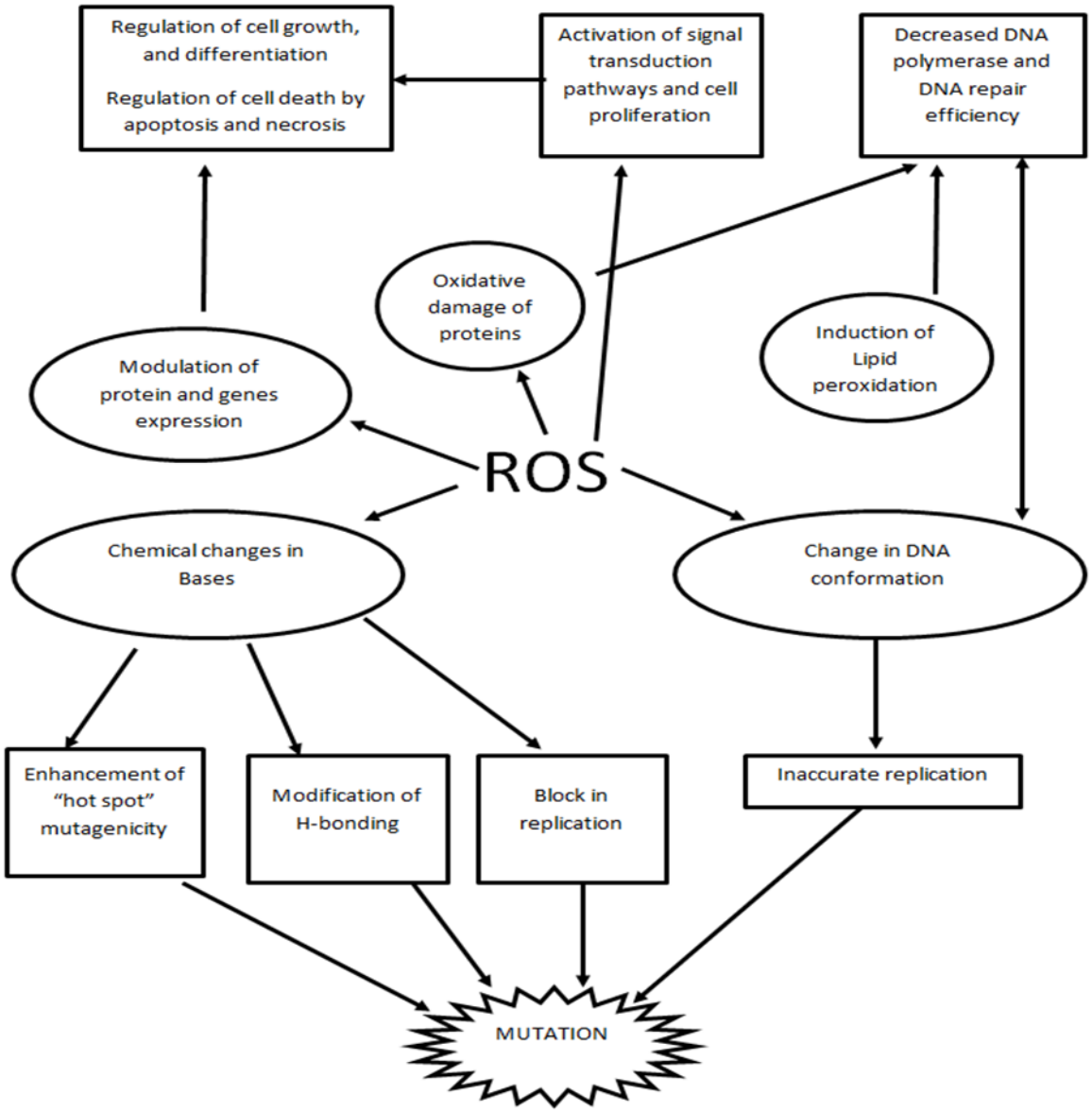


Figure 2:6: ROS induced oxidative damage. The figure shows the interrelationship between ROS, oxidative damage and human disease (Compiled by author according to Mates *et al.,* 1999.

2.4.3 Oxidative stress and HIV/AIDS

There is sufficient evidence suggesting HIV-infected patients are under chronic oxidative stress (Pace & Leaf, 1995; Allard *et al.,* 1998; Jaruga *et al.,* 2002; Gil *et al.,* 2003; Wanchu *et al.,* 2009), which might be related to increased activation of polymorphonuclear leukocytes or from pro-inflammatory cytokines produced by activated macrophages during the infection, or decreased intake of antioxidants (Peterhans *et al.,* 1987; Valko *et al.,* 2007; Coaccioli *et al.,* 2010; Morris *et al.,* 2012). Furthermore, a role of viral protein *Tat* in intracellular increases of ROS, that is thought to induce apoptosis via FAS/CD95 interactions, thereby contributing to depletion of CD4+ T-cells, has been reported (Gil *et al.,* 2003).

Several studies have reported depleted levels of antioxidants such as glutathione, ascorbic acid, tocopherols, carotenoids, selenium and antioxidant enzymes (SOD, GPx & catalase) in HIV-infected individuals (Gil *et al.,* 2003; Pasupathi *et al.,* 2009: Suresh *et al.,* 2009; Morris *et al.,* 2012). Increased levels of lipid peroxidation products such as MDA/TBARS and conjugated dienes and more importantly, increased free radical production by neutrophils, have also been observed (Reeves, *et al.,* 2002). The subsequent overload of H_2O_2, together with deficiencies in the antioxidant glutathione and antioxidant enzymes such as, CAT and GPx lead to overproduction of hydroxyl radicals and lipid peroxides, which are thought to promote apoptosis of CD4+T-lymphocytes in HIV infection (Hockenbery *et al.,* 1993; Kotler, 1998; Gil *et al.,* 2003; Reinehr *et al.,* 2008; Morris *et al.,* 2012). Dobmeyer *et al.* presented the evidence that oxidative stress plays a fundamental role in initiation of apoptosis in HIV infection (Dobmeyer *et al.,* 1996). This study reported that ROS generated under circumstances similar to those that occur during opportunistic infections, promotes apoptosis and that this effect could be ameliorated by antioxidants and/or antioxidant enzymes such as catalase, SOD, GSH, NAC and anti-TNF-α. Gougen & Montagnier theorized that the majority of the T-helper (CD4+) cell depletion occurs essentially via apoptosis and not by direct HIV infection (Gougen & Montagnier, 1993). This has been confirmed in subsequent studies in HIV-infected individuals (Dobmeyer *et al.,* 1996).

Oxidative stress is thought to significantly contribute to the reduction of CD4+ lymphocyte count in HIV-infected individuals, aiding in the progression to AIDS (Wanchu, *et al.,* 2009). Moreover, lymphocytes of AIDS patients have been shown to be deficient of glutathione, an important endogenous antioxidant, (Venketaraman *et al.,* 2006; Wanchu *et al.,* 2009; Morris *et al,* 2012), making them susceptible to oxidative stress (Willcox *et al.,* 2004). Furthermore, changes in the antioxidant defence system including depleted levels of ascorbic acid, vitamin E, superoxide dismutase and glutathione have been reported in lymphocytes including the T-cell subsets of HIV-infected persons (Staal *et al.,* 1992; Pasupathi *et al.,* 2009; Suresh *et al.,* 2009; Gil *et al.,* 2011). Suresh *et al.* noted elevated levels of serum malondialdehyde and hydroperoxides in these persons, suggesting oxidative stress during HIV infection (Suresh *et al.,* 2009). A change in the levels and in the normal activity of superoxide dismutase, catalase, and glutathione peroxidise have also been noted in HIV-infected persons (Polyakov *et al.,* 1994).

Several studies have reported a severely disturbed redox balance in untreated HIV-infected individuals (Israel & Gougerot-Pocidalo, 1997; Romero-Avira & Roche, 1998; Allard *et al.,* 1998; Gil *et al.,* 2003). In one of the studies, Gil *et al.* investigated the relationship between redox status indices of GSH, MDA, peroxidation potential, total antioxidant status, glutathione peroxidase, superoxide dismutase, total hydroperoxide (TH), DNA fragmentation and relative CD4, CD95, CD38/CD8 T-lymphocytes counts in HIV/AIDS patients in comparison with

healthy individuals (Gil *et al.,* 2003). The study indicated both a reduction of GSH levels and an increase in MDA and TH levels in the plasma of HIV-infected patients. The authors also reported that increased oxidative stress occurs in addition to persistent redox imbalance during successful HAART in a similar study (Gil *et al.,* 2011). An increase of DNA fragmentation in lymphocytes and significant differences in global indices of total antioxidant status was noted in these patients. Furthermore, reduction of GPx and an increase in SOD activity in erythrocytes of the HIV-infected patients was also noted (Gil *et al.,* 2003). Morris *et al.* in their study on causes of decreased glutathione levels in HIV infection, attributed this to increased pro-inflammatory cytokines, which increases ROS production, depleting intracellular GSH levels observed in the disease (Morris *et al.,* 2012). Wanchu *et al.* in a study on oxidative stress in HIV-infected individuals, found decreased GSH levels and increased lipid peroxidation in infected individuals when compared to uninfected healthy control individuals (Wanchu *et al.,* 2009)

In another study, Pace & Leaf reported that oxidative stress was thought to contribute to the pathogenesis of HIV disease, including viral replication, inflammatory responses, decreased immune cell proliferation, loss of immune cell function, apoptosis, chronic weight loss and increased sensitivity to drug toxicity (Pace & Leaf, 1995). Of specific significance, is the fact that HIV has only been isolated from *in vitro* cultures and that no HIV can be isolated, unless the cultures are subjected to oxidative stress. Therefore, oxidation is a prerequisite for HIV expression (Papadopulos-Eleopulus *et al.,* 1992). More importantly, ROS are known to activate NF-kB that is obligatory for HIV replication, therefore enhancing pathogenesis of the disease (Sen & Packer, 1996). It has been demonstrated that HIV replication is enhanced under oxidative conditions *in vitro* (Nabel & Baltimore, 1987; Sen & Packer, 1996). ROS are capable of directly activating (inhibitor of kappa B) IkB kinase (IKK), which phosphorylates the C-terminal ankylin repeats of the IkBα proteins bound to NF-kB at serine residuals 32 and 36 (Hayden & Ghosh, 2008). This leads to ubiquitination and degradation of the IkBα, unmasking the DNA binding sites subsequently causing translocation of the NF-kB to the nucleus, where it binds to kB sites activating transcription of genes involved in cellular processes such as production of pro-inflammatory cytokines, inflammation, thereby promoting oxidative stress (Bonizzi & Karin, 2004). *In vitro* inhibition of viral replication has been established by experiments with both water and lipid soluble antioxidants (Kalebic *et al.,* 1991; Harakeh & Jariwalla, 1991; Suzuki *et al.,* 1992). The activity of antioxidants nonetheless, differs with different cell culture systems and thus do not emerge as strong anti-HIV agents based on the high concentrations used *in vitro* (Pace & Leaf, 1995). More specifically, antioxidants such as vitamin C and NAC have been shown to inhibit NF-kB activation *in vivo* and *in vitro,* thereby ameliorating oxidative stress (Aillet *et al.,* 1994; Baeuerle *et al.,* 1994; Harakey & Jariwalla, 1997; Bellezzo *et al.,* 1997). Several

observations, including depletion of anti-apoptotic/antioxidant protein Bcl-2 in HIV-infected CD4+ T-cells, reduced apoptosis in HIV-infected cells treated with antioxidants and presence of apoptotic cytokines such as TNF-α, IL-1 & IL-6 secreted by activated macrophages in AIDS patients, all strengthen the proposition that apoptosis is involved in depletion of CD4+ T-cells in HIV/AIDS infection (Romero-Alvira & Roche, 1998).

2.4.4 The mechanism of apoptosis

Apoptosis or programmed cell death is a normal physiological process involved in tissue homeostasis, but is increased during oxidative stress (Pace & Leaf, 1995; Kotler, 1998).The physiological importance and quantitative significance of ROS-dependent receptor-mediated apoptosis in relation to the classical receptor/ligand-induced apoptotic signalling are poorly understood and requires further investigation (Circu & Aw, 2010). Apoptosis involves several biochemical proceedings culminating in cell membrane blebbing, shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. Apoptosis is regulated by various cell signals, originating either extracellularly or intracellularly and activated through two separate pathways: the extrinsic (death receptor pathway) and intrinsic (mitochondrial) pathways. The extrinsic pathway is activated by the binding of death receptors FAS/CD95, TNFR1, DR4 and DR5 with their respective ligands i.e. Fas ligand (FasL), TNF, and TNF-related apoptosis-inducing ligand (TRAIL/Apo-2) ligands as shown in figure 2.7. The binding of receptors to their ligands, lead to protein-protein interactions at cell membranes, initiating a cascade of activation of membrane-proximal or initiator caspases (caspase-8 and caspase-10), which in turn activates caspase-3 and caspase-7 (executor caspases) (Ashkenazi & Dixit, 1999). Apoptotic signalling begins with the linking of death domains, containing adaptor protein within the death domain occurring at the c-terminal domain of the receptor.

Emerging evidence indicates a possible direct role of ROS in mediating death receptor activation and apoptosis stimulation via ROS-induced receptor clustering and formation of lipid-raft-derived signaling platforms as shown in figure 2.7 (Circu & Aw, 2010). Oxidative stress-induced apoptosis mechanisms involve loss of mitochondrial transmembrane potential, release of cytochrome c to the cytoplasm, loss of Bcl-2, down-regulation and degradation of mitochondrially encoded mRNA, rRNA, and diminished transcription of the mitochondrial genome (Zamzani *et al.,* 1997). Likewise, apoptosis may be initiated by genes in response to DNA damage or endoplasmic reticulum stress, death signals (Fas ligand) received at the membrane or when proteolytic enzymes such as granzymes enters the cells directly (Israels & Israels, 2002).

The intrinsic pathway is activated by the disruption of mitochondrial membrane and subsequent release of mitochondrial proteins such as cytochrome c into the cytoplasm

(Figure 2.7), which is vital for the assembly of the caspase–activating complex or apoptosome comprising of cytochrome c, Apaf-1, dATP & procaspase. As a result, caspase-activating complex activates caspase-9, which initiates the apoptotic caspase cascade (Danial & Korsmeyer, 2004). The release of cytochrome c from the cytosol is regulated by Bcl-2 family proteins. The Bcl-2 family proteins are classified depending on their roles in apoptosis into proapoptotic (Bid and Bax) and antiapoptotic Bcl-2 & Bcl-xL (Ryter *et al.,* 2007; Circu Aw, 2010).

Apoptosis is characterized by certain morphological features which include loss of plasma membrane asymmetry. In apoptotic cells, the membrane phospholipid PS is translocated from the inner to the outer leaflet of the plasma membrane, as a result exposing the PS to the external environment. Annexin V which is a 35-36 kDa Ca2+ dependent phospholipid-binding protein has a high affinity for PS, and binds to cells with exposed PS. Annexin V, which can be conjugated to fluorochromes, therefore can serve as a sensitive probe for flow cytometry analysis of cells undergoing early apoptosis, since externalization of PS occurs in the earlier stages of apoptosis (Vermes *et al.,* 1995). Various mediators including ROS, pro-inflammatory cytokines, metals, radiation, pro-apoptotic genes etc. are known to activate various pathways of apoptosis (Circu & Aw, 2010).

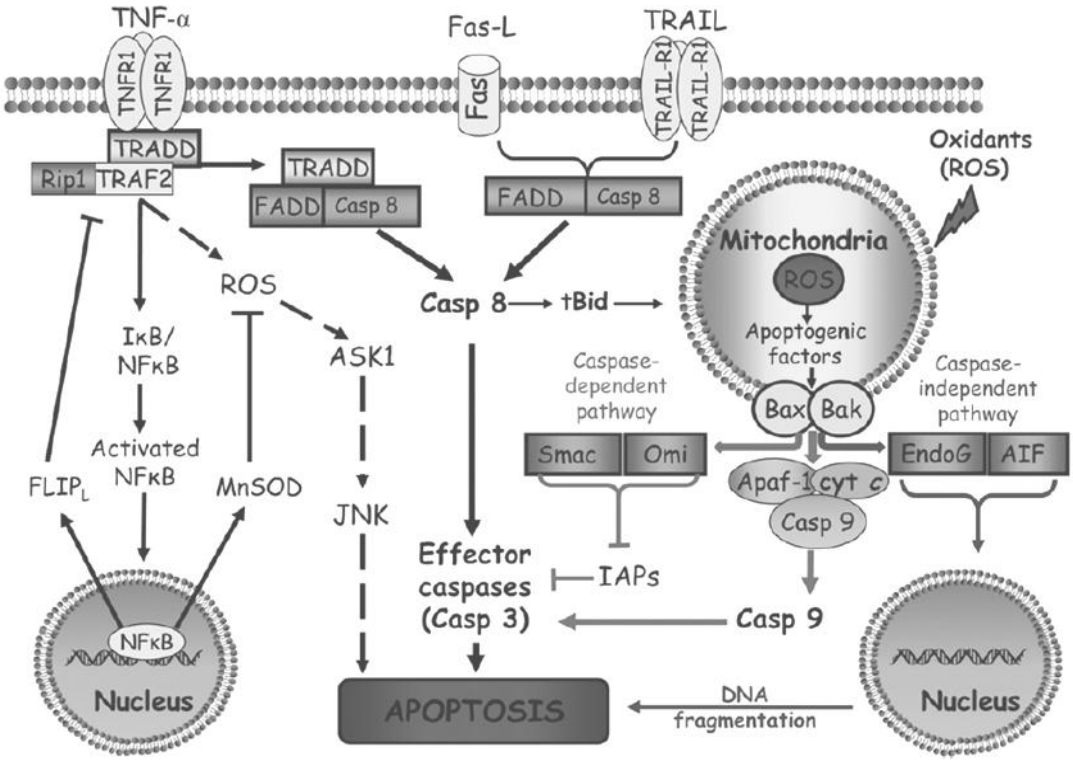


Figure 2:7: Death receptor mediated and mitochondrial pathways of apoptosis. (Source; Circu & Aw, 2010).

Depending on where they exert their effects, mediators of apoptosis, can be broadly classified into five categories; 1) cell surface mediators, 2) the cytosol mediators, 3) the

cytoskeleton mediators, 4) the mitochondrion and 5) the nucleus mediators (Kanna & Jain, 2002). Table 2.2 summarises example of mediators of apoptosis.

The cell surface mediators are the death receptors that include; Fas (CD 95), TNF-R1, CAR1, DR3, DR4 and DR5. Figure 2.7 illustrates the interactions between these death receptors with their ligands which lead to activation of various pathways ultimately leading to apoptosis. The interaction of these death receptors with their ligands may lead to initiation of a death signal depending on the presence of intracellular death domains and association with Fas-associated death domain (FADD) (Circu & Aw, 2010). Another key player in apoptosis is a class of cysteine-dependent and glutathione redox status sensitive enzymes called caspases. Importantly, they are synthesized as precursors and one of their functions is to inactivate proteins that are important for cell survival. FADD is capable of communicating with caspase-8, which triggers a cascade of caspases activation, therefore committing the cell to apoptosis. So far 13 different caspases have been identified (Israels & Israels, 2002).

Table 2:2: Mediators of apoptosis (Source: Kanna & Jain, 2000)

Inducers of oxidative stress	**Examples**
ROS	Superoxide, hydroxyl, hydrogen peroxide radicals
Metals	Iron, cadmium, mercuric chloride
Pathophysiologic conditions	Hyperglycaemia, ischemic heart disease, Alzheimer's and Parkinson's
Inducers of apoptosis involving oxidative stress	Examples
Pro-oxidants	H_2O_2, diamide, etoposide and semiquinones
Ionizing radiation	Gamma UV radiation
Protein synthesis inhibitor	Cycloheximide
Apoptotic stimuli	Fas (CD95), TNF α, ceramide, glutamate, growth factor withdrawal (IL-2, IL-3, nerve growth factor and serum starvation)
Physiologic stimuli	Glucocorticosteroids, calcium, TNF α, glutamate
Pathophysiological conditions	Serum starvation, IL-2 and IL-3 withdrawal, hyperglycaemia, ischemia and reperfusion
Pro- apoptotic genes	P53, Bax, c-myc
Organic solvents and metabolites	Benzene and their metabolites and 2,5-hexanedione
Pesticides	DDT, endosulfan, dieldrin and 2,3,7,8 tetrachrolodibenzo-p-dioxin
Drugs	Actinomycin D, cisplatin, camptothecin and staurosporine

2.4.5 Oxidative stress, immune activation and immune response

The chronic immune activation during HIV infection promotes increased ROS generation (Gil *et al.,* 2003; Awodele *et al.,* 2012), which activates nuclear transcription factors such as NF-kB leading to viral gene expression, initiating a vicious cycle of activation, oxidative stress and viral replication (Ma, 2010; Gendron *et al.,* 2011). In addition, the cells of the immune system are remarkably sensitive to oxidative stress since their plasma membranes contain high levels of polyunsaturated acyl lipids, which is vulnerable to peroxidation (Kotler, 1998;

Block *et al.,* 2002; Roberts *et al*., 2010). As a result, ROS may modify protein structures, making them susceptible to spontaneous fragmentation, proteolysis and ultimately impairing their function (Gil *et al.,* 2013). Peroxidation of the polyunsaturated acyl chain in the cell membranes leads to loss of membrane integrity and altered membrane fluidity, consequently leading to impairment of intracellular signalling and the overall cell function (Chew & Park, 2009). The subsequent oxidative modifications of DNA leads to gene mutations and deletions, which has been implicated in pathophysiology of many diseases involving mitochondrion dysfunction, aging (Devasagayama, 2004) and cellular senescence (Block *et al.,* 2002; Roberts *et al.,* 2010). Pasupathi *et al.* associated the increased production of ROS to chronic immune system activation, which depleted antioxidant defences, causing damage to cellular components as indicated by increased lipid peroxidation products, oxidized proteins and altered DNA sequences (Pasupathi *et al.,* 2009). A higher incidence of diseases associated with oxidative stress such as cancer, cardiovascular disorders, neurodegeneration and waste syndrome have been reported in HIV patients (Gil *et al.,* 2013).

Translocation of microbial products from GIT to the systemic circulation is thought to activate the innate immune system resulting in generation of pro-inflammatory cytokines and ROS, increased oxidative stress, depletion of antioxidant defences and susceptibility to apoptosis (Brenchley *et al.,* 2006; Haynes, 2006; Morris *et al.,* 2012). Interestingly, a unique feature of HIV infection is that it targets mainly the CD4+ T-cell pool and to a lesser extent macrophages and dendritic cells, which are vital immune cells (Hunt, 2010). More specifically, during the chronic phase of HIV infection, the main target of the virus is the CCR5+ CD4+ activated T-lymphocytes (Siliciano & Siliciano, 2000; Veazey *et al.,* 2000). The majority of these cells (about 80%) inhabit the lymphoid tissues such as lymph nodes in particular MALT such as GIT associated lymphoid tissue (GALT) (Haynes, 2006; Hunt, 2010). Importantly, these mucosal CD4+T-cells consist primarily of memory CD4+ T-cells expressing the HIV co-receptor CCR5, therefore are perfect targets for the HIV virus (Veazey, *et al.,* 2000; Appay *et al.,* 2008). The “leaky gut” phenomenon during early HIV infection, results in loss of mucosal integrity leading to translocation of microbial products such as LPS, flagellin and CpG DNA (toll like receptor ligands) across the epithelial surface (Brenchley *et al.,* 2006). These microbial products are known to activate innate immune cells such as neutrophils, macrophages/monocytes and dendritic cells directly to produce ROS and pro-inflammatory cytokines e.g. TNF-α, IL-6 and IL-1β. In the early stages of HIV infection, ROS e.g. H_2O_2 production is markedly increased, which is thought to contribute to the increased depletion of CD4+ T-cells via apoptosis (Pugliese *et al.,* 2005).

Considerably high plasma levels of LPS correlating directly with markers of immune activation have been demonstrated in HIV infection, indicating microbial translocation and

immune activation (Brenchley *et al.,* 2006). LPS induces activation of innate immune cells such as neutrophils, monocytes and dendritic cells via toll like receptor 4 (TLR4) resulting in increased oxidative stress, depletion of antioxidant defense mechanisms and increased susceptibility to apoptosis (Brenchley *et al.,* 2004; Haynes, 2006).

It is a well-established fact that chronic activation of CD8 and CD4+ T-cells characterises HIV-1 infection and that this consistent activation is associated with depletion of CD4+ T-cells and increased risk of faster disease progression to AIDS (Hazenberg *et al.,* 2003). Importantly, markers of immune activation are now considered more valuable predictors of disease progression to AIDS than the CD4 count and viral load (Giorgi *et al.,* 1993; Liu *et al.,* 1997; Fahey *et al.,* 1998). In addition, a complex interplay between HIV replication, immune activation, inflammation and oxidative stress exists (Le douce *et al.,* 2010) making these conditions closely intertwined and interlinked (Gendron *et al.,* 2010; Ma, 2010). Oxidative imbalance in the early phase of HIV infection, which is characterised by low serum and tissue antioxidants and antioxidant enzymes such as low GSH and SOD and high lipid peroxidation products (MDA) has been reported (Friis-Moller, 2007: Awodele *et al.,* 2011). The immune system's function is influenced by its glutathione redox status and potential. As a consequence, oxidative stress and decreased glutathione levels in lymphocytes *in vitro*, cause changes in cell function, abnormal cytokine production, and a dysfunctional proliferation response (Fidelus, 1990; Maly, 1990; Hamilos, 1992). The increased activation of polymorphonuclear leukocytes during infections or due to the effect of TNF-α produced by activated macrophages during HIV infection is closely associated with increased production of ROS and oxidative stress (Das *et al.,* 1990). Therefore, chronic oxidative stress is associated with an abnormal T-lymphocyte function in HIV-infected patients (Droge, *et al.,* 1991; Droge, 1993).

Oxidative stress leads to over expression of TNF-α which activates HIV replication in T-lymphocytes, and mononuclear cells, which leads to up regulation of interleukin-2 (IL-2) receptor, and other cytokines, resulting in decreased immune cell proliferation (Hamilos, 1992; Droge, 1993). However, various glutathione-replenishing agents have been shown to restore *in vitro* proliferative response in HIV-infected cells and in cells in which glutathione had been depleted chemically in cell cultures (Zmuda & Friedenson, 1983; Hamilos *et al.,* 1989; Eylar *et al.,* 1992; Iwata *et al.,* 1994). ROS are known to activate monocytes/macrophages potentiating them to produce increased pro-inflammatory cytokines therefore playing an important role in inflammatory process in HIV infection (Chaudri & Clark, 1989; Morris *et al.,* 2013).

2.4.6 Oxidative stress and inflammation

Inflammation has been shown to directly induce oxidative stress and endothelial dysfunction in HIV-infected individuals, which might predispose them to thrombotic complications (Baliga *et al.,* 2005). Although inflammation is an important non-specific defence response to tissue injury by either a pathogen or a wound, however if not properly regulated it can be detrimental (Janeway *et al.,* 2005). It is well know that excessive ROS are produced during inflammation (McGregor *et al.,* 2006), which are linked to the pathophysiology of chronic diseases such as atherosclerosis and chronic inflammatory diseases (Mayne, 2003).

The inflammatory response is divided into two phases; an acute phase which forms part of innate immunity and a chronic phase forming part of adaptive immunity (Medzhitov *et al.,* 2008). Acute inflammation is the immediate response of the body to injury or cell death and it begins when injured tissue cells releases chemical signals that activate the endothelium of the nearby capillaries (Israels & Israels, 2002; Prescott *et al.,* 2002). As a result, the activated endothelial cells display selectin or cell adhesion molecules, P-selectin and E-selectin, which randomly attract and attach neutrophils to the endothelial cells slowing them down. The neutrophils encounter the inflammatory mediators that activate adhesion receptors (integrins) on the cells. Importantly, the neutrophil integrins attach tightly to endothelial adhesion molecules and stops rolling (Prescott *et al.,* 2002). As a result, neutrophils undergo changes in shape, squeeze through the endothelial wall into interstitial tissue fluid, migrate to the site of injury, and attack the pathogen or the cause of tissue injury.

The recruited activated neutrophils and macrophages shed arachidonic acid (A A) from the membrane phospholipids mediated by the action of phospholipase A_2. Consequently, A A after undergoing a series of eicosanoids conversions is ultimately converted to prostaglandins responsible for typical pain, vasodilation, erythema and fever that are associated with inflammatory reaction (Israels & Israels, 2002). Depending on the severity and nature of tissue damage other leukocytes e.g. lymphocytes, monocytes, and macrophages may follow the neutrophils (Prescott *et al.,* 2002). The phagocytic cells (monocytes, tissue macrophages and neutrophils) are important early defence against invading pathogens. Once the invading pathogens are ingested by the phagocytes, they are taken to lysosomes where they are destroyed. Macrophages and neutrophils also contain oxygen-dependent enzymes (NADPH oxidase system) that produce noxious ROS which are efficient in killing the attacking pathogen (Kotler, 1998). The expression of NADPH oxidase NOX2 (*gp91* phox) responsible for ROS production during inflammatory process is induced by NF-kB (Israels & Israels, 2002). This occurs during respiratory burst due to increased oxygen use and ATP production and further promotes oxidative stress, tissue injury and inflammation (Prescott *et al.,* 2002). Inflammatory cytokines such as TNF-α, IL-1, IL-6, are known to stimulate HIV transcription, replication and rapid depletion of CD4+ T-cells via

apoptosis, making them important factors in the progression of HIV to AIDS (Roederer *et al.,* 1990; Staal *et al.,* 1990; Villinger & Ansari, 2010; Morris *et al.,* 2012). In addition, excessive TNF-α may cause inflammatory damage and toxicity impairing the hosts' immune responses independent of CD4 depletion. As a result, the chronic inflammatory state in HIV infection causes prolonged production of ROS leading to chronic oxidative stress (Merrill *et al.,* 1989; Cheung *et al.,* 2008).

Inflammation has been associated with a higher mortality risk in HIV infection even in patients with high CD4 count (Julg & Goebel, 2006; Fichtenbaum, 2010). In their study, Tien *et al.* found that inflammatory biomarkers fibrinogen and C-reactive proteins (CRP) were strong and independent predictors of mortality in HIV-infected patients (Tien *et al.,* 2010). Furthermore, excessive production of pro-inflammatory cytokines such as TNF-α may induce inflammatory damage and toxicity, ultimately leading to dysfunctional immune response according to Morris *et al. (*Morris *et al.,* 2012). More specifically, the chronic infection characteristic of HIV infection, leads to a sustained production of ROS and a chronic state of inflammation, which promotes chronic oxidative stress (Merrill *et al.,* 1989; Cheung *et al.,* 2008). The complexity of inflammation and oxidative stress in HIV is summarised in figure 2.8.

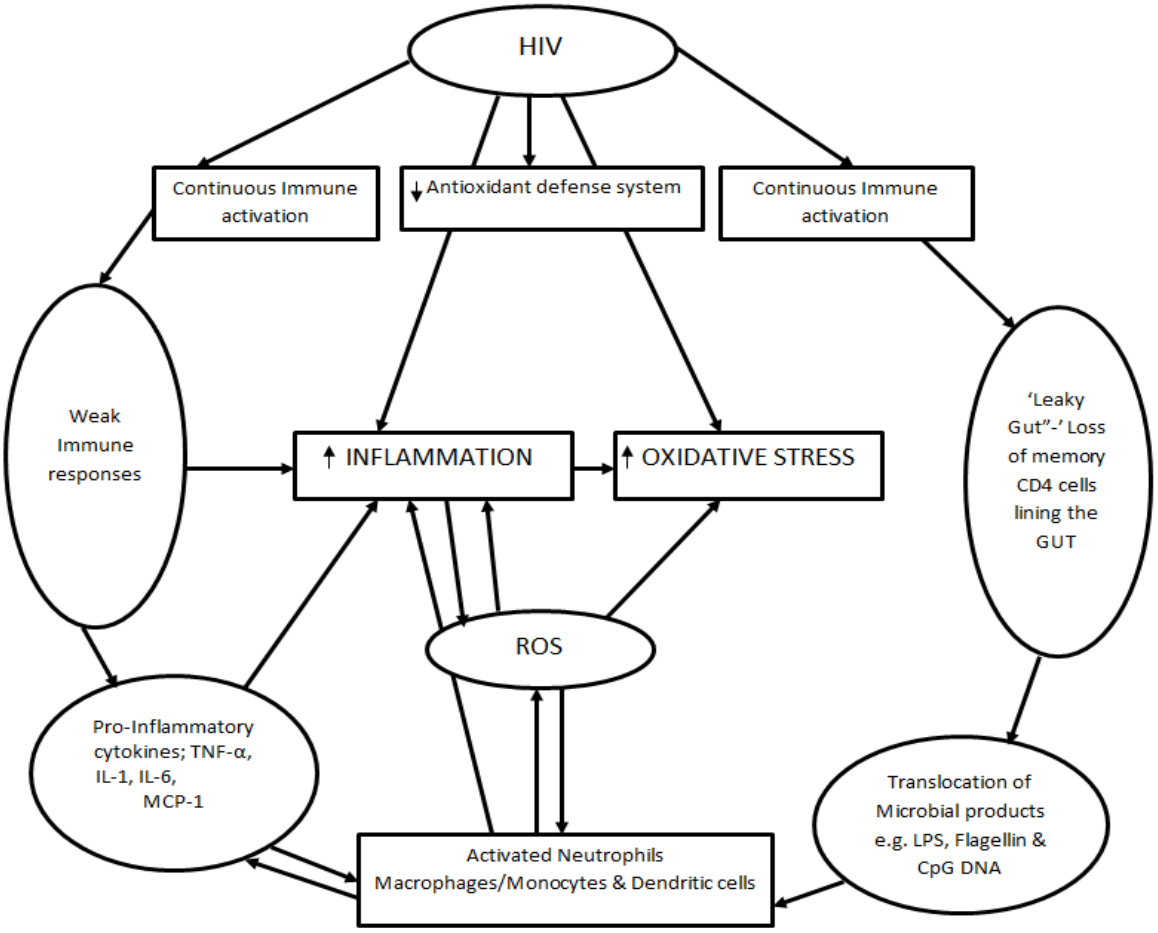


Figure 2:8: Oxidative stress and inflammation in HIV infection. The figure shows the proposed link between inflammation, oxidative stress and HIV (compiled by the author according to Appay & Sauce, 2008; Haynes, 2006; Brenchley *et al.,* 2004.

2.5 Consequences of oxidative stress in HIV

2.5.1 Oxidative stress and signaling pathways

In order to understand the consequences of oxidative stress and apoptosis in HIV, it is important to comprehend the signalling pathways activated by oxidative stress. Apoptosis or programmed cell death is regulated by various molecules including cytokines such TNF-α, Fas ligand (FasL), ROS and mitogen-activated protein kinesis (MAPK) such as extracellular signal-related protein kinase (ERK), c-Jun N-terminal kinase/ stress activated (JNK) (Akgul & Edwards, 2003). Some of the pathways activated by oxidative stress are summarised in Figure 2.9. Oxidative stress is proposed to activate an intracellular mediator, the small G protein Ras (Figure 2.9), which in turn activates MAPK via a network of protein kinases (enzymes that modify other proteins by chemically adding phosphate groups to them) such as ERK kinase, (MEK) kinase (MEKK) and subsequently activating MEK1, a regulator of the MAP kinase ERK1/2. It has been shown that other MAPKs including BMK1, JNK, and p38

may be regulated by oxidative stress (Kyaw *et al.,* 2004; Circu & Aw, 2010). Among the targets of these MAP kinases are various transcription factors including Elk-1 (ternary complex factor, TCF), c-Jun, ATF-2, and CHOP. It has also been reported that ROS activates tyrosine kinases and non-receptor protein kinases (Src family & JAK) initiating MAPK, NF-*k*B, nuclear erythroid 2-related factor-2 (Nrf2) and PI3K signalling pathways (Kyaw *et al.,* 2004). Of specific importance to this study is the NF-kB signalling pathway and the fact that GSSG (oxidised form of GSH during oxidative stress) has been shown to actívate stress-activated protein kinase (SAPK), JNK and MAPK pathways, thereby directly inducing apoptosis (Filomeni *et al.,* 2005).

