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**Metabonomics profile and corresponding immune parameters
of HIV infected individuals**

by

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Dedication

This thesis is dedicated to my parents, Mr Quinten B. Williams and Mrs Martha E. Pretorius. Thank you for instilling in me the basic morals, principles and values of life. From an early age you have left me to be “Miss Independent” – leaving me to pursue that which made me happy. The sacrifices you have made and which you are still making have not gone unnoticed but is appreciated beyond what words can express.

To my brothers; Grouchkin and Aidan as well as sister, Danica – I hope that my achievements will serve as a trigger that will encourage you to pursue your dreams and to succeed in all that you may set out to do.



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I, Aurelia Alvina Williams declare that the thesis, which is herewith submitted for the degree Ph.D. Biochemistry at the University of Pretoria, is my own work and has not previously been submitted for a degree at this or any other tertiary institution.

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“Data does not equal information; information does not equal knowledge; and, most importantly of all, knowledge does not equal wisdom. We have oceans of data, rivers of information, small puddles of knowledge, and the odd drop of wisdom ”-

Henry Nix

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LIST OF IMPORTANT ABBREVIATIONS

| | |
|-------------|-----------------------|
| α | Alpha |
| β | Beta |
| β_2m | Beta-2 microglobulin |
| γ | Gamma |
| $^{\circ}C$ | Degree Celsius |
| $\times g$ | Centrifugal Force |
| kDa | KiloDalton |
| μg | Microgram |
| μl | Microlitre |
| μM | Micromolar |
| mg | Milligram |
| ml | Millilitre |
| mm | Millimeter |
| mM | Millimolar |
| M | Molar |
| ng | Nanogram |
| nm | Nanometer |
| ω | Omega |
| pg | Picograms |
| % | Percentage |
| v/v | Volume per Volume |
| NH_4Cl | Ammonium Chloride |
| $KHCO_3$ | Potassium Bicarbonate |
| KCl | Potassium Chloride |
| NaCl | Sodium Chloride |
| H_2SO_4 | Sulphuric Acid |
| Tris-HCl | Tris Hydrochloride |

| | |
|------------------|---|
| ACK | Ammonium Chloride Potassium |
| AIDS | Acquired Immunodeficiency Syndrome |
| AMDIS | Automated Mass Spectral Deconvolution and Identification System |
| APC | Allophycocyanin |
| APCs | Antigen Presenting Cells |
| ART | Antiretroviral Therapy |
| ATP | Adenosine Triphosphate |
| AZT | Zidovudine |
| B cells | B Lymphocytes |
| bp | Base Pair |
| BE-ANCH | 5'-TCCTGGCTGTGGAAAGATACCTA-3' |
| BECO5 | 5'-GGCATCAAACAGCTCCAGGCAAG-3' |
| BECO3 | 5'-AGCAAAGCCCTTTCTAAGCCCTGTCT-3' |
| BSTFA | N,O-Bis (Trimethylsilyl) Trifluoroacetamide |
| C-SPEC | 5'-AGACCCCAATACTGCACAAGACTT-3' |
| Ca ²⁺ | Calcium |
| CBA | Cytometric Bead Array |
| CCR5 | Chemokine Receptor Type 5 |
| CD | Cluster of Differentiation |
| CD3 | Cluster of Differentiation 3 |
| CD4 | Cluster of Differentiation 4 |
| CD8 | Cluster of Differentiation 8 |
| CDC | Centre for Disease Control |
| CE | Capillary Electrophoresis |
| CMI | Cell-Mediated Immune |
| CoQ10 | Coenzyme Q10 |
| CRFs | Circulating Recombinant Forms |
| CSA | Centre for the Study of AIDS |
| CSF | Cerebrospinal Fluid |
| CT | Computer Tomography |

| | |
|-------------------|--|
| CTL | Cytotoxic Lymphocyte |
| CV | Coefficient of Variation |
| CXCR4 | Chemokine Receptor Type 4 |
| dH ₂ O | Distilled Water |
| dNTPs | Deoxynucleotide Triphosphates |
| DAPI | 4',6-Diamidino-2-Phenylindole |
| DCs | Dendritic Cells |
| DEPPD | N, N-diethyl-para-phenylendiamine |
| DEXA | Dual Energy X-Ray Absorptiometry |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic Acid |
| EI | Electron Impact |
| EIAs | Enzyme Immunoassays |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| <i>env</i> | Envelope |
| ER | Endoplasmic Reticulum |
| ES | Effect Size |
| ESI-UPLC-MS | Electrospray Ionization Ultra Performance Liquid Chromatography Mass Spectrometry |
| eV | Electron Volt |
| FACS | Fluorescence Activated Cell Sorter |
| Fas L | Fas Ligand |
| FBS/FCS | Fetal Bovine/Calf Serum |
| FITC | Fluorescein Isothiocyanate |
| FMO | Fluorescence Minus One |
| FSC | Forward Scatter |
| <i>gag</i> | Group-Specific Antigen |
| GC | Gas Chromatography |
| GC-MS | Gas Chromatography Mass Spectrometry |
| GFP | Green Fluorescent Protein |
| Glut4 | Glucose Transporter type 4 |

| | |
|---------------|--|
| gp 41 | Glycoprotein 41 |
| gp 120 | Glycoprotein 120 |
| HAART | Highly Active Antiretroviral Therapy |
| HCA | Hierarchical Clustering Analysis |
| HCl | Hydrochloric Acid |
| HCMV | Human Cytomegalovirus |
| HCV | Hepatitis C Virus |
| HDL | High Density Lipoprotein |
| HIV | Human Immunodeficiency Virus |
| HIV- | HIV Seronegative |
| HIV+ | HIV Seropositive |
| HIV-1 | Human Immunodeficiency Virus Type-1 |
| HIV-2 | Human Immunodeficiency Virus Type-2 |
| HMDB | Human Metabolome Database |
| HML | Human Metabolite Library |
| HRP | Horseradish Peroxidase |
| HTLV-III | Human T Cell Lymphotropic Virus type III |
| ICA | Independent Component Analysis |
| ICCS | Intracellular Cytokine Staining |
| IDSA | Infectious Diseases Society of America |
| Ile | Isoleucine |
| IgG | Immunoglobulin G |
| IFN | Interferon |
| IFN- γ | Interferon Gamma |
| IL | Interleukin |
| IL-1 | Interleukin 1 |
| IL-2 | Interleukin 2 |
| IL-4 | Interleukin 4 |
| IL-6 | Interleukin 6 |
| IL-10 | Interleukin 10 |
| IL-17A | Interleukin 17A |

| | |
|----------------------|--|
| IN | Integrase |
| IR | Insulin Resistance |
| IRt | Ion Retention time |
| LAV | Lymphadenopathy Associated Virus |
| LC | Liquid Chromatography |
| LDA | Linear Discriminant Analysis |
| LDL | Low Density Lipoprotein |
| LTNPs | Long Term Nonprogressors |
| LTRs | Long Terminal Repeats |
| MALDI-MS | Matrix-Assisted Laser Desorption Ionization Mass Spectrometry |
| MDMs | Monocyte Derived Macrophages |
| MHz | Megahertz |
| MET-IDEA | Metabolomics Ion-based Data Extraction Algorithm |
| MFI | Mean Fluorescence Intensity |
| MHC | Major Histocompatibility Complex |
| MRC | Mitochondrial Respiratory Chain |
| MRI | Magnetic Resonance Imaging |
| MRS | Magnetic Resonance Spectroscopy |
| MS | Mass Spectrometry |
| MSI | Metabolomics Standards Initiative |
| mtDNA | Mitochondrial DNA |
| MTT | 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide |
| m/z | Mass-to-Charge |
| <i>nef</i> | Negative Replication Factor |
| NF- κ β | Nuclear Factor-Kappa Beta |
| NIAID | National Institute of Allergy and Infectious Diseases |
| NICD | National Institute for Communicable Diseases |

| | |
|------------|---|
| NIST | National Institute of Standards and Technology |
| NK | Natural Killer |
| NMR | Nuclear Magnetic Resonance |
| NRF | National Research Foundation |
| NWU | North-West University |
| OIs | Opportunistic Infections |
| PBLs | Peripheral Blood Lymphocytes |
| PBMCs | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |
| PCs | Principal Component |
| PC1 | Principal Component 1 |
| PC2 | Principal Component 2 |
| PCA | Principal Component Analysis |
| PCR | Polymerase Chain Reaction |
| PE | Phycoerythrin |
| PerCP | Peridinin Chlorophyll Protein Complex |
| PHA-P | Phytohemagglutinin from <i>Phaseolus Vulgaris</i> |
| PI | Propidium Iodide |
| PKC | Protein Kinase C |
| PLA2 | Phospholipase A2 |
| PLS-DA | Partial Least Squares Discriminant Analysis |
| PMA | Phorbol 12-Myristate 13-Acetate |
| p7 | 7 kiloDalton (kDa) non-glycosylated nucleocapsid protein |
| p17 | 17 kDa non-glycosylated matrix protein |
| p24 | 24 kDa non-glycosylated core protein |
| <i>pol</i> | Polymerase |
| PR | Protease |
| QC | Quality Control |
| <i>rev</i> | Anti-Repression Transactivator Protein |
| RNA | Ribonucleic Acid |