NF-kB is an inducible and ubiquitously expressed transcription factor that regulates the expression of hundreds of genes involved in cell survival, differentiation, growth, apoptosis and inflammation (Amiri & Richmond, 2005). It occurs as an inactive complex in the cytosol, bound to IkBα, which mask the NF-kB's DNA binding domains. More importantly, ROS are capable of directly activating IkB kinase (IKK) which phosphorylates the C-terminal ankylin repeats of the IkBα proteins bound to NF-kB at serine residuals 32 and 36 (Hayden & Ghosh, 2008). This leads to ubiquitination and degradation of the IkBα unmasking the DNA binding sites subsequently causing translocation of the NF-kB to the nucleus where it binds to kB sites and activates gene transcription involved in cell processes such as pro-inflammatory cytokines inflammation, thereby promoting oxidative stress (Bonizzi & Karin, 2004). Since NF-kB is a critical player in the regulation of the immune response to infection, its activation leads to proliferation of pro-inflammatory cytokines, provoking a vicious cycle of oxidative stress and inflammation. ROS, TNF-α, LPS and IL-1β are known to activate NF-kB, which is also a prerequisite for HIV replication (Brach *et al.,* 1992).

A previous study has indicated that chronic activation of TNF-α signalling pathway promotes transcription of the virus, initiation of apoptosis and suppression of haematopoiesis, therefore playing an important role in HIV-1 pathogenesis (Matsuyama, *et al.,* 1991). Matsuyama *et al.* identified c-Jun N-terminal Kinase (JNK) as the effector molecule in the H_2O_2 induced cell death downstream of receptor interacting protein (RIP) and tumour necrosis factor receptor associated factor-2 (TRAF2) (Matsuyama *et al.,* 2005). They proposed a pathway involving RIP, TRAF2 and JNK1 as novel signalling pathway regulating oxidative stress or ROS-induced cell death. Depending on the various signalling pathways activated and target, ROS can have a variety of damaging effects which are discussed in the following section.

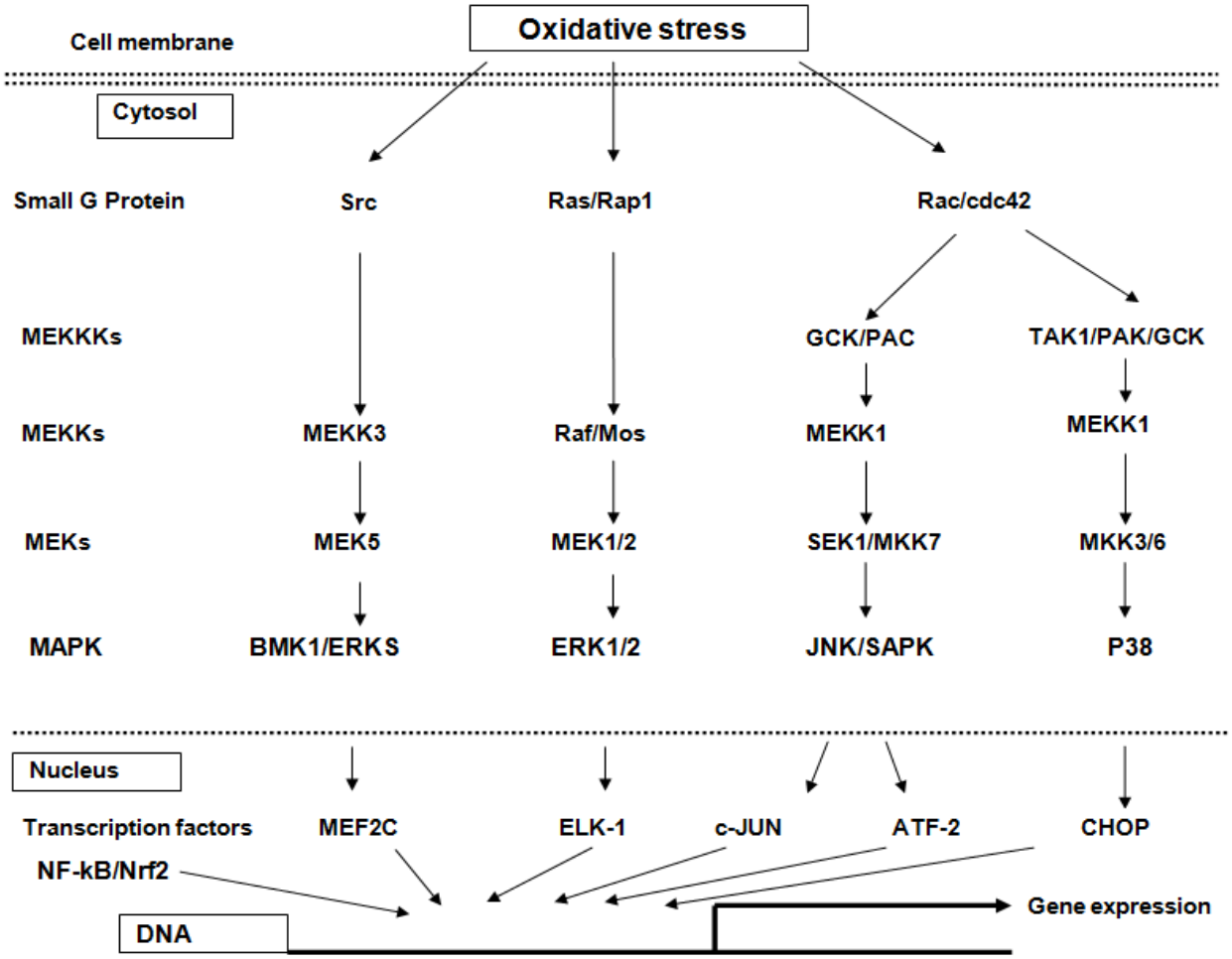


Figure 2:9: Oxidative stress induced signalling pathways. The figure shows the different mitogen activated protein kinases (MAPK) activated by oxidative stress. Adapted from Kyaw *et al.,* 2004.

2.5.2 Oxidative damage in HIV

As previously mentioned, excessive ROS leads to oxidative stress, which causes damage to biomolecules such as DNA, carbohydrates, proteins and uric acids (Devasagayam *et al.,* 2004). This oxidative damage is marked especially in lipids which constitute the cell membrane. More specifically, ROS can react directly with proteins, oxidising peptide bonds or side chains of amino acids to form products of carbonyl groups (1^{o} oxidation modification) or can react with lipids to form products that reacts with proteins (2^{o} oxidation modification), amino acids or peptide bonds for example covalent modification of proteins hydroxyl-nonenal (HNE), which is a very reactive unsaturated aldehyde produces lipid peroxidation (Devasagayam *et al.,* 2003). The carbonyl groups produced can be estimated in the laboratory by carbonyl assays such as enzyme-linked immunesorbent assay (ELISA), fluorometry and immunoblot assays. DNA oxidative modifications and damage involves oxidation of specific bases, which might induce mutations, deletions, strand breaks, DNA cross-linkages, modification of base pairs microsatellite instability, chromosomal aberrations, altered gene expression and cytotoxicity or neoplastic growth (Devasagayam *et al.,* 2004), all

of which have been implicated in various pathological conditions (Khansari *et al.,* 2009; Gil *et al.,* 2013. Hydroxyl radicals attack on DNA base can produce several different primary products of oxidation such as 8-OH-2'-deoxyguanosine (8-OH-dG), which is the most common marker for oxidative DNA damage in living systems (Evans *et al.,* 2004) and 8-OH-adenine (8-OH-Ade) among others.

Several methods for analysis of oxidative DNA damage analysis such as high performance liquid chromatography (HPLC), gas chromatography mass spectrophotometry (GC-MS), ELISA and radioactive immune assay (RIA), immunodetection, comet assay are available. Lipid peroxidation, a marker of oxidative damage, involves attack by ROS on polyunsaturated fatty acids (PUFAs) generating very complex group of oxidation products. The process starts when an electron is removed from lipids by free radicals initiating a chain reaction which generates more free radicals (Devasagayam *et al.,* 2003; Devasagayam *et al.,* 2004). As a result, oxidative stress in living systems can be estimated by measuring ROS directly or the degree of protein, DNA oxidation as mentioned previously or by measuring products of lipid peroxidation in the systems (Laguerre *et al.,* 2007). However, lipid peroxidation remains the most convenient marker of oxidative stress in living systems.

The estimation of the lipid peroxidation process in general is not easy as it is an intricate process that occurs in several stages (Nalsen, 2006). Specifically, both MDA and conjugated dienes, which are by-products of lipid oxidation reactions, can be estimated in plasma, serum or RBC's lysates. Lipid peroxidation has been traditionally estimated by the increase in TBARS which mirrors the production of MDA, an oxidation product of lipid peroxidation and conjugated dienes. Conjugated dienes on the other hand are PUFAs produced as by-products in the process of lipid peroxidation reaction. Plasma TBARS assay is based on the reaction of thiobarbituric acid (TBA) with malondialdehyde in the plasma and the absorbance of the MDA-TBA adducts is read at 530 nm (Khoschsorur *et al.,* 2009). During oxidation of PUFA side chain, the double bonds are converted into conjugated double bonds resulting in conjugated dienes which absorb UV light at 234 nm. The concentration of dienes can therefore be calculated using an extinction coefficient. However, due to the unspecificity of the TBARS assay, it is advisable that at least two different assays for lipid peroxidation should be applied for a reliable estimation (Hwang & Kim, 2007).

Several studies have reported increased lipid peroxidation in HIV infection (Pauspathi *et al.,* 2009; Suresh *et al.,* 2009; Awodele *et al.,* 2012). In their investigation of levels of oxidative stress parameters in HIV, Awodele *et al.* reported a significantly higher MDA levels in naïve patients when compared to HIV patients on HAART (Awodele *et al.,* 2012). However, in their study (REACH cohort), Stephensen *et al.* reported no association of HIV infection with

oxidative damage in adolescents between 14 - 23 years, indicating that young age and stage of disease were determinants of oxidative damage in HIV (Stephensen *et al.,* 2005).

2.5.3 Oxidative stress and apoptosis in HIV

It is a well-known fact that HIV patients are under chronic oxidative stress due to either over utilization of antioxidant defence system or reduced intake of antioxidants (Pace & Leaf, 1995; Kotler, 1998). That ROS can cause harm to the cells and tissues is also known and when the damage is extensive, it is to the advantage of the tissues to get rid of the cell to protect the surrounding cells. Consequently, ROS are capable of triggering both apoptotic and necrotic cell death depending on the severity of the oxidative stress (Saito *et al.,* 2006). Oxidative stress is thought to be a co-factor for the induction of apoptosis in neutrophils and T lymphocytes during HIV/AIDS infection (Pitrak *et al.,* 1996; Dobmeyer *et al.,* 1997; Gil *et al.,* 2013). Importantly, studies suggest that a reduction in CD4+ T-lymphocyte subsets during HIV might be due to apoptosis (Gougeon & Montagnier, 1993). Although apoptosis or programmed cell death is a normal physiological process involved in tissue homeostasis, it is increased during oxidative stress (Kotler, 1998). Several factors are known to induce apoptosis. These factors include; receptor-mediated signals, viral proteins, withdrawal of growth factors, direct viral toxicity, anti-tumour drugs and under certain conditions for example aberrant immune activation or oxidative stress damage to DNA (Appay & Sauce 2008; Cummins & Badley, 2010; Hunt, 2010). Other factors thought to stimulate apoptosis in CD4+ T-cells include viral proteins (gp120) (Westerndorp *et al.,* 1995), improper secretion of inflammatory cytokines by activated macrophages (TNF-α) (Wanchu *et al.,* 2009; Morris *et al.,* 2012) and toxins produced by opportunistic microorganisms (Repetto *et al.,* 1996).

According to Hockenbery *et al.*, an overload of H_2O_2 during oxidative stress in conjunction with deficiencies in catalase and glutathione peroxidase possibly leads to overproduction of hydroxyl radicals and lipid peroxides and consequently induces apoptosis (Hockenbery *et al.,* 1993). In view of that, the addition of TNF and H_2O_2 to HIV-infected cells *in vitro* increased cell death, whereas addition of antioxidants decreased apoptosis in the cells (Malorni *et al.,* 1993; Sandstrom *et al.,* 1993). Since most of the agents that induce apoptosis are either oxidants or activators of cellular oxidative metabolism, the generation of ROS consequently could induce apoptosis. The pathology of ROS is linked to oxidation of nucleic acids and chromosome breaks, when excessive of which promotes apoptosis (Greenspan & Aruoma, 1994; Devasagayama *et al.,* 2004; Gil *et al.,* 2013). Furthermore, vulnerability of lymphocytes to ROS-induced apoptosis is increased in HIV infection from early stages of the disease (Bundres *et al.,* 1993; Favier *et al.,* 1994; Jarstrand & Akerlund, 1994; Kotler, 1998; Block *et al.,* 2002; Pugliese *et al.,* 2005; Roberts *et al*., 2010). Further research needs to be done to study why lymphocytes from HIV-infected individuals exhibit an enhanced vulnerability to

oxidative stress. It has been shown that reactive oxygen radical scavengers, such as GSH, *N*-acetylcysteine (NAC), and catalase could prevent the initiation of apoptosis, while SOD, which degrades superoxide radicals to H_2O_2 decrease apoptosis significantly in a dose-dependent way (Dobmeyer *et al.,* 1997). As stated previously, caspases, important participants in apoptosis are cysteine-dependent and glutathione redox status sensitive enzymes (Staal *et al.,* 1992; Kotler 1998; Circu & Aw, 2010). Interestingly, cysteine starvation of caspases has been shown to induce apoptosis (Armstrong *et al.,* 2004). In addition, NAC, a well-recognized thiol antioxidant, which is converted to glutathione after uptake, as well as glutathione, has been reported to completely block activation-induced death and associated DNA fragmentation in T-cell hybridomas, therefore implicating redox regulation in the processes (Sandstrom *et al.,* 1994; Akerlund *et al.,* 1995; Morris *et al.,* 2013).

However, the significance of modulating oxidative stress on the clinical progression of HIV disease remains to be established, although trials to evaluate the clinical importance of oxidative stress in HIV are in advanced stages (Pace & Leaf, 1995; Wanchu *et al.,* 2009; Gil *et al.,* 2013). Results from recent clinical trials have indicated beneficial effects of antioxidant vitamins supplementation such as suppression of viral load (Hurwitz *et al.,* 2007), improved mitochondrial function (Milazzo *et al.,* 2010), CD4+ T-lymphocyte counts (Yousefi *et al.,* 2011) and suppression of progression of HIV (Morris *et al.,* 2013). In a recent study, Morris *et al.* reported that NAC and liposomal formulation of glutathione (*l*GSH) supplementation improves macrophage functions in HIV (Morris *et al.,* 2013).

2.5.4 Oxidative stress, immune activation and antioxidant depletion in HIV

The significant depletion of memory CD4+ T-cells lining the GIT mucosa in early HIV infection results in breakdown of the mucosa and on-going translocation of microbial products such as LPS across the epithelial surface (Brenchley *et al.,* 2004; Brenchley *et al.,* 2006). As a result, LPS induces activation of innate immune cells such as monocytes and dendritic cells resulting in increased oxidative stress; depletion of antioxidant defence mechanisms and an increased susceptibility to apoptosis. Importantly, CD4+ T-lymphocytes express toll like-receptor-4 (TLR4), which is a receptor for LPS. Specifically, TLR4 activates various signalling pathways such as mitogen-activated protein kinases (MAPK), p38 and JNK, which induce activation of transcription factor NF-kB with subsequent production of pro-inflammatory cytokines, chemokines, antimicrobial peptides and other defence molecules such as ROS (Kabelitz, 2007). As mentioned previously, the pro-inflammatory cytokines produced are capable of activating innate immune cells to produce more ROS. Subsequently, ROS and pro-inflammatory cytokines produced induce activation of both the extrinsic and intrinsic pathways of apoptosis. With the generation of excessive ROS and cytokines, adaptive immune system becomes activated, antioxidant defences such

glutathione gets depleted as they try to scavenge for the free radicals, thereby initiating a vicious cycle of oxidative stress and immune activation (Morris *et al.,* 2013).

The role of *in vitro* stimulation with LPS on T-cell activation in HIV has been explored previously. However, majority of published studies have used CD38 and HLA-DR as the activation biomarkers and were conducted on HIV-infected patients who were on antiretroviral treatment (Tincati *et al.,* 2010). A previous study by Bellezzo *et al.* reported activation of NF-kB in kupffer cells, which was inhibited by NAC and α-tocopherol (α-TOC). In this study NAC and α-TOC suppressed LPS-induced TNF-α mRNA levels and secretion (Bellezzo *et al.,* 1998). Only a few studies on CD25 as an activation marker have been published despite CD25 being a well-established activation biomarker on activated T and B-cells.

Recently, the use of HAART has led to decline in viral load, qualitative and quantitative improvement in immune functions of HIV patients and constant progress in reducing morbidity and mortality (Corbeau & Reynes, 2011; Plaeger *et al.,* 2013). However, since the ART therapies do not eliminate HIV completely, the problem of insufficient immune reconstitution in some patients persists (Coaccioli *et al.,* 2010; Corbeau & Reynes, 2011). Unfortunately, hepatotoxicity and mitochondrial toxicity in patients on HAART has been reported recently meaning that the outcomes of treatment will largely depend on immune system's ability to recover and control residual virus (Gil *et al.,* 2012). In spite of sufficient viral suppression, chronic inflammation and immune activation persist in HIV-infected patients on HAART (Plaeger *et al.,* 2012), which independently predicts faster disease progression to non-AIDS related morbidity and mortality (Nixon & Landay, 2010). Therefore, reducing immune activation, inflammation and oxidative stress with interventions such as nutritional and antioxidant interventions becomes important (Hurwitz *et al.,* 2007; Aquaro *et al.,* 2008; Edeas *et al.,* 2010; Gil *et al.,* 2013). In their study to characterize oxidative stress in different clinical conditions, Gil *et al.* showed a significant beneficial change in antioxidant status (↑GSH, ↓MDA, ↓CD95 receptor) by nutritional and antioxidant supplementation with Vimang in HIV-infected Cuban patients (Gil *et al.,* 2013).

HIV infection has been shown to affect levels of antioxidant enzymes such as glutathione peroxidase (Stephensen, 2007). Furthermore, HIV *Tat* gene has been shown to induce H_2O_2 signaling resulting in enhanced T-cell activation and death signal expression (Campbell, 2005). Therefore, it is likely that both the depletion of endogenous antioxidants such as glutathione and the increased production of H_2O_2 as a result of chronic inflammatory processes, contribute to the deaths of CD4+ T-lymphocytes in HIV infection (Kotler, 1998; Gil *et al.,* 2013).

However, it is a well-known fact that factors such as temperature, duration and concentration have a bearing on results of these studies, thereby necessitating optimization in order to have reliable results.

2.6 The effects of temperature, time and concentration on the activity of LPS and antioxidants in immune activation and oxidative stress

Previous studies involving LPS-induced activation of cells have utilised varying concentrations of LPS (100 ng/ml - 5 µg/ml), NAC (5 - 50 µM), and vitamin C (25 - 50 nM), therefore there is a need for optimization (Dobmeyer *et al.,* 1996; Shang *et al.,* 2003; Yamanda *et al.,* 2006). Different markers such as Annexin V/7-AAD, CD4, CD25 and CD69 monoclonal antibodies have been used previously as convenient markers of early and late apoptosis and immune activation. Therefore, it is important to optimize the *in vitro* effects of temperature and concentration and concentrations of LPS and selected antioxidants on LPS-induced immune activation in asymptomatic HIV infection.

LPS is a polysaccharide, cell wall component expressed by gram negative bacteria, which is a strong immunostimulatory molecule associated with T-cell and monocyte activation (Brenchley *et al.,* 2006). Annexins are a group of calcium-dependent phospholipid binding proteins occurring abundantly in eukaryotic cells. Annexin V is Ca^{+} dependent, phospholipid binding proteins, which is capable of specifically binding to PS strongly even when conjugated with fluorescein isothiocyanate (FITC) (Raynal & Pollard, 1994; Vermes *et al.,* 1995). This phenomenon has been applied in cell analysis. Annexin V-FITC and 7-AAD are used in combination to detect early apoptosis (Koopman *et al.,* 1994). The principle of this assay is based on the ability of Annexin V to bind to PS and on the specificity of 7-AAD to bind DNA guanine-cytosine base pair (Zelenin *et al.,* 1984). Early detection of apoptosis was based on morphological changes of the cell like cell shrinkage, nuclear condensation and pyknosis, which were complimented with biochemical changes such cleavage of the DNA between the nucleosomes, resulting in appearance of DNA bands on agarose gels. However, it was discovered that not all cells undergoing apoptosis cleave their DNA strands between nucleosomes and that those that does, do so only at latter stages in the apoptotic pathway (Israels & Israels, 2002). Hence other parameters had to be used to detect early stages of apoptosis.

One of these parameters is the appearance of PS on the surface of the cells. During the early phase of apoptosis, although the integrity of the cell membrane is maintained, the cells loose the asymmetry of their membrane phospholipids thereby exposing PS at the surface of the cell (Martin *et al.,* 1995). PS is a negatively charged phospholipid which is usually located in the inner leaflets of the plasma membrane. Importantly, the exposure of the PS at the surface of the cell forms one of the earliest and specific signals for recognition and removal

of apoptotic cells by macrophages via PS receptor (Chaurio *et al.,* 2009; Kim *et al.,* 2005). Annexin V-FITC or PE is used to quantitatively determine the percentage of cell within a population that are actively undergoing apoptosis. The interaction of Annexin V and PS is calcium-dependent, thus it is critical to avoid buffers containing EDTA or other calcium chelators during Annexin V staining (Raynal & Pollard, 1994; Vermes *et al.,* 1995).

Since cell membrane integrity is lost as the apoptosis process progresses, distinction between early apoptotic and late apoptotic or necrotic cells is possible using a DNA specific dye like 7-AAD. The baseline levels of apoptosis and necrosis varies widely within a population and therefore even in the absence of induced apoptosis, most cell populations will show a little percentage of cells that are positive for apoptosis. It is recommended therefore that unstained or untreated cells population be included to define the baseline levels of apoptotic cells and dead cells (Vermes *et al.,* 1995; Koopman *et al.,* 1994).

During LPS-induced stimulation, activated monocytes respond to LPS by secreting inflammatory cytokines such as IL-1, 6 & TNF-α which leads to increased ROS production. Furthermore, LPS is known to activate various signalling pathways including the ERK ERK, P38, and SAP/JNK, in addition to PI3 Kinase and IKK/NF-κB pathways in peripheral blood via the Toll-like receptor-4 (TLR4) complexes (Chow *et al.,* 2005; Shankey *et al.,* 2006; Hedley *et al.,* 2008). Importantly, macrophages/monocytes, neutrophils as well as CD4+ T-cells express TLR4 (Kabelitz, 2007), the principle signalling component of the LPS receptor complex (CD14, LBP & TLR4) on their surfaces, thus can be activated directly by LPS or indirectly by pro-inflammatory cytokines from activated monocytes, neutrophils or ROS (Xu *et al.,* 2005; Caramalho *et al.,* 2003). Flow cytometry can be used to monitor these signalling pathways activation. The same approach can be used to study or monitor the impact or activity of targeted inhibitors or of specific signalling pathways inhibitors. Using fluorescently labelled monoclonal antibodies to assess protein and antigen expression, cellular patterns and activation states can be identified (Alvarez *et al.,* 2009).

Studies on humans infected with HIV indicated that most of the depletion of the CD4+T-cells characterising HIV infection occurs in the mucosal tissues during acute phase of the infection either as direct targets of the cytopathic effects of the virus or indirectly by immune activation induced-apoptosis (Veazey *et al.,* 2003; Brenchley *et al.,* 2004; Haynes, 2006). The optimum concentrations of LPS on *in vitro* activation and apoptosis of immune cells in whole blood need to be established. As mentioned previously, studies involving LPS-induced activation of cells have utilised varying concentrations of LPS (100 ng/ml - 5 µg/ml), NAC (5 - 50 µM), and vitamin C (25 - 50 nM) therefore the need for optimization (Dobmeyer *et al.,* 1996; Shang *et al.,* 2003; Yamanda *et al.,* 2006). Kim *et al*. in their study on analysis of cellular senescence

induced by LPS, demonstrated a significant ($p < 0.005$) decrease of cellular viability at 15 µg/ml or greater concentration of LPS (Kim *et al.,* 2012).

Antioxidants such as NAC and vitamin C have been shown to inhibit cytotoxicity; free radicals-induced DNA damage, ROS generation, immune activation and apoptosis (Alul *et al.,* 2003). Therefore, optimizing a functional assay for LPS induced activation and amelioration of this activation by antioxidants is important. However, antioxidants such as vitamin C at higher concentrations are pro-oxidants and therefore toxic to cells or can cause activation of the cells (Halliwell & Gutteridge, 1989; Godin & Wahaieb, 1988). Therefore, antioxidant concentrations optimum for inhibition of the LPS-induced cell activation and apoptosis should be established before the start of analysis. Importantly, incubation of cells with 0.2 mg/ml of vitamin C for 24 hours was shown to cause a 39% increase in apoptotic cells, suggesting toxicity of vitamin C (Bergman *et al.,* 2004). Therefore, the importance of optimizing the concentrations of NAC and vitamin C prior use cannot be over emphasized.

By using fluorescently labelled monoclonal antibodies to assess protein and antigen expression, cellular patterns and activation states can be identified (Alvarez *et al.,* 2009). Some of the monoclonal antibodies includes; CD45, CD 25, CD69, CD4, Annexin V & 7-AAD which can be used in LPS stimulation and antioxidants optimization. CD45 also known as leukocyte common antigen (LCA) is a member of the protein tyrosine phosphate family expressed on all hematopoietic cells except mature erythrocytes and platelets (Braford, 1994). Different isoforms of CD45 arising from splicing of variable splicing of exon 4, 5, and which are specific to the activation and maturation state of the cells and cell type exists (Virts *et al.,* 1997). CD45 plays an important role in T-cell receptor (TCR) and B-cell receptor (BCR) signal transduction. CD4 monoclonal antibody is a single-chain transmembrane glycoprotein expressed on T-helper lymphocytes. It is a member of the immunoglobin g (Ig) superfamily, present on most thymocytes, where it is co-expressed with CD8 (Littman, 1987). CD4 is also expressed on all monocytes/macrophages and dendritic cells although dimly. CD4 acts a co-receptor with the TCR during T-cell activation and thymic differentiation by binding MHC class II. More importantly, CD4 is the main receptor for HIV-1 envelops protein gp-120 (Hannet, 1992).

CD69 is a 60 kDa cell surface glycoprotein also known as activation inducer molecule. CD69 antigen is one of the earliest activation markers to be expressed and is found on activated T, B-lymphocytes and platelets, but absent on resting lymphocytes (Testi, 1994; Sanchez-Madrid, 1995). CD69 is expressed on activated macrophages, NK cells and other cells such as neutrophils, eosinophils and platelets (Testi, 1994). The use CD69 fluorochrome conjugated monoclonal antibody has allowed the phenotyping, characterization, and enumeration of activated cells in HIV/AIDS and other immune system disorders using flow

cytometry (Maino *et al.,* 1995). CD 25 antigen is a 55 kDa single chain glycoprotein which in association with CD122 and CD 132 is the receptor for IL-2. It is found on activated T and B-cells, on activated monocytes, macrophages and T-cell clones (Anderson *et al.,* 1995). It is also found on CD4 T-regulatory cells. It is a late marker of activation. The use of CD45, CD4, CD25and CD69 fluorochrome conjugated monoclonal antibody has allowed the maximizing of gate purity and lymphocyte recovery, thus immunophenotyping, characterization, and enumeration of lymphocytes in HIV/AIDS and other immune system disorders can be done using flow cytometry (Aandahl *et al.,* 2004; WHO, 2007).

7-AAD emits light in the far-red of the spectrum and can be separated from phycoerythrine (PE) or FITC or other tandem dyes, thus monoclonal antibodies conjugated with these fluorochromes can be used to identify different cell types simultaneously (Rabinovitch, 1986). However, cells lose their viability if left in the presence of 7-AAD dye for too long; therefore it is recommended that the incubation with Annexin V and 7-AAD be carried out on ice to sustain cells viability (Keeney, 1999).

2.7 Biomarkers of antioxidant status and lipid peroxidation

Baseline levels of TAS, markers of lipid peroxidation and redox cellular status of plasma have previously been used to indicate oxidative stress in HIV infection (Pasupathi *et al.,* 2009; Suresh *et al.,* 2009). The TAS determination in human blood plasma is necessary when investigating the relationship between diet, oxidative stress and human disease and when estimating the potential to resist oxidative stress (Rice-Evans & Miller, 1994). It considers the cumulative effect of all antioxidants present in the blood and body fluids and can be used as an early biomarker of oxidative stress in HIV-infected patients (Suresh *et al.,* 2009). The three commonly used assays for TAS are; ORAC, trolox equivalent antioxidant capacity (TEAC/ABTS) and ferric reducing antioxidant power (FRAP) assays. The principle of all these assays is to produce free radicals at a known rate and then study the ability of a sample to hinder this radical production by a certain end point (Cao *et al.,* 1995; Cao & Prior, 1998; Cao *et al.,* 1998). ORAC is based on the fact that the fluorescence of an oxidizable substrate e.g. fluorescein changes with respect to time upon damage caused by a peroxyl or hydroxyl attack. This assay involves a pro-oxidant produced by a generator such as 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) and an oxidizable substrate capable of fluorescence. The free radical induces oxidative damage which is inhibited in the presence of an antioxidant (Huang *et al.*, 2005). This inhibition is measured (in terms of fluorescence) and related to the antioxidant capacity of the antioxidant. FRAP assay is based on electron transfer reactions where antioxidants are used as reductants in a redox-linked colorimetric method. The change in absorbance is directly proportional to the concentration of antioxidants. One FRAP unit has been randomly defined as the reduction of 1 mole of Fe (III)

to Fe (II) by Benzie & Strain (Benzie & Strain, 1996). The FRAP assay is fast, reproducible with single antioxidants in pure solutions and with mixtures in aqueous solutions. However, in FRAP there are no free radicals. It is simply a redox reaction to measure the ability of antioxidants in a sample to reduce ferric iron (III) to ferrous iron (II). Hence, FRAP is not a direct measure of the antioxidant capacity of a potential antioxidant. At low pH, reduction of a ferric-tripyridyltriazine (Fe^{III}-TPTZ) complex to the ferrous form, which has an intense blue colour, can be monitored by measuring the absorption at 593 nm (Benzie & Strain, 1996). Although ORAC and FRAP are fast, reproducible and reliable assays they have their own limitations which necessitates caution when interpreting results; they do not include measurements of blood albumin levels and metal binding proteins like transferrin and lactoferrin.

As mentioned previously, oxidative stress in living systems can be estimated by measuring the degree of lipid peroxidation in the systems (Laguerre *et al.,* 2007). Lipid peroxidation has been traditionally estimated by the increase in TBARS which mirrors the production of MDA, an oxidation product of lipid peroxidation and CDs, polyunsaturated fatty acids produced as by-products in the process of lipid peroxidation reaction. Plasma TBARS assay is based on the reaction of thiobarbituric acid (TBA) with malondialdehyde in the plasma and the absorbance of the MDA-TBA adducts is read at 530 nm (Khoschsorur *et al.,* 2009). During oxidation of polyunsaturated fatty acid (PUFA) side chain, the double bonds are converted into conjugated double bonds resulting in conjugated dienes which absorb UV light at 234 nm (Recknagel & Glende, 1984). The concentration of dienes can thus be calculated using an extinction coefficient.

2.8 Antioxidants, oxidative stress and HIV infection

Whereas several studies have indicated a weakened antioxidant defence system in HIV/AIDS infection (Allard *et al.,* 1998), however, the question of whether antioxidants have potential therapeutic benefits if they are capable of inhibiting oxidative stress-induced apoptosis of CD4+ T-cells and disease progression, still remains (Wanchu *et al.,* 2009). Importantly, the antioxidant defence system depends on the integrity of an enzymatic system that needs sufficient intake of trace minerals including selenium, copper, zinc and manganese and adequate amounts of vitamin A, C, E and β-carotene in the cytoplasm and lipid membrane of the cells. Previous studies have indicated that HIV/AIDS patients may have deficiencies in some of these trace minerals and vitamins such as selenium and vitamin A (Cirelli *et al.,* 1991; Simba *et al.,* 1993; Allard *et al.,* 1998; Allard *et al.,* 2008). Allard *et al* showed an increased oxidative stress in HIV-positive patients when compared to seronegative controls and decreased plasma concentrations of various antioxidant vitamins and selenium (Allard *et al.,* 2008). Whether antioxidant supplementation will have any effect

on oxidative stress, viral replication and disease progression remains to be established. Some investigators have suggested that the best way to prevent immune depression in HIV infection is to reduce oxidative stress, which is key at the earliest stage (Baruchel & Wainberg, 1992; Pace & Leaf, 1995) in particular at stage II where free radical production is highest (Wanchu, *et al.,* 2009). More specifically, a role of antioxidants such as NAC, vitamin C in HIV therapies has been proposed (Harakeh & Jariwalla, 1995; Garland & Fawzi, 1999; Aquaro *et al.,* 2008; Suresh *et al.,* 2009; Edeas *et al.,* 2010) and in addition, antioxidants might slow down the rate of apoptosis which has been implicated in HIV/AIDS progression (Gougen & Montagnier, 1993; Akerlund *et al.,* 1996; Herzenberg *et al.,* 1996; Marmor *et al.,* 1997). The ability of antioxidant supplements to reduce DNA damage and disease progression in HIV infection has been reported (Akerlund *et al.,* 1996; Jaruga *et al.,* 2002)

2.8.1 Vitamin C

Decreased levels of serum vitamins and minerals have been linked with a higher risk of disease progression and mortality in HIV infection (Allard *et al.,* 1998; Gil *et al.,* 2013). Furthermore, supplementation with micronutrients such as vitamin C has been shown to delay HIV progression and reduce mortality in HIV-infected individuals not receiving HAART (Fawzi *et al.,* 2004; Drain *et al.,* 2007).

Vitamin C is a six-carbon lactone synthesised in the liver of majority of mammalian species except humans, non-human primates and guinea pigs (Nishikimi & Yagi, 1996). It is a water-soluble, chain-breaking antioxidant which occurs primarily as a monovalent anion at physiological pH. The ability of vitamin C to donate electrons confers strong antioxidant properties to it. Its strong antioxidant effects have been reported *in vitro* in numerous studies (Packer & Colman, 1999; Padayatty *et al.,* 2003). More importantly, the ability of vitamin C (ascorbate) to donate one electron and form semi-dehydroascorbate, which is an ascorbyl radical, of little reactivity and to donate a second electron, resulting in the formation of dehydroascorbate confers its strong antioxidant activity (Padayatty *et al.,* 2003). Glutathione through chemical reduction and via glutathione-dependent enzymes is directly involved in the recycling of ascorbate. Since humans cannot synthesise vitamin C, which is essential for iron absorption, synthesis of bile, collagen and maintenance of skin elasticity due to lack of L-gulonolactone oxidase as they lack, they must obtain it from if from dietary intakes (Packer &Colman, 1999; Padayatty *et al.,* 2003; Pavlovic & Sarac, 2010). The chemical structure of vitamin C is shown in Figure 2:10.

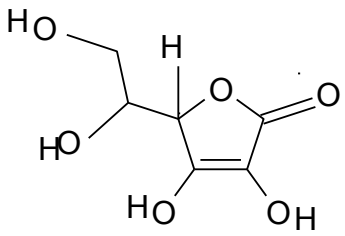


Figure 2:10: Chemical structure of vitamin C (Astley, 2003).

Interestingly, vitamin C's ability to regenerate vitamin E in the cell membranes enables it to synergistically participate with vitamin E in protecting cells from oxidative stress (Packer & Colman, 1999). As a consequence, vitamin C removes the tocopherol radical formed by vitamin E activity within the membrane and vitamin C recycles the tocopherol radical produced from the lipid radical back to tocopherol (Padayatty *et al.,* 2003).

Ascorbate also participates in the reduction of Fe^{3+} (III) or Cu^{2+} (II) to Fe^{2+} (II) or Cu^{+} (I) and the ascorbyl radical during fenton reaction with the Fe^{2+} or Cu^{+} produced capable of catalysing the production of H_2O_2, eventually generating the highly reactive hydroxyl radicals. This reaction has been associated with the pro-oxidant activity of vitamin C and as a consequence, high doses and long-term mega doses of vitamin C may be toxic as they can cause oxidation in a living system, there great care and consideration should be taken when designing doses or experiments (Carr & Frei*,* 1999; Lee *et al.,* 2001).