| | |
|---------------|---|
| ROS | Reactive Oxygen Species |
| RPMI | Rosewell Park Memorial Institute |
| RSD | Relative Standard Deviation |
| RT | Reverse Transcriptase |
| SA | South African |
| SD | Standard Deviation |
| SIMCA | Soft-Independent Methods of Class Analogy |
| SIV | Simian Immunodeficiency Virus |
| SMD | Serum Metabolome Database |
| SSC | Side Scatter |
| TAG | Triacylglycerol |
| <i>tat</i> | Trans-Activator of Transcription |
| T cells | T lymphocytes |
| Th | T Helper |
| Th1 | T Helper Type-1 |
| Th2 | T helper Type-2 |
| Th17 | T helper Type-17 |
| TIA | Technology Innovation Agency |
| TIC | Total Ion Chromatogram |
| TMCS | Trimethylchlorosilane |
| TNF | Tumor Necrosis Factor |
| TNF- α | Tumor Necrosis Factor Alpha |
| TE | Tris-EDTA |
| UMP | Uridine Monophosphate |
| UNAIDS | The Joint United Nations Programme on HIV/AIDS |
| UP | University of Pretoria |
| URL | Uniform Resource Locator |
| USA | United States of America |
| <i>vif</i> | Virion Infectivity Factor |
| VIP | Variables Important In Projection |



| | |
|------------|------------------------------|
| VLDL | Very Low Density Lipoprotein |
| <i>vpr</i> | Viral Protein R |
| <i>vpu</i> | Viral Protein U |
| WHO | World Health Organization |
| www | World Wide Web |

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- My co-supervisor, **Prof Carolus Reinecke**: This research project like the many others one reads of turned out to be far more complex than originally thought. Thank you for always encouraging me and reminding me of the “light at the end of the tunnel”. A BIG thank you for availing yourself, your laboratory, staff (Peet Janse van Rensburg, Roan Louw) and students (Marli Dercksen, Zander Lindique) to assist me where needed.
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- **Dr Wendy Burgers**: for providing aliquots of the Gag peptide pool.
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- **My family and friends:** Thank you for your support which was shown through the many communication channels available: emails, text messages, prayer, phonecalls etc. It sure lifted my spirit and reminded me to "keep my eye on the ball."
- **Ntakadzeni Edwin Madala:** for always sharing his knowledge on Metabonomics and for being the good friend that he is.
- **Colleagues and members of the HIV research group at UP (especially Pascaline Fonteh):** It has been a stressful yet memorable journey. Here is to the "not so happy moments" that were overcome, the laughter and good times that were shared and a toast to future successes. **Wayne Barnes**, thank you for always lending an extra hand with flow cytometry and for reading through my write-ups on more than one occasion.
- **BD Biosciences' Applications and Sales Specialists, Nandi Mbatha and Marisha Meyer** for technical assistance with CBA analysis.

PREFACE

The research presented in this thesis has been published in a peer-reviewed journal, reported in a feature article and presented at several conferences. A copy of the published manuscript has been attached at the end of this document. Additional manuscripts which are in preparation are also listed.

Publication(s)

Published Manuscript(s)

- Aurelia Williams, Gerhard Koekemoer, Zander Lindeque, Carolus Reinecke and Debra Meyer (2011). Qualitative serum organic acid profiles of asymptomatic HIV-infected individuals not on antiretroviral treatment. *Metabolomics*. In Press. DOI: 10.1007/s11306-011-0376-2.

Interview(s)

Feature Article:

- “Apoptosis: New Tools to Tease Out Complex Pathways”, June 2010. Accessible through: <http://www.biocompare.com/Articles/FeaturedArticle/1162/Apoptosis-New-Tools-to-Tease-Out-Complex-Pathways.html>

Conferences

Portions of the work in this thesis were presented at:

- The 49th Annual Meeting of the Infectious Diseases Society of America (IDSA) which was held on the 20-23rd October 2011 in Boston, United States of America (USA). Poster presentation by Aurelia Williams, Christiane Bremnaes and Debra Meyer (2011) entitled: A Cellular Epitope (R7V) Stimulates IFN- γ Production in HIV-1 Infected Non-Progressors.

- The 5th SA AIDS Conference in Durban (South Africa) which took place on the 7-10th June 2011. Poster presentation by Aurelia Williams, Gerhard Koekemoer, Carolus Reinecke and Debra Meyer (2011) entitled: Characterizing the immune and metabolic profiles of HIV-infected biofluids.
- The HIV and AIDS Research Indaba held at the University of Pretoria (UP), Pretoria, South Africa. The Indaba was hosted by the CSA and was held on the 26-27th February 2009. An oral presentation entitled: “Mass Spectrometry-based Metabolomics for analyzing the downstream effects of HIV infection” was delivered.
- The Cell Death in Infectious Diseases and Cancer Conference which took place at Muldersdrift, South Africa in June 2009. I co-authored an abstract (Mass Spectrometric Metabolomics of HIV-induced Apoptosis) for this invited talk.

Awards

- I was awarded travel support by the IDSA and HIV Medical Association to attend the IDSA’s 49th annual meeting in Boston, MA, USA (20-23rd October 2011). The funding for this award was provided through the Offices of AIDS Research at the National Institutes of Health. This award was also granted in recognition of excellence in HIV research.
- I received a scholarship from the organizing committee of the 5th SA AIDS conference to attend the conference which was held in Durban (South Africa) from the 7-10th of June 2011.
- Following the submission of an abstract entitled: “Flow Cytometry and Mass Spectrometry for the rapid and quantitative detection of HIV-induced immunological and metabolic changes” I was awarded a partial scholarship to attend the “Infectious Diseases in Africa: Measurement of Immune Responses and 3rd African Flow Cytometry Workshop”. The workshop was organized by the National Institute for Communicable Diseases (NICD), Duke University and California Department of Public Health in collaboration with the National Institutes of Health and the Offices of AIDS Research, NIAID. It took place in Johannesburg during November 2009.
- In 2008 I sourced additional funding for running costs toward my project from the CSA at the University of Pretoria. Additional funding to the value of R15 000 was awarded.

Additional manuscripts in preparation:

- Aurelia Williams, Francois Steffens, Carolus Reinecke and Debra Meyer (2012). Serum Th1/Th2/Th17 cytokine profiles of treatment naive HIV-infected individuals: a cytokinomics/multivariate approach. To be submitted to: Biochemical and Biophysical Research Communications.
- Lungile Sitole, Aurelia Williams and Debra Meyer (2012). Metabonomics analysis of HIV-infected biofluids. To be submitted to: Biochemical and Biophysical Research Communications or HIV & AIDS Reviews.
- Aurelia Williams, Khanyisile Kgoadi, Paul Steenkamp and Debra Meyer (2012). Ultra performance liquid chromatography mass spectrometry analysis of HIV-infected biofluid. To be submitted to Metabolomics.

SUMMARY

Metabonomics profile and corresponding immune parameters of HIV infected individuals

by

Aurelia Alvina Williams

Supervisor: Professor Debra Meyer

Co-supervisor: Professor Carolus Reinecke

Department: Biochemistry

Degree: Ph.D. Biochemistry

Background: Immunological events due to infection by the human immunodeficiency virus (HIV) perturb mitochondrial function which augments virus-induced metabolic imbalances. Organic acids, established biomarkers of mitochondrial dysfunction have not yet been studied as indicators of HIV-induced changes in this organelle.

In this study, mass spectrometry (MS) was used to determine the organic acid profile and flow cytometry the corresponding immune changes in biofluids of clinically stable patients, with the aim of identifying HIV-influenced molecules which could potentially be developed into diagnostic and/or prognostic markers.

Methodology and Results: Gas chromatography mass spectrometry (GC-MS) was used to determine HIV-induced mitochondrial dysfunction by means of organic acid profiling of sera, peripheral blood mononuclear cells (PBMCs) and urine. The Metabolomics Ion-based Data Extraction Algorithm (MET-IDEA) proved more suitable for data analysis than other software packages. The biofluids analyzed differed in the type of metabolites identified but provided related biological information. An overlap in the metabolic profiles of HIV seronegative (HIV-) and seropositive (HIV+) groups was observed. When cases in the advanced stage of the disease were included an improved separation between the groups was observed. Metabolites altered as a result of HIV infection were representative of disrupted

mitochondrial metabolism, changes in lipid, sugar, energy and neurometabolism as well as oxidative stress. Metabolite detection was found to be influenced by viral load.

Corresponding immune parameters were measured by detecting oxidative stress, apoptosis and cytokine changes. As expected, the HIV+ individuals experience constant oxidative stress. Significantly higher amounts of reactive oxygen species (ROS, $p = 0.004$) were detected in infected sera. Apoptosis in the HIV+ cells was significantly higher than that occurring in the HIV- cells ($p < 0.0001$). When gating T cells, a greater percentage apoptosis was measured in the CD8 positive cell population ($p = 0.0269$). Since the CD4 cells of the patient group were not depleted these cells were able to produce the soluble factor needed for apoptosis to occur in CD8 cells. *In vitro* stimulation of the infected PBMCs with viral peptides led to an increase in the percentage T cells which produced intracellular interferon gamma (IFN- γ). The T helper type 1 (Th1), Th2 and Th17 cytokine profile in aliquots of HIV- and HIV+ sera measured using Cytometric Bead Array (CBA) technology and analyzed using multivariate statistics, correctly classified over 70 % of the cases as HIV- or HIV+. Interleukin (IL)-6 and IL-10 were found to be the key immune markers altered during HIV infection. Analyzing cytokines in this manner follows a cytokinomics approach.

Conclusion: Organic acids detected agree with the oxidative, apoptotic and cytokine responses. The impact of HIV on the metabolic signature and immune system is detectable in the early asymptomatic phase of infection by using MS, flow cytometry and spectroscopy. The observed changes share a biochemical relationship and are supportive of the link between the metabolic and immune systems. The data was collected using different forms of spectroscopy and spectrometry and these approaches may therefore have a future in the management of HIV infection and the acquired immunodeficiency syndrome (AIDS).

CHAPTER 1

INTRODUCTION

1. Introduction

HIV infections have had devastating global effects, e.g. an increase in workforce deaths which translate into low productivity and low economic gain, as well as an increase in the number of orphans to name but a few (Ashford, 2006; www.avert.org/aids-impact-africa.htm). Although existing therapeutic interventions significantly reduce HIV-related mortalities (Panos et al., 2008), disease eradication remains a distant goal. A vast amount of time and resources are spent on the development of a vaccine and better alternatives to antiretroviral therapy (ART). Even if these developments succeed the metabolic and immunological effects associated with HIV infection will remain problematic for many years because for those individuals already infected a vaccine will not be able to protect against infection. The only alternative for infected individuals is to make use of ART, but these drugs do not restore ongoing HIV-induced immunological imbalances (Gaudieri, 2011; Nixon and Landay 2010). In fact, ART can augment virus-induced metabolic imbalances. Available therapies prevent the infection of new cells, but cannot inhibit virion release from already infected cells (Siliciano and Siliciano 2010). As a result, these drugs can also not inhibit the subsequent immune activation associated with virion release. Not all the effects of HIV are known, or can be predicted. In the meantime, the host has to face the numerous consequences (depletion of immune cells, oxidative stress, cytokine dysregulation etc) that follow infection. Studying various biochemical pathways for the identification of HIV-induced changes could lead to the detection of HIV-specific biomarkers. Knowledge of the metabolic and corresponding immune profiles of HIV-infected individuals prior to ART may be valuable in this regard.

HIV is known for infecting CD4+ T lymphocytes resulting in a range of symptoms (Rosenberg and Fauci 1991) namely; immune activation, immunodeficiency and a dysregulation in cytokine production (Landay, 1998). The immune system is complex with cells, cytokines and other molecules often working together to elicit protective responses. However, while initiating immune responses (i.e. development of Th1 and cytotoxic responses) tissue damage occurs (Saric et al., 2010) and contributes to the development of metabolic imbalances. A change in metabolism is also induced as a general response by the host to deal with infection (Beisel, 1972). In addition, the HI virus directly induces metabolic

change (Safrin and Grunfeld 1999; Hommes et al., 1990). Examples include changes in body composition, fat distribution as well as changes in lipid, glucose, energy and protein metabolism (Slama et al., 2009; Salas-Salvado and Garcia-Lorda 2001; Wanke, 1999). Other complications of HIV infection which are underrecognized or neglected include heart disease (Dubé et al., 2008), the accumulation of iron (Boelaert et al., 1996), the occurrence of oxidative stress, a dysregulation in T cell signalling (Schweneker et al., 2008), malnutrition (Hattingh et al., 2009) as well as organ/organelle dysfunction to name but a few. Particular metabolic changes which result from HIV's perturbation of mitochondrial function will be the focus of this research.

Mitochondria forms part of various biochemical pathways and when compromised in structure and function results in various metabolic complications. The functional status of these organelles is particularly well-researched immunologically as well as in the metabolic sense. Organic acids are established biomarkers of mitochondrial dysfunction and are representative of biochemical pathways of intermediary metabolism (e.g. the Krebs cycle, Hoffmann and Feyh 2005). Despite the relationship between these molecules and mitochondria, as well as the link between HIV and mitochondria, organic acids have up to now not been profiled to show HIV-induced mitochondrial damage. Little is known about HIV-induced organic acid changes. Literature on other HIV-induced metabolic events is also limited especially for subtype C infections which are so predominant in South Africa. More focus has been directed to the metabolic effects associated with ART (Dubé et al., 2008; Chen et al., 2002) than to the sole metabolic effects of the virus. Although information about HIV's effect on the host metabolism exists, literature on the subject matter is outdated as can be seen by articles referenced hereafter (Pascal et al., 1991; Hommes et al., 1990).

To characterize metabolic and immune changes sensitive technologies such as MS-based metabonomics and multi-parametric flow cytometry are increasingly being used and improved upon. Metabonomics measures changes in metabolite levels following a biochemical perturbation as a result of disease, drugs and toxins (Goodacre et al., 2004; Lindon et al., 2003; Nicholson et al., 1999) and in essence measures the metabolic responses of living systems to biological stimuli (Kamleh et al., 2009). In context to the work presented here the stimulus is represented by HIV. Flow cytometry is a technique which measures a multitude of physical characteristics of single cells. Basic phenotype characteristics linked to infection can thus be measured. These techniques are covered in greater detail in Sections 2.10.1 and 2.10.3. In the first ¹H nuclear magnetic resonance (NMR)-based metabonomics investigation on HIV-infected biofluid by this group, Hewer et al (2006) reported on the use of metabonomics to distinguish between uninfected and HIV-infected individuals as well as AIDS patients on ART. Since then there has been ongoing

interest in the area of virus-induced metabolic changes by our research laboratory (Williams et al., 2011; Philippeos et al., 2009) and that of others (Ghannoum et al., 2011; Hollenbaugh et al., 2011; Hattingh et al., 2009). In studies such as that of Hattingh et al (2009), metabolic changes are often characterized using conventional biochemical techniques/assays which are laborious to perform and not very sensitive. This is in contrast to the work of authors such as Wikoff et al (2008) who applied a more sensitive technique, liquid chromatography mass spectrometry (LC-MS) and revealed changes in the cerebrospinal fluid (CSF) metabolome of rhesus macaques infected with the simian immunodeficiency virus (SIV). There are disadvantages associated with using less sensitive techniques i.e. low dynamic ranges, high instead of low detection limits etc. Limitations of current diagnostic tools (limited detection of HIV during seroconversion) and prognostic markers (variable CD4 counts) further support the need to develop new methodologies and novel biomarkers for the characterization of HIV infection and HIV-induced changes. Metabonomics offers several advantages over convention. Firstly, the metabolic profiles of individuals can be obtained non-invasively, is characteristic of the individual's phenotype and therefore associated with less variation. This makes metabonomics more accurate than existing diagnostic and prognostic tools. Collecting data is fast and high-throughput analysis is possible. Metabonomics techniques are more sensitive (Wikoff et al., 2008) and allow a range of molecules to be profiled at once in keeping with the complex and interconnected nature of metabolic pathways. Although other "omics" technologies such as proteomics have contributed significantly to understanding pathological consequences of HIV infection (Pendyala and Fox 2010) the advantages and possible contributions of metabonomics investigations lag behind.

In the case of immune-based analysis there has been a tendency in the literature to measure one analyte at a time using the conventional enzyme linked immunosorbent assay (ELISA). Due to the complexity of the immune system as well as the availability of multi-parametric flow cytometers researchers now multiplex (measure more than one parameter) in order to profile a range of immune changes in limited volumes of sample (Keating et al., 2011; Tang et al., 2011; Nixon and Landay 2010; Roberts et al., 2010; Tang et al., 2008). The aim is to extract more usable biological information at one time. Multiplexing however generates large data sets which become difficult to analyze and interpret. Similar to ELISA assays where one cytokine is detected and analyzed at a time the datasets comprising of multiple analytes are often still analyzed using univariate analysis. Because the importance of a molecule may change when analyzed in combination with other variables (Philippeos et al., 2009) multi- instead of univariate statistics may more accurately describe HIV-induced immunological changes, an aspect addressed in this study.