In vitro suppression of virus production and cell fusion in HIV-infected T-lymphocytes cell (Harakeh *et al.,* 1990), inhibition of antigen-induced and spontaneous T-cell apoptosis (Pavlovic *et al.,* 2005), Fas-induced monocyte apoptosis (Perez-Cruz *et al.,* 2003), increased activity of natural killer cells (Vojdani *et al.,* 2000) and inhibition of reactivation in TNF-α stimulated cells by vitamin C have been reported (Harakeh *et al.,* 1995). In a previous study, clinical improvement was reported in AIDS patients who willingly consumed high doses of ascorbic acid (Cathcart, 1984). Since vitamin C is an essential water soluble antioxidant, as it can scavenge for superoxide radical, singlet oxygen, H_2O_2 and hydroxyl radical directly (Packer & Colman, 1999), it has also been shown to be a potent inhibitor of apoptosis, albeit in erythrocytes (Mahmud *et al.,* 2010). It has been reported that supplementation of vitamin E and C in HIV-infected persons reduces oxidative stress and could reduce viral load (Allard *et al.,* 1998).

2.8.2 N-acetyl cysteine (NAC)

NAC is a cysteine analogue that replenishes intracellular GSH and glutathione peroxidase. NAC is a well-recognized thiol antioxidant, which is converted to glutathione after uptake (Sandstrom *et al.,* 1994). This antioxidant that scavenges peroxides has been shown to inhibit apoptosis (Hockenbery *et al.,* 1993).and protect alveolar cells from H_2O_2 induced apoptosis by scavenging ROS and suppressing c-Jun- N-terminal kinase (Fang *et al.,* 2010).

More specific, NAC and glutathione have been shown to completely block activation-induced death and associated DNA fragmentation in T-cell hybridomas, therefore implicating redox regulation in the processes (Sandstrom *et al.,* 1994).

Although NAC acts directly as a free radical scavenger by reducing hypochlorus acid produced by neutrophils to kill target pathogens and cells, however, this direct antioxidant activity of NAC could inhibit the natural mechanisms of neutrophils cytolysis. Cell studies however, have indicated that NAC essentially boosts intracellular killing of bacteria by protecting the neutrophils and macrophages from free radical generated during phagocytosis (Oddera *et al.,* 1994).

2.8.3 Vitamin E and other antioxidants

Vitamin E, a lipid soluble, chain breaking antioxidant, is important for the appropriate functioning of the immune system, as it increases humoral and cell mediated responses, antibody production, phagocytic response and resistance to viral and infectious diseases (Allard *et al.,* 1998, Watson, 1994). Vitamin E, broadly classified as tocopherols and tocotrienols is the major chain breaking, lipid soluble antioxidant present in body tissues. More importantly, it provides the first line of defence to cell membranes against oxidative stress by trapping peroxyl radicals at early stages of free radical attack (Lien *et al.,* 1999). Although vitamin E (α-tocopherol) and vitamin C react synergistically, vitamin E is considered more lipophilic and the more potent antioxidant with respect to lipid peroxidation and penetration to specific sites in bio membranes. Oxidative stress in HIV increases utilization of vitamins, which leads to deficiency, leading to further weaken of the immune system (Chandra, 1997). Supplementation with vitamin E and C was shown to reduce oxidative stress and produced a trend towards a reduction in viral load in HIV-infected persons (Allard *et al.,* 1998).

As previously discussed in section 2.4.1, other exogenous antioxidants, including vitamins such as vitamin A (retinol), vitamin C (ascorbic acid), carotenoids and polyphenols (Papas, 1999; Podsedec, 2007; Valko *et al.,* 2007) exist.

2.8.4 Antioxidants: potential for ameliorating immune activation and apoptosis?

Several antioxidants including NAC, glutathione and alpha-lipoic acid have been shown to play a significant role in the inhibition of apoptosis and viral activation as well as replication in HIV infection (Hockenberry *et al.,* 1993; Gougen & Montagnier, 1993). The dispensation of the ARV zidovudine in combination with antioxidants has been reported to increase its therapeutic capability (Romero-Alvira & Roch, 1998). Although the contemporary use of HAART has greatly reduced morbidity and mortality of HIV infection (UNAIDS, 2011; Gil *et al.,* 2013), increased toxicity, complications and considerable failure to reconstitute immune system (De Clercq, 2009; Corbeau & Reynes, 2011) have necessitated a more cautious re-

evaluation of HIV therapies and examining of other creative alternative interventions (Aquaro *et al.,* 2008; Edeas *et al.,* 2010; Gil *et al.,* 2013). Furthermore, the initial objective of HIV treatment was to manage opportunistic infections and keep the individual alive, which later shifted to suppressing HIV replication and managing drug side effects and complications that may arise (De Clercq, 2009; Gil *et al.,* 2013; UNAIDS, 2011). However, today's emphasis is on improving overall health and enabling people with HIV to live long.

Immune activation and inflammation-induced oxidative stress is thought to contribute significantly to the apoptosis and subsequent depletion of CD4+ T-cells. Therefore, strategies targeting amelioration of inflammation-induced oxidative stress driven apoptosis should be explored as therapeutically viable strategies. Antioxidants, anti-inflammatories, in combination with HAART provide potential viable therapeutic strategies. Highleyman, (2010) has proposed three ways in which this can be approached: (i) reducing T-cell activation, (ii) altering production and activity of cytokines and inflammatory mediators such ROS and (iii) changing underlying risk factors through lifestyle modifications.

Although free radicals have been implicated as a major cause of oxidative damage in living tissues, it is important to note that they are not always harmful. They also perform important biological functions like normal phagocytosis and apoptosis, signal transduction, gene transcription among others. Thus, extra care should be taken when dealing with antioxidants to alleviate oxidative stress. A proper redox balance must always be maintained in order not to disrupt the normal body functioning. The antioxidant status and redox status of the individual must always be considered before any antioxidant intervention or supplementation can be recommended. The strategy recommended in this review is to treat oxidative stress at the earliest possibility by measuring oxidative stress markers in the blood and tailoring treatment based on these tests.

The link between oxidative stress, inflammation and immune activation and their roles and impacts on immunohaematopoietic cells has been explored in this literature review. The potential of antioxidants to ameliorate inflammation-induced oxidative stress and its implications such as apoptosis of CD4+ T-lymphocytes, in HIV has also been highlighted. Glutathione, NAC, vitamin C and E as potential ameliorating interventions has been part of the focus of this review, since they are the most potent naturally occurring antioxidants in the cell membranes that protect the cells from oxidative stress induced-damage.

ROS participates in chronic inflammation, HIV replication and the apoptosis seen in HIV infections. The estimated duration from infection with HIV to development of AIDS is 8-10 years, however, it can be as few as 2 to 3 years, but overall it varies from individual to individual depending on various factors such as host's immune system and dietary factors (Appay & Sauce, 2009; Hunt, 2010). Due to the large numbers of people already infected

with HIV requiring ARV treatment and the fact that only a few of them are receiving such treatments due to various constraints, other means of delaying progression to full blown AIDS will have important implications in the management of the disease. For this reason, reducing the impact of oxidative stress in immune cells might present an additional strategy for slowing down HIV progression and prognosis and thus increase the survival rate of infected persons. The importance of finding ways to reduce oxidative stress at the earliest stage in the infected immune-hematopoietic cells becomes paramount. The general question is whether reduced oxidative stress in immunohaematopoietic cells with a resultant reduced apoptosis will delay the progression from HIV infection to AIDS?

The discovery of a cure for HIV/AIDS has remained elusive and is still a serious challenge for scientists. As scientists continue to seek cure for HIV, we must continue relentlessly to find cost effective ways of prolonging the lives of the already infected. Out of the many people infected with HIV only a small proportion can access ARV treatment due to cost and logistical constraints and despite viral suppression, chronic inflammation still persists. The mechanism by which the chronic inflammation persists and continues to promote pathology remains unclear (Plaeger *et al.,* 2012). Therefore, other interventions to reduce inflammation, oxidative stress and immune activation in infected individuals which is a key factor in progression of the infection from HIV to AIDS and to death would be important in supplementing stakeholders' effort of managing the disease.

2.9 Significance of literature review

In general the immune system of HIV-infected individuals encounters many challenges; it has to cope with a massive CD4+T-cells depletion through apoptosis or direct infection and contain HIV replication and associated pathogens which more often than not is overwhelming. It has also been indicated in this review that HIV-infected individuals are under chronic oxidative stress. An insight into the inflammatory and oxidative stress status of HIV-infected individuals will enable long term profiling of each patient with a view of individualized therapy. This approach may have a direct impact on patient care in resource limited settings like sub-Saharan Africa.

The basic objectives of this literature review was to highlight the previous studies on inflammation, immune activation and oxidative stress and its role in progression and pathogenesis of HIV infection, to highlight the relationship between inflammation, oxidative stress and apoptosis and its impact on CD4+ T-lymphocytes in HIV infection. Potential ways to ameliorate oxidative stress using selected micronutrients which could delay the progression and thereby increase survival rate of infected persons have also been discussed. In this review, the interrelationship between oxidative stress, inflammation and apoptosis and their possible role and impact on apoptosis and depletion of the CD4+ T-

lymphocytes was analysed. A brief overview of some of the immunohaematopoietic cells targeted by HIV has also been discussed.

The above objectives were based on the following hypotheses; chronic inflammation and a dysfunctional enzymatic antioxidant defense system (SOD, CAT & GPx) will lead to an overall increase in H_2O_2 levels which have been implicated in the death of cells from HIV-infected individuals; specific antioxidant intervention will ameliorate HIV inflammation-induced oxidative stress and thereby limit the death of the cells from HIV-infected individuals which may represent a valuable adjunct in the management of patients with chronic HIV infection.

By addressing one of the underlying mechanisms by which dietary antioxidants (in combination) can modulate inflammation-induced oxidative stress, in specific immune cells (CD4+ T-cells) from HIV-infected individuals, results from this study will contribute to our current knowledge in the field of HIV management.

2.9.1 Research gaps

Confirmation from various studies has indicated that HIV-infected patients are under chronic oxidative stress and that oxidative stress plays a key role in HIV pathogenesis (Pace & Leaf, 1995; Pasupathi *et al.,* 2009; Suresh *et al.,* 2009; Wanchu *et al.,* 2009; Awodele *et al.,* 2012; Morris *et al.,* 2012). Numerous studies on oxidative stress and HIV have been undertaken and all have consistently implicated alterations in the antioxidant defence system and increased ROS production during infection in the disease progression (Allard *et al.,* 1998; Gil *et al.,* 2003; Pasupathi *et al.,* 2009; Suresh *et al.,* 2009; Coaccioli *et al.,* 2010). These studies have also concluded that micronutrients such as antioxidants, could offer a cost effective supplementary therapy to HIV patients to prolong and improve survival rate. However, despite the numerous studies done on HIV and oxidative stress, several gaps in these studies exist that warrants further research.

Several aspects still need to be addressed and include;

1) Immunomodulatory effects of antioxidants and clarification of the role of antioxidants on oxidative stress and apoptosis in HIV infection;

2) The relationship between HIV-induced inflammation and respiratory burst/oxidative stress

3) The role of antioxidant enzymes such as; superoxide dismutase, catalase and glutathione peroxidase in apoptosis; for example: do increased SOD activity and reduced GPx activity enhance apoptosis?

4) Why lymphocytes of HIV-infected people show increased susceptibility to oxidative stress - the role of micronutrient/antioxidant deficiency; decreased SOD activity, H_2O_2 or dysfunctional antioxidant defence system.

This review has addressed some of the above research gaps such as determining the impact of inflammatory induced oxidative stress on the apoptosis of CD4+ T-lymphocytes in HIV infection and further, investigation of potential ameliorating interventions and future directions. Due to a limitation of small sample size, this study has only tried to provide an insight into the research gaps highlighted above but has not closed them.

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CHAPTER 3

A preliminary study of the neutrophil respiratory burst in untreated asymptomatic HIV-infected individuals as an *in vitro* indication of response to immune stimulation

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A preliminary study of the neutrophil respiratory burst in untreated asymptomatic HIV-infected individuals as an *in vitro* indication of response to immune stimulation

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Abstract

HIV infection is associated with on-going and aberrant activation of both the innate and adaptive immune systems. The enrolment and activation of neutrophils at the site of infection in order to eliminate pathogens is a key aspect of innate immunity. Activated neutrophils and macrophages produce H_2O_2 and other oxidants during the respiratory burst as a result of increased oxygen use and ATP production. This further promotes tissue injury and inflammation. Enhanced respiratory/oxidative burst may produce excessive ROS resulting in oxidative stress as a by-product of inflammation, which is thought to contribute to the depletion of the CD4+T-lymphocytes in HIV infection.

The aim of this preliminary study was to determine the neutrophil respiratory burst response in asymptomatic HIV-infected individuals and compare it with uninfected controls. This was a preliminary study design whereby blood samples of 14 consenting, untreated HIV positive individuals and 12 HIV negative controls was analysed for respiratory burst. The neutrophil respiratory burst (phagoburst test) was analysed using flow cytometry. The results were correlated with CD4 count, viral load and other markers of immune system activation and inflammation in these individuals and control samples.

Neutrophils from HIV-infected individuals at rest and in response to low stimulating agent, bacterial N-formyl peptides (fMLP), showed significantly ($P = 0.041$) higher levels of burst response than in the uninfected controls. No differences were detected with the more potent stimulating agents, phorbol myristate acetate (PMA) and *E. coli.* The CD38/8 was significantly ($P = 0.0069$) different between this two groups as was the CD4 count ($P<0.001$). In summary,

neutrophils from HIV-infected individuals at rest showed a “primed” response to low stimulating agent (fMLP), which was significantly different from control group. The HIV-infected group showed significantly higher immune activation levels (%CD38/8) compared to the controls. This may indicate that patients with untreated asymptomatic HIV infection have higher levels of oxidative stress *in vivo.* Therefore, respiratory burst might be an important contributing factor in inflammation-associated oxidative stress in HIV infection and increased depletion of CD4+ T-lymphocytes in the asymptomatic stage of HIV infection.

Keywords; Neutrophils, respiratory burst, HIV, reactive oxygen species, neutrophil activation, apoptosis, phagocytosis.

3.1 Introduction

A major characteristic of HIV infection is the continuous systemic immune activation and chronic inflammation resulting in weakened immune responses, pro-inflammatory cytokine production and uncontrolled viral replication in activated CD4+ T-cells (Haynes, 2006). The significant depletion of memory CD4+ T-cells lining the GIT mucosa in early HIV infection results in loss of mucosal integrity and on-going translocation of microbial products such as LPS across the epithelial surface (Brenchley *et al.,* 2004). LPS induces activation of innate immune cells such as monocytes and dendritic cells resulting in increased oxidative stress; depletion of antioxidant defence mechanisms and an increased susceptibility to apoptosis (Brenchley *et al.,* 2006; Cassol *et al.,* 2010).

Neutrophils continuously traverse blood vessels scrutinizing for bacterial infection and inflammation. When they encounter an infection they leave the circulation and migrate to the site of infection where they phagocytose and kill invading microbes by releasing packaged anti-microbial systems (Prescot, 2002). The recruited phagocytes kill the invading pathogens by a combination of two mechanisms; the oxygen-independent and the oxygen-dependent response or respiratory burst (Prescott *et al.,* 2002). The oxygen-independent mechanism is mediated by highly toxic cationic proteins and enzymes e.g. myeloperoxidase (MPO), defensins, lactoferrin, lysozyme, gelatinase, etc contained in azurophilic, gelatinase and specific granules (Segal, 2005). The oxygen-dependent mechanism (the respiratory or oxidative burst) involves a sequential reduction of oxygen by an NADPH oxidase leading to the production of toxic oxygen metabolites such as H_2O_2, hydroxyl radicals and singlet oxygen (Parkin & Cohen, 2001). Once neutrophils ingest bacteria, oxygen consumption of neutrophils is enhanced 100-fold, most of which is converted to ROS in what is referred to as respiratory burst (Israel & Israel, 2002). Respiratory burst occurs via the membrane bound NADPH oxidase. The NADPH oxidase system is inactive, but is activated when the neutrophils are stimulated by chemokines, immune

complexes, C5a, or N-formyl oligopeptides shed by microbes (Taylor *et al.,* 1993; Cruse & Lewis, 2010). The process of recruitment, activation and respiratory burst of the neutrophils and macrophages at the sites of infection is well regulated, however, if prolonged, can lead to inflammation and damage to the surrounding tissues and cells. Previous studies of neutrophils from HIV-infected individuals have indicated increased baseline levels of superoxide especially before AIDS sets in (Elbim *et al.,* 2001). Enhanced respiratory burst of neutrophils may produce excessive ROS in HIV infection, consequently causing oxidative stress, which is thought to contribute to the depletion of the CD4+ T-lymphocytes (Aquaro *et al.,* 2008).

Activated neutrophils and macrophages during inflammation and infection, are major source of H_2O_2 and other oxidants as a result of increased oxygen intake and ATP production. This promotes further tissue injury and more inflammation (Kotler, 1998; Prescot *et al.,* 2002. Inflammation is an important non-specific protective response to harmful stimuli e.g. a pathogen, damaged cells or irritants (Prescott *et al.,* 2002). However, an unregulated inflammatory process becomes harmful such as the one that occurs in HIV infection. Inflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), are known to stimulate HIV transcription and replication and thus may be important factors in the progression of HIV to AIDS (Aquaro *et al.,* 2008). Neutrophil respiratory burst response and its impact on CD4+ T-cell group of immune cells has not been described previously in the context of asymptomatic HIV infection. Therefore, this preliminary study determined neutrophil respiratory burst response in asymptomatic HIV-infected and compared with uninfected controls. The results were correlated with CD4 count, neutrophil count, viral load, and total antioxidant status.

3.2 Material and methods

Monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and dibasic sodium phosphate ($Na_2HPO_4.12H_2O$), hydrochloric acid (HCl), sodium Phosphate, (NaH_2PO_4), per chloric acid ($HClO_4$), sodium di-hydrogen orthophosphate dehydrate ($NaH_2PO_4.2H_2O$), di-sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$), metaphoshoric acid (MPA), trichloric acetic acid, 5,5' Dithiobis-(2-nitrobenzoic acid) were purchased from Merck Chemicals (South Africa). Fluorescein sodium salt ($C_{20}H_{10}Na_2O_5$), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), were purchased from Sigma-Aldrich (South Africa). The phagoburst assay kit was purchased from Orpegen Pharma (Germany). The fluorescence was read on a Fluoroskan plate reader (Thermo Electron Corporation). The absorbance was read in a Multiskan plate reader (Thermo Electron Corporation, Germany). A FACSCalibur four colour flow cytometer (Becton Dickinson San Jose, USA), with one laser, Cell Quest Pro and FCS Express V3 softwares were used in this study.

3.2.1 Study population & design

In this preliminary study, twelve eligible untreated asymptomatic HIV positive individuals (4 males and 8 female) and 14 negative controls (6 males 8 females), average age 31 years (range 21-51) were sourced from the well-established clinics attached to the Institute of Infectious Diseases, Desmond Tutu HIV centre. There was no significant age difference between the two groups. The average CD4 count for the HIV-infected group was 439 cells/mm^3 while that of the uninfected control was significantly higher ($P>0.001$) at 929 cells/mm^3. The patients' demographics are summarized in Table 3.1. Informed consent was obtained from each participant and each participant was assigned a random number. Confidentiality was maintained during the duration of the study. Inclusion criteria for the study participants were; 21 years or older, patients with chronic HIV infection and CD4 count > 200 not started on ARV's or any other chronic medication nor antioxidant supplementation, minerals and vitamins supplements, aspirin or any other drugs e.g. anti-inflammatory, with established antioxidant properties. Exclusion criteria include patients with TB or other co-infections and those receiving antiretroviral therapy, anti-TB treatment or other antibiotic treatment, use of any chronic/life sustaining medication, antioxidant supplementation, minerals and vitamins supplements, aspirin or any other drug e.g. anti-inflammatory, with established antioxidant properties. Ethics approval was obtained from both the clinical site: University of Cape Town: REC: REF: 417/2006 and University of Stellenbosch (laboratory site): N07/09/197. This preliminary sub-study was added as an addendum to the ethics approval received for the original project N07/09/197.

3.2.2 Sample processing

The blood samples were collected from the antecubital vein in the forearm in both (Ethylenediaminetetraacetic acid) EDTA and Heparin vacutainers. The blood samples were transported to the laboratory within two hours of collection. Plasma samples for the total antioxidant status assay were processed appropriately by centrifuging them at 1200 g at 4 ^oC for 10 minutes, aliquoting 500 µl of plasma and storing at -80 ^oC till the day of analyses. All the antioxidant status samples were analysed in triplicates. EDTA blood samples were used for CD4 count and viral load while plasma samples were used for analysis of antioxidant status.

3.2.3 Laboratory investigation

3.2.3.1 Markers of disease and immune activation

CD4+ T-cell counts were determined by staining whole blood with BD MultiTEST™ CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent in BD TruCOUNT™ tubes according to the manufacturer's instructions and analysed on a BD FACSCalibur™ flow cytometer (BD

Biosciences, San Jose, CA). HIV-1 RNA quantifications were performed using 1.0 mL of plasma with use of the Nuclisens Easy Q HIV-1 v.1.2 kit (BioMerieux Inc., Boxtel, Netherlands).

CD38 expression on CD8+ T-cells (CD38/8) was determined by flow cytometry. Whole blood samples were incubated with the monoclonal antibodies; CD8 Per-CP; CD38 APC; CD3 FITC (BD Biosciences, San Jose, CA) and analysed on a BD FACSCalibur instrument using BD Cell Quest Pro (Version 2) software. Lymphocytes were gated on forward vs. side scatter, CD3 and CD8 expression.

3.2.3.2 Total antioxidant status assay

The total antioxidant status measured as ORAC was determined using the modified fluorescein assay by Huang *et al.* which is based on the principle that the fluorescence of an oxidizable substrate e.g. fluorescein, changes with respect to time upon damage caused by a peroxyl or hydroxyl attack (Huang *et al.,* 2005).). In this assay, fluorescence was measured at 1 min intervals at an emission wavelength of 563 nm and excitation wavelength of 540 nm using a Fluoroskan plate reader (Thermo Electron Corporation Germany) until zero fluorescence occurred. The final assay mixture consisted of AAPH (peroxyl radical generator), sample (target of the free radical attack), trolox as the standard control and an oxidizable substrate capable of fluorescence. All the dilutions were prepared using phosphate buffer pH 7.0. In order to initiate the reaction, AAPH was added and the mixture incubated at 37 ^{o}C. Fluorescence was measured at 1 min intervals at an emission wavelength of 563 nm and excitation wavelength of 540 nm using a Fluoroskan plate reader (Thermo Electron Corporation Germany) until zero fluorescence occurred.

3.2.3.3 Phagoburst assay

The phagoburst assay was done according to Rothe *et al.* method, which is based on the oxidation of DHR 123 to R 123 by ROS, which is then measured using flow cytometer (Rothe *et al.,* 1988). When stimulated, phagocytes produce ROS to destroy bacteria. The ROS produced can be monitored by addition of DHR 123 which in presence of ROS is converted to R 123. The percentage of the phagocytic cells expressing R 123 can then be measure using flow cytometer. The reaction was stopped by addition of lysing solution, which removes erythrocytes and results in a partial fixation of leukocytes. After one washing step with washing solution, DNA staining solution was added to exclude aggregation artefacts of bacteria or cells and to stain DNA. The percentage of cells having produced ROS was then analysed as well as their mean fluorescence intensity (enzymatic activity) using flow cytometry.

The cells were analysed by flow cytometry using the blue-green excitation light (488 nm argon-ion laser and FACSCalibur Cell Quest Pro software. During data acquisition a "live" gate was set in the red fluorescence histogram on those events which had at least the same DNA content as a human diploid cell (i.e. exclusion of bacteria or platelet aggregates having the same scatter light properties as leukocytes. The percentage of cells having produced ROS was analysed as well as their mean fluorescence intensity (amount of cleaved substrate, activity). For that purpose, the granulocytes were gated in the software program in the scatter diagram (Linear FSc. vs. Linear SSc.) as shown in figure 3.1 and its green fluorescence histogram (FL1) was analysed. A control sample was used to set a marker for fluorescence-1 (FL1) so that less than 1 - 3% of the events were positive. The percentage of positive cells in the test samples could then be determined by counting the number of events above this marker position. The mean fluorescence correlated with oxidation quantity per individual leukocyte.

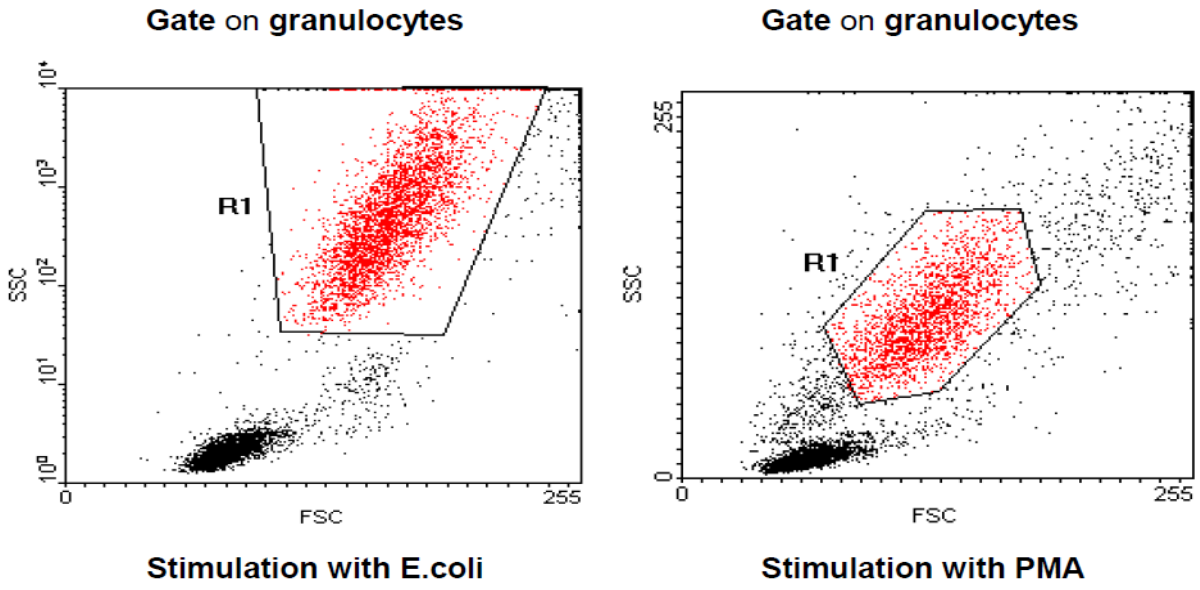

Figure 3:1: Typical gating strategy (Source, ORPEGEN Pharma; Burst test Test Kit Instructions)

3.3 Statistical analysis

Data analysis was done in consultation with a stastician using various statistical techniques. Comparisons between groups (e.g. HIV+ vs. HIV-) were done using Mann-Whitney's non-parametric test. No assumptions were made. Spearman's rank correlation test was done. The results were reported as medians with interquartile ranges. A 5% or lower (P≤0.05) significance level was used to determine significant findings.

3.4 Results

3.4.1 Demographics of the study population

The demographic characteristics of the study group are summarized in Table 3.1. The study group consisted of 12 HIV-infected individuals and 14 uninfected controls. The HIV-infected participants' mean age was 29.5 and 25 for the uninfected control group. There was no significance difference (P=0.09) in age between the two groups. The HIV-infected group had a well maintained CD4 count averaging 443; were clinically well and had a median viral load of 6300. The uninfected control group had a significantly (P=0.002) higher CD4 count with an average of 905 and were also clinically well.

Table 3:1: Demographic characteristics of both the HIV-infected and uninfected control participants.

Parameter	HIV+ (*n*=12)	Controls (*n*=14)	P value
Male: Female	4:8	6:8	
Median age (yrs.)	29.5(27-45)	25(24-34)	0.09
Range	21-56	21-51	
Median CD4 cells/mm	443(323-543)	905(624-1079)	0.0002**
Median Viral load (copies/ml)	6300(37-19000)	ND	
Median Log Viral load	4.0(1.6-4.5)		

All the values in columns are medians (interquartile range) of HIV-infected (*n* = 12) and uninfected controls (*n* = 14). **Medians significant at P<0.05.

3.4.2 Neutrophil respiratory burst in HIV-infected group and HIV negative group

The results of the neutrophil respiratory burst of the HIV-infected and control groups are summarized in Table 3.2. The median % of resting neutrophils and after stimulation with a low stimulant fMLP was significantly (P = 0.001 & P = 0.002 respectively) different. The response to stronger stimulants PMA and *E. coli* of the two groups was not significantly different between the two groups.

Table 3:2: Neutrophil respiratory burst in HIV-infected group and HIV negative group

	Median Resting Neutrophils (%)	Median fMLP (%)	Mean PMA (%)	Mean *E.coli* (%)
HIV+	4.6(2.4-9.5)	13.7(5.7-17.5)	99.9	99.1
Controls	2.6(2.0-3.5)	5.9(3.4-8.9)	99.9	98.8
P - value	**0.041****	0.046**	0.78[a]	0.714[a]

The values are medians (interquartile ranges) percentages of neutrophils. ** Median significantly different at (P<0.05); [a] Not significant at P<0.05.

3.4.3 The total white cell count, absolute neutrophil count, CD38/8 viral load and total antioxidant status

Table 3.3 summarizes the results of other markers of disease and total antioxidant status measured as ORAC. As expected the CD4 count between the HIV-infected and control groups was significantly (P = 0.000) different. CD38/8 was significantly (P = 0.006) increased in HIV-infected group. The differences in the other markers of disease and total antioxidant status were not significant.

Table 3:3: Total white cell count, absolute Neutrophil count, CD38/8, CD4 count, viral load and TAS (ORAC)

	Total WCC	Mean Abso. Neut (%)	CD 38/8(%)	Mean CD4 Count cells/mm^3	Median Viral load (copies /ml)	Mean TAS (ORAC; µmol TE/L)
HIV+(*n=14*)	5.58±0.9	3.6±1	55.1(43.2-69.6)	443(323-543)	220	918.1(726.3-1073)
Controls (*n=12*)	6.79±2.7	4.3±2	32.6(27.6-40.4)	905(624-1079)	-	888.5(661-88.5)
P- value	0.155[b]	0.54[b]	**0.0069*** **	**0.00014*** **		0.74[b]

The values for cell counts are either median (interquartile range) for percentage cells count and mean ± S.D for absolute counts. *** Medians significantly different (P>0.05); [b] Medians not significantly different at P<0.05.

A negative correlation (r = -0.6; P<0.05)) between fMLP stimulated neutrophils and CD4 count was noted.

3.5 Discussion

HIV infection is associated with on-going and aberrant activation of both the innate and adaptive immune systems. The central feature of innate immune response is the recruitment and activation of neutrophils at the site of infection, with subsequent generation of ROS (Elbim *et al.,* 2001). In this preliminary study, the neutrophil respiratory burst response in asymptomatic HIV infection was investigated as a possible indication of baseline levels of inflammation and *in vitro* response to immune stimulation. The aim was to determine whether the neutrophil respiratory burst response is enhanced in HIV infection and whether the resultant oxidative stress could be a contributing factor to the overall depletion of CD4+ T-lymphocytes associated with asymptomatic stages of the disease. Using the phagoburst test assay, the neutrophil respiratory burst was measured in HIV-infected individuals (*n = 12*) and compared with that of controls (*n = 14*). Interestingly, no differences in the response were detected with the more potent stimulating agents PMA and *E.coli,* suggesting that at this stage of the infection, neutrophils are not yet exhausted and retain the capacity to respond to these strong stimuli. Importantly, this test demonstrated that neutrophils from HIV-infected individuals at rest and in response to low

stimulating agent (fMLP), showed significantly (P = 0.041) higher levels of burst (13 ± 7) response than in the uninfected controls (3 ± 2); suggesting a 'pre-activation' or priming by ROS or pro-inflammatory cytokines *in vivo*. The significantly higher expression of CD38/8 may indicate that patients with asymptomatic HIV infection have higher immune activation and higher levels of oxidative stress *in vivo.* This was also demonstrated by statistically higher baseline resting levels and a 'primed' response to a low stimulating agent. The total antioxidant status (ORAC) showed no significant differences at this stage of the infection as shown in Table 3.3.

The enhanced respiratory burst in unstimulated/resting and neutrophils stimulated with fMLP is suggestive of a pre-activation or priming of the cells by ROS or pro-inflammatory cytokines *in vivo* which could be from other cells such as activated macrophages or T-cells. Supporting evidence of possible pre-activation and priming of neutrophils by TNF comes from a study by Elbin *et al.* (1996). The authors showed that TNF, granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 8 (IL-8) strongly prime a sub-population of neutrophils which strongly bound fMLP and produced large amounts of H_2O_2 in response to fMLP. Elbin *et al.* showed that in the process of priming, resting phagocytes acquire a state of pre-activation that enables them to produce a more powerful bactericidal response at the infection site (Elbin *et al.,* 1996). Tumour necrosis factor is therefore not only a pro-apoptotic cytokine but also a primer of neutrophils in a similar manner to that of IL-8, GM-CSF, IL-1 α & β (Elbim & Gougerot-Pocidalo, 1996).

Additional evidence of pre-activation, priming and increased respiratory burst comes from a study by Bandres *et al.* who showed an enhanced respiratory burst and phagocytosis by neutrophils and monocytes of men with stage 1 HIV infection (Bandres *et al.,* 1998). However, the results from previous studies on respiratory burst have been contradictory. They range from decreased burst in monocytes (Dobmeyer *et al.,* 1995; Michailidis *et al.,* 2012), decreased oxidative burst in AIDS, which correlated with CD4 count (Elbim *et al.,* 1996) to increased phagocytosis and oxidative burst (Bandres *et al.,* 1993) to normal oxidative burst in HIV (Nottet *et al.,* 1993). In this study there was normal respiratory burst in both HIV positive and negative controls patients, however, there was enhanced oxidative burst of resting and fMLP stimulated neutrophils in HIV positive patients than in negative controls suggesting a preactivation or priming by the inflammatory cytokines (TNF, IL-1, 8). This was in agreement with of our findings in our preliminary study.

Previous study findings may have been contradictory due to the isolation of neutrophils from their whole blood environment (Elbim & Lizard, 2008). In line with this, Dobmeyer *et al.* in their study showed a significant decrease in respiratory burst response of asymptomatic HIV infection

patients in contrast to those with AIDS and this correlated well with CD4 count (Dobmeyer *et al.,* 1995). In this study, we used whole blood in order to maintain the phagocytes in their normal environment as much as possible. The contradictions could also have arisen from the stage of the HIV infection investigated. Thus it was important to correlate the respiratory burst response findings with CD4 count. The staging of the disease is critical in the interpretation of the results. There seems to be either normal or increased respiratory burst in early or stage one of HIV infection (Dobmeyer *et al.,* 1995; Michailidis *et al.,* 2012). As the disease progresses there appears to be a decrease in respiratory burst as the ROS and inflammatory cytokines cause the dysfunction and increased apoptosis of the neutrophils (Elbim *et al.,* 1996; Michailidis *et al.,* 2012).

The results of our study not only confirms enhanced neutrophil respiratory burst in HIV infection but also reported a negative correlation ($r = -0.6$; $P<0.05$)) of fMLP stimulated neutrophils with CD4 count showing a possible link between the respiratory burst (at rest and fMLP), immune activation and apoptosis of the CD4+ T-lymphocytes in HIV infection. It has previously been reported that the cytochrome release from the mitochondrial is associated with exhaustion of glutathione and that Bcl-2 driven blockage of cytochrome C release hinders depletion of glutathione concentration, thus modifying the redox environment towards a reducing one which is unfavorable for apoptosis (Circu *et al.,* 2008: Circu & Aw, 2010).

In order to determine the levels of immune activation, we investigated the expression of CD38/8, a marker of activation on CD8+ T-lymphocytes. A significant ($P = 0.0018$) difference in expression of the activation marker between the two groups (HIV positive individuals and uninfected controls) was noted. The significantly higher expression of CD38/8 may indicate that patients with asymptomatic HIV infection have higher immune activation and higher levels of oxidative stress *in vivo.*

Thus far, only a few studies on respiratory burst response have been performed on neutrophils in HIV infection. Most of the previous studies were performed on monocytes (Spear *et al.,* 1990; Dobmeyer *et al.,* 1995; Elbim *et al.,* 1996). A limitation of our study was that the phagoburst assay only measures the neutrophils respiratory burst. It does not measure other cells such as activated macrophages and the 'NOX' family members which are also known to have NADPH oxidase system thus capable of respiratory burst response. Another limitation of this study was its preliminary nature which provides 'snap-shot' insight into neutrophil respiratory burst response towards various stimulants at the time of patient sampling. Follow-up studies may be of value. In addition, although the neutrophil respiratory burst assay is not specific for HIV infection only; it was of value here in demonstrating the significant differences in HIV at this

stage compared with the uninfected group. Other potentially confounding factors were that specific tests for diagnosing underlying subclinical infections were not performed and smoking and alcohol habits were not documented.

3.6 Conclusions

In summary, neutrophils from HIV-infected individuals showed a "primed" response at rest and to low stimulating agent (fMLP), which was statistically different from controls. This may indicate that patients with asymptomatic HIV infection have higher levels of oxidative stress *in vivo*. Thus the respiratory burst may be an important contributing factor in inflammation-associated oxidative stress in HIV infection and increased depletion of CD4+ T-lymphocytes in the asymptomatic stages of HIV infection.

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CHAPTER 4

Enhanced lipid peroxidation is associated with decreased antioxidant capacity and a lower CD4 count in untreated asymptomatic HIV-infected individuals in South African participants

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Enhanced lipid peroxidation is associated with decreased antioxidant capacity and a lower CD4 count in untreated asymptomatic HIV-infected individuals in South African participants

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Contributorship: SM researched literature, wrote the first draft and acquired data of this study. JM was involved in concept and design, protocol development reviewing and intellectual input. AA was involved in concept and design, protocol development, reviewing and intellectual input of the study. HI was involved in concept and design, protocol development, gaining of ethical approval, participants' recruitment, reviewing and intellectual input.

Abstract

Chronic inflammation and immune activation are hallmarks of HIV infection, resulting in chronic oxidative stress with over-utilization of antioxidant defences, which may contribute to the loss of immune cells and faster disease progression. Low levels of antioxidants in HIV positive individuals have been associated with frequent opportunistic infections and increased mortality risk. The purpose of this study was to determine the baseline total antioxidant status, oxidative stress biomarkers, antioxidant enzymes and glutathione redox

status in untreated HIV positive South African participants and compare them with uninfected control group.

In a cross-sectional study, blood samples of 20 untreated HIV positive individuals and 20 controls were analysed for the total antioxidant status (ferric reducing antioxidant potential, oxygen radical absorbance capacity & trolox equivalent antioxidant capacity), lipid peroxidation [(MDA, measured as TBARS) & CDs] glutathione redox status (GSH:GSSG ratio) and activity of antioxidant enzymes [SOD, CAT & GPx] in red blood cells. The results were also correlated with CD4 counts, viral loads and markers of inflammation in these groups.

There was a significant increase in catalase activity (0.36 (0.28-1.3) vs. 0.25 (0.21-0.29) µg/mg/min: P = 0.0028), and lipid peroxidation markers (CDs – 195.6 (144.1-238.0) vs. 137 (131.9-151.3) µm/L: P = 0.0164) and a decrease in the antioxidant status (TEAC – 4853 (2391-7121) vs. 7512 (7231–7711) µmole TE/L: P = 0.0022) in HIV positive individuals. There was a positive correlation between (r = 0.79; P<0.01) the antioxidant status and CD4 counts and negative correlation (r = -0.8; P = 0.008) between redox status and MDA levels.

Increased oxidative stress with corresponding decrease in antioxidant capacity in untreated HIV positive individuals with lower CD4 count has implications for untreated patients with relatively well-maintained CD4 counts and who are already showing the effects of oxidative stress.

Keywords: Oxidative stress, total antioxidant status, antioxidant enzymes, glutathione redox status, lipid peroxidation.

4.1 Introduction

A major characteristic of HIV infection is chronic inflammation and immune activation, which may result in chronic oxidative stress with over-utilization of the endogenous antioxidant defences. This may contribute to the loss of immune cells and disease progression. Low levels of antioxidants in HIV-infected individuals have been associated with frequent opportunistic infections, faster disease progression and an increased risk of mortality [1,2,3,4]. A dysfunctional antioxidant defense system which includes a decreased TAS and decreased levels and activities of vitamin C, E, carotenoids, SOD, GPx, CAT and elevated markers of lipid peroxidation such as MDA/TBARS and conjugated dienes, have all been reported in HIV-infected individuals [5;6;7].

Previous studies have proposed a role of oxidative stress in the stimulation of HIV replication and the progression of HIV infection to AIDS [8,9]. Importantly, the total antioxidant capacity (TAC) has been proposed as an early novel biomarker of oxidative stress in HIV infection and may be utilized as a monitoring tool of tissue damage by free radicals. TAC therefore, may be useful to monitor and optimize antioxidant therapy in HIV infection [5,6,7]. A

significant decrease in activity of erythrocyte antioxidant enzyme activity has been observed in AIDS patients when compared to HIV-infected and healthy subjects [6]. The central role glutathione, a tripeptide of glycine, glutamate and cysteine, plays in cellular redox homeostasis and the subsequent importance of this redox balance in cell apoptosis, is well established in various cell types. This makes glutathione redox status a reliable marker of oxidative stress. Importantly, caspases, key players in apoptosis are cysteine-dependent and glutathione redox status sensitive enzymes [10,11,12]. Staal *et al.* (1992) observed significantly lower GSH levels in CD4+ and CD8+ T cells from HIV-infected individuals compared with uninfected control individual, the greatest decrease occurring in the later stages of the infection. These results suggest that intracellular GSH levels could be an important aspect in HIV infection and progression to AIDS [10].

The baseline levels of markers of total antioxidant status, markers of lipid peroxidation and glutathione redox status of plasma and blood samples, have previously been used as indicators of oxidative stress in HIV infection, but not in the context of asymptomatic infection. In this study, we investigated how the baseline antioxidant status and oxidative stress markers relate to markers of disease and inflammation in untreated HIV infection. Therefore, the baseline total antioxidant status, oxidative stress biomarkers, activity of antioxidant enzymes and the glutathione redox status in asymptomatic untreated HIV-infected individuals in comparison to uninfected controls were determined and correlated with other markers of the disease. To our knowledge, this is the first study to report on these parameters in the context of asymptomatic untreated HIV infection in South African participants. Using a battery of assays, that included, FRAP, ORAC & trolox equivalent antioxidant capacity (TEAC), the total antioxidant status was determined, while MDA/TBARS using HPLC methodology and conjugated dienes were used as markers of lipid peroxidation.

4.2 Materials and Methods

4.2.1 Reagents

Monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and dibasic sodium phosphate ($Na_2HPO_4.12H_2O$), hydrochloric acid (HCl), sodium phosphate, (NaH_2PO_4), perchloric acid ($HClO_4$), hydrogen peroxide, chloroform, methanol, cyclohexane, sodium di-hydrogen orthophosphate dehydrate ($NaH_2PO_4.2H_2O$), di-sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$), metaphoshoric acid (MPA), trichloric acetic acid, 5,5' dithiobis-(2-nitrobenzoic acid) were purchased from Merck Chemicals (South Africa). Fluorescein sodium salt ($C_{20}H_{10}Na_2O_5$), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), L-ascorbic acid and iron (III) chloride salt, GSH, GR, NADPH solution and sodium azide solution, nicotinamide adenine dinucleotide phosphate (NADPH), 2,4,6-Tri [2-pyridyl]-s-triazine (TPTZ),1-methyl-2-vinyl-

pyridinium trifluoromethane sulfonate (M2VP), 6-hydroxydopamine (6-HD) and GSSG were purchased from Sigma-Aldrich (South Africa).

The BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in sodium hydroxide) and BCA reagent B (4% cupric acid), and albumin standard (bovine serum albumin, 2.0 mg/ml in 0.9% saline and 0.05% sodium azide) working stock solution were purchased from Thermo scientific (South Africa). The fluorescence was read on a Fluoroskan plate reader (Thermo Electron Corporation, Germany). The absorbance was read in a Multiskan plate reader (Thermo Electron Corporation, Germany).

4.2.2 Study population and design

In this cross-sectional study, twenty untreated HIV positive individuals (6 males; median age 32 (27-42); 14 females, mean age 29 (27-34) and 20 controls (7 males mean age 28 (26-30); 13 females mean age 27 (24-38) were sourced from the same HIV testing and prevention primary health clinic in Crossroads, Cape Town (South Africa). In this phase of the study, only 20 HIV positive participants and 20 uninfected controls were used as this was part of larger study investigating the impact of inflammation-induced oxidative stress in asymptomatic untreated HIV infection. There was no significant difference in age between the two groups. The patients' demographics are summarised in Table 3.1. The HIV positive group had a significantly ($P = 0.0003$) lower CD4 count compared to the control group. Eleven of the HIV positive participants had CD4>500, while 9 had CD4<500 but greater than 200. Informed consent was received from all the participating subjects. Inclusion criteria for the study participants were 21 years or older, individuals with HIV infection and CD4 counts >200 and not taking anti-retrovirals (ARV's) or any other chronic medication or antioxidant supplements. Exclusion criteria included patients with tuberculosis (TB) or other co-infections and those receiving antiretroviral therapy, anti-TB treatment or other antibiotic treatment, antioxidant supplementation, minerals and vitamins supplements, aspirin or any other drug e.g. anti-inflammatory, with established antioxidant properties. Ethics approval was obtained from both the clinical site: University of Cape Town: REC: REF: 417/2006 and laboratory site, University of Stellenbosch: N07/09/197.This sub study was added as an addendum to the ethics approval received for the original project N07/09/197 and performed in accordance to Helsinki Declaration of 1975, as revised in 2000.

4.2.3 Sample processing

The blood samples were collected in the morning from non-fasting participants from the antecubital vein in the forearm in (ethylenediaminetetraacetic acid) EDTA, sodium citrate and Heparin vacutainers and transported to the laboratory on ice. Plasma samples for the total antioxidant status assays were processed appropriately by centrifuging them at 1200 g at 4 ^{o}C for 10 min, aliquoting 500 µl of plasma and storing them at -80 ^{o}C till the day of analyses.

The samples for the GSSG analysis were prepared by mixing 100 µl of whole blood with 10 µl of M2VP to derivatize GSH and stored at -80 ^{o}C. Erythrocyte lysates were prepared by centrifuging EDTA whole blood (as described above), removing the white buffy layer (leukocytes) and washing the RBC's 3 times in phosphate buffer (PBS). The red blood cells were lysed in 4 times their volume ice cold distilled water and then centrifuged at 1200 g for 15 min at 4 ^{o}C. The supernatant (erythrocyte lysate) was collected and frozen at -80 ^{o}C until analysed. EDTA blood samples were used for CD counts and viral loads, while serum samples from the sodium citrate tubes, were used for analysis of inflammatory markers (fibrinogen and D-dimers). The study was carried out from 12th of September 2011 to 20th of December 2011. Recruitment of participants took 11/2 month (12th of September to 25th of October). About 10 ml and 5 ml of blood were taken into heparin, EDTA or citrate tubes respectively.

4.2.4 CD 4 counts, viral loads, fibrinogen and D-Dimers

Heparin blood samples (50 µl) were mixed with 20 µl of the multitest reagent of antibody mix and incubated for 15 min in the dark at room temperature. Red blood cells were lysed with 450 fluorescent activated cell sorters (FACS) lysing solution from Beckton Dickinson. The cells were washed once in PBS (1300 g), resuspended and analysed by flow cytometry (FACSCalibur B.D Biosciences., San Jose, CA, USA).

EDTA blood was used for quantification of viral load. The plasma viral load was determined by the amplification of viral RNA on duplicate samples using PCR and detecting the real time molecular beacons according to assay kit's manufacturers (BioMerieux Inc., Boxtel, Netherlands) specification.

Fibrinogen, a marker of inflammation, was analysed using the Hemos IL- Fibrinogen C method using ACL TOP (Beckman Coulter, Inc., USA) that utilises excess of thrombin to convert fibrinogen to fibrin in diluted plasma. Sodium citrated blood was centrifuged at 2000 g for 15 min and plasma aliquoted. Plasma was mixed with fibrinogen reagent and absorbance read at 405 nm.

D-dimer, a marker of fibrinogen breakdown and clot formation, thus an indirect marker of inflammation, was estimated by spectrophotometry using the IL-D-dimer method, which is an automated immunoassay for quantitative determination of D-dimers in plasma. Plasma from sodium citrate blood samples, was mixed with latex reagent and buffer all supplied by Beckman Coulter (SA) and agglutination, measured as decrease in absorbance was read at 405 nm using ACL TOP from Beckman Coulter Inc.,(USA).

4.2.5 Measurement of total antioxidant status (TAS), antioxidant enzymes and lipid peroxidation

To measure the plasma TAS ORAC, FRAP and TEAC assays were used. ORAC was determined using the method described by Ou *et al.* (2001) [13]. In this assay, fluorescence was measured at 1 min intervals at an emission wavelength of 563 nm and excitation wavelength of 540 nm using a fluoroskan plate reader (Thermo Electron Corporation Germany) until zero fluorescence occurred. FRAP was determined using the assay method of Benzie & Strain (1996) [14]. The reaction mixture was incubated at 37 ^{o}C for 30 min and absorbance read using a multiskan plate reader (Thermo Electron Corporation, Germany). TEAC was determined using Re *et al.* (1999) method [15]. The reaction mixture was left at room temperature for 30 min before taking a reading after which, the absorbance was read in a multiskan plate reader (Thermo Electron Corporation). The activity of CAT and SOD in RBC lysates was analysed using the modified method of Ellerby & Bredesen (2000) [16]. Glutathione peroxidase activity was analysed using the method of Flohe & Gunzler which measures GPx activity indirectly [17]. All the RBC's antioxidant enzyme activities were expressed per milligram of proteins per millilitre of the sample. Therefore, total proteins of the RBC lysates were determined using the BCA assay method. Lipid peroxidation was measured as CDs and TBARS/MDA. The level of plasma conjugated dienes was estimated according to the method of Recknagel & Glende (1984) [18]. Plasma TBARS were measured using Khoschsorur (2000) method [19]. Agilent technology 1200 series HPLC (Germany) system was used in the analysis of plasma TBARS. The glutathione redox analysis was done according to of Asensi *et al.* (1999) method [20].

4.3 Statistical analysis

The data was analysed using the Graphpad Prism version 5 statistical analysis software. Analysis of variance (ANOVA) was used to determine whether the means of the two groups (HIV positive and control groups) differed significantly. Comparisons between the groups (HIV positive and control groups) were done. Mann Whitney non-parametric test and spearman's correlation were applied. Results were reported as medians with interquartile range. A 5% or lower significance level was used to judge significant findings ($P \leq 0.05$).

4.4 Results

4.4.1 Demographics of study population

The participants' demographic characteristics are summarized in Table 4.1. The group included 20 HIV positive individuals; (6 males; mean age 32 (27-42); 14 females, mean age 29 (27-34) and 20 controls (7 males mean age 28 (26-30); 13 females mean age 27 (24-38). Using unpaired t-test, there was no significant difference in age between the two groups ($P = 0.187$). The HIV positive group had a well maintained CD4 count averaging 458 (median 427 (310-734) and clinically well with a median viral load of 43959 median 2240 (628.5 - 40998).

The control group had a high CD4 count with an average of 861 and also clinically well. The CD4 count of the two groups was significantly (P = 0.0001) different, the other parameters were not. Thirteen of the HIV-infected participants had CD4 > 350, while 7 had CD4 < 350 but greater than 200.

Table 4:1: Demographics characteristics of both the HIV positive and controls.

Parameter	HIV positive group (*n*=20)	Controls (*n*=20)	P -values
Male: Female (%)	6:14	7:13	
Median age (yrs.)	30(27-35)	28(24-32)	0.187
Range	22-47	21-58	
Median CD4 cells/mm^3	427(310-734)	810(700-997)	**0.0003*** **
Median Viral load (copies/ml)	2240(628.5-40998)	ND	
Log Viral load	3.7(2.9-5.0)		

All the values in columns are median (interquartile ranges) of HIV positive (*n* = 20) and controls (*n* = 20).*** CD4 counts were significantly different at P = 0.05.

4.4.2 Plasma total antioxidant status and levels of lipid peroxidation products

The total antioxidant status and lipid peroxidation products of the forty study participants are shown in Table 4.2. There was a significant (P<0.005) difference in TAS as measured by TEAC assay between the two groups. The ORAC and FRAP showed no significant differences. However there was a statistically significant difference in TEAC between the groups. Plasma conjugated dienes concentration were significantly (P<0.05) higher in HIV positive individuals when compared with control individuals.

Table 4:2: Plasma total antioxidant status of study participants measured as FRAP, ORAC and TEAC and lipid peroxidation markers (MDA & CDs).

	Total antioxidant status			Lipid peroxidation markers	
	FRAP (µmole/L)	**ORAC (µmol TE/L)**	**TEAC (µmole TE/L)**	**MDA (µmol/L)**	**CD (µm/L**
HIV positive	421.7(385.5-493.1)	344.1(269.9-492.9)	4853(2391-7121)	1.3(1.1-1.7)	195.6(144.1-238.0)
Controls	418.9(378.7-497.2)	441.2(299.1-688.2)	7512(7231-7711)	1.0(0.8-1.3)	137.6(131.9-151.3)
P -values	0.9871	0.0775	0.0022**	0.0533	0.0164**

All values in columns are medians (interquartile ranges) (all samples analysed in triplicates and repeated. Plasma total antioxidant measured as TEAC was significantly higher; ** at P = 0.05 in HIV positive group when compared with controls. CDs were significantly different between the groups.

4.4.3 Red blood cell (RBC) antioxidant enzymes and Erythrocyte redox status

Table 4.3 show the RBC antioxidant enzyme activities and glutathione redox status as indicated by GSH:GSSG ratio of the study population. Catalase activity was significantly (P = 0.003) increased in HIV positive individuals when compared to the controls, while no significant differences were shown for SOD and GPx.

The ratio was significantly (P = 0.014) lower in the HIV positive individuals, indicating utilisation or conversion of GSH to GSSG, thus increased oxidative stress.

Table 4:3: RBC enzyme activity and glutathione redox status of the participants

	RBC enzyme activity			Glutathione redox status		
	CAT (µg/mg/min)	**SOD (µg/mg/min)**	**GPx (µg/mg/min)**	**GSHt (µM)**	**GSSG (µM)**	**Ratio**
HIV positive	0.36(0.28-1.3)	1.0(0.79-1.2)	0.06(0.05-0.07)	1128(944.1-1241)	135.1(68.0-162.6)	7.9(7.2-20.3)
Controls	0.25(0.21-0.29)	0.75(0.44-1.31)	0.07(0.05-0.16)	1206(1080-1388)	104.8(62.6-126.2)	11.8(9.9-17.7)
P-values	0.0028**	0.117	0.1520	0.093*	0.22	0.014**

**Medians significantly different at P< 0.005 between the two groups. Catalase activity was significantly (P =0.0028) between the two groups the total glutathione (GSH) GSSG and glutathione redox ratio are shown in Table3. * The total glutathione and GSSG in HIV-positive group although lower than the control group, the difference was not significant. However, the redox ratio was significantly (P = 0.014) different.

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4.4.4 Other markers of disease in HIV infection

Table 4.4 shows the values of other markers of inflammation, viral load and CD4 counts of these groups. When compared with the controls, as expected, the HIV positive individuals had significantly (P<0.05) lower CD4 counts. D-dimers, an indirect (marker of fibrinogen breakdown and clot formation) and marker of inflammation was significantly (P<0.05) higher in the HIV positive individuals than in controls.

Table 4:4: Other markers of disease in HIV infection

Other markers of disease in HIV infection				
	D-dimers (mg/L)	**CD4 count (cells/mm^3**	**Median Viral load (copies/ mL**	**Log viral load**
HIV positive	0.24(0.2-0.4)	427(310-734)	2240(628.5-40998)	3.7(2.9-5.0)
Controls	0.2(0.20-0.22)	810(700-997)	N/A	N/A
P- value	0.0306**	0.0003***		

The table shows the D-dimers CD4 count and viral load in this cohort expressed as medians (interquartile range): ** indicates that the medians were significantly different at P<0.05. *** CD4 count was significantly different (P<0.001).

A negative correlation (r = -0.63; P<0.05) between SOD and redox ratio was noted in the untreated HIV positive group. There was a high negative correlation (r = -0.8; P = 0.008) between the glutathione ratio and MDA. The viral load was inversely correlated (r = - 0.58; P = 0.03) with redox status. There was a negative correlation between D-dimers and catalase (r = -0.701; P<0.05); a positive correlation between total antioxidant status and CD4's (r = 0.79; P<0.01), GPx and viral load (r = 0.73; P<0.05), SOD and fibrinogen (r = -0.68; P<0.05), and SOD and redox status (r = -0.63; P<0.05).

4.5 Discussion

HIV infection is characterized by chronic inflammation and persistent immune activation, which could lead to chronic oxidative stress and deficiencies in antioxidant defenses. In this study, we determined the baseline total antioxidant status, oxidative stress biomarkers, antioxidant enzyme activities and glutathione redox status in asymptomatic untreated HIV positive individuals and compared with those of controls.

Results from this study showed increased catalase activity, increased levels of lipid peroxidation products (MDA & CDs) and decreased total antioxidant status (TEAC) in the untreated HIV positive individuals when compared to the control group. The increase in catalase activity in this phase of HIV infection could be in response to heightened levels of oxidative stress as evidenced by the increased levels of lipid peroxidation markers as shown

in this study. Catalase eliminates H_2O_2 in the cells; while the antioxidant capacity of GSH protects the cells from oxidative stress [21,22,23]. Catalase is a ubiquitous antioxidant enzyme that occurs in high levels in human red blood cells. It is a convenient marker of oxidative stress in living systems [24, 25]. It protects the cells from H_2O_2 generated within them and therefore plays a vital role in acquiring tolerance to oxidative stress in the adaptive response of cells [26]. Thus, the increased catalase activity in the HIV positive individuals could be indicative of increased oxidative stress in those individuals. Previous studies have shown a significantly decreased catalase activity in AIDS as compared to HIV infection and healthy individuals supporting our finding of increased catalase activity in asymptomatic untreated HIV-infected individuals [27]. The increase in catalase in asymptomatic HIV infection could be attributed to an increased synthesis of these enzymes precursors as a response to increased oxidative stress.

In this study a significant ($P = 0.002$) decrease in total antioxidant status (TEAC) was observed. There was no significant differences in TAS measured as FRAP and ORAC. FRAP assay is based on ability of antioxidants to reduce Fe (III) to Fe (II), thus unspecific and not a direct measure of antioxidant capacity [14]. FRAP assay cannot measure antioxidant capacity of certain antioxidants because the iron (II) does not react with SH (sulphur and hydrogen) group containing antioxidants (e.g. lipoic acid and GSH) [28]. Although ORAC is a simple, sensitive and reliable assay, sometimes its use is disappointing due to lack of discrimination among health and diseased individuals. The assay is influenced by proteins of biological fluids; furthermore, high levels of bilirubin and uric acid are major contributors of increased serum and plasma ORAC values. Similar or ORAC values not significantly different in normal individuals and in diabetes, cancer and HIV positive individuals have been reported [28]. These shortcomings of FRAP and ORAC assay could explain lack of significant differences in our study.

Lipid peroxidation markers (MDA/TBARS and CDs) measure oxidative damage by ROS, and increased levels in the untreated HIV positive individuals would indicate increased levels of oxidative stress in these individuals. Chronic inflammation and persistent immune activation in HIV infection, induces chronic oxidative stress, which may result in over-utilization of the antioxidant defences, as previously mentioned. This explains our finding of lower total antioxidant status (measured as TEAC) in the untreated HIV positive individuals than in the controls group. In this study we investigated the baseline levels of lipid peroxidation products (MDA measured as TBARS and CDs. Increased oxidative stress in HIV infection is supported by our finding of significantly higher levels of MDA/TBARS ($P = 0.053$), catalase ($P = 0.0028$) and conjugated dienes ($P = 0.016$) and lower total antioxidant status (TEAC) in HIV positive individuals when compared to controls. Due to the non-specificity of the TBARS assay, two different assays for lipid peroxidation were used for a more reliable estimation and both indicated increased lipid peroxidation in the current study.

The ratio of GSH in relation to GSSG (redox status) is a reliable marker of oxidative stress in living systems [22,27]. Usually GSH:GSSG ratio is approximately 90% to 10% in humans and a change in the ratio or a decrease in GSH/increase in GSSG, indicates oxidative stress. In this study, the lower total glutathione (tGSH) and GSH:GSSG ratio in HIV positive individuals compared to the controls indicated an increased oxidative stress. Another important finding in this study was the high negative correlation ($r = -0.8$; $P = 0.008$) between the glutathione ratio and MDA. Therefore, the higher the levels of lipid peroxidation, the lower the GSH redox potential. This has important implications considering that this group is not yet on any ARV treatment, and seemingly clinically well with reasonably well-maintained CD4 counts, but are already showing adverse effects of oxidative stress as indicated by higher levels of lipid peroxidation and decreased GSH redox status. Glutathione being a major intercellular free radical scavenger is thought to inhibit activation of NF-kB which is involved in the transcription of HIV-1. Depletion of glutathione leads to a glutathione redox imbalance. Consequently, the arising oxidative stress may lead to activation of NF-kB, increased HIV transcription and replication [9,27]. This further promotes increased oxidative stress as indicated by increased levels of lipid peroxidation products (MDA and CDs). Several studies have indicated significantly lower GSH levels in CD4+ and CD8+ T-cells from HIV positive individuals, with the greatest decrease occurring in the later stages of the infection [10,29]. These results suggest that intracellular GSH levels could be a key aspect in HIV infection and progression to AIDS. Interestingly, viral load was inversely correlated ($r = -0.58$; $P = 0.03$) with redox status, suggesting the higher the viral load the lower the redox potential in these patients.

SOD is an important antioxidant enzyme as it protects the cells from the oxidative stress-related damage of superoxide radicals. In the current study, there were no significant differences between the two groups investigated, although the median SOD activities were higher in untreated HIV positive individuals than in the control group. The lack of significant differences in SOD activity between the two groups in indicates an intact antioxidant enzymes defence mechanism in this relatively asymptomatic phase of HIV infection. SOD requires the presence of GSH to be effective. Hence, in the current study, there was a negative correlation between SOD and GSH:GSSG ratio. Another important antioxidant enzyme, GPx also protects the cells from oxidative damage by catalysing the removal of hydroperoxides at the expense of glutathione. Although lower GPx median values in HIV positive individuals were shown in the current study, it was not significant, again indicating an intact antioxidant enzymes defence mechanism in this relatively asymptomatic phase of HIV infection. A dysfunctional antioxidant enzyme defence appears to occur only at later stages of the HIV infection.

In their study of activity of blood antioxidant enzymes in HIV infection, Kostyushov, *et al.* found a much more pronounced increase in lipid peroxidation (MDA) and a decrease in total

antioxidant status and activity of blood serum antioxidant enzymes (SOD, CAT and GPx) in patients with manifested forms of AIDS [30]. The stage of HIV infection is also important, thus the more advanced the infection, the more the increase in lipid peroxidation the more the decrease in total antioxidants (TEAC) and antioxidant enzymes such as GPx and SOD. Our finding of increased catalase activity, increased levels of lipid peroxidation products (MDA &CDs) and decreased total antioxidant status in the HIV positive individuals in this asymptomatic phase of the disease confirms what has been described in previous studies.

D-dimers have been shown to be an important marker of underlying inflammation and have strong predictive value for adverse outcomes in HIV infection [28]. The median value of D-dimers in HIV positive individuals was higher than in control group indicating increased levels of inflammation in HIV infection. In addition, there was a negative correlation between D-dimers and catalase ($r = -0.701$; $P<0.05$); suggesting that the higher the levels of inflammation, the more oxidative stress is placed on the system; which results initially in an increase in protective antioxidant enzymes in this asymptomatic phase of the infection but decreases later as the disease progresses.

In this study we found a positive correlation between total antioxidant status and CD4s ($r = 0.79$; $P<0.01$), GPx and viral load ($r = 0.73$; $P<0.05$), SOD and fibrinogen ($r = -0.68$; $P<0.05$), and SOD and redox status ($r = -0.63$; $P<0.05$). Antioxidant enzymes belong to a group of thiol enzymes. In order to function effectively, they require the presence of thiols. Therefore they utilize free glutathione-SH group for their catalytic activity. Glutathione also supplies -SH groups during redox reactions to protect the cells against hydroxyl radicals and other ROS, explaining the finding of the negative correlation between SOD and redox status in the current study.

Demonstration of decreased TAS, increased catalase activity and lipid peroxidation in asymptomatic untreated HIV infection, indicates that catalase enzyme is essential in protection of cells against severe oxidative stress. The findings also indicate that TAS might be critical in protecting the immune system from oxidative damage as a result of ROS insult.

A limitation of this study was its cross-sectional nature, which provides ‘snap-shot’ insight into oxidative stress levels at the time of patient sampling; nevertheless it was of value in demonstrating the significant differences in HIV at this stage compared with the uninfected group. In addition, we were not able to do specific tests for diagnosing underlying subclinical infections and smoking and alcohol habits were not documented. Another limitation of the study was the fact that ages spanned 22 - 42 years and antioxidant status is known to change with age [31]. Nevertheless, the majority of the participants fell between the ages of 25 and 35, with median ages for the two groups being very close ($P = 0.187$).

It will be important to follow up these markers in longitudinal cohort studies to determine their true surrogate value with treatment intervention strategies.

4.6 Conclusions

The current study showed in this South African study population, an increased oxidative stress as evidenced by increased catalase activity, MDA and CDs' with corresponding decrease in antioxidant capacity in untreated HIV-infected individuals with lower CD4 count. MDA levels were inversely correlated with glutathione redox status in asymptomatic untreated HIV infection. There was also a direct correlation between total antioxidant status (ORAC) and CD4 count. In addition, a higher viral load was linked to a lower GSH redox potential.

This has important implications for patients not yet on treatment who have relatively well-maintained CD4 counts who are already showing effects of oxidative stress.

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4.6.2 References

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CHAPTER 5

Effects of temperature, time and concentration on LPS-induced whole blood activation and antioxidant intervention in untreated asymptomatic HIV infection: An optimization study

CHAPTER 5

Effects of temperature, time and concentration on LPS-induced whole blood activation and antioxidant intervention in asymptomatic untreated HIV infection: An optimization study

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Abstract

Immune activation is a hallmark of HIV infection. Chronic immune activation is implicated in the depletion of CD4+ T-lymphocytes via apoptosis and therefore contributes to disease progression. LPS as a result of the breakdown of the intestinal mucosa is an important contributing factor in the on-going stimulation of the immune system. Annexin V/7-AAD viability dye has been used previously as a way of discriminating early apoptosis from late apoptosis and necrosis.

The aims of this study were to investigate and optimize the *in vitro* effects of time, temperature and concentration on LPS-induced immune activation in asymptomatic untreated HIV infection. The effect of varying concentrations of selected antioxidants (vitamin C & NAC) on LPS-induced activation was also determined. This was achieved by stimulation of whole blood samples (*n* = 20) with different concentrations of LPS ranging from 100 ng/ml to 5 µg/ml, and incubation of the samples with vitamin C (10 µM to 50 µM) or *N*-acetyl cysteine (5 mM) to 50 mM) for 1 hour or 24 hours. This was to develop a functional assay for LPS-induced activation and apoptosis measurement using flow cytometry.

The optimum concentration of LPS which gives maximum *in vitro* activation of whole blood was determined to be 2 µg/ml after overnight incubation with LPS. LPS-induced activation was shown to be inhibited at low temperatures (working on ice) when compared to room temperature. Low doses of vitamin C (10 mM) and NAC (5 µm) individually or as a cocktail were shown to confer protection to LPS-induced activation. Higher concentrations of

antioxidants above these optimum concentrations were shown to cause more activation and apoptosis than protection to these cells.

Antioxidants in high concentrations may be toxic to CD4+ T-cells; however at optimum doses they inhibit LPS-induced immune activation thereby protecting the cells from activation induced apoptosis.

Keywords: Optimization, LPS, Annexin V, 7-AAD, vitamin C, N-acetyl cysteine, whole blood activation.

5.1 Introduction

HIV infection is associated with chronic and aberrant activation of both the innate and adaptive immune systems. The resultant chronic inflammation and oxidative stress predispose patients to inflammation-associated complications and progression to AIDS. Chronic inflammation induces the up-regulated expression of markers of activation on immune cells and these become detectable on the surface of these cells as early and late immune activation markers (Gil *et al.,* 2003; Deeks *et al.,* 2004). The breakdown of the gastrointestinal barrier resulting in translocation of microbial products such as LPS into the systemic circulation is thought to be an important contributing factor underlying immune activation in HIV infection. LPS is known to activate various signalling pathways including the extracellular signal-regulated protein kinase (ERK), P38 and stress activated protein kinase/c-Jun N terminal kinase (SAPK/JNK), in addition to phosphoinositide 3 kinase (PI3) kinase and inhibitor of kappa B kinase/nuclear factor of kappa B (IKK/NF-kB) pathways in peripheral blood via toll-like receptor 4 (TLR4) complexes (Chow *et al.,* 2005; Shankey *et al.,* 2006; Hedley *et al.,* 2008). The effects of activation of these pathways can be measured by flow cytometry. The same approach can be used to study or monitor the impact of targeted inhibitors of these activation pathways.

CD45 also known as leukocyte common antigen (LCA) is a member of the protein tyrosine phosphate family expressed on all hematopoietic cells except mature erythrocytes and platelets (Braford, 1994). Different isoforms of CD45 arising from splicing of variable splicing of exon 4, 5, and which are specific to the activation and maturation state of the cells and cell type exist (Virts *et al.,* 1997). It plays an important role in TCR and BCR signal transduction. The use of CD45 fluorochrome conjugated monoclonal antibody has allowed the maximizing of gate purity and lymphocyte recovery, thus immunophenotyping, characterization, and enumeration of lymphocytes in HIV/AIDS and other immune system disorders can be done using flow cytometry (WHO, 2007).

CD4 is a single-chain transmembrane glycoprotein expressed on T-helper lymphocytes. It is a member of the immunoglobin g (Ig) superfamily. It is present on most thymocytes, where it

is co-expressed with CD8 (Littman, 1987). It is also expressed on all monocytes/macrophages and dendritic cells though dimly. It acts a co-receptor with the TCR during T-cell activation and thymic differentiation by binding MHC class II. CD4 is the main receptor for HIV-1 envelope protein gp-120 (Hannet, 1992). The use of CD4 fluorochrome conjugated monoclonal antibody has allowed the phenotyping, characterization, and enumeration of T-helper lymphocytes (CD4 antigen expressing cells) in HIV/AIDS and other immune system disorders using flow cytometry (WHO, 2007). Macrophages/monocytes, neutrophils as well as CD4+ T-cells express TLR4, the receptor for LPS on their surfaces, thus can be activated directly by LPS (Kabelitz, 2007; Xu *et al.,* 2005; Caramalho, *et al.,* 2003).

CD69 is a 60 kDa cell surface glycoprotein also known as activation inducer molecule. CD69 antigen is one of the earliest activation markers to be expressed. It is found on activated T, B lymphocytes and platelets, but is absent on resting lymphocytes (Testi, 1994; Sanchez-Madrid, 1995). It is also expressed by activated macrophages, NK cells and other cells such as neutrophils, eosinophils and platelets (Testi, 1994). The use CD69 fluorochrome conjugated monoclonal antibody has allowed the phenotyping, characterization, and enumeration of activated cells in HIV/AIDS and other immune system disorders using flow cytometry (Maino *et al.,* 1995).

CD25 antigen is a 55 kDa single chain glycoprotein, which in association with CD122 and CD 132 is the receptor for IL-2. It is found on activated T and B-cells, on activated monocytes, macrophages and T-cell clones (Anderson *et al.,* 1995). The use of CD25 fluorochrome conjugated monoclonal antibody has allowed the phenotyping, characterization, and enumeration of activated cells in HIV/AIDS and other immune system disorders using flow cytometry (Aandahl *et al.,* 2004).