Due to the established link between the metabolic and immune systems ([Matarese and La Cava 2004](#)) co-analysis of the two systems is becoming increasingly important for a better understanding of HIV/AIDS pathogenesis. Despite this link, these two target systems have mainly been analyzed independently of each other, often by characterizing one molecule at a time. Taking all the above aspects into consideration an **objective** was set to characterize HIV-induced metabolic and immune changes in the biofluid of treatment naive individuals. By investigating this an association between organic acids and HIV was established, possible biomarkers capable of distinguishing uninfected from infected samples identified, the monitoring of the disease facilitated, metabolic changes in the absence of ART investigated, MS applied to the study of HIV-infected biofluid, the influence of multivariate statistics on immune data evaluated, consequences other than the commonly reported immunodeficiency addressed and a biochemical link established between the measured metabolic and immune parameters for the experimental groups used here. This was achieved through the novel integrated application of MS-based metabonomics and flow cytometry.

Although a MS-based metabolomics investigation of saliva from HIV+ individuals ([Ghannoum et al., 2011](#)) and CSF from SIV-infected monkeys ([Wikoff et al., 2008](#)) has been done, these approaches have had limited application to blood/blood products of HIV-infected individuals. [Hollenbaugh et al \(2011\)](#) recently investigated the metabolic profile of CD4 and macrophage cells. Unlike the work presented here that is representative of chronic HIV infection, the cells analyzed by these authors were infected with HIV *in vitro* and representative of acute infection. In the articles listed above the investigations were primarily untargeted i.e. the entire metabolome was screened for metabolic changes whilst the research presented in this thesis was targeted i.e. the organic acid metabolome was investigated. In addition to blood-based biofluids the urinary organic acid metabolome of HIV-infected individuals was also determined. To our knowledge there is no literature reporting on metabonomics-based analysis on the urine of HIV+ patients. Studies in this laboratory where metabonomics has been applied to human samples have mainly utilized NMR ([Philippeos et al., 2009](#); [Hewer et al., 2006](#)). In practise, both metabonomics and flow cytometry are thus applied in research but an integrated analysis of HIV's effect on the metabolic and immune systems of clinically stable patients has not been done using such sensitive, multi-parametric, analytical technologies.

This study began by investigating the organic acid profile of HIV- and HIV+ sera, PBMCs and urine in parallel to immune-based analysis. The GC-MS datasets were best analyzed with MET-IDEA. Following multivariate statistics overlapping as well as separated metabolic profiles were obtained for the various batches of biofluid analyzed. The overlapping and non-

overlapping profiles were attributed to differences in viral load, instrument sensitivity and masking of the metabolic stress by high numbers of uninfected cells in the vicinity of infected ones. Metabolites found to make a significant contribution to the metabolic profiles were related to disrupted mitochondrial metabolism, changes in lipid, sugar, energy and neurometabolism as well as oxidative stress. Immune parameters (oxidative, apoptotic and cytokine levels) were found to differ significantly between the HIV- and HIV+ groups. HIV+ cells stimulated with antigen induced the production of cytokines having antiviral activity. Where serum cytokine profiles were analyzed using multivariate statistics more than 70 % of cases were correctly classified allowing the two groups to be distinguished from each other and cytokine markers representative of HIV infection to be identified.

The data obtained from the integrated MS and flow cytometry analysis serves to facilitate a better understanding of virus-host interactions, mechanisms of viral infection and HIV/AIDS pathogenesis. In the long-term it may assist in the design of better-suited diagnostic, prognostic, therapeutic as well as improved HIV/AIDS management strategies.

The following chapter contains background information on HIV and AIDS, the functioning of the immune and metabolic systems as well as HIV's interrelated effects on these systems. This is followed by the rationale of this work, the **hypothesis**, **objectives (Section 2.9)** and techniques with which the proposed research questions will be answered. The experimental design and practical considerations are highlighted in Chapter 3 whilst Chapters 4 and 5 respectively explain the metabolic and immunological methodologies employed, the results obtained and a discussion to these findings. This is followed by a conclusion of the findings, the significance and limitations of the project as well as future recommendations for this type of analysis. Supplementary data and published manuscripts relevant to this work are provided in the appendix.

CHAPTER 2

LITERATURE REVIEW

2. HIV/AIDS and its effect on the immune and metabolic systems

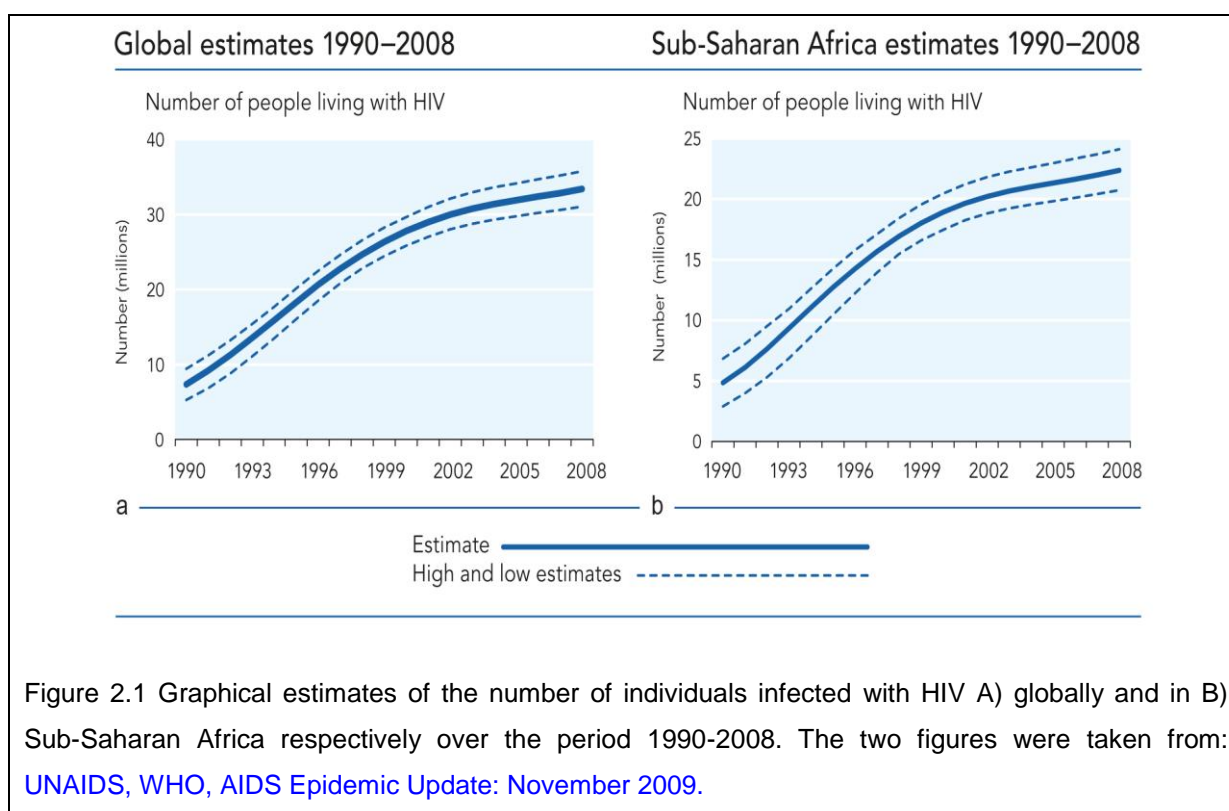
2.1 History of HIV/AIDS

Initially known as the Lymphadenopathy Associated Virus (LAV) and the Human T cell Lymphotropic virus type III (HTLV-III) respectively, HIV as we know it today has become one of the most thriving infectious agents to date. There is still a lot of uncertainty about the origins of the virus but the most plausible explanation is that it was transferred from SIV-infected monkeys to humans through zoonosis ([Hahn et al., 2000](#); [Gao et al., 1999](#)).

Even though the earliest samples infected with HIV dates back to 1959 (www.avert.org/origin-aids-hiv.htm) and computer models dates the virus' existence to as early as 1884 and the early 1900s ([Hahn et al., 2000](#)), HIV was only isolated and shown to be the causative agent of AIDS in the 1980s ([Barré-Sinoussi et al., 1983](#); [Gallo et al., 1983](#)). Following confirmation of the link between HIV and AIDS tests to diagnose infected patients were developed and drugs with activity against the virus synthesized. Zidovudine (AZT) was the first anti-HIV drug to be licensed in 1987 followed by the protease inhibitors in 1995 ([Fauci, 2008](#)). Present therapies prolong the life-span of infected individuals and have subsequently resulted in HIV infection becoming a chronic condition in the developed world. Chronic HIV infection as well as ART induce metabolic alterations in the host and as a result there has also been an increase in incidences of chronically infected individuals presenting with HIV-induced metabolic disturbances. Because the therapies are not a cure, efforts toward vaccine development, treating HIV infections and preventing new infections continue. Detecting HIV infection early enough contributes to the success of currently available therapies. However, accurate and reliable markers for the diagnosis of HIV infection, monitoring of disease progression and assessing the success of therapeutic interventions are still lacking ([Zhang and Versalovic 2002](#)). This is because many molecules such as the p24 antigen, serum neopterin, β -2 microglobulin (β 2m), albumin, cytokines etc ([Neaton et al., 2010](#); [Nixon and Landay 2010](#); [Touloumi and Hatzakis 2000](#)) that are influenced by HIV infection fail to meet the basic requirements of a biomarker. Biomarkers per definition provide information on the history of infection and are present in all infected individuals. The levels of the biomarkers also changes with disease progression and when interventions are

administered but these molecules are not altered when therapy fails (Kanekar, 2010). In addition; the mechanisms of HIV infection, the functioning of the metabolic and immune systems as well as the role of various molecules (such as antibodies, CD4 T cells, etc) are not entirely understood (Keane and John 2011) and as such hampers the characterization of biomarkers. The CD4 count and viral load (though not infallible) are two parameters which have met the criteria of a biomarker and are currently used in the clinic.