Annexin V/7-AAD have been used previously as markers of early and late apoptosis. Annexin V is Ca^{+} dependent, phospholipid binding protein that is capable of binding specifically to PS even when conjugated with a fluorochrome such as FITC (Raynal & Pollard, 1994; Vermes *et al.,* 1995). The ability of Annexin V to bind to PS and specificity of 7-AAD to bind DNA guanine-cytosine base pair, enables them to be used in combination to discriminate between early apoptosis, late apoptosis and necrosis (Zelenin *et al.,* 1984; Koopman *et al.,* 1994). The interaction of Annexin V and PS is calcium-dependent, thus it is critical to avoid buffers containing EDTA or other calcium chelators during Annexin V staining. The baseline levels of apoptosis and necrosis varies widely within a population and therefore, even in the absence of induced apoptosis, most cell populations will show a small percentage of cells that are positive for apoptosis. Unstained or an untreated cell population

is included to define the baseline levels of apoptotic cells and dead cells (Vermes *et al.,* 1995; Koopman *et al.,* 1994). 7-AAD emits light in the far-red of the spectrum and can be separated from phycoerythrine (PE) or FITC or other tandem dyes, thus monoclonal antibodies conjugated with these fluorochromes can be used to identify different cell types simultaneously (Rabinovitch, 1986). It is important to note that cells lose their viability if left in the presence of 7-AAD dye for too long. It is therefore recommended that the incubation with Annexin V and 7-AAD be carried out on ice in order to maintain cell viability (Keeney, 1999).

Previous studies involving LPS-induced activation of cells have utilised varying concentrations of LPS (100 ng/ml - 5 µg/ml), NAC (5 - 50 µM), and vitamin C (25 - 50 nM), therefore it was important to optimize the concentrations for use in the current study (Dobmeyer *et al.,* 1996; Shang *et al.,* 2003; Yamanda *et al.,* 2006). Annexin V/7-AAD, CD4, CD25 and CD69 have been used previously as markers of early and late apoptosis and immune activation. In this study, we evaluated and optimized the *in vitro* effects of temperature and concentrations of LPS and selected antioxidants on LPS-induced immune activation in asymptomatic HIV infection.

5.2 Materials and techniques

5.2.1 Blood sample preparation

Twenty blood samples of consenting, untreated HIV positive individuals and 20 uninfected controls were used for optimization of LPS-induced activation assay and to assess the ability of selected antioxidants to inhibit this activation. Whole blood samples were stimulated with increasing concentrations of LPS (100 ng/ml - 5 µg/ml) at room temperature, 35 ^{o}C or on ice, incubated with or without antioxidants such as vitamin C (10 - 50 nM), and glutathione precursor (NAC) (5 - 50 µM), for 1 hour or 24 hours. CD69, CD25, Annexin V and 7-AAD expression on CD4+ T-cells were measured by flow cytometry.

5.2.2 Materials and methods

5.2.2.1 Study population and design

In this preliminary study, twenty untreated, asymptomatic HIV positive individuals and 20 uninfected controls (32 females and 8 males) were sourced from a single HIV testing and prevention primary health clinic in Crossroads, Cape Town (South Africa). The median age of the participants was 32 years (range 24 - 42). There was no significant difference (P = 0.27) in age between the two groups. The HIV-infected group had a significantly (P = 0.0003) lower CD4 count compared to the control group. The patients' demographics are summarised in Table 5.1. Informed consent was taken from all the participating subjects. Inclusion criteria for the study participants were 21 years or older, individuals with HIV infection and CD4 count>200; not on antiretrovirals (ARV's) or any other chronic medication or antioxidant supplements. Exclusion criteria included patients with tuberculosis (TB) or other co-infections

and those receiving antiretroviral therapy, anti-TB treatment or other antibiotic treatment, antioxidant supplementation, mineral and vitamins supplements, aspirin or any other drug e.g. anti-inflammatory.

An FC 500 flow cytometer (Beckman Coulter, Miami, Florida, USA) with two lasers, five fluorescence channels, CXP and FCS express V3 software were used in this study. Flow-check Fluorospheres, flow-set Fluorospheres, CD4-PE, CD4-APC CD69-APC, CD25-PE, Annexin V-FITC/7AAD-PE kit were obtained from Beckman Coulter, Miami, Florida (US.A). Vitamin C, NAC and LPS stock solution (from Salmonella at 1 mg/ml) were all purchased from Sigma Aldrich (S.A). A working solution of LPS (1 mg/ml) was made from the powder (Stock) by adding 1 ml PBS and reconstituting it by mixing well. Different concentrations of LPS were then made from this working solution.

5.2.3 Instrument set up

Alignment of the lasers was performed with a mixture of Flow-check and Flow-check beads. The appropriate voltages were determined and standardized with a mixture of Flow-set and Flow-set beads. Full matrix colour compensation (as shown in Table 5.3) was done using FITC, PE, APC & PerCP/PC5 stained whole blood cells prepared using the lyse and wash method as described below. A panel was created for test analysis using the cytometer settings established with Flow-Set and full matrix colour compensation.

CHAPTER 5

Instrument settings for optimization (LPS, Annexin V/7-AAD, activation& apoptosis assay)

FC 500 instrument settings

Table 5:1: Detectors for FC 5OO

Name	volts	Gain
FS	661	2.0
SS	759	20.0
FL1	264	1.0
FL2	445	1.0
FL3	465	1.0
FL4	588	1.0
FL5	577	1.0
Discriminator	FS	50

Table 5:2: Acquiring set up; discriminator

FS	50
SS	OFF
FL1	OFF
FL2	OFF
FL3	OFF
FL4	OFF
FL5	OFF
AUX	OFF

Table 5:3: Compensation

	FL1	FL2	FL3	FL4	FL5
FL1		0.6	0.0	0.0	0.0
FL2	14.7		15.5	16.9	0.0
FL3	9.8	40.8		8.8	0.0
FL4	0.0	13.9	30.4		0.0
FL5	0.0	0.0	0.0	0.0	

5.3 Laboratory investigation

5.3.1 Effects of concentration of LPS on activation and apoptosis

LPS is known to activate multiple signalling pathways in cells through its interaction with TLR4. The effects of different concentrations of LPS (100 ng/ml, 1 µg/ml, 2 µg/ml & 5 µg/ml) was evaluated to determine the optimal concentration that induces maximum activation and apoptosis (measured as %CD25 or %CD69 and Annexin V/ 7-AAD expression on CD4+ T-

cells). The samples were incubated for varying durations ranging from 10 minutes, 1 hour and overnight incubation to determine the optimum incubation time that would give the best results. In order to achieve this, 50 µl of blood was added into labelled tubes after which 20 µl of the different concentrations of LPS with or without the antioxidants were added. After the 10 minutes, 1 hour or overnight incubation, the samples were processed using either of the two different staining protocols (on ice and at room temperature) as discussed below.

5.3.2 Effect of temperature on LPS and 7-AAD activity

In order to determine the effects of temperature on LPS and 7-AAD activity, three different protocols were tested on a number of samples to determine the optimal method for activation and apoptosis analysis of cells. In the first protocol, the samples were always kept on ice during processing. The staining buffer was prepared by adding 1.25 ml foetal bovine saline (FBS) in 500 ml of phosphate buffered saline (PBS). The lysing solution was prepared by diluting 10 X fluorescein activated cell sorting (FACS) lyse solution. This was done by mixing 5 ml of the stock lyse solution with 45 ml reagent grade water. The FACS tubes were labelled appropriately and to each of the tubes 50 µL of whole blood was added. The antibodies mix (10 µl) of CD4, CD69 or CD25, Annexin V and 7-AAD were added to the tubes, vortexed gently, kept on ice and incubated for 15 minutes in the dark. The RBC's were lysed by adding 500 µl of FACS lysing solution, vortexed gently, kept on ice and incubated for 15 minutes in the dark. After lysing, 250 µl of ice cold staining buffer and 250 µl of binding buffer were added and the mixture centrifuged at 300 g for 5 minutes at room temperature. From the mixture 750 µl of supernatant was removed, pellets resuspended and 200 µl of staining buffer and 200 µl Annexin V binding buffer added. The preparation was analysed on flow cytometer within 30 minutes.

In the second protocol, the samples were not kept on ice but the processing was carried out at normal room temperature. The staining buffer and lysing solution were prepared as described above. The samples were processed for flow cytometer analysis as previously described. In this protocol, the 7-AAD was added only 5 - 15 minutes before analysis on flow cytometer due to adverse effects on the viability of cells if left in the presence of 7-AAD for too long. The first two protocols tested the effects of temperature and different concentration of LPS on T-cell activation.

In the third protocol, the effects of different concentration of antioxidants (vitamin C and NAC) on LPS stimulation of T-cells were tested. The reaction mixture consisting of whole blood, LPS and antioxidants was incubated at 37 ^{o}C for varying durations of time.

5.3.3 Effects of time on LPS-induced activation and apoptosis and antioxidant intervention

The samples were incubated at 37 ^{o}C with selected antioxidants for different duration ranging from 1 hour and overnight incubation to determine the optimum incubation times that give the best results. In order to achieve this, 50 µl of blood was added into the labelled tubes and 20 µl of vitamin C or 20 µl of NAC or 40 µl of both vitamin C and NAC were added, vortexed gently and incubated for 20 minutes. Different concentrations of the vitamins (vitamin C; 10 µM, 25 µM, 35 µM, 50 µM, and for NAC; 5 mM, 10 mM, 25 mM & 50 mM), were analysed to determine the optimum concentration of the antioxidants for inhibition of activation and/or apoptosis. After 20 minutes incubation of the whole blood with antioxidants, 20 µl of different concentrations (100 ng/ml, 1 µg/ml, 2 µg/ml, 5 µg/ml) of LPS was added. After the 1 hour or overnight incubation the samples were processed with the two different staining protocols (on ice and at room temperature) as discussed earlier and analysed on flow cytometer.

5.4 Data acquisition

5.4.1 Data acquisition and analysis for LPS optimization

For LPS optimization, plot quadrants were set using unstained cells for every sample such that the negative Annexin-V cells and 7-AAD negative population lay in the first decade of the Y and X axis. A sequential gating strategy, by first gating on lymphocytes for CD4+ T-cells and then gating on CD4 T-cells for the other markers as shown on figures 5.1 and 5.2 below was employed. This was in order to detect CD4+ T-cells expressing CD69, CD25, Annexin V or 7-AAD. A total of 300,000 events were acquired in order to analyse a minimum of 2000 CD4+ T-cells. CXP and FCS express V3 softwares were used to analyse flow cytometry data.

5.4.2 Data acquisition and analysis for vitamin C and *N*-acetyl cysteine

A sequential gating strategy on lymphocytes was employed in order to count at least 2000 CD4+ lymphocytes. The gating strategy is shown in figure 5.1 below. A minimum of 300 000 events were acquired to ensure consistency and a statistically relevant number of CD4+ T-cells were counted. CXP and FCS Express V3 softwares were used to analyse flow cytometry data.

5.5 Results

The participants' demographics are summarized in table 5.1. The group included 20 HIV-infected and 20 uninfected controls most of whom were females (Fisher's test P = 0.36). There was no significant (P>0.05) difference between the two groups in terms of age. The mean age was 35.4 years for HIV-infected group and 29 years for the HIV negative group. The HIV-infected group had a significantly (P = 0.006) lower CD4 count compared to the uninfected control group. The HIV-infected group had a well maintained CD4 count averaging 417 cells/mm^3 and were clinically well. Median viral load was 45705 copies/mL. The HIV negative group had a high CD4 count with an average of 809 cells/mm^3 and was also clinically well.

Table 5:4: Demographics characteristics of both the HIV-infected and uninfected controls

Parameter	**HIV-infected group (*n*=20)**	**HIV negative controls (*n*=20)**	***p*-values**
Male: Female	9:11	5:15	
Mean age (yrs.)	35.4	29.0	0.27
Range	24-56	23-46	
Mean CD4 cells/mm^3 ± SD	417±182	809±197.9	**0.006*** **
Median Viral load (copies/ml)	48360	ND	
Log Viral load	4.2±1.1		

All the values in columns are means ± S.D of HIV-infected (*n* = 20) individuals and HIV negative controls (*n* = 20). *** Statistically significant at P<0.05.

The protocols used in the optimization, gating strategies employed and examples of dot plots are shown in the figures below. The dosage response curves of LPS, Vitamin C and NAC are also shown below;

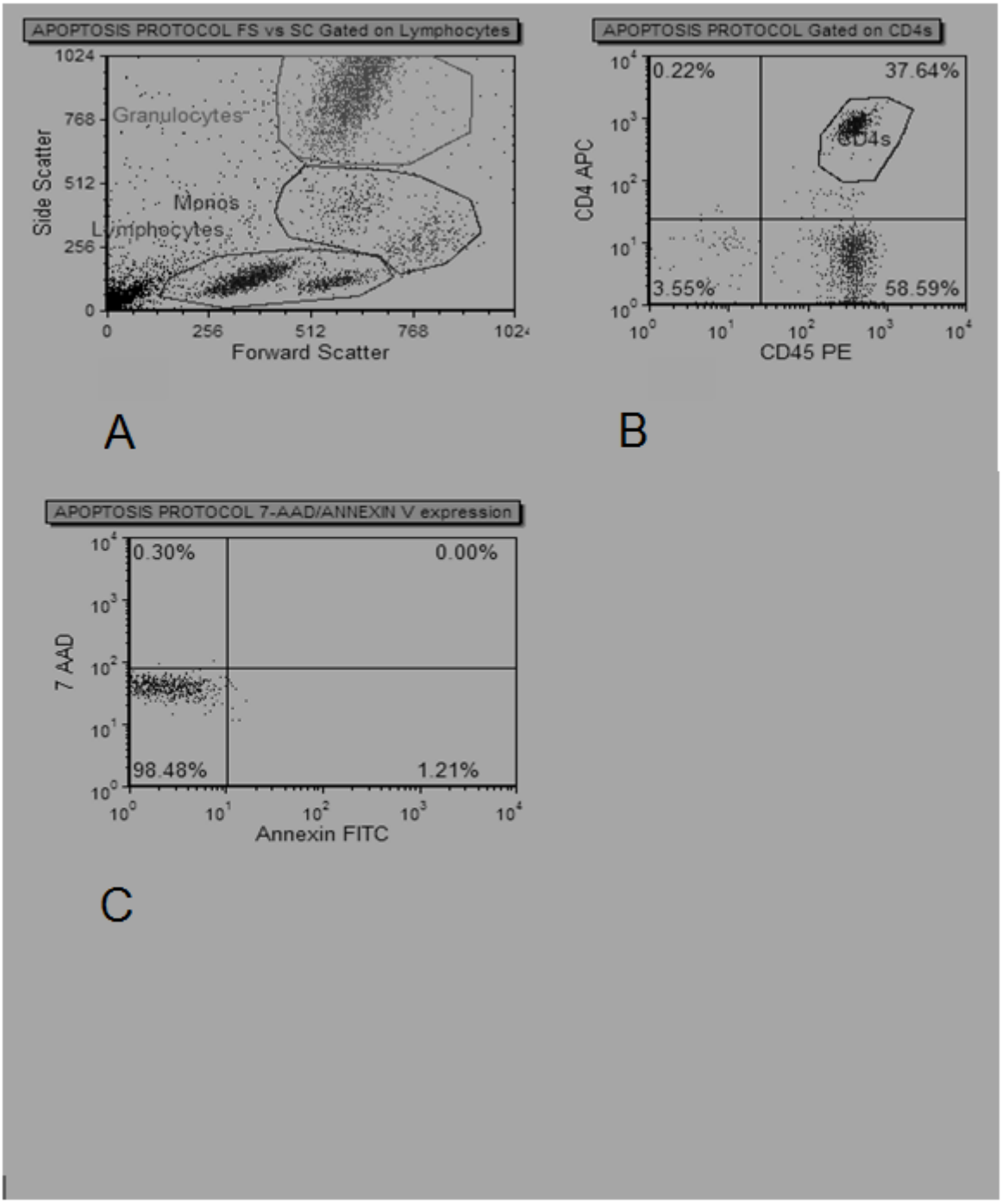

Figure 5:1: Apoptosis protocol. The figure shows the panel that was used to analyse the samples for activation and apoptosis. Lymphocytes were gated in the first plot (Plot A), then a CD4 gate (Plot B) was created based on the initial lymphocyte gate and the cells expressing either Annexin V, 7-AAD or both (Plot C) were analysed. 300, 000 events were acquired but only 25% of the dots are shown in the plot.

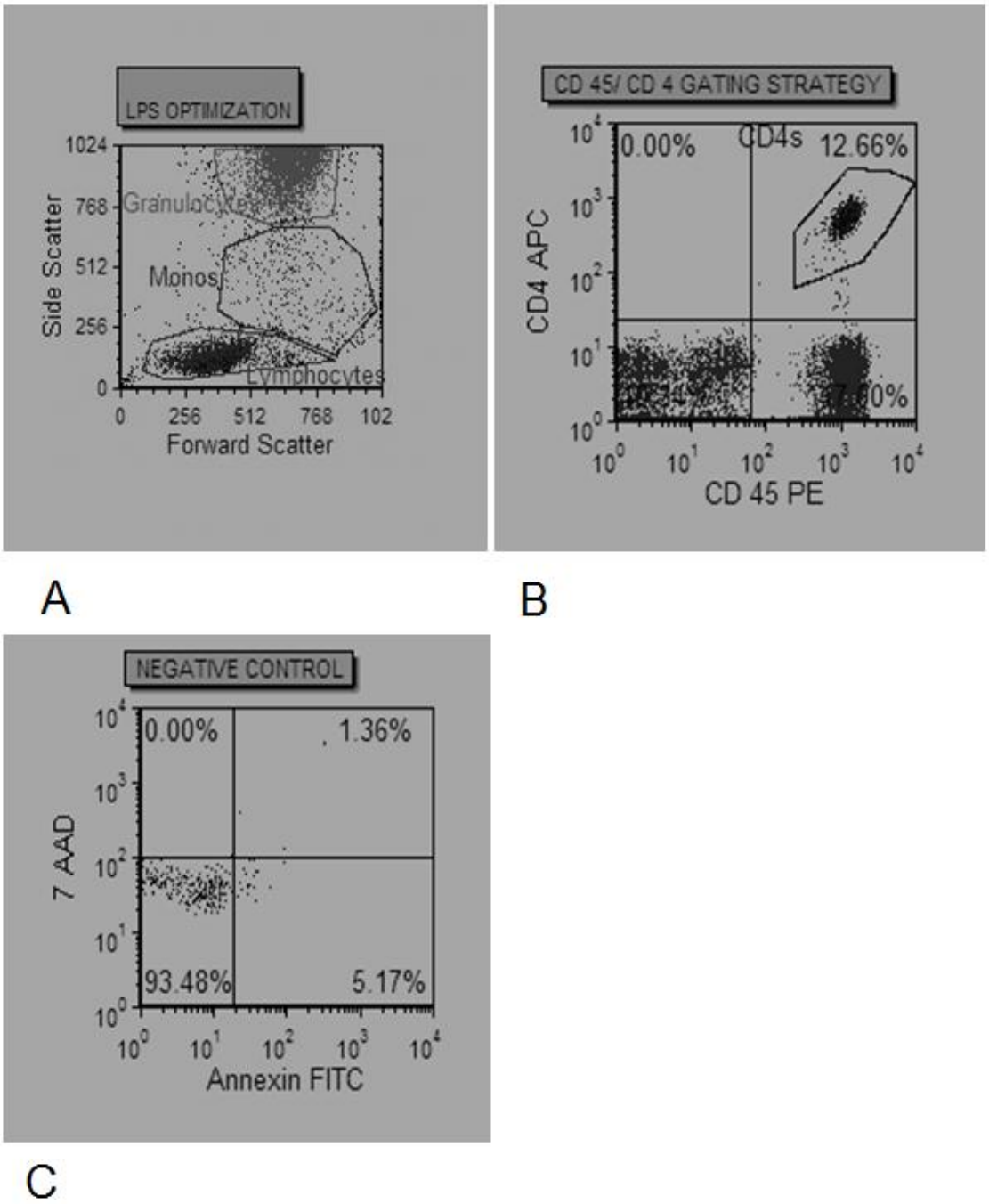

Figure 5:2: Gating strategy. The figure shows the gating strategy employed in the acquisition of data for forward side scatter (Fig. A), CD4 lymphocytes (Fig. B), Annexin V/ 7-AAD (Fig. C) A negative control/unstained sample (For 7-AAD and Annexin V) that was used with every sample to set the quadrants is also shown (Fig. C). A total of 300,000 events were analysed. 7-AAD being a bright flourochrome is brightly expressed as seen in the negative control (Figure 5.0.2 above) and thus the quadrant was adjusted appropriately upwards.

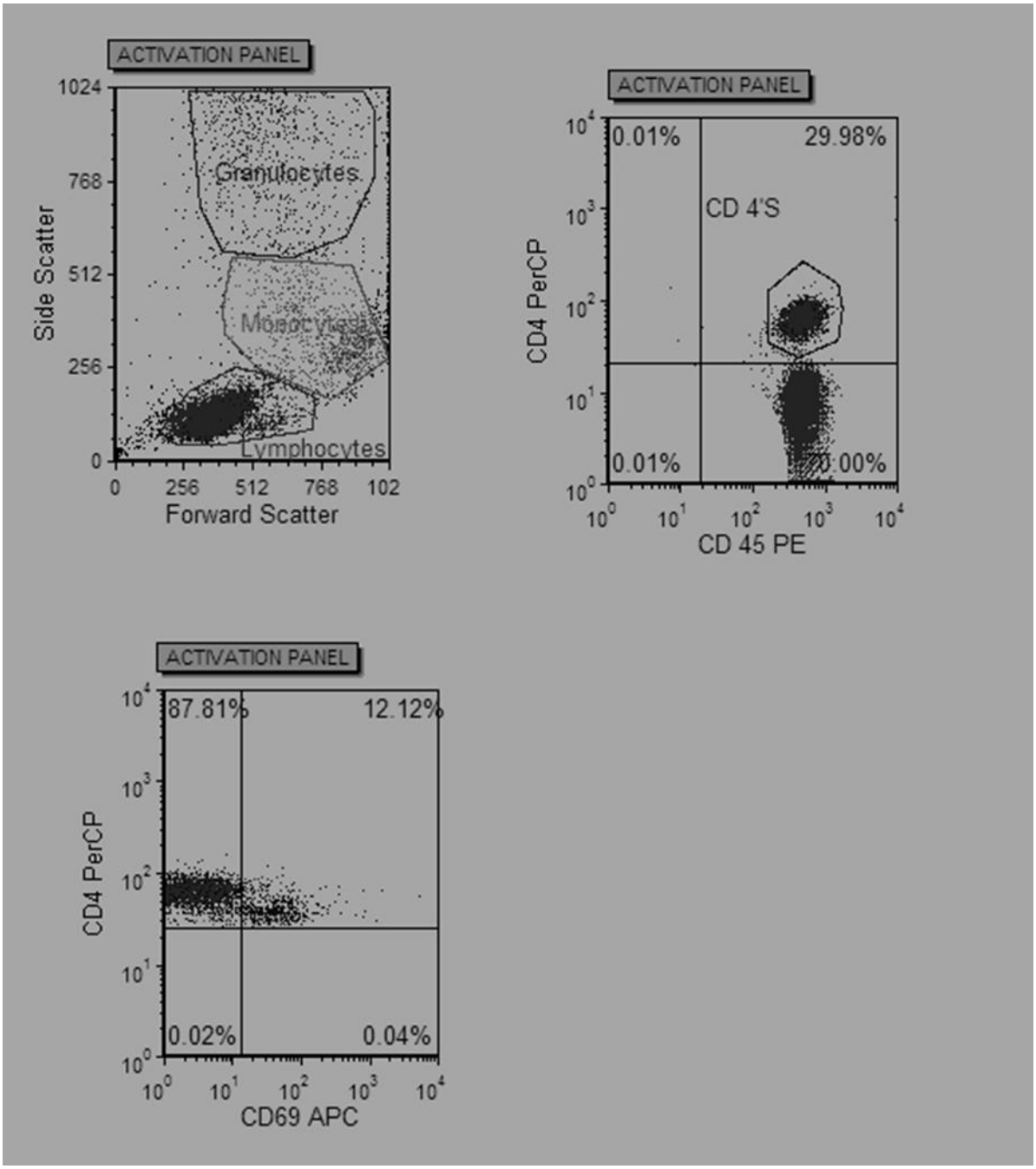

Figure 5:3: Activation panel. The figure shows the panel that was used for LPS (2 µg/ml) induced activation (1 hour) of whole blood. Plot A shows the Side Scatter vs. Forward Scatter. The lymphocytes were gated and CD4+ T lymphocytes gated from the lymphocyte gate. The third plot shows the percentage of CD4+ T cells expressing CD69. A similar panel (except that CD25 was used as the activation marker in place of CD69) that was used for overnight activation is not shown.

After analyzing several samples activated with increasing concentration (100 ng/ml, 1000 ng/ml, 2000 ng/ml and 5 µg/ml) of LPS, 2 µg/ml showed maximum activation and was chosen as the optimum concentration to use for LPS induced activation of whole blood. 7-AAD viability dye has adverse effects on cells when left for too long with cells. It is

recommended that the samples be on ice when working with 7-AAD (Keeney, 1999). However, working on ice is known to arrest activation progress through stages of viability, apoptosis and necrosis of the cells (Maya & Meyer, 2011). Therefore, the effect of activating the cells on ice and at room temperature was investigated. After choosing 2 µg/ml as the appropriate concentration to use for LPS activation, the effect of activation of whole blood on ice and at room temperature was investigated.

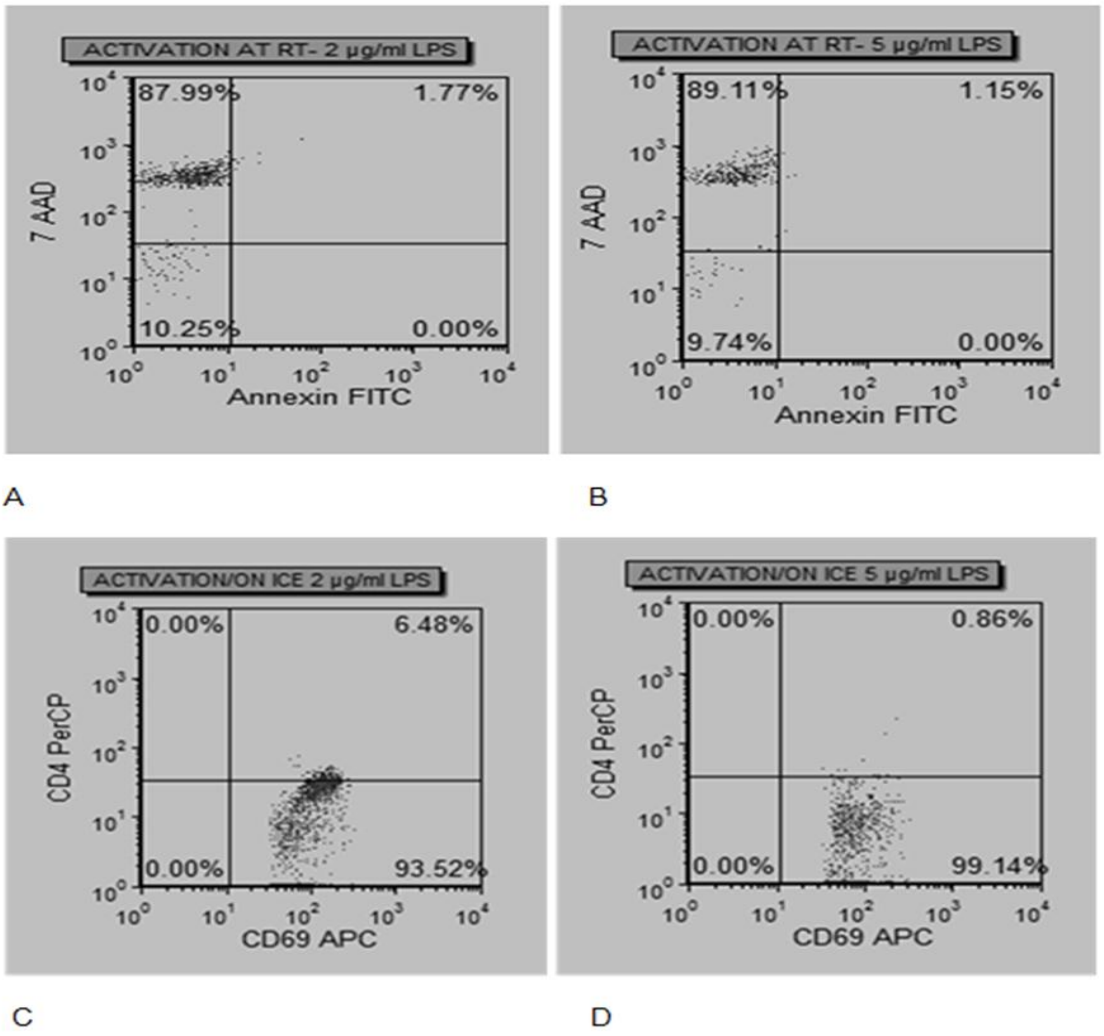

Figure 5:4: Comparison of LPS activation of whole blood on ice and at room temperature. The figure shows arrest/Inhibition of LPS-induced activation by Ice compared to room temperature. The figure shows a higher inhibition of activation at 5 µg/ml LPS concentration. LPS concentration at 2 µg/ml (in both ice and at room temperature) showed maximum activation and therefore was selected as the optimum concentration to use for LPS activation. A total of 300,000 events were analyzed.

The monoclonal antibodies were added at the beginning (protocol of activation on ice), while with the activation at room temperature protocol, 7-AAD was added after the lysing and washing of the cells stage and the cells analyzed as soon as possible. This was to minimize the adverse effects on the viability of the cells left in the presence of the 7-AAD dye for too long.

Vitamin C and NAC on their own (without LPS) and at high concentrations induced cell activation and death. This indicates that although in low doses, antioxidants are capable of inhibiting LPS-induced activation, on their own and in high concentrations they can be toxic to the cells.

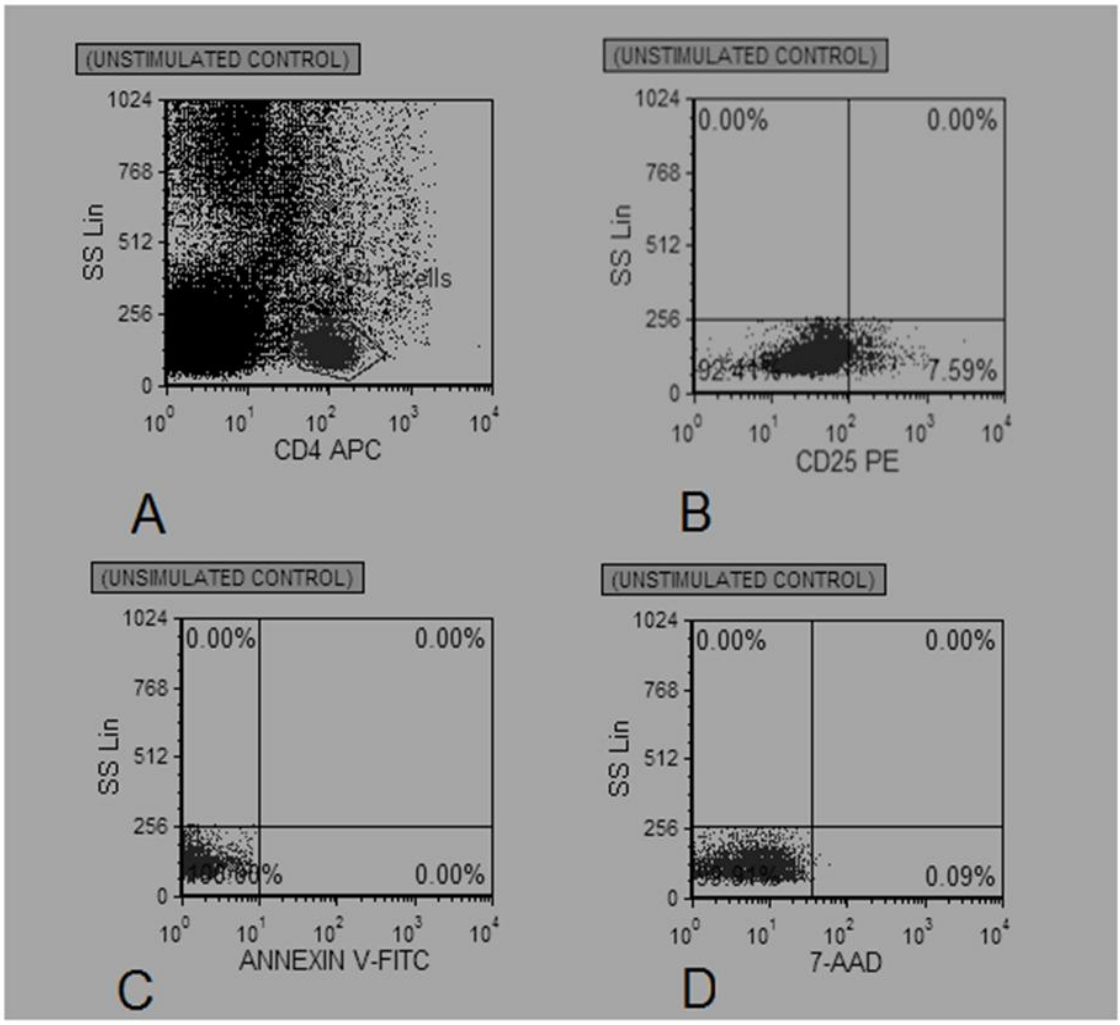

Figure 5:5: Example of dot plots used for LPS induced cell activation and death dot plot. Figures A, B, C & D show various markers of activation and apoptosis using unstimulated sample).

The dose-response curve of LPS-induced activation and vitamin C and NAC response curve are shown below;

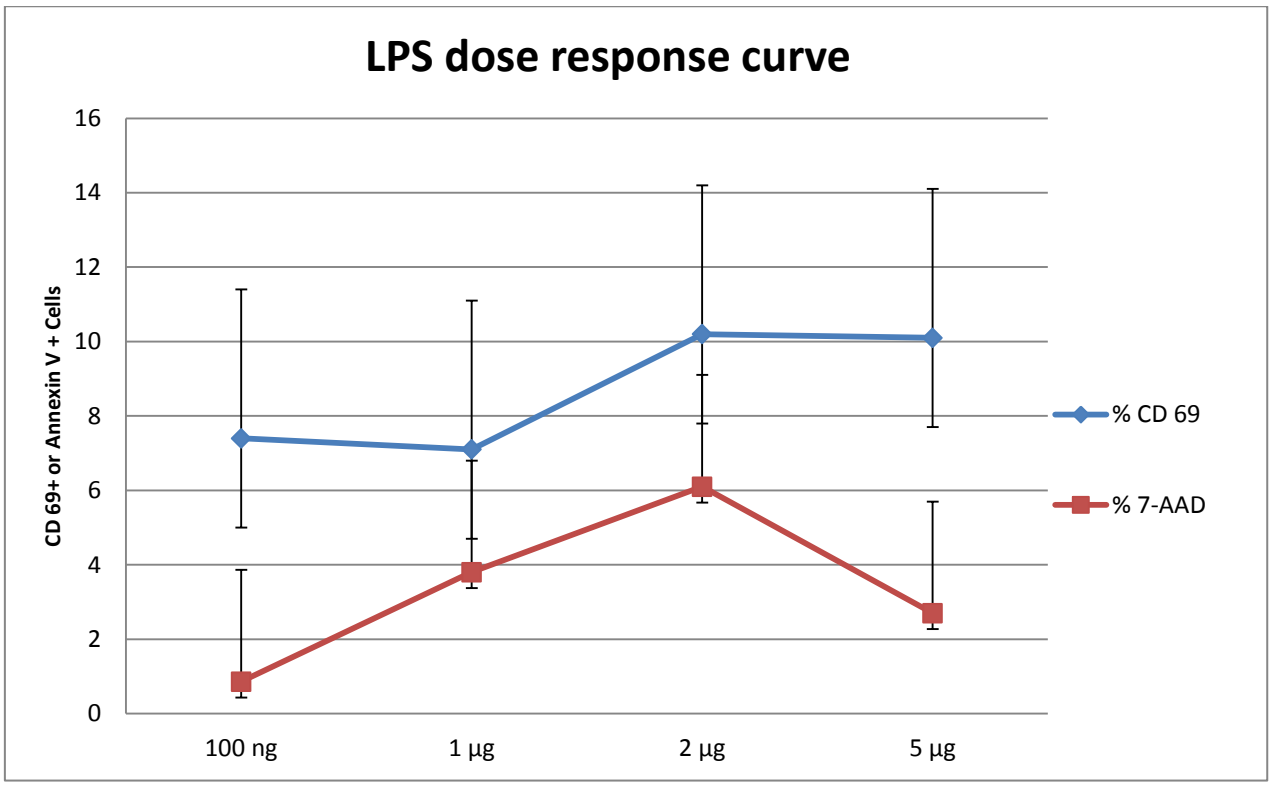

Figure 5:6: LPS dose response curve; the figure shows response of whole blood to different concentrations of LPS. At 2 µg/ml LPS concentration Annexin V expression, a marker of apoptosis was considerably increased. There was also a high level of activation (%CD69 expression).