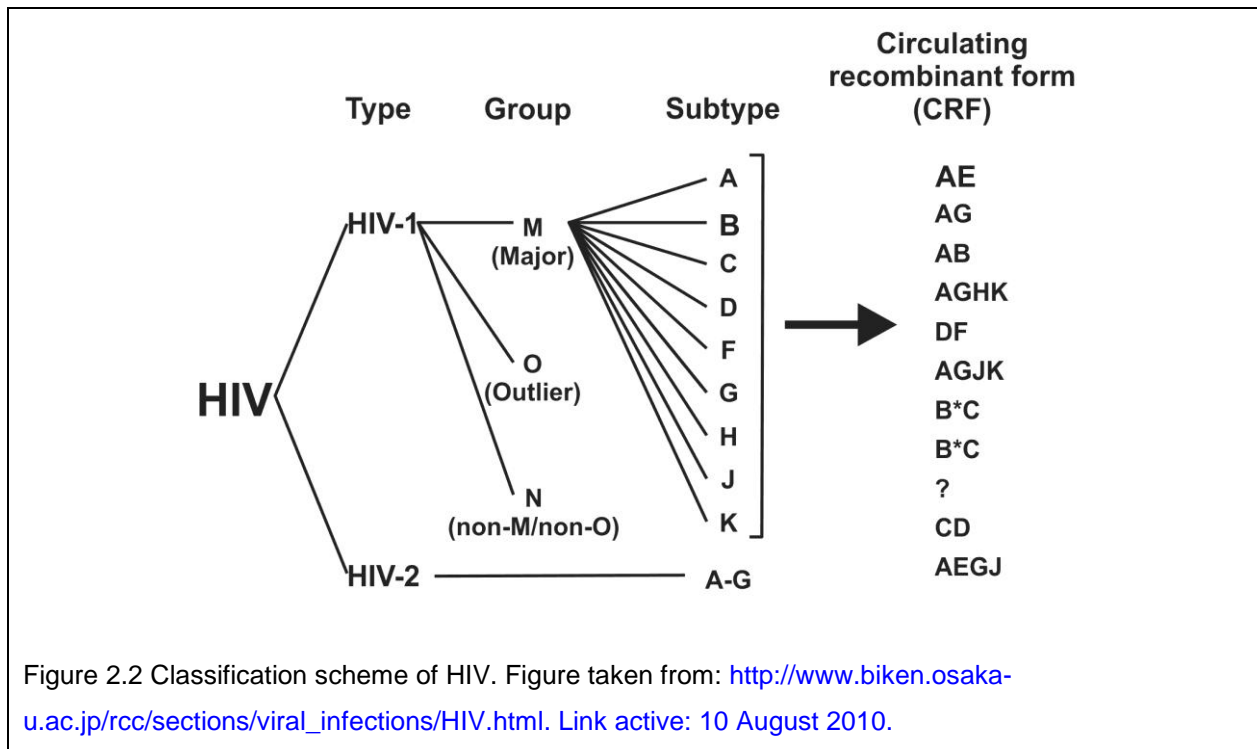
In 2008 an estimated 33.4 million individuals were reported to be infected with HIV globally (Figure 2.1, a) with 67 % of the infections occurring in sub-Saharan Africa (Figure 2.1, b). Women represent a large fraction of this estimate because of the relationships they have with elderly men for financial and social security as well as the desire for material things. This gender is also more susceptible to heterosexual transmission because of their exposure to sexual and physical violence (UNAIDS Report, 2002 and 2009). Although HIV infections were primarily dominant in homosexual men, injecting drug users and patients receiving transfusions (Karim and Karim 2002) the prevalence and geographical distribution has changed due to an increase in travel/migration (Nepal, 2002). Since HIV prevalence remains high the consequences of infection (metabolic and immunological in this case) needs to be addressed.



Highly active antiretroviral therapy (HAART) has assisted in stabilizing HIV prevalence but the development of HIV to AIDS remains common because for many individuals treatment is too expensive. There is also limited access to prevention and treatment services. Even if these services are available individuals are afraid to approach these centres because of the stigma and discrimination still associated with HIV infection (Abdool Karim et al., 2007). In treated individuals progression to AIDS occurs as a result of non-adherence to medication largely due to the associated side effects of these drugs. This results in viral resistance toward the various therapies which are currently available on the market (Salas-Salvado and Garcia-Lorda 2001). Many individuals are still not aware of their infection status and as a result continue to spread the virus (Abdool Karim et al., 2007). In addition to the above factors the survival of the pathogen is also attributed to its structural and genetic make-up as well as its complex life cycle.

2.2 Classification

HIV is an enveloped virus which infects the cells of the immune system. It belongs to the Retroviridae family and is classified under the lentivirus genus because it takes long to produce an effect in the host (Lourenço and Figueiredo 2008). It is genetically diverse with two predominant types namely, HIV-1 and HIV-2 (Buonaguro et al., 2007). HIV-1 is divided into **major/main** (M), **outlier** (O) and **non-M/non-O** (N) groups (Buonaguro et al., 2007). These groups are further divided into various subtypes with the most prevalent being subtypes A, B and C. The majority of HIV-1 infections reported are as a result of Group M subtype C viruses. Infection with HIV-2 is less common than HIV-1. This strain is also less virulent and as a result progression to AIDS is slower in comparison to HIV-1 infection. Adding to the groups and subtypes, circulating recombinant forms (CRFs) of the virus also exists. CRFs occur when an individual is infected with more than one subtype of HIV at one time. Recombinant viral strains having a mosaic genome are thus formed. Only when recombinant virus is detected in at least three individuals having different epidemiological backgrounds is it classified as a CRF (Buonaguro et al., 2007). This study primarily focused on HIV-1 which will be referred to as HIV throughout the text. The classification system of HIV as described above is shown in Figure 2.2.



2.3 Virion Structure

The HI virion depicted in Figure 2.3 is spherical with an inner nucleocapsid core that contains two single stranded ribonucleic acid (RNA) molecules, viral enzymes and other cellular factors (Sierra et al., 2005; Hirsch and Curran 1990). Surrounding the nucleocapsid core is a lipid bilayer membrane from which glycoproteins (gp 120 and gp 41) that are essential for virus-host interactions protrudes. Various proteins such as major histocompatibility (MHC) class 1, derived from the host cell during virion budding, are also contained in the lipid bilayer.

2.4 HIV-1 Genome

Once a host cell is infected with HIV proviral double stranded deoxyribonucleic acid (DNA) is the dominant form of genetic material that is detected. HIV has a 9.2 kb genome and is characterized by four main regions which include the long terminal repeats (LTRs), the group-specific antigen (*gag*), polymerase (*pol*) and envelope (*env*) genes respectively (Sierra et al., 2005, Figure 2.4). The LTR regions do not code for viral proteins but assist in controlling viral replication whilst the *gag*, *pol* and *env* genes code for structural proteins (HIV Sequence Compendium, 2008).

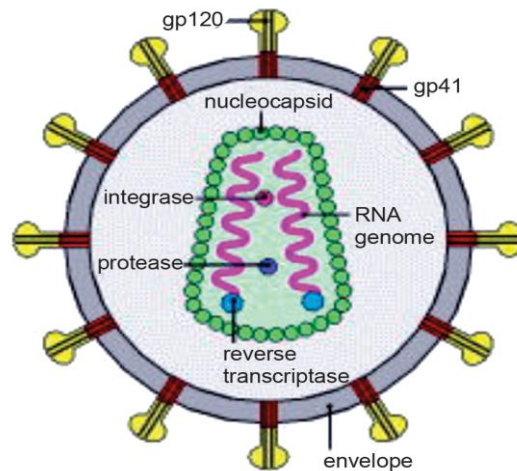


Figure 2.3 An illustration of a mature HIV-1 virion and its components. Picture taken from: [Janeway et al., \(2001\) Immunobiology 5th edition. New York and London: Garland Science; Figure 11.22.](#)

Proteins encoded by the *gag* gene include the non-glycosylated 24 kiloDalton (kDa) capsid protein (p24), the 17kDa matrix protein (p17) and the 7kDa nucleocapsid protein (p7). Viral proteins encoded by the *pol* gene includes: reverse transcriptase (RT), integrase (IN) and protease (PR) whilst the *env* gene codes mainly for gp 120 and gp 41 ([Sierra et al., 2005](#)). There are additional regulatory and accessory genes i.e. *vpu*, *vif*, *vpr*, *nef*, *tat* and *rev* which code for proteins that control infectability, viral copying and the onset of disease. The role of some of these proteins is explained in the section on the life cycle of HIV (Section 2.5).