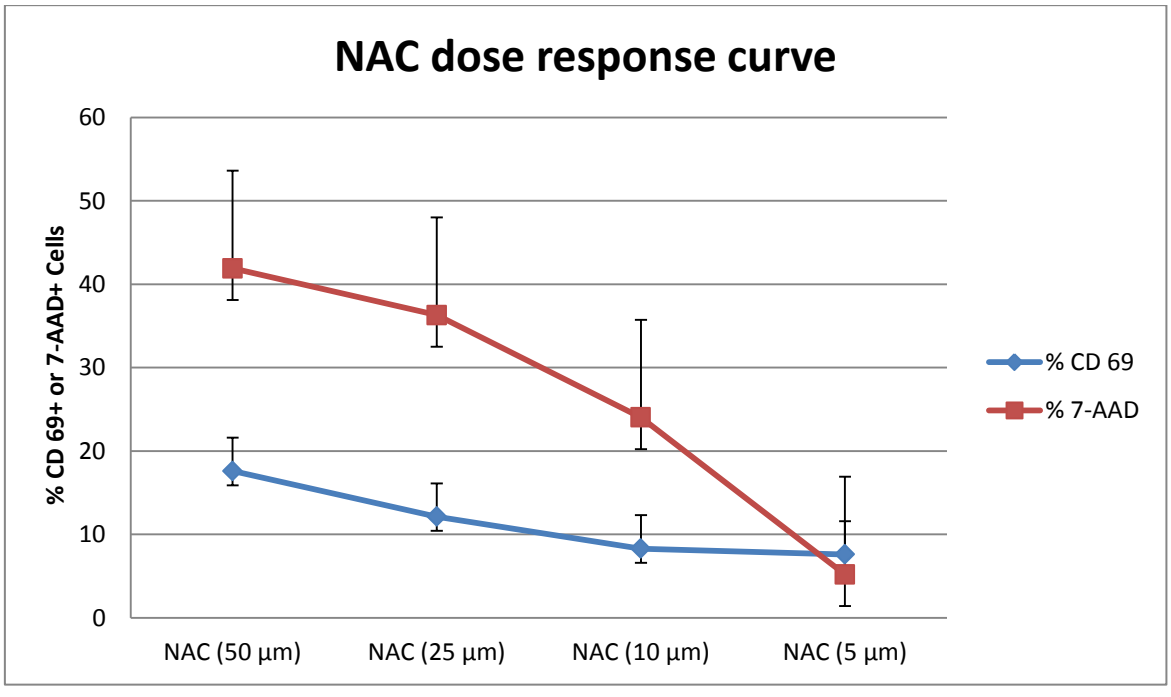

Figure 5:7: NAC dose response curve; the figure shows the LPS induced cell activation and apoptosis in response to various concentrations of NAC. At 5 µM, NAC greatly decreased activation (%CD69) and cell death (%7-AAD expression).

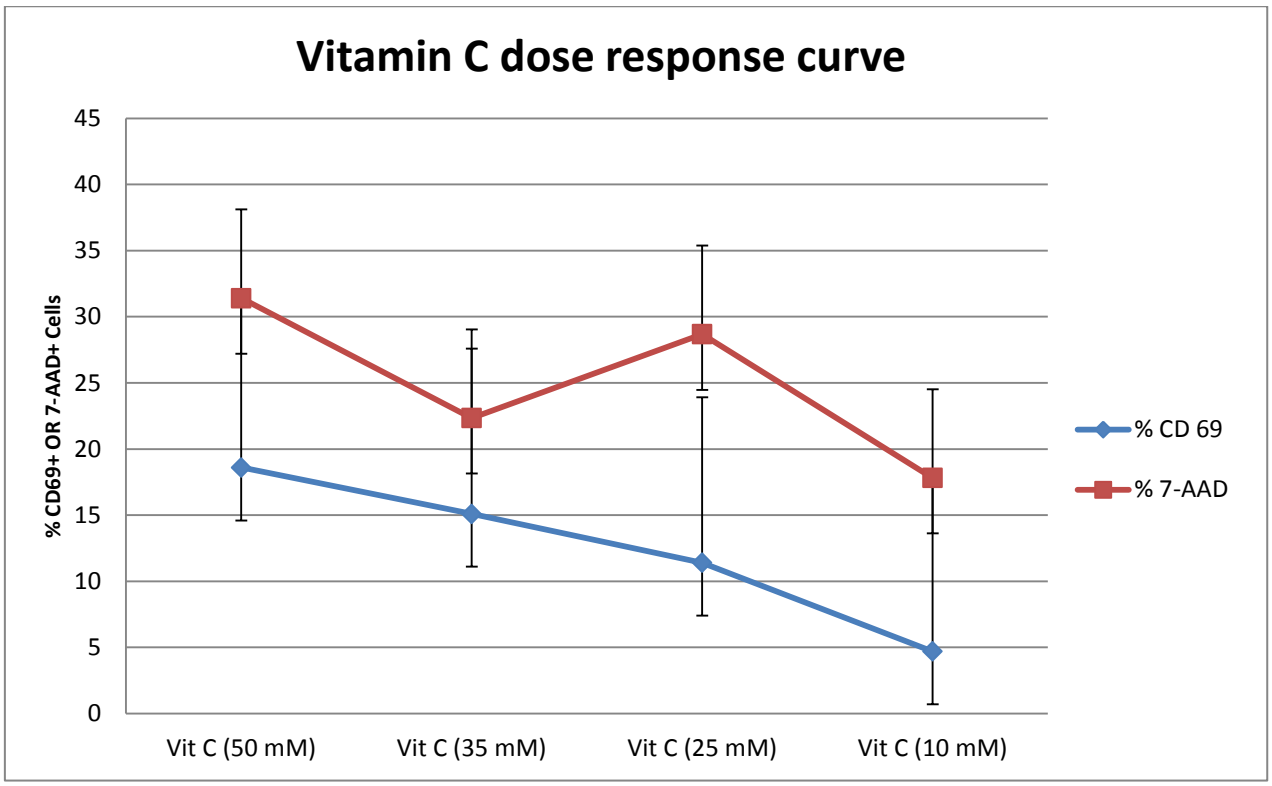

Figure 5:8: Vitamin C dose response curve: The figure shows the LPS induced activation and apoptosis in response to various concentrations of Vitamin C. At concentrations above 10 mM activation (%CD69 expression) and cell death (%7-AAD expression) was substantially increased.

5.6 Discussion

In this phase of the study, the effects of temperature, time and concentration of LPS on activation and apoptosis of whole blood was determined, as well as the optimal concentrations of antioxidants for inhibition of these processes. Studies on humans infected with HIV indicated that most of the depletion of the CD4+T-cells occurs in the mucosal tissues during the acute phase of the infection either as direct targets of the cytopathic effects of the virus or indirectly by immune activation induced-apoptosis (Veazey *et al.,* 2003; Brenchley *et al.,* 2004; Haynes, 2006). The loss of gastrointestinal mucosal integrity results in the translocation of microbial products such as LPS into the systemic circulation and is thought to be an important contributing factor to the on-going immune activation in HIV infection. LPS is a polysaccharide, cell wall component expressed by gram negative bacteria. It is a strong immuno-stimulatory molecule associated with T-cell and monocyte activation (Brenchley *et al.,* 2006). Macrophages/monocytes, neutrophils as well as CD4+ T-cells express TLR4 (Xu *et al.,* 2005; Kabelitz, 2007), the principle signaling component of the LPS receptor complex (CD14, LBP & TLR4) on their surfaces, thus these cells can be activated directly by LPS or indirectly by pro-inflammatory cytokines from activated monocytes or neutrophils or ROS (Xu *et al.,* 2005; Caramalho, *et al.,* 2003).

The use of fluorescently labelled monoclonal antibodies to assess protein and antigen expression enables the identification of cellular patterns and activation states (Alvarez *et al.,* 2009). Monoclonal antibodies such as CD45, CD25, CD69, CD4, Annexin V & 7-AAD were used in the LPS, NAC and vitamin C optimization processes. The effect of various concentrations of LPS on the activation of CD4+ T-cells in whole blood was explored. The results indicated that the optimal LPS concentration for inducing activation in whole blood was 2 µg/ml. Thereafter, the duration for maximum LPS induced activation and apoptosis was investigated. Previously authors have used varying duration of time ranging from 15 minutes to 20 hrs (Schafer *et al.,* 1999; Gercia-Verdugo *et al.,* 2007). In this study, incubation durations of I hour and 24 hours were investigated. Overnight incubation of whole blood for 20 hours was shown to induce optimum cell activation and apoptosis as determined by expression levels of CD25, Annexin V and 7-AAD; in comparison with 1 hour as expressed by CD69, Annexin V and 7-AAD. Previous studies involving LPS-induced activation of cells have utilised varying concentrations of LPS (100 ng/ml - 5 µg/ml), NAC (5 - 50 µM), and vitamin C (25 - 50 nM) therefore optimization was needed for the current study (Dobmeyer *et al.,* 1996; Shang *et al.,* 2003; Yamanda *et al.,* 2006). Kim *et al.* in their study on analysis of cellular senescence induced by LPS, demonstrated a significant ($P<0.005$) decrease of cellular viability at 15 µg/ml or greater concentration of LPS (Kim *et al.,* 2012).

Antioxidants such as NAC and vitamin C have been shown to inhibit cytotoxicity; free radical-induced DNA damage, ROS generation, immune activation and apoptosis (Alul *et al*., 2003). Therefore, optimizing a functional assay for LPS-induced activation and amelioration with the use of by antioxidants was important. The optimal antioxidant concentrations for inhibition of the LPS-induced cell activation and apoptosis were also determined. It was shown, that the optimal concentrations of vitamin C and NAC capable of *in vitro* inhibition of LPS-induced cell activation and apoptosis were 10 mM and 5 µM respectively. Concentrations higher than these were shown to be toxic to the cells as they induced greater levels of apoptosis and cell death. Antioxidants such as vitamin C and NAC at higher concentrations are known to be pro-oxidants and therefore may become toxic to cells, possibly as a result of greater levels of activation-induced death. (Halliwell & Gutteridge, 1989; Godin & Wahaieb, 1988; Bayday *et al.,* 2007). Incubation of cells with 0.2 mg/ml of vitamin C for 24 hours was shown to cause a 39% increase in apoptotic cells; suggesting toxicity of vitamin C (Bergman *et al.,* 2004). Therefore, it was vital to optimize the concentrations of vitamin C and NAC used in this functional assay. NAC and vitamin C independently and as a cocktail were shown to induce activation of the cells in the absence of LPS, but had an inhibitory effect on activation when cells were incubated with LPS. This showed that selected antioxidants such as NAC and vitamin C are capable of inhibiting LPS induced immune activation and apoptosis. This study has demonstrated that higher concentrations were toxic and able to cause activation and

even death of the cells. Thus, at higher doses vitamin C is likely to have a pro-oxidant effect which causes activation and even death of cells.

It is also recommended that the incubation with Annexin V and 7-AAD be carried out on ice to sustain cells viability (Keeney, 1999). Therefore, two different protocols were tested on a number of samples (in duplicates) to determine the optimal method for activation and apoptosis analysis of cells. The first protocol was carried out on ice while the other protocol was at room temperature. The protocol which was carried out at room temperature worked best for this study. Working on ice was shown to inhibit LPS activation which we were trying to induce when compared to working at room temperature. The recommended protocol is to work at room temperature, but to add 7-AAD, 5 - 15 mins. before analysis on flow cytometer due to adverse effects on the viability of cells if left in the presence of 7-AAD for too long

A limitation of this study was the lack of a specific marker for regulatory T-cells (T-regs) such as FoxP3 to distinguish the activated CD4 cells from the T-regs.

5.7 Conclusion and recommendation

In summary, this study showed that at high concentrations, antioxidants may be toxic to cells as shown by increased levels of activation and apoptosis. The study indicated that LPS in low concentrations, though capable of inducing activation of immune cells, may not be sufficient to induce apoptosis of the cells. Therefore, the optimum concentration of LPS that induced both activation and apoptosis in this assay was determined. In addition, antioxidants, though important for good health, may be potentially toxic to cells in high doses and therefore, optimal concentrations are important to establish for the limitation of cell activation and death.

5.7.1 Acknowledgments

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CHAPTER 6

Modulation of LPS-induced CD4+ T-cell activation and apoptosis by antioxidants in untreated asymptomatic HIV infection: An *in vitro* study

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Modulation of LPS-induced CD4+ T-cell activation and apoptosis by antioxidants in untreated asymptomatic HIV infected participants: An *in vitro* study

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Abstract

Persistent immune activation characterises HIV infection and is associated with depletion of CD4+ T-cells and increased risk of disease progression. Early loss of gut mucosal integrity results in the translocation of microbial products such as LPS into the systemic circulation. This is an important source of on-going immune stimulation.

The purpose of this study was to determine levels of CD4+ T-cell activation (%CD25 expression) and apoptosis (%Annexin V/7-AAD) in asymptomatic, untreated HIV infection at baseline and after stimulation with LPS and incubation with or without vitamin C and N-acetyl cysteine.

LPS induced a significant ($P<0.03$) increase in %CD25 expression, Annexin V and 7-AAD in HIV positive individuals. NAC in combination with vitamin C, significantly ($P = 0.0018$) reduced activation and early apoptosis of CD4+ T-cells to a greater degree than with either antioxidant alone.

Certain combinations of antioxidants could be important in reducing the harmful effects of chronic immune activation and thereby limit CD4+ T-cell depletion. Importantly, we showed that CD4+ T-cells of the HIV positive group responded better to a combination of the antioxidants at this stage than the controls. Therefore, appropriate intervention at this

asymptomatic stage, could rescue the cells before repetitive activation results in the death of CD4+ T-cells.

Keywords: HIV infection; immune activation; lipopolysaccharide, apoptosis, Annexin V

6.1 Introduction

HIV infection is characterized by chronic immune activation and inflammatory cytokine production [l,2]. The consistent activation of CD4+ and CD8+ T-cells is associated with depletion of CD4+ T-cells and increased risk of disease progression to AIDS [3]. Furthermore, markers of immune activation have been shown to be stronger predictors of progression to AIDS than either the CD4 counts or viral loads [4,5,6]. In particular, increased T-cell activation has been associated with AIDS suggesting that activated T-cells are susceptible to apoptosis [7]. In addition, elevated levels of CD38, a marker of immune activation on CD4 and CD8+ T-cell predicts a rapid decrease of CD4+ T-cells and a shorter survival rate, independent of HIV viral loads [8,9].

The significant depletion of memory-type CD4+ T-cells lining the GIT mucosa in early HIV infection results in the breakdown of the mucosa and on-going translocation of microbial products such as LPS across the epithelial surface [10,1]. LPS induces activation of innate immune cells such as monocytes and dendritic cells resulting in increased oxidative stress; depletion of antioxidant defence mechanisms and an increased susceptibility to apoptosis [1,11]. In addition, CD4+ T-lymphocytes have been shown to express toll like-receptor-4 (TLR4), which is a receptor for LPS [12,13]. Triggering of TLR4 activates various signalling pathways such as mitogen-activated protein kinases (MAPK), p38 and JNK, which induce activation of transcription factor NF-kB and subsequent production of pro-inflammatory cytokines, chemokines, antimicrobial peptides and other defence molecules such as ROS [13]. The pro-inflammatory cytokines such as TNF-α, are capable of activating innate immune cells to produce more ROS. Reactive oxygen species and pro-inflammatory cytokines in turn induce activation of both the extrinsic and intrinsic pathways of apoptosis. The role of *in vitro* stimulation with LPS on T-cell activation in HIV has been explored only minimally [14,15]. The first study to date in HIV used CD38 and HLA-DR as the activation markers and were conducted on HIV positive patients who were on antiretroviral treatment [15]. Few studies have investigated the inhibitory effects by antioxidants on immune activation and apoptosis in asymptomatic, untreated HIV infection. In the current study, we investigated the effects of vitamin C and NAC on LPS–induced up-regulation of interleukin receptor-2 receptor alpha chain, (CD25), as a marker of LPS-induced activation of CD4+T-cells after overnight incubation, in untreated HIV infection.

In this study therefore, levels of immune activation and apoptosis were measured before and after stimulation with LPS and incubation with selected antioxidants (vitamin C and NAC) in

untreated HIV positive individuals. These levels were compared to a control group. We developed an assay that demonstrates the response of CD4+ T-cells to LPS-induced stimulation and further, showed the inhibitory effect of antioxidants in this process.

6.2 Materials and Methods

6.2.1 Study population and design

In this cross-sectional study, twenty untreated, asymptomatic HIV positive individuals and 20 controls (32 females and 8 males) were sourced from a single HIV testing and prevention primary health clinic in Crossroads, Cape Town (South Africa). The median age of the participants was 32 years (range 22 - 42). There was no significant difference ($P = 0.43$) in age between the two groups. The HIV positive group had a significantly ($P = 0.0003$) lower CD4 count compared to the control group. The patients' demographics are summarised in Table 6.1. Informed consent was taken from all the participating subjects. Inclusion criteria for the study participants were 21 years or older, individuals with HIV infection and CD4 counts>200; not on antiretrovirals (ARV's) or any other chronic medication or antioxidant supplements. Exclusion criteria included patients with tuberculosis (TB) or other co-infections and those receiving antiretroviral therapy, anti-TB treatment or other antibiotic treatment, antioxidant supplementation, mineral and vitamins supplements, aspirin or any other drug e.g. anti-inflammatory.

Ethics approval was obtained from both the clinical site, University of Cape Town: REC: REF: 417/2006 and laboratory site, University of Stellenbosch HREC N07/09/197.

6.2.1 Reagents

Flow-check Fluorospheres, Flow-set Fluorospheres, CD4-PE, CD4-APC, CD25-PE, Annexin V-FITC/ 7AAD-PE kit were obtained from Beckman Coulter, Miami Florida Inc. (USA). FC 500 cytometer with two lasers from Beckman Coulter, Miami Florida, (USA) was used to acquire the data. L-ascorbic acid stock powder or vitamin C ($C_6H_8O_{6;}$ Molecular weight 176.12 g/mol; 25 g powder) and N-acetyl -L- cysteine stock powder ($C_5H_9NO_3S$; 25 g powder) were purchased from Sigma-Aldrich (South Africa).

6.2.2 Sample preparation

Blood was drawn into two 10 ml tubes with Heparin, one 5 ml tube with EDTA (for viral load) and one 5 ml tube with citrate (for D-dimers). Samples were then couriered from the clinic to the laboratory within two hours of collection.

Heparinized whole blood samples were incubated with antioxidants for 20 min, then stimulated with LPS, incubated overnight and analysed on flow cytometer. Briefly, 100 µl of blood was added into the labelled tubes and 30 µl of vitamin C (10 mM) or 20 µl of NAC (5 µM), vortexed gently and incubated for 20 minutes. An additional tube was prepared with the

'cocktail' of both antioxidants. After 20 minutes incubation of the samples at 37 ^{o}C with 5% carbon dioxide (CO_2), 20 µl of LPS (2 µg/ml) was added. The dosages (2 µg/ml LPS, 10 mM vitamin C and 5 µM NAC) used in this study were chosen for these experiments after a rigorous optimization study on the effects of temperature, time and concentration on LPS-induced whole blood activation and antioxidant intervention in asymptomatic untreated HIV infection previously done in our lab (data not shown) that these doses were chosen. The samples were incubated overnight and analysed on flow cytometer.

For each sample, 100 µl of blood was added to appropriately labelled Beckman's flow tubes and 10 µl of monoclonal antibody mix added. The sample was vortexed gently and incubated at room temperature for 15 minutes in the dark after which 500 µl of fluorescein activated cell sorting (FACS) lysing solution was added. The sample was vortexed gently and incubated for 15 minutes at room temperature. After incubation, 250 µl of ice cold staining buffer and 250 µl of binding buffer were added. The sample was spun at 300 g for 5 minutes after which 750 µl of supernatant was removed. The pellets were re-suspended, 200 µl of staining buffer and 200 µl of binding buffers added and analysed on flow cytometer.

6.2.3 Flow cytometry analysis

An FC 500 flow cytometer (Beckman Coulter, Miami Florida, USA) with two lasers, five fluorescence channels and CXP analysis software were used in this study. Alignment of the lasers was performed with a mixture of Flow-check and Flow-check beads. The appropriate voltages were determined and standardized with a mixture of Flow-set and Flow-set beads. Full matrix colour compensation was done using FITC, PE, APC & PerCP/PC5 stained whole blood cells prepared using the lyse and wash method. A panel was created for test analysis using the cytometer settings established with Flow-Set and full matrix colour compensation. CXP and FCS Express V3 softwares were used to analyse the flow cytometry data.

6.2.4 Data acquisition and analysis for apoptosis

For apoptosis, plot quadrants were set using unstained cells for every sample such that the negative Annexin-V cells and 7-AAD negative population lay in the first decade of the Y and X axis. A sequential gating strategy, by first gating on lymphocytes for CD4+ T-cells and then gating on CD4+ T-cells for Annexin V vs. 7-AAD. This was in order to detect Annexin V + 7-AAD negative cells (apoptotic cells), Annexin V negative 7-AAD positive cells (dead cells) and Annexin V positive 7-AAD positive (secondary apoptotic or necrotic) CD4+ T-cells. A total of 300,000 events were acquired in order to analyse a minimum of 2000 CD4+ T-cells. CXP and FCS express V3 softwares were used to analyse flow cytometry data.

6.2.5 Markers of disease and immune activation

CD4+ T-cell counts were determined by staining whole blood with Becton Dickinson (BD) MultiTEST™ CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent in BD TruCOUNT™ tubes according to the manufacturer's instructions and analysed on a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). HIV-1 RNA quantifications were performed using 1.0 mL of plasma with use of the Nuclisens Easy Q HIV-1 v.1.2 kit (BioMerieux Inc., Boxtel, Netherlands). CD38 expression on CD8+ T-cells (CD38/8) was determined by flow cytometry. Whole blood samples were incubated with the monoclonal antibodies; CD8-Per-CP; CD38-APC; CD3-FITC (BD Biosciences, San Jose, CA) and analysed on a BD FACSCalibur instrument using BD Cell Quest Pro (Version 2) software. Lymphocytes were gated on forward vs. side scatter, CD3 and CD8 expression.

6.2.6 D-dimers

D-dimer, a marker of fibrinogen breakdown and clot formation, thus an indirect marker of inflammation, was determined by spectrophotometry using the IL-D-dimer method. This is an automated immunoassay for quantitative determination of D-dimers in plasma. Plasma from sodium citrate blood samples, was mixed with latex reagent and buffer all supplied by Beckman Coulter (Miami, Florida, USA) and agglutination, measured as decrease in absorbance was read at 405 nm using ACL TOP from Beckman Coulter (Miami, Florida, USA).

6.3 Statistical analysis

The data was analysed using the Graphpad Prism version 5 statistical analysis software. Comparisons between the groups (HIV + and HIV-) were done. Analysis of variance (ANOVA) was used to determine whether the means of the two groups (HIV + and HIV-) differed significantly. Mann Whitney non-parametric test and spearman's correlation were applied. Results were reported as medians with interquartile ranges. A 5% or lower significance level was used to determine significant findings ($P \leq 0.05$).

6.4 Results

6.4.1 Demographics of study population

The participants' demographics are summarized in Table 6.1. The group included 20 HIV positive and 20 controls most of whom were females (Fisher's test $P = 0.36$). There was no significant ($P > 0.05$) difference between the two groups in terms of age. Both groups had similar mean ages: 31 years for HIV positive group and 30 years for the control group. The HIV positive group had a significantly ($P = 0.0012$) lower CD4 count compared to the control group. The HIV positive group had a well maintained CD4 count averaging 464 cells/mm^3 (median- 411cell/mm^3) and was clinically well. Median viral load was 45705 copies/mL. The

control group had a high CD4 count with an average of 746 cells/mm^3 and was also clinically well.

Table 6:1: Demographics characteristics of both the HIV positive and controls

Parameter	HIV positive group (*n* = 20)	Controls (*n* = 20)	P-values
Male: Female	5:15	3:17	
Median age (yrs.)	31.8(27-35)	30.3(22-35)	0.43
Range	21-51	21-48	
Median CD4 cells/mm^3	411(265-634)	753(564-870)	0.0012**
Median Viral load (copies/ml)	45705(2174-157294)	ND	
Log Viral load	4.0(3.1-5.4)		

All the values in columns are median (Interquartile range) of HIV positive (*n* = 20) individuals and controls (*n* = 20). ** Median significant at P<0.05.

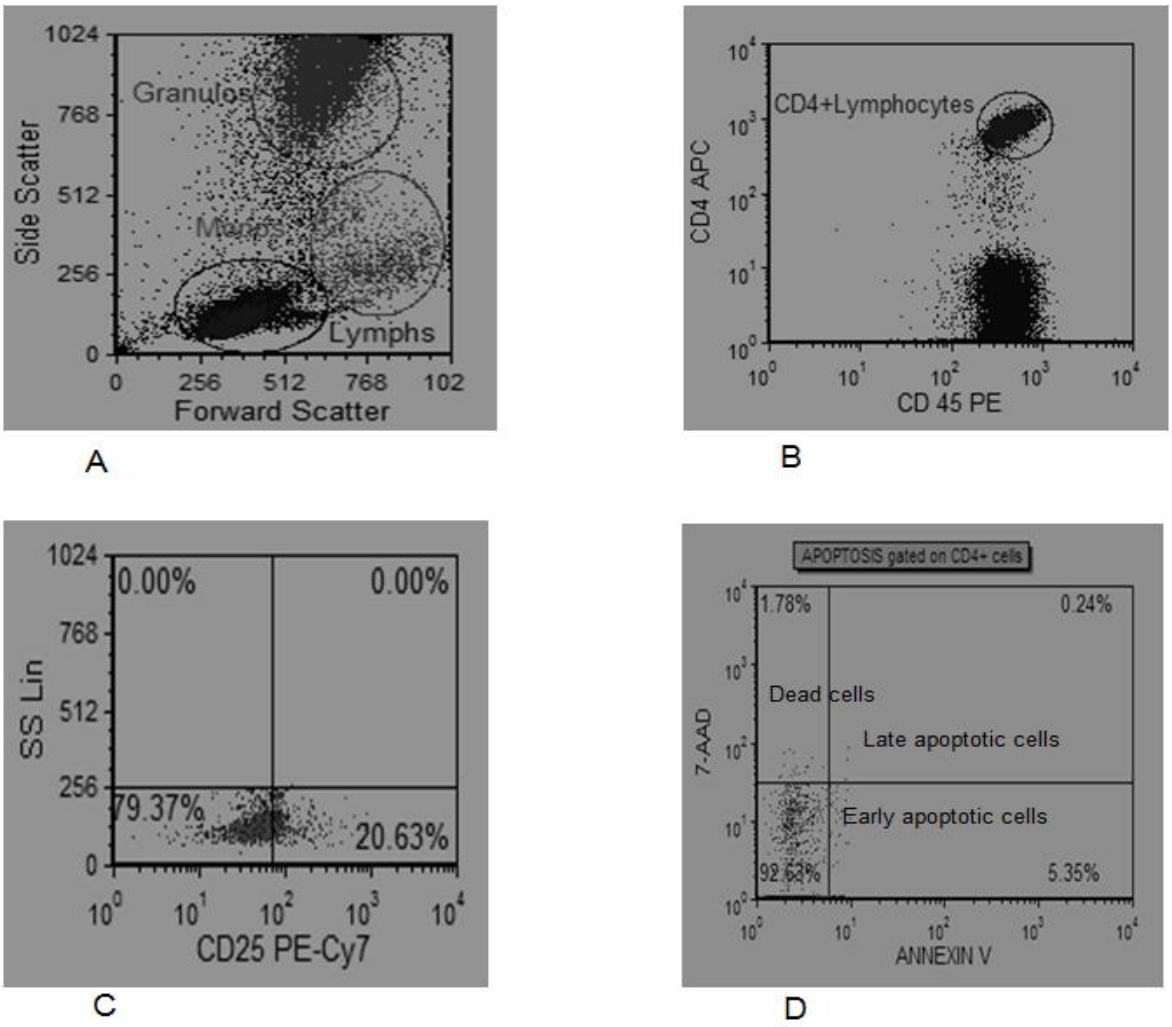

Figure 6:1: Gating strategy for activation (CD25) and apoptosis (Annexin V/7-AAD). Plots A (side scatter vs. forward scatter), B (bright CD4+ cells gated from the CD45+ leukocytes), C CD25+ cells gated from the CD4 gate & D (early, late apoptosis and dead cells gated from CD4+ cells gate) shows dot plots of whole blood used to set the quadrants.

6.4.2 %CD25 expression in the HIV positive and control group

The %CD25 expression before and after stimulation with LPS and incubation with vitamin C and NAC of the forty study participants is shown in Table 6.2 and illustrated in figure 2. Baseline (unstimulated) levels of activation were not significantly different between the two groups (P = 0.40), however after stimulation the HIV positive group showed statistically significant increase in activation (P = 0.03) when compared to the controls, which was not significant (P = 0.16). A significant difference was noted with incubation with LPS and vitamin C alone and NAC alone: in the control group. Optimal levels of inhibition of activation in the HIV group were achieved with the combination of NAC + vitamin C (P = 0.0018).

Table 6:2: The %CD25 expression before and after overnight stimulation with LPS and incubation with vitamin C and/or NAC

%CD25	HIV+ group	Controls	P-value between HIV and control	P-value for LPS activation and inhibition by antioxidants in HIV group	P-value for control
unstimulated	7.9(7.1-14.4)	10.2(7.8-14.4)	0.4047		
LPS stimulated	12.5(10.3-17.6)	11.6(9.6-15.2)	0.6823	0.0337**	0.16
LPS +Vit C	15.2(11.7-17.6)	8.6(7.2-11.9)	0.0003**	0.4	0.01**
LPS +NAC	9.0(7.1-14.2)	9.9(6.9-13.1)	0.9033	0.0416**	0.07
LPS + cocktail	6.0(4.2-13.1)	9.1(7.8-14.6)	0.0501	0.0018**	0.22

All the values are median (interquartile range) percentages of cells expressing CD25 of the HIV positive and control groups; unstimulated and stimulated with LPS and incubated overnight **Medians significant at P<0.05.

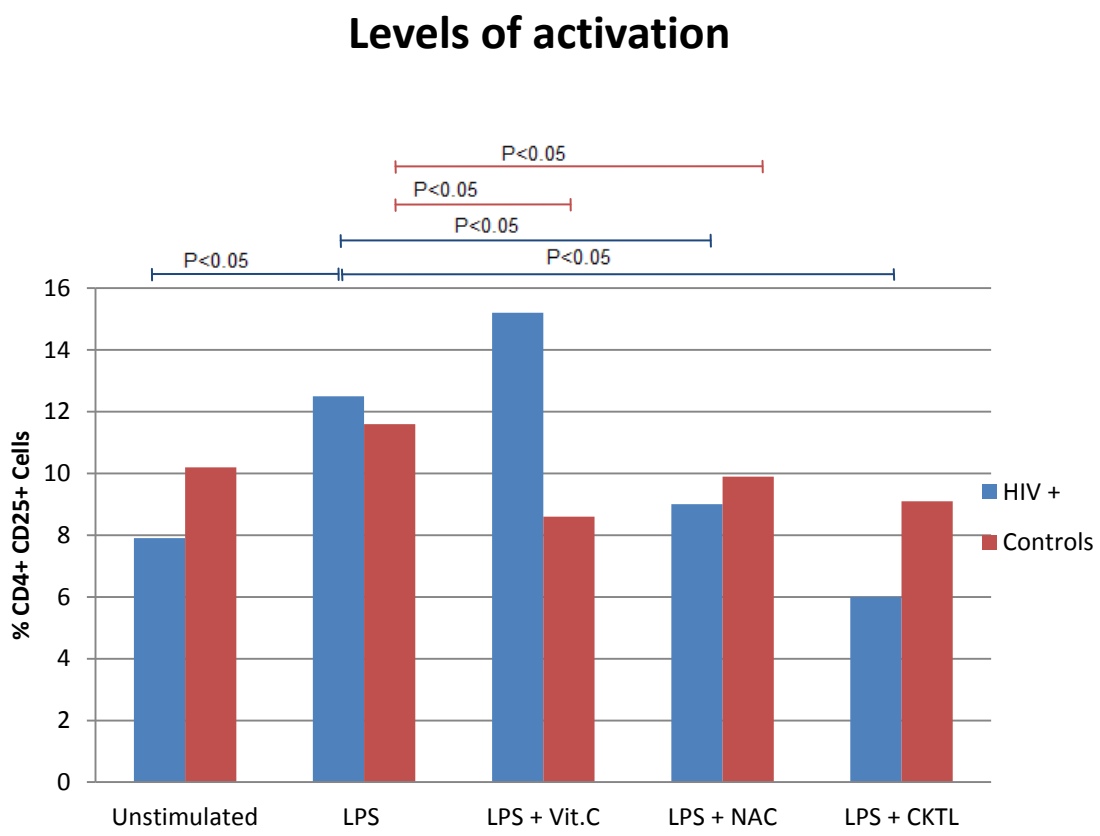

Figure 6:2: %CD25 expression on CD4+ T-cells; The figure shows the median %CD25 expression on CD4+ T-cells under different experimental conditions for both HIV positive and control groups. LPS induced a significant increase in CD25 expression in HIV infection (P = 0.03) and this increase was similar to that of the controls (P = 0.68). Red and black lines at the top of the bars indicate significance.

6.4.3 The %Annexin V/7-AAD staining for early and late apoptosis between the HIV positive and control groups

The %Annexin/7-AAD staining before and after stimulation with LPS and overnight incubation with vitamin C and NAC is summarised in Table 6.3 and figure 3 & 4. For early apoptosis, at baseline, the levels of Annexin V+/7AAD- staining were not significantly (P>0.05) different between the two groups, however, a significant difference was noted after stimulation with LPS (P = 0.007) in the control group. There was a significant difference after incubation with LPS, and a combination of NAC and vitamin C in both groups. Importantly the combination of vitamin C and NAC significantly (P<0.0001) reduced the Annexin V+/7-AAD- staining cells back to its unstimulated levels. NAC and vitamin C in combination significantly (P = 0.007, P = 0.002) decreased the staining of Annexin V+/7-AAD- cells. For late apoptosis, there was no significant difference (P>0.05) before stimulation with LPS and after stimulation with LPS and incubation with the antioxidants individually or in combination, meaning that these antioxidants are effective in limiting early apoptosis. This could help in retaining their functionality and protect them from early death.

Table 6:3: The %Annexin V+/7-AAD-staining of the HIV positive and the controls

%Annexin V	HIV positive group	Control	P-value(HIV & Controls	P-value for LPS activation and inhibition by antioxidants in HIV+ group	P-value for controls
unstimulated	1.6(0.9-4)	1.9(1.2-2.3)	0.49		
LPS-activated cells	3.3(1.6-5.6)	2.5(2.0-3.2)	0.17	0.11	0.007**
LPS + VIT C	3.6(1.9-4.2)	2.6(2.1-3.4)	0.26	0.84	0.44
LPS + NAC	3.4(1.2-4.3)	2.6(2.2–3.4)	0.55	0.54	0.66
LPS + CKTL	1.8(1.0-3.7)	1.9(1.5-2.4)	0.96	0.02**	0.008**

All the values are median (interquartile range) percentages of cells staining with Annexin V/7-AAD- of the HIV positive and control groups; unstimulated and stimulated with LPS and incubated overnight. **Medians significant at P<0.05.