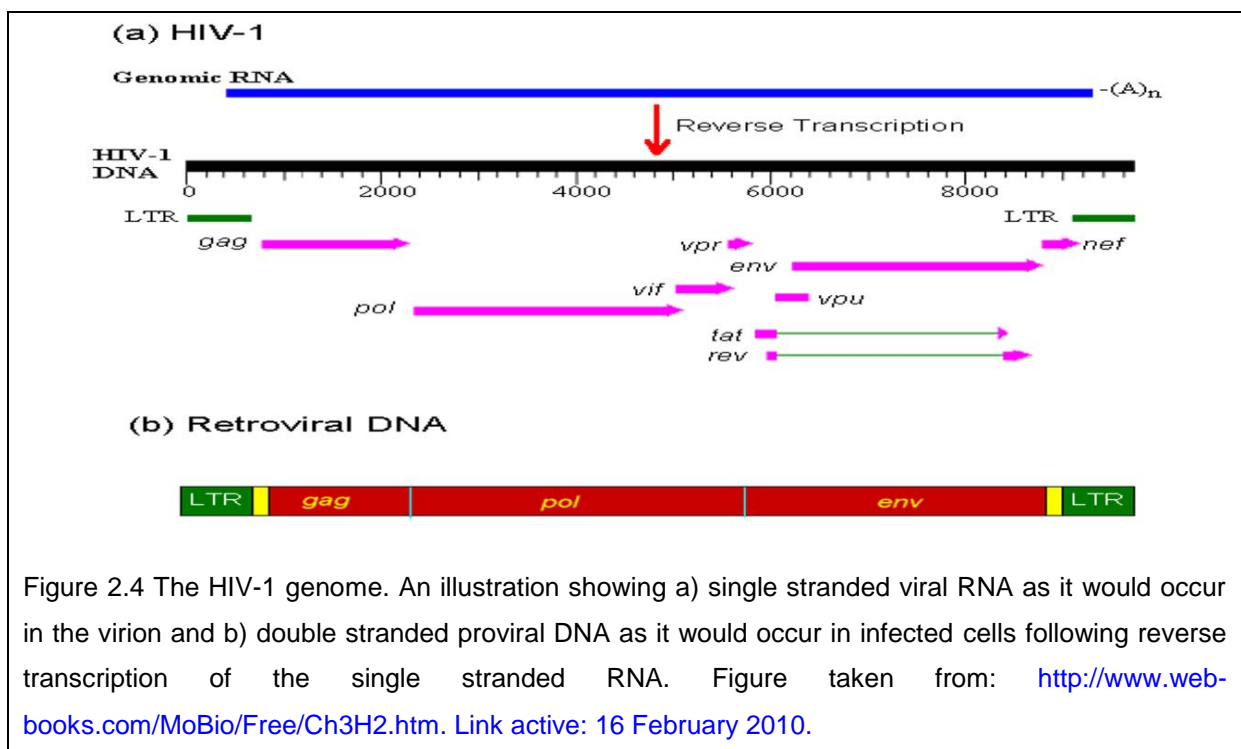
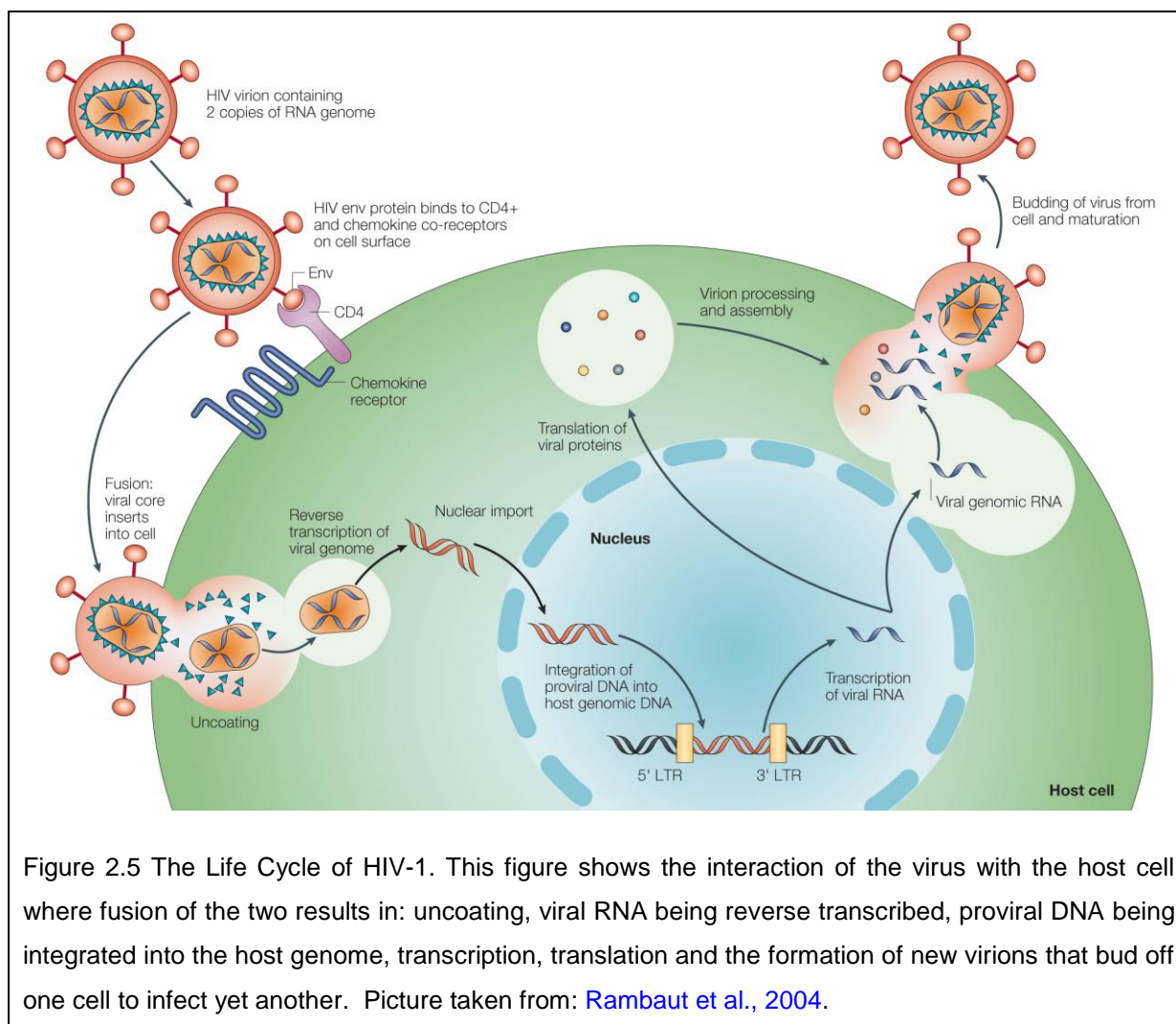


Figure 2.4 The HIV-1 genome. An illustration showing a) single stranded viral RNA as it would occur in the virion and b) double stranded proviral DNA as it would occur in infected cells following reverse transcription of the single stranded RNA. Figure taken from: <http://www.web-books.com/MoBio/Free/Ch3H2.htm>. Link active: 16 February 2010.

2.5 HIV-1 Life Cycle

HIV primarily infects T helper lymphocytes that express the CD4 receptor molecule on their surface. The life cycle of the virus shown in Figure 2.5 begins with the attachment of the virus to the host cell i.e. there is an interaction between the protruding extracellular glycoproteins of the virion, gp 120 and the cells' CD4 receptor. Upon binding to CD4 gp 120 undergoes a conformational change which enables it to bind to either chemokine receptor type 4 (CXCR4) or chemokine receptor type 5 (CCR5, [Harrison, 2005](#)). These are the main coreceptors utilized by HIV to facilitate viral entry into host cells. There is then fusion of the viral envelope and cellular membranes releasing the nucleocapsid core into the cytoplasm of the cell. RT reverse transcribes the single stranded viral RNA into double stranded proviral DNA which then enters the cell's nucleus and becomes integrated into the host's genome through the enzyme, integrase. Proviral transcription and translation then takes place followed by packaging of new, immature virions that bud off the infected cell. During the budding process the virus incorporates some of the host's proteins into its envelope. This has various implications relevant to HIV disease diagnosis, vaccine design, prognosis and the development of therapeutics ([Bremanaes and Meyer 2009](#)). Cleavage of larger protein segments into smaller units is achieved through the protease enzyme after budding to finally produce mature virions which take part in the infection and replication process all over again ([Sierra et al., 2005](#)). CD4+ T cells targeted by HIV are responsible for the induction of all other immunological responses but due to infection and persistent viral replication the numbers of CD4 cells are eventually reduced leading to a state of immunodeficiency ([Rosenberg and Fauci 1991](#)). Various factors such as secondary infections, genetics, viral factors, host factors, behavioural changes, immune status, concentration of virus at infection, route of infection, kind of cells infected, number of cells infected, viral heterogeneity, time of infection and receptor expression influence disease progression ([Lederman et al., 2004](#); [Touloumi and Hatzakis, 2000](#); [Pedersen et al., 1989](#)). All these factors cause the development and progression of disease to vary ([Touloumi and Hatzakis, 2000](#); [Pantaleo et al., 1993](#)) hence some individuals die within months while others succumb much later.



2.6 Clinical Course of HIV-1 infection

Upon exposure to the HI virus a number of clinical parameters as shown in Figure 2.6 can be measured: First, **primary infection** occurs which is measured by an increase in viral replication (high plasma viremia) as well as a sudden decline in CD4+ T cells. During this stage viral RNA levels equates to or exceeds 5×10^6 RNA copies/ml plasma ([Weber, 2001](#); [Touloumi and Hatzakis 2000](#)). The virus spreads throughout the body settling at different sites whilst the individual presents symptoms associated with seroconversion, the time period between infection and antibody production. Immune responses are then elicited resulting in decreased levels of HIV RNA. The **chronic asymptomatic stage**, so named because the individual shows no clinical signs or symptoms of infection, then follows. Unless infection is confirmed it is during this stage that the infected individual may be perceived to be a “normal” healthy individual. During this stage which lasts approximately 12 years ([Weber, 2001](#)) the immune response causes a lowering of plasma viremia and restoration of CD4+ T cell levels. Cell numbers are however never restored to a level higher than what it was pre-infection. Although viremia is reduced HIV is not entirely eliminated from the host

system. Viral replication therefore continues with a concurrent slow decline in CD4+ T cells and an activated immune state thus persists (Fauci et al., 1996). With continuing viral replication, a loss in the number of immune cells and a loss of immune function, **the symptomatic stage** of infection develops where the individual presents with HIV-associated symptoms. The individual becomes susceptible to numerous opportunistic infections and cancers. When immunity is completely compromised i.e. CD4 counts drop to a value below 200 cells/ μ l blood, symptoms become severe, susceptibility to opportunistic infections increases, cancers and **AIDS** develops, ultimately resulting in patient deaths (Pantaleo and Fauci 1996; www.avert.org). Based on symptoms or lack thereof, individuals are classified into these four stages or groups by the Centre for Disease Control (CDC) and the World Health Organization's (WHO) classification systems for HIV infection (WHO, 2006; CDC, 1986). These classification systems are continuously revised when more information regarding HIV infection becomes available. During the four stages of HIV infection differences in the biochemical profiles of the individuals probably occur but are rarely reported on. If these pathways are analyzed HIV-specific biomarkers capable of facilitating disease diagnosis and prognosis may be identified. These biomarkers may also allow for monitoring cases where corrective therapy has been administered.

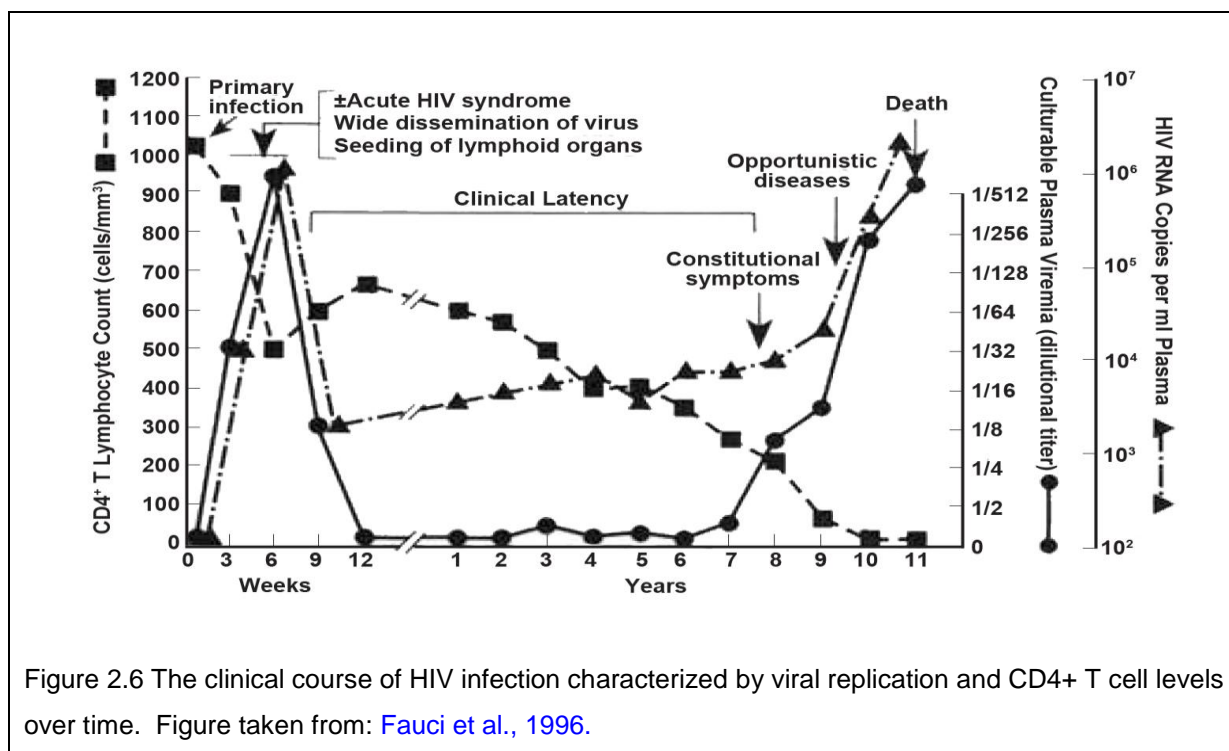


Figure 2.6 The clinical course of HIV infection characterized by viral replication and CD4+ T cell levels over time. Figure taken from: Fauci et al., 1996.

Because the clinical course of HIV is so different for each individual basic decisions about life and the therapeutic options to use are complicated (Mellors et al., 1997). Evaluating biochemical pathways/systems affected by HIV will provide additional information about already known HIV-induced changes or predict new changes ahead of clinical symptoms in the individual. The metabolic and immune pathways represent two such systems that were assessed. Background information on the immune system is presented because of the effect of HIV on this system and *vice versa*. Immune responses against HIV can also enhance virus-induced metabolic imbalances. Both the immune and metabolic systems will therefore be discussed next, starting with the former.

2.7 The Immune System and HIV

The immune system is capable of mounting protective responses against HIV even in the face of a direct attack of the virus on the system. This system consists of many organs (thymus, bone marrow, spleen, lymph nodes) and is made up of a complex network of interdependent cells including T lymphocytes (T cells), natural killer (NK) cells, B lymphocytes (B cells), granulocytes, macrophages and dendritic cells (DCs). The T cells commonly affected by HIV infection is divided into two subsets namely, the CD4⁺ T helper cells and the CD8⁺ T killer/cytotoxic cells. The CD4⁺ T cells are important for regulating the immune response and for activating other immune cells such as the CD8⁺ T cells. In turn, the cytotoxic CD8 cells are responsible for killing target cells which express foreign antigen.