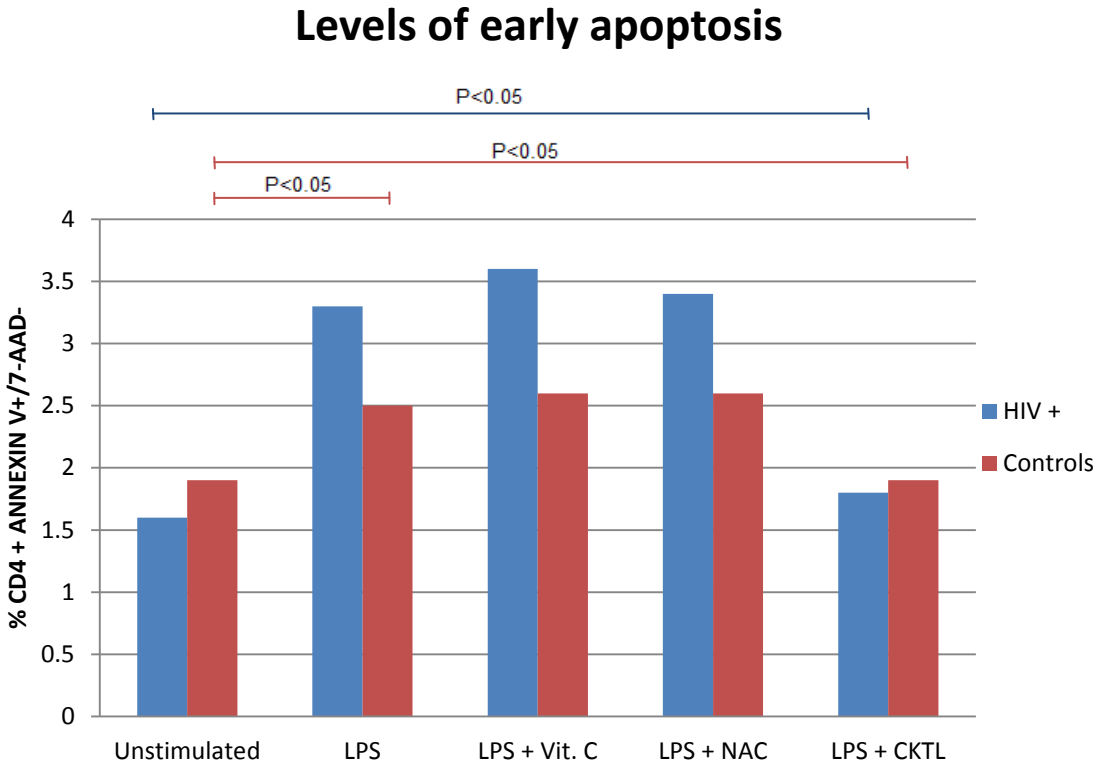

Figure 6:3: %Annexin V staining: The figure shows the median %Annexin V staining on CD4+ T-cells under different experimental conditions for both HIV positive and control groups. LPS induced a significant increase in Annexin V staining in the controls (P = 0.007) and this increase was significantly reduced in both HIV positive and controls (P = 0.02; P = 0.008 respectively) by a combination of vitamin C and NAC. Red and blue lines at the top of the bars indicate significance.

Table 6:4: The %Annexin V+7-AAD+ staining of the HIV-positive and control group

%7-AAD	HIV positive group	Control	P-value between HIV and control	P-value for LPS activation and inhibition by antioxidants in HIV group	P-values for the controls
Baseline	0.7(0.4-2.2)	0.4(0.3-0.5)	0.02**		
LPS-activated cells	1.9(0.7-3.2)	0.6(0.4-0.7)	0.0029**	0.10	0.14
LPS + VIT C	1.2(0.7-2.7)	0.7(0.4-1.0)	0.0048**	0.89	0.27
LPS + NAC	2.4(1.0-3.4)	0.6(0.4-0.8)	0.0003**	0.64	0.79
LPS + CKTL	1.8(1.3-2.1)	0.7(0.6-1.2)	0.0001***	0.76	0.56

The values are medians (interquartile range) percentages of cells staining for both Annexin V and 7-AAD of the HIV positive and control groups; unstimulated and stimulated with LPS and incubated overnight. **Medians significant at P<0.05. *** Medians significant at P≤0.0001.

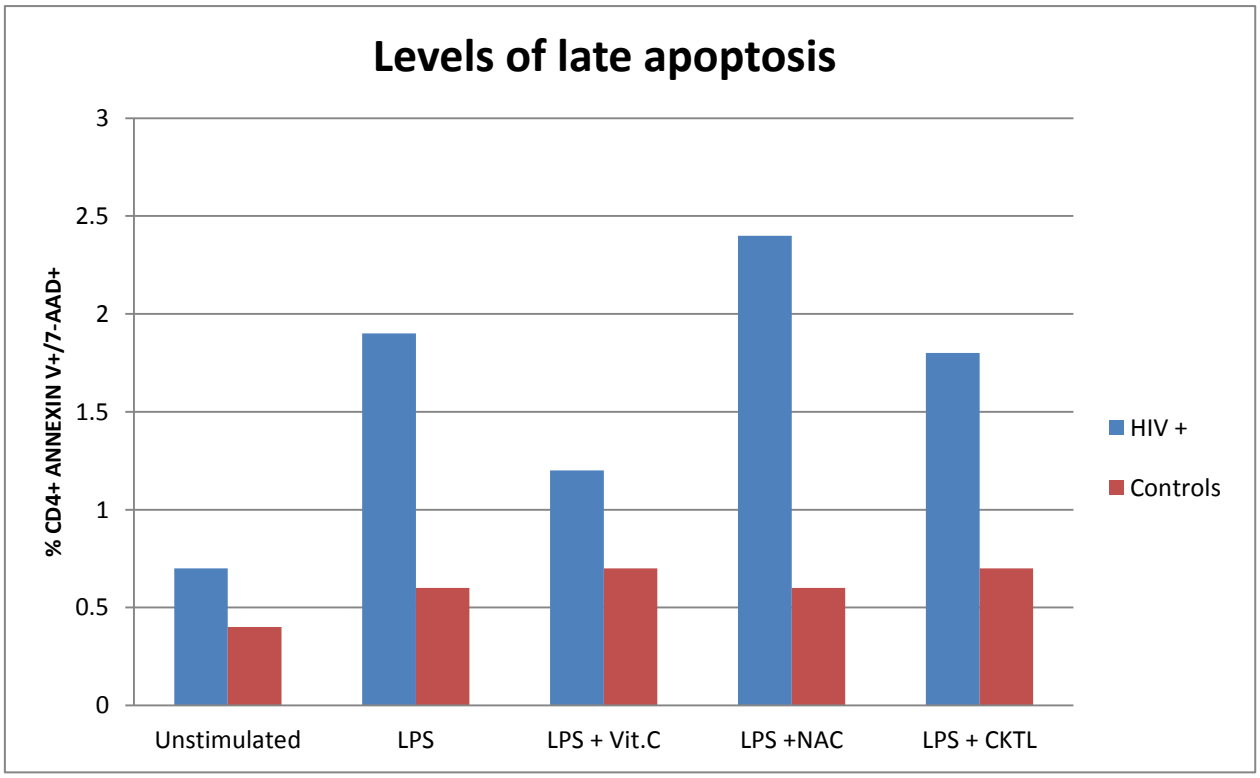

Figure 6:4: %Annexin V+/7AAD+ staining on CD4+T-cells: **The figure shows the median %Annexin V+/7-AAD+ staining on CD4+ T-cells under different experimental conditions for both HIV positive and control groups. LPS did not induce a significant increase in Annexin V+/7-AAD+ staining in both groups (P>0.05).**

6.4.4 Other markers of disease in HIV infection

Table 6.5 shows the values of other markers of disease and immune activation in the cohort. When compared with the controls as expected, the HIV positive individuals had significantly (P<0.05) lower CD4 counts. D-dimers, an indirect (marker of fibrinogen breakdown and clot

formation) marker of inflammation was significantly (P<0.05) higher in HIV positive patients than in controls.

Table 6:5: Other markers of disease in HIV infection in both the HIV-positive and control group

	HIV positive group	Control group	P-value
D-dimers (mg/L)	0.23(0.2-0.3)	0.21(0.20-0.26)	<0.0001**
CD38/8 (%)	27.6(17.5-44.0)	11.6(7.3-15.8)	<0.0001**

The table shows the D-dimers and %CD38/8 in these groups expressed as median (interquartile range): ** indicates that the medians were significantly different at P<0.05. CD38/8 was significantly different (P<0.0001).

There was a strong inverse correlations between CD4 count and viral load (r = -0.62; P = 0.03) and negative correlation between CD4 count and %CD38/CD8+ (r = -0.48; P = 0.05). However, there was no correlation between CD4 counts and D-dimers

6.5 Discussion

Persistent immune activation characterises HIV infection and is associated with the depletion of CD4+ T-cells, increased risk of disease progression and higher mortality. The breakdown of gut mucosal integrity in early HIV infection results in translocation of microbial products such as LPS into the systemic circulation. This is an important source of on-going immune stimulation. In this study therefore, we developed an assay to determine the ability of CD4+ T-cells in HIV to be activated by LPS *in vitro* and further, to be inhibited by selected antioxidants.

LPS induced a significant increase in CD25 (activation marker) expression in HIV infection when compared to that of the controls (P = 0.68). Thus, at this stage of the infection, with relatively well maintained CD4 counts and no clinical symptoms; CD4+ T-cells in HIV infection retains the ability to be activated, which was significantly reduced by NAC and a combination of NAC and vitamin C. After incubation with antioxidants and stimulation with LPS, interestingly, the HIV positive group showed good responses to NAC alone and a "cocktail" of NAC and vitamin C; with CD25 levels returning to below baseline values; however, a similar effect could only be demonstrated in the control group with vitamin C alone. The combination of vitamin C and NAC was required to; achieve optimal inhibition of the LPS induced-activation.

LPS induces activation of innate immune cells such as monocytes and dendritic cells resulting in increased production of pro-inflammatory cytokines consequently inducing oxidative stress. This leads to depletion of antioxidant defence mechanisms and an increased susceptibility to apoptosis. CD25 is a relatively late marker of activation [16]. In the study by Tincati *et al.* on the effects of *in vitro* LPS stimulation on T-cells in patients on HAART, significantly higher CD4+ & CD8+ expressing HLA-DR and CD38+ expressing cells were detected in low and intermediate responders compared to the HIV negative group

confirming a sustained immune activation in HIV infection [15]. The current study confirms Tincati *et al*. findings of increased LPS-induced immune activation as measured by CD25 expression in HIV infection when compared to controls. Furthermore, there was a significantly ($P<0.05$) increased immune activation expressed as CD38 in the HIV positive group when compared to the controls.

Annexin V staining was also significantly increased after stimulation with LPS in HIV-positive and control group, but was more pronounced in the HIV positive group. NAC alone and in combination with vitamin C, significantly reduced early apoptosis of CD4+ T-cells to a greater degree than vitamin C alone. Moreover, LPS induced a significant increase in 7-AAD staining in HIV infection, which was significantly reduced by the antioxidants either alone or in combination.

This study demonstrates that LPS was capable of inducing CD4+ T-cell activation and apoptosis *in vitro* as indicated by increased CD25, Annexin V and 7-AAD, which was ameliorated by the combination of antioxidants. In addition, NAC alone significantly reduced LPS-induced activation and apoptosis of CD4+ T-cells in HIV infection. Early work demonstrated that NAC administration to HIV positive individuals was able to slow down CD4 decline in HIV infection [17]. NAC and glutathione have been shown to completely block activation induced death and associated DNA fragmentation in T-cell hybridomas, therefore implicating redox regulation in the processes [18]. In addition, NAC has been shown to directly scavenge free radicals by decreasing hypochlorus acid produced by neutrophils [19,20]. Cell studies have indicated that NAC enhances intracellular killing of bacteria by protecting the neutrophils and macrophages from free radicals generated during phagocytosis [21]. In support of the current study findings, NAC has been shown previously to inhibit LPS-mediated activation; however this work was performed in rats kupffer cells [14]. The effect of NAC on T-cell activation and apoptosis particularly in the context of HIV infection is not well documented and therefore the findings of this study may be of value in the future management of persons living with HIV.

An important finding of this study was that vitamin C had no effect on LPS-induced activation when used alone in HIV; but when used in combination with NAC (cocktail); it showed a significant reduction in CD4+ T-cell activation levels. Previous studies have reported clinical improvement in AIDS patients who willingly consumed high doses (500 mg, 800 mg and 1 800 mg) of ascorbic acid [22,23,24,25]. In addition vitamin C has been shown to inhibit NF-kB activation via multiple stimuli including IL-1 and TNF in endothelial cell lines ECV30VS and in primary HUVECS [26]. Several studies have shown that, vitamin C inhibited T-cell pathways of apoptosis, which includes up-regulation of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) protein expression levels [27,28,29]. Although vitamin C exhibits strong antioxidant properties, it has been demonstrated *in vitro* that it can also act as a pro-oxidant in the

presence of free transition metals [30,31]. In this setting it generates hydroxyl radicals in a fenton-like reaction. This could explain the cause of activation of CD4+ T-cells when vitamin C was used alone in the current study. In support of this, Bergman *et al.* demonstrated a 39 % increase in apoptotic cells when cells were incubated with 0.2 mg/ml vitamin C for 24 hrs [32]. Previous data on clinical trials using vitamin C have been conflicting. Some authors have suggested that supplementation with vitamin C is toxic [33,34]. In this study, only low concentrations (10 mM) of vitamin C were utilized. Previous work in our laboratory (results not shown) had demonstrated that higher concentrations were toxic and able to cause activation and even death of the cells. Thus, at higher doses vitamin C is likely to have a pro-oxidant effect which causes activation and even death of cells.

Therefore, the current study has developed an important assay that demonstrates the response of CD4+ T-cells to LPS-induced stimulation and further, showed the effects of antioxidants in this process. The study was able to demonstrate that at this stage of HIV infection, CD4+ T-cells were able to respond to LPS-induced stimulation and antioxidants; therefore do not appear 'exhausted' at this stage of the disease. However, it should be noted that LPS induced more death in the form of Annexin V+/7-AAD+staining in the HIV group, than the control; suggesting that the cells may have been "primed" for death previously *in vivo.* Vitamin C alone did not inhibit LPS-induced activation in the HIV group as it did in the controls; suggesting that the use of vitamin C alone in HIV infection would not be of value. The combination of NAC with vitamin C produced the greatest level of inhibition of early apoptosis; suggesting a potential beneficial effect of this cocktail in the management of this stage of the infection. It is at this early stage of HIV infection that the 'cocktail' is most effective and this study has demonstrated beneficial effects of the cocktail in limiting immune activation and early apoptosis. This way immune cells can be rescued before irreversible damage to the cells occurs.

Limitations of the study were that specific tests for diagnosing underlying subclinical infections could not be performed and smoking and alcohol habits were not documented. Longitudinal cohort studies will be important to determine the value of intervention with the combination of anti-oxidants as described in this study, in HIV positive persons with CD4 counts > 350 cells/mm^3

6.6 Conclusion

This is an important assay that demonstrated the response of CD4+ T-cells to LPS-induced stimulation and showed the inhibitory effects of antioxidants. Certain combinations of antioxidants could be important in reducing the harmful effects of chronic immune activation and thereby limit CD4+ T-cell depletion. Importantly, the study showed that CD4+ T-cells of the HIV positive group responded better to a combination of vitamin C and NAC. Therefore

appropriate intervention at this asymptomatic stage, could rescue the cells before exhaustion and senescence sets in.

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CHAPTER 7

Modulation of LPS-induced immune activation and oxidative stress by vitamin C and N-acetyl cysteine in untreated asymptomatic HIV individuals: an *in vitro* study

CHAPTER 7

Modulation of LPS-induced oxidative stress by vitamin C and N-acetyl cysteine in untreated asymptomatic HIV individuals: an *in vitro* study

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Contributorship: SM researched literature, wrote the first draft and acquired data of this study. JM was involved in concept and design, protocol development reviewing and intellectual input. AA was involved in concept and design, protocol development, reviewing and intellectual input of the study. HI was involved in concept and design, protocol development, gaining of ethical approval, participants' recruitment, reviewing and intellectual input.

Abstract

Chronic immune activation, with resultant inflammation, induced oxidative stress and increased susceptibility to apoptosis, characterises HIV infection.

The purpose of this study was to determine whether a combination of selected antioxidants (vitamin C & NAC) were capable of limiting LPS-induced oxidative stress in asymptomatic untreated HIV-infected South African participants.

After treatment with NAC & vitamin C and activation with LPS, ORAC and TEAC values significantly decreased, while a significant ($P<0.05$) increase in CDs was noted in the HIV-infected group, but with no effect on the MDA levels in this group. NAC and vitamin C decreased TBARS levels, which was more significant in controls. A decrease in GSH and GSSH with a resultant increase in GSH: GSSG ratio in both the HIV-infected and controls was observed after activation with LPS and treatment with NAC & vitamin C.

The results indicate that treatment with these antioxidants rendered some protection against the induced oxidative stress, as supported by the improved redox status of glutathione and that *ex vivo* treatment of HIV-infected whole blood with vitamin C and NAC could be beneficial in that the treatment with LPS did not compromise the total antioxidant status at the same level as it did in controls.

Keywords: Lipopolysaccharide, oxidative stress, antioxidants, vitamin C, *N*-acetyl cysteine, glutathione.

7.1 Introduction

HIV infection is characterised by chronic inflammation, immune activation and oxidative stress, which predisposes patients to thrombotic complications and progression to AIDS. LPS, produced from the breakdown of the gastrointestinal barrier ("leaky gut" phenomenon) during early HIV infection, induce activation of innate immune cells such as monocytes and dendritic cells, resulting in increased production of pro-inflammatory cytokines and consequently inducing oxidative stress [1,2]. Reactive oxygen species participate in GIT epithelial cell injury, contributing to the breakdown of the GIT barrier. Furthermore, ROS, formed as a result of the activation of cells such as neutrophils, macrophages and monocytes, promote inflammation and continuous immune activation in HIV infection [1]. This leads to depletion of antioxidant defence mechanisms and increased susceptibility to apoptosis.

HIV infection has been shown to affect levels of antioxidant enzymes such as GPx [3]. Furthermore, lymphocytes of HIV/AIDS patients are known to be deficient of GSH, an important endogenous antioxidant, therefore making them susceptible to oxidative stress [4]. HIV protein-Tat has been shown to induce H_2O_2 signalling, resulting in enhanced T-cell activation and death signal expression [5]. Therefore, it is likely that both the depletion of endogenous antioxidants such as GSH and the increased production of H_2O_2 as a result of chronic inflammatory processes, contribute to the depletion of CD4+ T-lymphocytes in HIV infection [6].

Whereas several studies have linked a weakened antioxidant defence system to the increased oxidative stress found in HIV/AIDS infection whether as a result of increased utilization or reduced intakes of antioxidant micronutrients [7], the impact of direct stimulation

of the innate immune system on CD4+ T-cell death, has not been well described in the literature to date. More importantly, stage II of HIV infection has been associated with higher free radical production [8,9,10]. Allard *et al.* showed an increased oxidative stress in HIV-positive patients than in seronegative control subjects and decreased plasma concentrations of various antioxidant vitamins and selenium [11]. Some investigators have suggested that the best way to prevent immune depression in HIV infection is to reduce oxidative stress, which is key at the earliest stage [12,13].

Therefore, reducing oxidative stress, may limit the susceptibility of immune cells to death by apoptosis. Dobmeyer *et al.* presented the evidence that oxidative stress plays a fundamental role in initiation of apoptosis in HIV infection [14]. They noted that ROS generated under circumstances similar to those that occur during opportunistic infections, promotes apoptosis and further, that this effect could be ameliorated by antioxidants. Montagnier theorized that the majority of the T-helper (CD4) cell depletion occurs essentially by apoptosis and not by direct HIV infection and this has been confirmed in subsequent studies in patients [15,14]. It has also been shown that T-cell activation induces death ligand expression and that increased superoxide generated in the mitochondria results in up-regulation of FasI [16].

The effects of vitamin C and NAC on LPS-induced stimulation of the immune system and oxidative stress in asymptomatic HIV infection have not been well documented. Therefore, in this study, baseline levels of oxidative stress markers and antioxidant status after stimulation with LPS and incubation with vitamin C and NAC were determined in asymptomatic untreated HIV-infected individuals. These markers were compared with those of an HIV negative group and correlated with other markers of the disease.

7.2 Materials and methods

7.2.1 Reagents

Monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and dibasic sodium phosphate ($Na_2HPO_4.12H_2O$), hydrochloric acid (HCl), sodium phosphate, (NaH_2PO_4), per chloric acid ($HClO_4$), hydrogen peroxide, chloroform, methanol, cyclohexane, sodium di-hydrogen orthophosphate dehydrate ($NaH_2PO_4.2H_20$), di-sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_20$), metaphoshoric acid (MPA), trichloric acetic acid, 5,5' dithiobis-(2-nitrobenzoic acid) were purchased from Merck Chemicals (South Africa). Fluorescein sodium salt ($C_{20}H_{10}Na_2O_5$), 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), L-ascorbic acid and iron (III) chloride salt, GSH, GR, NADPH solution and sodium azide solution, nicotinamide adenine dinucleotide phosphate (NADPH), 2,4,6-Tri [2-pyridyl]-s-triazine (TPTZ), 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP), 6-hydroxydopamine (6-HD), vitamin C, N-acetyl cysteine (NAC), LPS (from Salmonella) and GSSG were purchased from Sigma-

Aldrich (South Africa). The fluorescence was read on a Fluoroskan plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). The absorbance was read in a Multiskan plate reader (Thermo Fisher Scientific, Waltham, Mass., USA).

7.2.2 Study population and design

In this cross-sectional study, 20 untreated asymptomatic HIV positive participants and 20 uninfected controls (32 females and 8 females) were recruited from the same HIV testing and prevention primary health clinic in Crossroads, Cape Town (South Africa). The clinic uses the national HIV counselling and testing (HCT) testing algorithm with accredited rapid tests. The median age of the participants was 32 years (range 21 - 57). There was no significant difference (P = 0.43) in age between the two groups. The patients' demographics are summarised in table 7.1. The HIV-infected group had a significantly (P = 0.0003) lower CD4 count compared to the uninfected control group. Informed consent was obtained from all the participants, with inclusion criteria for the study comprised of 21 years or older, individuals with HIV infection and CD4 counts of >200 cells/mm^3 and not taking ARVs or any other chronic medication or antioxidant supplements. Volunteers with tuberculosis (TB) or other co-infections and those receiving antiretroviral therapy, anti-TB treatment or other antibiotic treatment, minerals and vitamins supplements, aspirin or any other drugs e.g. anti-inflammatory, with established antioxidant properties, were excluded from the study.

Ethics approval was obtained from both the clinical site, University of Cape Town: REC: REF: 417/2006 and laboratory site, University of Stellenbosch: N07/09/197. This sub study was added as an addendum to the ethics approval received for the original project N07/09/197.

7.2.3 Sample preparation

Fresh heparin, EDTA and sodium citrate blood was collected from the antecubital vein and transported on ice to the laboratory for immediate processing. For baseline redox status determinations, plasma were obtained from 1 ml heparinised whole blood, centrifuged at 1200 g at 4 ^oC for 10 minutes. The plasma samples were aliquoted and frozen at -70 ^oC until the day of analysis (within 2 months). Fresh EDTA blood was aliquoted immediately for CD4, CD8 counts and viral loads, while citrate blood was used for fibrinogen and D-dimers determination, which was done within 4 hours of collection.

7.2.3.1 GSSG sample preparation

A 100 µL portion of EDTA-treated whole blood was added to 10 µL M2VP and mixed gently and frozen at -80^oC. The samples were thawed and mixed immediately and incubated at room temperatures for 2-10 minutes thereafter, 290 µL of cold 5% MPA was added to the tube and vortexed for 15-20 seconds. The mixture was centrifuged at 10 000 x g for 10

minutes. A 25 µL portion of this MPA extract was added to the buffer in Eppendorf tubes and the extracts were placed on ice until use.

7.2.3.2 GSH sample preparation

A 50 µL portion of whole EDTA-treated blood was aliquoted into tubes and frozen at -80 ^{o}C. On the day of analysis, the samples were thawed and mixed immediately with 350 µL of 5% cold MPA and vortexed for 15-20 seconds, centrifuged at 10 000 x g for 10 minutes. A 5 µL portion of the MPA extract was added to 300 µL of buffer and placed on ice until use.

The baseline samples were treated with vitamin C and NAC only and incubated overnight. For LPS-stimulated samples, I ml of fresh whole blood, were incubated at 37 ^{o}C with 300 µl of vitamin C (10 mM) and 300 µl of NAC (5 µM). After 20 minutes, 200 µl of LPS (2 µg/ml) was added and samples were further incubated overnight at 37 ^{o}C. Thereafter, plasma was obtained by centrifugation at 1200 g at 4 ^{o}C for 10 min. All plasma samples were aliquoted and frozen at –80 ^{o}C until analysis, which was done within 2 months.

7.2.4 Laboratory analysis

7.2.4.1 CD4 counts and CD38/8

Heparin blood samples (50 µl) were mixed with 20 µl of the multitest reagent of antibody mix (CD4, CD45, CD8, CD38) and incubated for 15 min in the dark at room temperature. Red blood cells were lysed with 450 fluorescent activated cell sorter (FACS) lysing solution from Beckton Dickinson (San Jose C.A, USA). The cells were washed once in PBS (1300 g), resuspended in staining buffer and analysed on flow cytometer (FACSCalibur B.D Biosciences., San Jose C. A, USA). The results were expressed as percentage of the gated cells.

7.2.4.2 Viral loads

EDTA blood was used for the quantification of viral loads. The plasma viral load was determined by the amplification of viral RNA on duplicate samples using PCR and detecting the real time molecular beacons, according to the assay kit manufacturer's (BioMerieux Inc., Boxtel, Netherlands) specification. Seropositivity of the participants was determined at the HIV counselling and testing prevention clinic, which uses the national HCT testing algorithm, with accredited rapid tests. Based on WHO/CDC clinical and immunological classification (CD4 ≥ 350cells/mm^3 and no symptoms), the participants were classified asymptomatic, with 12 participants having a CD4 count of ≥350cells/mm^3, while 8 had a CD4 count lower than 350 but >200 cells/mm^3.

7.2.4.3 Fibrinogen

Fibrinogen, a marker of inflammation, was determined by the Hemos IL-fibrinogen C method using ACL TOP (Beckman Coulter SA), which utilises excess thrombin to convert fibrinogen

to fibrin in diluted plasma. Sodium citrate blood was centrifuged at 2000 g for 15 min to obtain plasma which was aliquoted. Plasma samples were mixed with fibrinogen reagent and the absorbance was read at 405 nm. The results were expressed in g/L with normal ranges being 2.0 – 4.0 g/l [17].

7.2.4.4 D-dimers

D-dimer, a marker of fibrinogen breakdown and clot formation, thus an indirect marker of inflammation, was estimated by spectrophotometry using the IL-D-dimer method (Legnani *et al.,* 2010). This method is an automated immunoassay for the quantitative determination of D-dimers in plasma. Plasma from sodium citrate blood samples, was mixed with latex reagent and buffer, (Beckman Coulter, Miami, Florida USA), allowing for agglutination and measured as a decrease in absorbance read at 405 nm using ACL TOP (Beckman Coulter, Miami, Florida, USA). The results were expressed in mg/L and the normal ranges are 0.00 – 0.25 mg/l [18].

7.2.5 Biochemical measurements

The total antioxidant status of the participants was determined using a battery of assays including; ORAC, TEAC, lipid peroxidation products (MDA/TBARS & CDs) and redox status of GSH.

7.2.5.1 Oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) was determined using the modified fluorescein method by Ou *et al.* which is based on the principle that the fluorescence of an oxidizable substrate e.g. fluorescein, changes with respect to time upon damage caused by a peroxyl radical [19]. Calculations were performed using a Microsoft Excel® spreadsheet.

7.2.5.2 Trolox equivalent antioxidant capacity

The Trolox equivalent antioxidant capacity (TEAC) was determined using the method described by Re *et al.* [20]. The ABTS radical (prepared by reacting 8 mM ABTS salt with 140 mM potassium peroxodisulphate) was prepared 24 hours before the assay while stored in the dark. The ABTS radical; solution was diluted with distilled water (1:20) to give an absorbance of 1.5 at 734 nm.

7.2.5.3 Lipid peroxidation

Lipid peroxidation was assessed by measurement of CDs and MDA. The level of plasma conjugated dienes was estimated according to the method of Recknagel & Glende [21]. Plasma MDA was determined using a HPLC method adapted from Khoschsorur *et al.* based on the reaction of thiobarbituric acid (TBA) with MDA [22]).

7.2.5.4 Glutathione redox status analysis (GSH:GSSG)

The total glutathione (GSH and GSSG) was measured according to the method described by Asensi *et al.* [23]. The glutathione redox analysis is based on the enzymatic recycling method with GR.

7.3 Statistical analysis

The data was analysed using the Graphpad Prism version 5 statistical analysis software. Analysis of variance (ANOVA) was used to determine whether the means of the two groups (HIV + and HIV-) differed significantly. No assumptions were made; Mann Whitney non-parametric test and Spearman's correlation were applied. Results were reported as medians (interquartile range). A 5% or lower significance level ($P<0.05$) was used to judge significant findings.

7.4 Results

7.4.1 Demographics of study population

Participant information and baseline clinical characteristics are summarized in table 7.1. The group included 20 HIV-infected and 20 uninfected controls, most of whom were females. The mean age of HIV-positive participants were 31 years and 30 years for the control group. The HIV-infected group had a well maintained CD4 count, averaging 464 cells/mm^3 (median 411 cells/mm^3), asymptomatic according to WHO classification and clinically well with a median viral load of 45705 copies/ml. The uninfected group had a significantly ($P = 0.0012$) higher CD4 count, with an average of 746 cells/mm^3 (median 753 cells/mm^3) and also clinically well. Table 7.2 shows the values of two markers of inflammation, of the two participant groups measured before treatment with antioxidants and activation with LPS. When compared with the HIV negative participants, the HIV positive group had significantly ($P<0.05$) lower CD4 counts. CD38/8 was significantly ($P<0.005$) higher in the HIV positive participants than in the uninfected control group.

Table 7:1: Demographic characteristics of both the HIV-infected and HIV-negative participants

Parameter	HIV Infected group (*n*=20)	Controls (*n*=20)	P-value
Male: Female	5:15	3:17	
Median age (yrs.)	31.8(27-35)	30.3(22-35)	0.430
Age range (yrs.)	21-51	21-57	
Median CD4 cell count (cells/mm^3)	411(265-634)	753(564-870)	0.001
CD4 count (<350, >200)	n=9		
CD4 count (>350)	n=11		
CD38/8	27.6(17.5–44.0)	11.6(7.3–15.8)	0.001**
Median Viral load (copies/ml)	45705(2174–157294)	ND	
Log Viral load	4.0(3.1-5.4)		

Some of the values in columns are medians (Interquartile range) of HIV-infected (*n* = 20) and controls (*n* = 20).

7.4.2 Other markers of disease in HIV infection measured in these participants

When considering D-dimers, an indirect marker of fibrinogen breakdown and clot formation, the levels were significantly (P<0.05) higher in the HIV-infected participants than in the uninfected control study participants, while fibrinogen, a marker of inflammation, a well-described acute phase reactant, was not significantly (P>0.05) different between the two groups (Table 7.2).

Table 7:2: Baseline levels of inflammatory markers (both direct and indirect) of the HIV-infected and uninfected controls

	HIV-positive	Controls	P-value
Fibrinogen (g/L)	2.7(2.3 3.1)	2.8(2.4-3.2)	0.32
D-dimers (mg/L)	0.23(0.2–0.3)	0.21(0.20–0.20)	0.02**

Values in columns are medians (Interquartile range).** Medians significant at P<0.05.

7.4.3 Plasma total antioxidant status

The TAS measured as ORAC and TEAC of the forty study participants are shown in table 7.3. There were no significant (P>0.05) differences in baseline ORAC and TEAC levels between the HIV positive and uninfected control group. However, after treatment with NAC and vitamin C and LPS activation, the baseline TAS (both ORAC & TEAC) decreased significantly (P≤0.005) between the two groups and between experiments i.e. baseline levels before and after stimulation with LPS. The decrease in TAS after treatment with NAC and vitamin C and activation with LPS was more pronounced (P<0.05) in the uninfected control group when compared with the HIV-infected group.

Table 7:3: Plasma total antioxidant status measured as ORAC and TEAC of study participants

	ORAC (unstimulated)	ORAC (NAC + Vit C & LPS- activated)	P-value	TEAC (unstimulated)	TEAC (NAC + Vit C & LPS-activated)	P-value
HIV +	903.3(630.0-994.3)	710.0(609.7-761.9)	0.05	7849(6128-8482)	4532(3435-4997).	0.000
HIV-	884.8(760.3-1179.0)	558.2(501.9-601.9)	0.0001	8048(6513-9068)	3429(2813-4142)	0.000
P-value	0.5	0.003		0.4	0.020	

All values in columns are medians (interquartile range) (all samples analysed in triplicates and repeated. P<0.05 considered significant, P>0.05 considered not significant.

7.4.4 Lipid peroxidation markers

Plasma levels of both lipid peroxidation markers, MDA and CDs are shown in table 7.4. The baseline levels of CDs in the two groups were similar and did not differ significantly (P>0.05). However, the CD levels significantly (P = 0.0075) increased in HIV positive participants after treatment with NAC and vitamin and overnight stimulation with LPS, while the levels in the uninfected controls did not increase significantly (P = 0.88). The increase, after LPS activation and antioxidant treatment, between the two groups was significantly (P = 0.0002) higher in the HIV-infected participants. When considering the baseline levels of MDA, there was no significant differences between the two groups (P = 0.32). Interestingly, after overnight stimulation with LPS and incubation with antioxidants, the MDA levels decreased significantly (P<0.0001) in the uninfected controls and only slightly, although not significant (P = 0.15) in HIV–infected individuals.

Table 7:4: Changes in participants lipid peroxidation markers after treatment with antioxidants (NAC & vit. C) and LPS activation

	CDs (unstimulated)	CDs (NAC + Vit. C & LPS-activated	P-value	MDA (unstimulated)	MDA (LPS-stimulated)	P-value
HIV +	121.1(101.9-142.1)	141.6(133.2-149.9)	0.007	3.4(3.0–4.0)	3.1(2.8–3.3)	0.150
HIV -	124.3(114.0–132.6)	121.0(116.3-132.3)	0.880	3.6(3.1-4.8)	2.3(2.1-2.8)	< 0.000**
P-value	0.98	0.000**		0.320	0.002**	

The values in columns are medians (Interquartile range), (all samples analysed in triplicates). ** Medians significant at P<0.05.

7.4.5 Erythrocyte glutathione redox status

Concentrations of erythrocyte GSH and GSSG as well as the ratios of these two glutathione forms (indicative of the redox status) of the study population are shown in the table 7.5. The baseline GSH and GSSG levels of the HIV positive and uninfected controls were similar and did not differ significantly (P>0.05). However, after treatment with NAC and vitamin C and LPS activation, both these levels, significantly (P<0.0001) reduced in both groups, which was

more pronounced in the HIV–infected group (P = 0.0001). When considering the baseline GSH:GSSG ratio, it was noted that this ratio was lower in the HIV-infected participants, although not significant. After treatment with NAC and vitamin C and activation with LPS, the glutathione ratios significantly increased in both groups, as a result of the significant decrease in GSSG levels.

Table 7:5: Levels of erythrocyte reduced (GSH) and oxidized (GSSG) glutathione and the ratios of the two forms in the study population at baseline and after antioxidant treatment and LPS activation

	Erythrocyte glutathione redox status					
	HIV+ (NAC & Vit. C) (unstimulated)	**HIV+ NAC+ Vit.C & LPS (stimulated)**	**P-value**	**HIV- (NAC & Vit. C (unstimulated)**	**HIV-(NAC+Vit. C & LPS) (stimulated)**	**P- value**
GSH (µM)	4383(3176–5592)	726.6(416.9–1133.0)	**<0.0001 ** **	4108(3271–5362)	938.4(456.8–1342.0)	**< 0.000** **
GSSG (µM)	680.9(423–708.6)	101.3(47.6–160.0)	**<0.0001 ** **	659.3(597.1–742.7)	98.4(63.8–183.4)	**< 0.000** **
GSH:GSSG ratio	5.7(3.9–5.8)	7.0(5.2-9.6)	**0.010** **	5.6(4. -6.1)	8.1(5.6–10.7)	**0.005** **

Values in columns are medians (interquartile range). ** Medians statistically significantly at P<0.05.

7.5 Discussion

LPS induces the activation of innate immune cells such as monocytes and dendritic cells, resulting in increased production of pro-inflammatory cytokines, consequently inducing oxidative stress, which could lead to the depletion of the antioxidant defence system and an increased susceptibility to apoptosis. Therefore in this study, we determined whether a combination of selected antioxidants (vitamin C & NAC) were capable of limiting LPS-induced oxidative stress, and thus protecting the cells from activation and oxidative stress-induced cell death, in asymptomatic untreated HIV-infected South African participants. Baseline ORAC and TEAC levels in both HIV–infected and uninfected controls were similar, however, after treatment with NAC and vitamin C and activation with LPS, the baseline TAS (both ORAC & TEAC) decreased significantly ($P \leq 0.005$) in both groups. When comparing the two groups after antioxidant treatment and LPS activation, the uninfected controls showed a significantly lower TAS (expressed as TEAC and ORAC). These results indicate that *ex vivo* treatment of HIV-infected whole blood with vitamin C and NAC could be beneficial, in that the treatment with LPS did not compromise the total antioxidant status at the same level as it did in the uninfected controls blood. It is not known whether this phenomenon will be the same in HIV-infected individuals and will still need to be confirmed. There were no significant ($P > 0.05$) differences between baseline plasma CD & MDA levels in HIV positive participants when compared to the uninfected group. However, after treatment with NAC & vitamin C and activation with LPS, a significant ($P < 0.05$) increase in CDs was noted in the HIV-infected group, but with no effect on the MDA levels in this group. The results thus indicate that in this study, the treatment with vitamin C and NAC did not protect against the LPS-induced oxidative stress when considering the early lipid peroxidation marker, CD. Although a protective effect was shown against the LPS-induced oxidative damage for the late lipid peroxidation marker, MDA, indicative of some protection against oxidative lipid damage. When considering the HIV negative participants, the CD levels remained the same, while the MDA levels significantly decreased after the treatment regime, also indicative of a protective effect, as expected in non-immuno-compromised cells.

Vitamin C supplementation has been shown to significantly reduce oxidative stress and inflammation, indicated by reduced F_2-isoprostanes, prostaglandin E_2 and monocyte chemotactic protein-1, albeit in healthy individuals [24]. By scavenging free radicals, thus preventing them from interacting with low density lipopolysaccharides (LDL), vitamin C was shown to inhibit LDL oxidation [25]. As discussed earlier, LPS is a well-known inducer of activation of innate immune cells. Immune cells such as monocytes and CD4+ T-lymphocytes express toll like-receptor-4 (TLR4) which is a receptor for LPS [26,27]. TLR4 activates various signalling pathways which induce activation of transcription factor NF-kB

with subsequent production of pro-inflammatory cytokines, chemokines, antimicrobial peptides and other defence molecules such as ROS. The pro-inflammatory cytokines such as TNF-α produced are capable of activating innate immune cells to produce more ROS, therefore causing increased oxidative stress as indicated by increased lipid peroxidation markers (CDs and MDA) and depletion of antioxidant defences as indicated by lower ORAC and TEAC after stimulation with LPS as seen in both the HIV-infected and uninfected controls in this study.

In the current study, the baseline GSH, GSSG and GSH:GSSG ratios of the two groups were similar and did not differ significantly. The high levels of GSH measured in both groups before LPS activation, can be explained as these are higher levels than previously reported in the literature for specifically South Africans [28]. At baseline the blood samples for the current study were treated with NAC, which is a cysteine analogue and a glutathione precursor and can account for the increased levels measured. After LPS-induced oxidative stress, the levels of oxidised glutathione significantly decreased in both groups, indicative of less oxidative damage to glutathione. This decrease was more pronounced in the HIV-infected cells, indicating that treatment with these antioxidants rendered some protection against the induced oxidative stress, as supported by the improved redox status of glutathione (measured as the ratio of reduced to oxidised glutathione). Deficiency in glutathione has been associated with increased mortality in HIV infection [29]. Furthermore, apoptosis depends on caspases, a class of cysteine-dependent and glutathione redox status sensitive enzymes. It has previously been reported that the cytochrome release from the mitochondrial is associated with exhaustion of glutathione and that Bcl-2 driven blockage of cytochrome C release, hinders depletion of glutathione concentration, thus modifying the redox environment towards a reducing one which is unfavorable for apoptosis [30,31]. NAC, a well-recognized thiol antioxidant which is converted to glutathione after uptake as well as glutathione have been shown to completely block activation induced death and associated DNA fragmentation in T-cell hybridomas, therefore implicating redox regulation in the processes [32]. The HIV positive individuals who had a lower antioxidant capacity (measured as ORAC and TEAC) and GSH as a consequence of which they had higher CDs and MDA after LPS stimulation and incubation with antioxidants. This indicates that higher antioxidant capacity is important in modulation of LPS-induced activation. Therefore the higher the immune activation the more the depletion of CD4+ T-cells.

A high correlation between MDA and viral load ($r = 0.58$; $P = 0.03$), CD4 and v/load ($r = -0.62$; $P = 0.03$), CD4 and CD38 ($r = -52$; $P = 0.04$). As expected, the CD4 count was significantly ($P = 0.0012$) different between the HIV-infected and uninfected controls. In addition, the D-dimers were significantly higher in HIV-infected group when compared to the

uninfected controls. Interestingly, the CD38/8 was significantly (P = 0.001) higher in HIV-infected group and correlated with CD4 count.

This study also demonstrated that a combination of vitamin C or NAC was capable of reducing the LPS-induced oxidative stress in both groups, probably via NF-kB signalling pathways. NF-kB is a ubiquitously expressed transcription factor that regulates the expression of hundreds of genes involved in cell survival, differentiation, growth, apoptosis and inflammation. It occurs as an inactive complex in the cytosol, bound to inhibitor of kappa B α (IkB-α) which masks the NF-kB's DNA binding domains. Reactive oxygen species are capable of directly activating IkB kinase (IKK), which phosphorylates the C-terminal ankylin repeats of the IkB-α proteins bound to NF-kB at serine residuals 32 and 36 [33]. This leads to ubiquitination and degradation of the IkB-α unmasking the DNA binding sites subsequently causing translocation of the NF-kB to the nucleus, where it binds to kB sites and activates the transcription of genes involved in cellular processes such as production of pro-inflammatory cytokines, inflammation, thereby promoting oxidative stress [34].

In HIV infection, oxidative stress may cause damage to immune cells, impairing the immune response to the virus and lead to faster disease progression [35,36]. Therefore, protection from oxidative damage in HIV infection might slow down the disease progression [36]. Antioxidants such as vitamin C and NAC have been shown to inhibit this NF-kB activation *in vivo* and *in vitro,* thereby ameliorating oxidative stress [37,38,39,40]. In this study, NAC and vitamin C were able to reduce MDA levels in both groups. CDs, being early markers of oxidative stress during lipid peroxidation process increased after stimulation and incubation with antioxidants. In a controlled clinical trial involving patients with HIV infection, Akerlund *et al.* showed a reduction in TNF levels in serum and interestingly a decreased rate of decline of CD4+ T-cell counts [41].

A limitation of this study was its cross-sectional nature which provides 'snap-shot' insight into oxidative stress levels at the time of patient sampling; nevertheless it was of value in demonstrating the significant differences in HIV at this stage compared with uninfected group. In addition, we were not able to do specific tests for diagnosing underlying subclinical infections and smoking (however, smoking did not affect serum TAS in untreated and treated patients [42] and alcohol habits were not documented.

It will be important to follow up these markers in longitudinal cohort studies to determine their true surrogate value with treatment intervention strategies.

7.6 Conclusion

The results of this study indicate that *ex vivo* treatment of HIV-infected whole blood with vitamin C and NAC could be beneficial in protecting against oxidative stress in HIV infection.

This *ex vivo* investigation was also able to demonstrate that at this stage of HIV infection, a response to oxidative stress is almost similar in HIV-infected and uninfected control groups. Although TAS was lower in HIV-infected group than that of uninfected controls, the response to LPS stimulation and incubation with antioxidants of both groups was in a similar manner. This suggests that antioxidant defence mechanisms of the HIV-infected group do not appear to be 'exhausted' or severely compromised at this stage of the disease and appropriate interventions at this stage might be protective against oxidative damage, as indicated by decreased MDA and increased glutathione redox ratio in this group. The increase in glutathione redox ratio might be important in protecting immune cells from oxidative stress–induced apoptosis.

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7.6.2 References

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CHAPTER 8

General discussion, conclusions, and recommendations

CHAPTER 8

8.1 General discussion, conclusions, and recommendations

8.1.1 A model of inflammation-induced oxidative stress and potential ameliorating interventions in HIV

As mentioned previously, a key occurrence in HIV infection is the continuous activation of the immune system and inflammation as a result of microbial translocation across the GIT mucosal lining during early HIV infection (Brenchley *et al.,* 2006). Microbial products such as LPS moving across the epithelial surface induce activation of the innate immune cells such as macrophages, neutrophils, monocytes and dendritic cells causing increased oxidative stress. As a result, activated immune cells especially macrophages and neutrophils produce high levels of ROS and TNF. Consequently, antioxidant defence mechanisms are depleted, which causes increased susceptibility of the CD4+ T-cells to apoptosis. The arising overload of ROS (specifically H_2O_2) and TNF in conjunction with deficiencies in enzymatic antioxidant defence mechanisms such as SOD, CAT and GPx and other antioxidants such as GSH, vitamin C and E are thought to promote the increased apoptosis and depletion of CD4+ T-lymphocytes seen in HIV infection (Hockenbery *et al.,* 1993; Kotler, 1998; Gil *et al.,* 2003; Reinehr *et al.,* 2008; Morris *et al.,* 2012).

Although more than 10 million people in low and middle income countries are currently receiving ARV's, 10 million more people living with HIV and who are qualified for treatment according to the new WHO guidelines, are still on a waiting list (UNAID, 2010). Currently, it is recommended internationally to commence ARVs at CD4 count of between 350 – 500 cells/mm^3 (WHO, 2013). However, in resource-poor countries this may be difficult to implement, since significant numbers of patients with CD4 count of less than 200 cells/mm^3 have not yet had access to antiretroviral therapies. Despite more than three decades of efforts, the discovery of a cure for HIV/AIDS has remained elusive and a challenge for scientists, especially in our geographical region of sub-Saharan Africa. It is becoming clear that immune activation and inflammation may be stronger indicators or predictors of HIV pathogenesis and prognosis than CD4 count and viral load (Giorgi *et al.,* 1993; Fahey *et al.,* 1998; Liu *et al.,* 1998; Brenchley *et al.,* 2004; Cassol *et al.,* 2010). An insight into the immune activation, inflammatory and oxidative stress status of patients will enable long term profiling of each patient with a view to individualized therapy. This approach may have a direct impact on patient care in resource-limited settings like sub-Saharan Africa.

In order to determine the impact of inflammation, immune activation and oxidative stress in asymptomatic untreated HIV infection, firstly, a preliminary study was conducted to establish baseline levels of oxidative burst and immune stimulation. In this preliminary study, the role of the neutrophil respiratory burst in asymptomatic untreated HIV infection as an *in vitro* indication of response to immune stimulation was investigated. The results of this study indicated that neutrophils from HIV-infected persons at rest and in response to low

stimulating agent (fMLP), had significantly (P = 0.041) higher levels of oxidative burst response compared to uninfected controls. These results suggested 'pre-activation' or priming by ROS or pro-inflammatory cytokines *in vivo,* which may indicate that patients with asymptomatic HIV-infection have higher levels of oxidative stress *in vivo,* as demonstrated by significantly higher baseline resting levels and a 'primed' response to a low stimulating agent. In addition to this, the significantly (P = 0.0069) higher expression of the cellular immune activation marker, CD38/8, may indicate that patients with asymptomatic HIV infection have higher immune activation and higher levels of oxidative stress *in vivo*. Based on results of this preliminary study, ROS and in particular the neutrophil respiratory burst, was considered to be a contributing factor in the persistent immune activation, inflammation and oxidative stress common in HIV infection. This may initiate or activate signalling pathways involved in apoptosis. The oxidative burst elicits a vicious cycle of oxidative stress, inflammation, immune activation and apoptosis leading to faster disease progression to AIDS.

One of the hypotheses of this study was that a dysfunctional enzymatic antioxidant defense system (SOD, CAT & GPx) would lead to an overall increase in H_2O_2 levels, which have been implicated in apoptosis of cells from HIV-infected individuals (Dobmeyer, *et al.,* 1996). The H_2O_2, which is membrane soluble can easily enter the cells and cause apoptosis (Dobmeyer, *et al.,* 1996). In addition, it has been reported that HIV–infected individuals are under chronic oxidative stress (Allard *et al.,* 1998; Jaruga *et al.,* 2002; Gil *et al.,* 2003; Wanchu *et al.,* 2009). Some previous studies have reported a dysfunctional enzymatic antioxidant activity, while others reported increased levels of the activity (Stephensen *et al.,* 2007; Kuller *et al.,* 2008; Pasupathi *et al.,* 2009: Suresh *et al.,* 2009). Therefore in the second phase of the study, we investigated how baseline levels of total antioxidants status relate with other markers of disease in asymptomatic, untreated HIV infection. There was increased oxidative stress as evidenced by increased catalase activity, MDA and CDs with corresponding decrease in antioxidant capacity in the HIV-infected individuals with lower CD4 count. This has important implications for patients not yet on treatment who have relatively well-maintained CD4 count and who are already showing the effects of oxidative stress.

This study also found a significant increase in catalase activity and a similar trend with the other enzymes (SOD & GPx) in this asymptomatic phase of HIV infection. From these results and supported by other studies, it is likely that the antioxidants enzymes increase initially in response to increased oxidative stress to protect the cells from induced oxidative damage. Later, as the disease progresses, the enzymatic antioxidant defenses are overwhelmed by ROS and thus become dysfunctional (Delmas-beauvieux *et al.,* 1996; Stephensen *et al.,* 2007; Kostyushov *et al.,* 2009). Therefore, in this asymptomatic phase of HIV infection, the

enzymatic antioxidant defenses are still functional and only become dysfunctional at a later stage of the infection, depending on other factors such as levels of oxidative stress, nutritional and immune status of individuals.

Previous studies involving LPS-induced activation of cells have utilised varying concentrations of LPS (100 ng/ml - 5 µg/ml), NAC (5 - 50 µM), and vitamin C (25 - 50 nM) therefore the need for optimization of the current study functional assay (Dobmeyer *et al.,* 1997; Shang *et al.,* 2003; Yamanda *et al.,* 2006). A decrease of cellular viability at higher concentrations (>15 µg/ml) of LPS as well as pro-oxidant activity of vitamin C have been reported (Carr & Frei, 1999; Lee *et al.,* 2001; Kim *et al*., 2012). Antioxidants have been shown to have an effect in inhibition of T-cell activation and apoptosis (Meier, 2002). Different concentrations of N-acetyl cysteine, vitamin C and E have been shown to have different effects on T-cell activation (Dobmeyer *et al.,* 1997; Shang *et al.,* 2003; Yamanda *et al.,* 2006). Thus, it was important to determine the dosage that was the most effective or had the desired effects. Combinations of different antioxidants at different concentrations also needed to be tested in order to determine their possible synergistic effects on T-cell activation and apoptosis. This may justify why dietary antioxidants rather than antioxidant supplements have been recommended previously (Krauss *et al.,* 2000; Norman *et al.,* 2003). Therefore, in the third phase of study we determined the optimal concentrations of LPS, NAC and vitamin C. The optimum concentration of LPS, which gave maximum *in vitro* activation of whole blood, was determined to be 2 µg/ml after overnight incubation with LPS. LPS-induced activation was shown to be inhibited at low temperatures (working on ice) when compared to room temperature. Low doses of vitamin C (10 mM) and NAC (5 µm) individually or as a cocktail, were shown to confer protection on LPS-induced activation. Higher concentrations of antioxidants above these optimum concentrations were shown to cause more activation and apoptosis than protection in these cells.

Our second hypothesis was that specific antioxidant intervention could ameliorate HIV inflammation-induced oxidative stress and thereby limit the death of the cells from HIV-infected individuals and may represent a valuable adjunct to the management of patients with earlier stages of HIV infection. Therefore, in the fourth phase of the study we investigated the potential of selected antioxidants (NAC and vitamin C) to ameliorate LPS induced-immune activation and oxidative stress in asymptomatic untreated HIV infection. The results of this study demonstrated a significantly ($P = 0.0075$) decreased antioxidant status, a dysfunctional glutathione redox status and significantly higher ($P = 0.03$) LPS-induced CD4+ T-cell activation in HIV-infected group when compared to uninfected participants.

8.1.2 What are the effects of vitamin C and the glutathione precursor (NAC) on immune activation, oxidative stress and apoptosis of CD4 T-cells in HIV infection?

Vitamin C had no effect on LPS-induced activation when used alone; but when used in combination with NAC (cocktail) it showed significant (P = 0.001) reduction in CD4+ T-cell activation levels. NAC significantly (P = 0.04) reduced LPS-induced activation of CD4+ T-cells in HIV infection. Stimulation of the whole blood with LPS induced a significant increase in the expression of Annexin V and 7-AAD. NAC and vitamin C individually and in combination were able to decrease the expression of Annexin V and 7-AAD significantly.

The antioxidant combination (NAC & vitamin C) decreased MDA values in HIV-infected individuals. A significant (P<0.0001) decrease in MDA levels was noted in the uninfected control group after LPS stimulation and incubation with antioxidants when compared to HIV-infected individuals. Another important finding of this study was the significant (P = 0.01) increase in glutathione redox ratio after incubation with NAC and vitamin and stimulation with LPS. There was a decrease in tGSH and GSSH, which was more pronounced in the HIV-infected group, however glutathione redox status ratio (GSH:GSSG ratio) in both the HIV-infected and uninfected control groups increased significantly after stimulation with LPS and incubation with antioxidants. A change in this ratio or decrease in GSH indicates an increase in GSSG, which in turn indicates lipid peroxidation and thus cellular oxidative stress (Sen, 1997; Schafer & Buettner, 2001).

In addition, this study demonstrated that a combination of vitamin C and NAC could reduce the LPS-induced activation *in vitro*. Importantly, this study demonstrated a significantly increased glutathione redox ratio after treatment with NAC and vitamin C and activation with LPS indicating that NAC, a precursor of glutathione and vitamin C might be important in the replenishment of GSH in HIV infection. This might be of specific importance in correction of the glutathione redox imbalance, which has been implicated in apoptosis. The ability of NAC to restore normal glutathione levels in lymphocytes and in so doing, reduce oxidative stress, has been associated with its health benefits in HIV (Malorni *et al.,* 1993). Oxidative stress and in particular ROS is linked to depletion of glutathione resulting in redox imbalance. As a result, the redox imbalance facilitates opening of permeability transition pore in the mitochondria leading to release of cytochrome C and activation of initiator caspases (3 & 7). It has been reported previously that cytochrome C released from the mitochondria is associated with exhaustion of glutathione and that Bcl-2 driven blockage of cytochrome C release hinders depletion of glutathione concentration, thus modifying the redox environment towards a reducing one which is unfavorable for apoptosis (Circu *et al.,* 2010).

8.1.3 Why do lymphocytes from HIV-infected people show increased susceptibility to oxidative stress?

Throughout this study, lower median values of glutathione redox status (GSH: GSSG ratio) were found in HIV positive individuals compared to negative controls, indicating decreased GSH levels. As previously mentioned, plasma membranes of the cells of immune system contain high levels of polyunsaturated acyl lipids, which are vulnerable to peroxidation making them highly sensitive to oxidative stress. Furthermore, lymphocytes of HIV/AIDS patients are known to be deficient in glutathione, therefore making them susceptible to oxidative stress (Willcox *et al.,* 2004; Guerra *et al.,* 2011).

LPS induces activation of innate immune cells such as monocytes and dendritic cells resulting in increased oxidative stress; depletion of antioxidant defence mechanisms and an increased susceptibility to apoptosis. As CD4+ T-lymphocytes express toll like-receptor-4 (TLR4), this receptor for LPS activates various signalling pathways such as mitogen-activated protein kinases (MAPK), p38 and JNK, which induce activation of transcription factor NF-kB with subsequent production of pro-inflammatory cytokines, chemokines, antimicrobial peptides and other defence molecules such as ROS (Kabelitz, 2007). The pro-inflammatory cytokines such as TNF-α that are produced, are capable of activating innate immune cells to produce more ROS. Reactive oxygen species and pro-inflammatory cytokines in turn induce activation of both the extrinsic and intrinsic pathway of apoptosis. This could explain in part the susceptibility of CD4+ T-lymphocytes to oxidative stress and persistent depletion of the cells during HIV infection.

8.1.4 Does increased SOD activity and reduced GPx activity in immune cells and RBC's in HIV infection, lead to increased oxidative stress and thus promotes enhanced apoptosis?

In the present study, there were no significant differences between the two groups investigated, although the mean SOD activities were higher in HIV-infected than in uninfected individuals. Although lower GPx mean values in HIV-infected individuals were shown in the present study, it was not statistically significant.

Catalase activity was shown to be increased during this asymptomatic phase of HIV-infection; possibly in response to heightened levels of oxidative stress as evidenced by the increased levels of lipid peroxidation markers demonstrated in this study. As discussed previously, results of this study and from others indicated that antioxidant enzymes initially increase in response to increased oxidative stress to protect the cells from induced oxidative damage, however as the disease progresses, the enzymatic antioxidant defenses are impaired by oxidative stress at a later stage of the infection depending on other factors such as the levels of oxidative stress, nutritional and immune status of individuals (Delmas-beauvieux *et al.,* 1996; Stephensen *et al.,* 2007; Kostyushov *et al.,* 2009). Based on the WHO immunological and clinical classification of HIV infection, the participants of this study

were at stage 1 (asymptomatic) with relatively well maintained CD4 count (averaging 460 cells/mm^3 and clinically well). Therefore, this study demonstrated that in this asymptomatic phase of HIV infection, the enzymatic antioxidant defenses were still functional and not overwhelmed by ROS. In addition, the neutrophil burst arm of this study demonstrated the ability of immune cells at this stage of HIV infection to respond similarly to that of uninfected controls in response to various stimulating agents.

Nevertheless, decreased antioxidant status was already evident in this clinically well group of HIV-infected individuals, suggesting that early intervention with selected antioxidants may serve a protective function against further oxidative damage to immune cells. Catalase and antioxidant defenses are likely to be important protective factors in inflammation-induced oxidative stress during asymptomatic HIV infection.

As previously mentioned, the initial objective of HIV treatment was to manage opportunistic infections and simply keep the individual alive, which then later shifted to suppressing HIV replication and managing drug side effects and complications that may arise. Today's emphasis is on improving overall health and enabling people with HIV to live long productive lives (Highleyman, 2010). Highleyman (2010) proposed three ways in which this can be approached: reducing T-cell activation, altering production and activity of cytokines and inflammatory mediators such as ROS and changing underlying risk factors through lifestyle modifications. In line with this, probiotics to reduce pro-inflammatory microbiota and increase anti-inflammatory bacteria in the gut have been proposed as a viable option to address the microbial translocation ("leaky gut phenomenon") (Kwon *et al.,* 2011). Consistent with this, the present study proposes and recommends a personalised/individualised model (summarised in Figure 8.1) of managing the persistent immune activation, inflammation and oxidative stress at the earliest possible time-point, before oxidative damage to the immune cells becomes pronounced. This way, the integrity of the immune cells can be maintained and the risk of progression to AIDS minimised. The present study recommends intervention with a "cocktail" of antioxidants vitamin C and NAC at this point, which were found to have inhibitory effects on oxidative stress and T-cells activation in conjunction with ARVs.

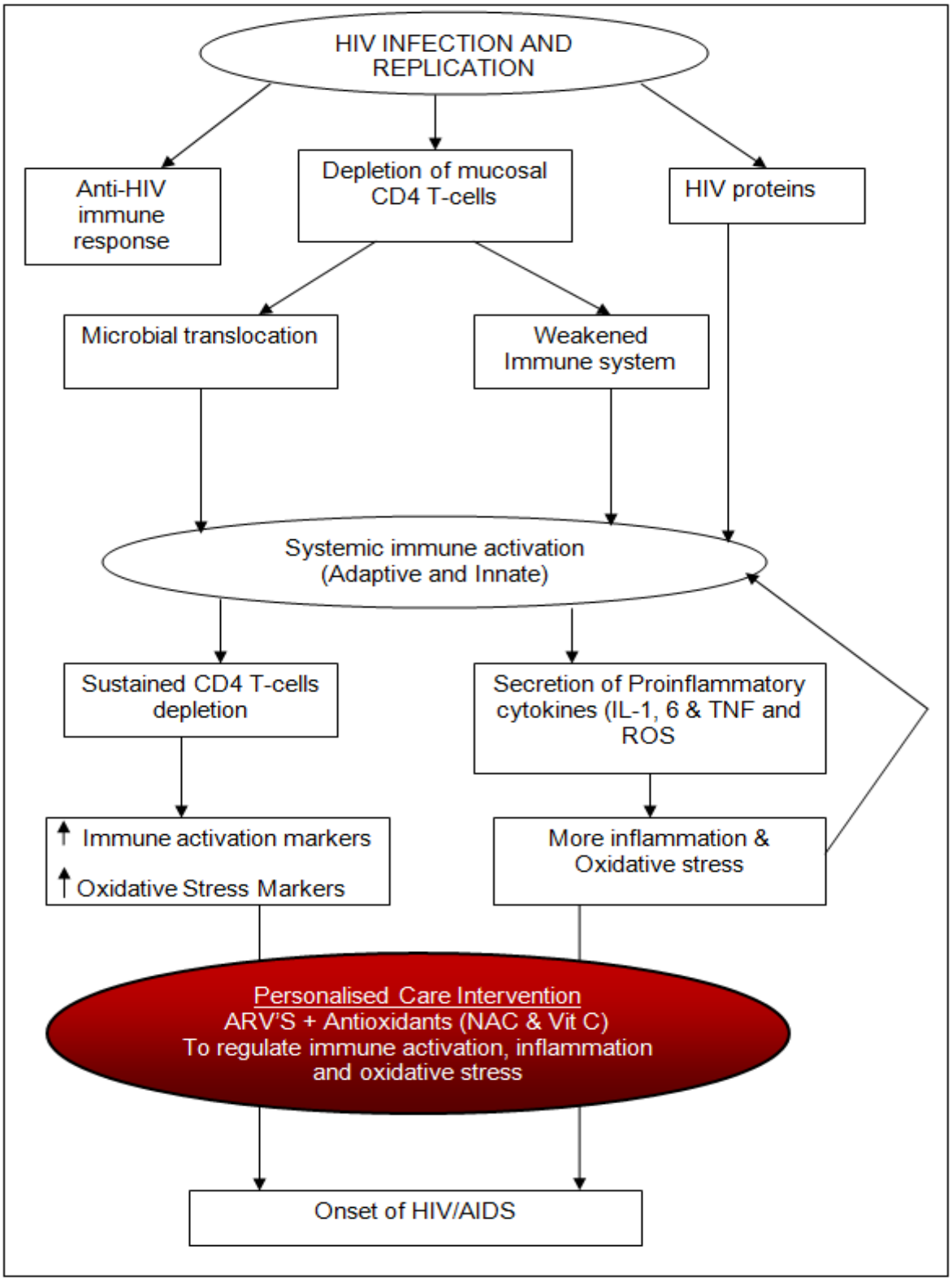

Figure 8:1: A model of inflammatory induced oxidative stress and potential ameliorating intervention (Adapted from Appay & Sauce, 2008).

8.2 Conclusions

In summary, this study has shown higher oxidative burst response in neutrophils of HIV-infected individuals at rest and in response to a low simulating agent, significantly higher catalase activity and lipid peroxidation with decreased total antioxidant status and increased

immune activation in asymptomatic untreated HIV infection, indicating increased oxidative stress in these individuals. This study has also shown increased baseline and LPS-induced immune activation, and that LPS-induced activation and oxidative stress can be modulated by specific combinations of antioxidants (vitamin C and NAC) in this untreated, clinically well HIV study population. The literature study indicated that most of the depletion of CD4+ T-cells occurs during the acute phase (Bundres *et al.,* 1993; Favier *et al.,* 1994; Jarstrand & Akerlund, 1994; Pugliese *et al.,* 2005). Our study has shown that even at this asymptomatic phase and with relatively well-maintained CD4 count, adverse effects of oxidative stress such as decreased TAS, increased lipid peroxidation, dysfunctional antioxidant enzymes, reduced glutathione redox potential and increased immune activation were already evident in individuals. Therefore, early intervention at this clinically asymptomatic stage of infection may be protective against further oxidative damage to immune cells.

Although ROS are produced constitutively by living systems to serve vital biological functions, evidence suggests that they play an important role in apoptosis and thus depletion of the CD4+ T-cells in HIV infection. They also fuel the process of inflammation, which induces immune activation, perpetuating a vicious cycle of inflammation and immune activation. Reactive oxygen species and inflammation act like 'fuel added to the immune activation fire', whose source is the HIV and thus very difficult to put out. At the moment ART remains the best treatment option for HIV/AIDS. The model described in this thesis is not a replacement therapy to ARV's, but a supplementary therapeutic strategy that targets lowering or minimizing immune activation, inflammation and oxidative stress responses in HIV infection. Through data from this study, it is hoped that individualised or personalised management and care strategies can be devised for HIV patients in resource-limited settings.

To the best of our knowledge, this study is the first to use an integrated approach involving not only plasma levels of antioxidant status, but also RBC antioxidant enzyme activities, oxidative damage (lipid peroxidation), inflammation, cellular levels of immune activation and potential ameliorating interventions in evaluating the problem of inflammation-induced oxidative stress in HIV infection. In addition, this is the first study to our knowledge that combines the effect of oxidative stress on apoptosis, total antioxidant status, the enzymatic antioxidant defence system and antioxidant intervention models in one study. The results from this study will certainly contribute to the current knowledge in the field of HIV and oxidative stress by addressing important mechanisms by which dietary antioxidants (in combination) can modulate oxidative stress in specific immune cells (CD4+ T-lymphocytes) from HIV-infected individuals. Therefore, this work represents a novel contribution to the field. Through data from this study, possible management strategies may be devised for HIV patients.

Currently, CD4 count and viral load remain the golden standard tests in HIV management. Recommendations for the commencement of ART are currently at a CD4 count of ≤500 cell/mm^3; however, the roll-out of these medications has only reached 50-60% (about 8, million by 2011) of those requiring therapy in Africa (WHO, 2013; WHO, 2012; UNAIDS, 2012). Furthermore, the current expansion of eligibility of commencement of ART criteria to ≤500 cell/mm^3 has added 10 million more patients to the waiting list of 15 million eligible patients, hence making universal access to ART difficult to attain in resource-poor settings (WHO, 2013). Therefore, other tests, particularly of inflammation, may be required to identify those patients at increased risk of disease progression or other inflammation-associated complications. In line with the findings of this study, it is recommended that integration of other tests such as inflammatory markers (fibrinogen) activation markers (CD38/8) redox status markers (tGSH, GSSG, GSH: GSSG ratio) total antioxidant status (TEAC), RBC antioxidant enzyme activity (SOD, CAT & GPx) lipid peroxidation (MDA/TBARS or CDs) with the routine CD4 count and viral load tests in HIV management and care be considered. In resource-limited settings such as sub-Saharan Africa, these tests, some of which have commercially available assay kits, are accessible and can give a quick insight into the inflammatory, immune activation and oxidative stress status of an individual. Based on the results, tailor-made personalised HIV care and management may be possible.

8.3 Future study recommendations

The preliminary nature of this study only provided ‘snap-shot’ insight into inflammation, immune activation and oxidative stress status of the participants at the time of patient sampling. Although it was of value in demonstrating the significant differences in HIV at this stage compared with uninfected group, it will be important to follow up these markers in longitudinal cohort studies to determine their true surrogate value with treatment intervention strategies. Future research can also be directed at investigating the role of genetic polymorphism in genes coding for apoptosis such as TRAIL, Bim, Fas and FasL, which are involved in the depletion of CD4+ T-cells and thus rapid progression to AIDS in certain individuals and not others. It would also be important to look at whether mRNA or genes involved in the increased surface expression of antigens are also up-regulated or down-regulated or whether their functions are affected at molecular level. In addition, polymorphisms in genes encoding for pro-inflammatory cytokines such as IL-6, TNF-α, INF-α that predispose individuals to inflammation and immune activation, should also be explored.

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CHAPTER 8

ADDENDUM 1:

RESEARCH OUTPUTS

PUBLICATION & CONFERENCE PRESENTATIONS

1. Mburu, S., Marnewick, J. L., Akin Abayomi., Ipp, H. 2013. Modulation of LPS- induced T-cell activation by antioxidants in asymptomatic HIV infection. *Clinical and Developmental Immunology,* Vol. 2013, ID631063, 2013.
2. Mburu, S., Marnewick, J. L., Akin Abayomi., Ipp, H. Modulation of LPS- induced T-cell activation by antioxidants in asymptomatic HIV infection. **Poster presentation** at Laboratory Medicine congress, 28th July - 30th July 2013. Laboratory Medicine congress, CTICC, Cape Town (S.A).
3. Mburu, S., Marnewick, J. L., Akin Abayomi., Ipp, H. Increased Catalase activity and Lipid peroxidation markers with decreased total antioxidant status in asymptomatic treatment naïve HIV infection. **Oral poster presentation** at PATHPOINT Congress 28th - 30th September 2012. Crystal Towers, Cape Town; (S.A).
4. Mburu, S, Marnewick, J. L., Akin Abayomi., Ipp, H. A preliminary study of the neutrophil respiratory burst in asymptomatic HIV patients as an indication of baseline levels of inflammation. **Oral presentation** at Stellenbosch University Research Day Presentation 17th August 2011. Cape Town; (S.A).
5. Mburu, S., Marnewick, J. L., Akin Abayomi., Ipp, H. A preliminary study of neutrophil respiratory burst in asymptomatic HIV infection as an indication of baseline levels of inflammation. **Poster presentation** at Laboratory Medicine Conference; 31st Oct-4thSept 2011. Sandton, Johannesburg (S.A).
6. Mburu, S, Marnewick, J. L., Akin Abayomi., Ipp, H. Impact of inflammation-induced oxidative stress on CD4+T-cells and potential ameliorating interventions; an in vitro HIV model. **Oral presentation** at Stellenbosch University Research Day Presentation 17th August 2013. Cape Town; (S.A).

CHAPTER 8

ADDENDUM 2:

ETHICS APPROVAL

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08 June 2010 **MAILED**

Dr RH Glashoff
Department of Pathology
8th Floor,clinical building
Stellenbosch University
Tygerberg campus
7505

Dear Dr Glashoff

"Modulation of apoptosis of CD4+ T cells from HIV-infected individuals with the neurotransmitter vasoactive intestinal peptide (VIP) and other biomediators."

ETHICS REFERENCE NO: N07/09/197

RE : AMENDMENT

Your letter dated 20 May 2010 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee. Please note that the progress report for this study is due on 19 August 2010; progress report templates is available on www.sun.ac.za/rds

The following projects are included:
- "Modulation of apoptosis of CD4T cells from HIV-infected individuals witht the neurotransmitter VIP and other mediators."
- "Identifying surrogate markers of the activation status of the immune system in chronic HIV-infection as correlates of risk of disease progression."
- "Impact of inflammatory induced oxidative stress on the integrity of immuno-haematopoetic cells and potential ameliorating interventions. An in vitro HIV model."
- "Monocyte/ neutrophil ativation study. An invitro HIV model."
- "Correlating CD8/ CD38 with Fas/ FasL expression in chronic HIV-infection."
- "Activated B lymphocytes in chronic HIV-infection: alternative markers for disease progression."
- "Platelet activation study: An in vitro HIV Model."
- "Development of an LPS-binding protein assay as surrogate marker of inflammation in HIV-infection."
- "DimethylArginine/ arginine study in chronic HIV-infection."
- "Development of an Intracellular signaling assay."
- "Development of RBC oxidant stress assay for chronic diseases."
- "DC maturation and activation: An in vitro HIV model."

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