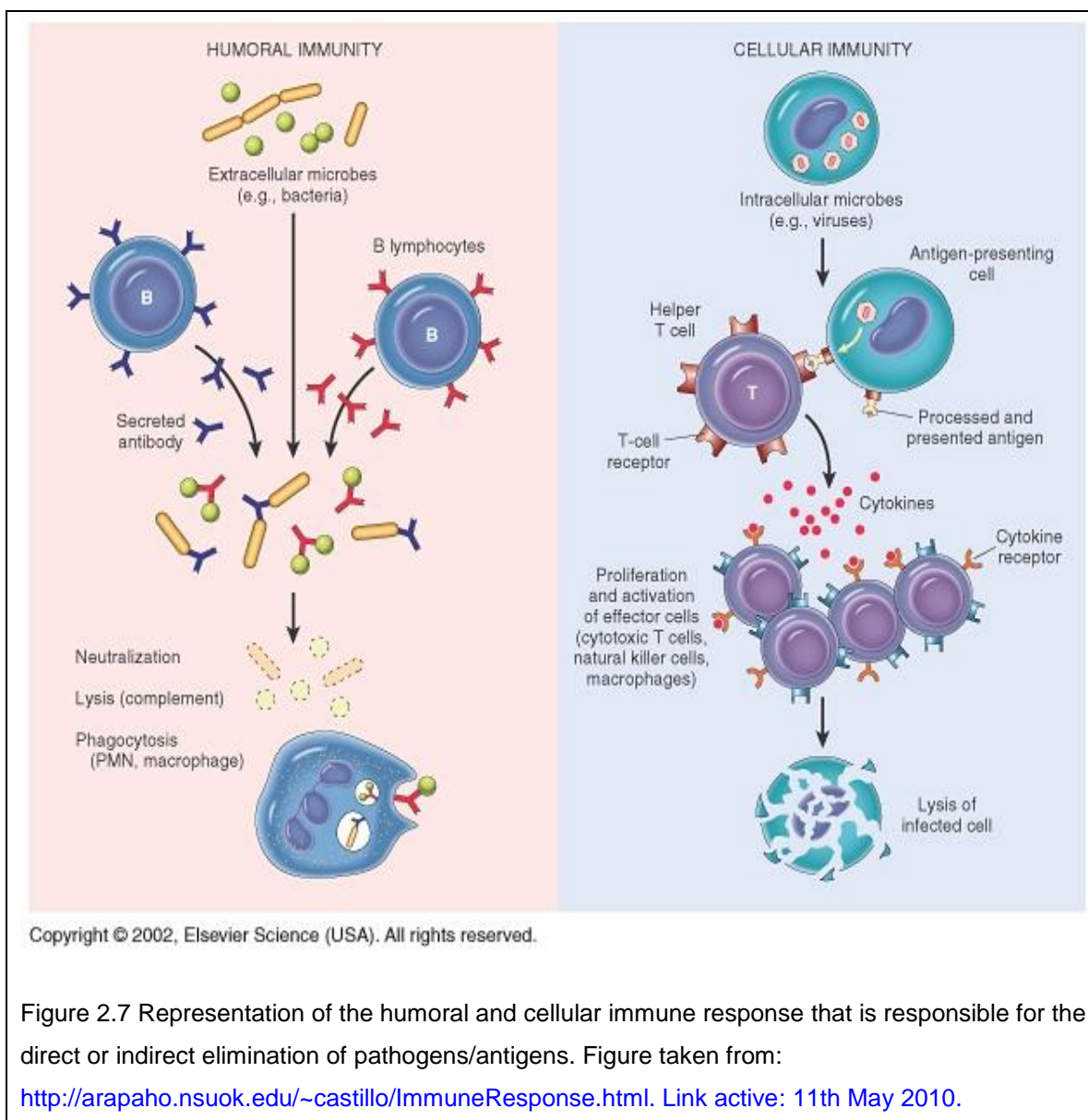
When infected with a pathogen such as HIV, antigen is presented on the surface of antigen presenting cells (APCs) to B cells or T cells to induce either a humoral or cell-mediated immune (CMI) response (Figure 2.7). Briefly, a humoral response is induced when an antigen that is bound to a MHC class II molecule is presented on the surface of an APC to a B cell (Goepfert, 2003). In the case of HIV infection the B cell proliferates and produces antibodies which renders the virus (cell-free) inactive and unable to bind the host cell receptors.

Another form of immunity against pathogen infection can be conferred by complement (Datta and Rappaport 2006). These are serum proteins which assist antibodies and phagocytic cells with marking pathogens for destruction by other immune cells. In the case of viral infections (e.g. HIV) complement is believed to bind directly to glycoprotein components on the surface of the virus to bring about non-specific immune responses and phagocytosis/lysis of the virus. Hyperactivation of the complement system can be as disruptive to the membrane of host cells (Datta and Rappaport 2006; Rus et al., 2005). Secretion of neutralizing antibodies and binding of complement to HIV surface molecules thus ensures direct inactivation of the virus. Complement also indirectly inhibits HIV

replication by lysing the cell reservoir which houses the virus. In addition to protein/antibody responses cellular activity in response to HIV infection is also prominent.

CMI responses occur when an antigen that is bound to an MHC class I molecule is presented on the surface of an APC to a T cell (Goepfert, 2003). CD4⁺ T cells recognize and respond to antigens encountered extracellularly and that are bound to MHC class I molecules while CD8⁺ T cells recognize and respond to antigens that have been synthesized intracellularly and that are bound to MHC class II molecules. Infection with virus, for example HIV, is representative of both endogenous and exogenous antigens thus CD4⁺ and CD8⁺ T cell responses are elicited (Goepfert, 2003). CMI responses particularly assist with reducing the amount of cell-associated virus in a host system. Following antigen presentation the T cells become activated, proliferate and secrete soluble factors called cytokines.

Cytokines are components of the immune system (Baruchel and Wainberg 1992) that serve as messengers (Baum et al., 2000) for the different cells within this system. Apart from serving as messengers these proteins influence disease progression, possess anti-HIV activity (Levy, 2001), activate other immune cells and ultimately shape the immune response (Salem et al., 2009). Based on the profile of cytokines produced or secreted T helper cells are defined as being either of the Th1 or Th2 lineages. Th1 cells clear intracellular and bacterial infections and are primarily associated with IFN- γ , TNF- α and IL-2 production as well as the induction of CMI responses. In contrast, the Th2 cells which eliminate parasitic infections and primarily induce humoral immunity are associated with IL-4, IL-6 and IL-10 production (Talat et al., 2011; Clerici et al., 1997). The progression of HIV to AIDS is associated with Th2 cytokines which contribute to the depletion of CD4 cells by augmenting apoptotic processes. This is in contrast to Th1 cytokines that have been shown to inhibit T cell apoptosis (Clerici et al., 1997; Clerici and Shearer 1994). A newly defined subset, the Th17 cells, which produce amongst others IL-17A, has also been identified (Korn et al., 2009; Wilson et al., 2007). Th17 cells have been shown to be elevated during early HIV infection but lowered during chronic infection (Prendergast et al., 2010; Yue et al., 2008).



2.7.1 HIV-induced apoptosis

Besides the above-mentioned cytokine phenotypes an array of other immunological changes is induced following HIV infection. Firstly, whole virus as well as viral proteins causes **hyperactivation** of the immune system (Ross, 2001) inducing a chronic state of inflammation within the host (Martin and Emery 2009). Failure by the immune system to control infection ultimately results in immunodeficiency measurable by a decrease in CD4+ T helper cells. The depletion of these cells is speculated to occur primarily through **apoptosis** which is an active, energy-requiring process that forms part of the normal development and maturation cycle of cells. Apoptosis is well-regulated and executed either through the extrinsic (death receptor pathway) or intrinsic pathways (mitochondrial pathway, Shedlock et al., 2008; Boya et al., 2004). For an illustration of the differences between these pathways see Figure 2.8. Briefly, extrinsic signals cause an upregulation in the expression of the tumor

necrosis factor (TNF) receptor, the expression of the type 1 transmembrane protein, Fas and Fas ligand (Fas L) respectively, other “death receptors” and their ligands (Badley et al., 2003). The binding of molecules (TNF- α , IFN- γ) to their receptors triggers intracellular signalling, caspase activation and apoptosis. In contrast, during the intrinsic pathway death signals act directly on mitochondria prior to the caspases being activated (Boya et al., 2004). The intrinsic pathway is the most common cell death pathway following intracellular infections. It is usually activated before the extrinsic pathway and is associated with mitochondrial damage (Genini et al., 2001), elevated ROS production and an increase in oxidative stress (Bayir and Kagan 2008). The intrinsic pathway is usually activated when cells are stressed (Lecoeur et al., 2008) and involves the translocation of pro-apoptotic proteins to mitochondria. These proteins cause a change in the organelles’ membrane potential causing the release of intermembrane space proteins (such as cytochrome c), apoptosis inducing factors and a range of metabolic intermediates (Lemasters et al., 1998). Externalization of phosphatidylserine then follows signalling early apoptosis. Late apoptosis is characterized by DNA fragmentation and degradation. When the cell is damaged necrosis becomes evident and the cell dies.

2.7.1.1 Detection of apoptosis

The various changes that cells undergo following HIV-induced apoptosis is measured through flow cytometry (elaborated on in Section 2.10.3) and include for example a loss in plasma membrane integrity, a decrease in mitochondrial membrane potential as well as morphological changes (Lecoeur et al., 2008). One of the primary changes involves the shrinking of cells. Using flow cytometry this is observed by a decrease in forward scatter (FSC) which relates to the size of cells (Vermees et al., 2000). Other changes are usually detected through the addition of fluorescent labels. In the context of HIV infection apoptosis may be beneficial or detrimental to both the host and pathogen respectively (Goldberg and Stricker 1999). For example, an increase in cell death through apoptosis may limit viral replication since the host reservoir becomes depleted. In contrast cell death may facilitate viral spread when damaged cells release their intracellular contents. The reverse also holds true i.e. apoptosis may be inhibited so that the host cells are not depleted and productive infection is maintained (Selliah and Finkel 2001).

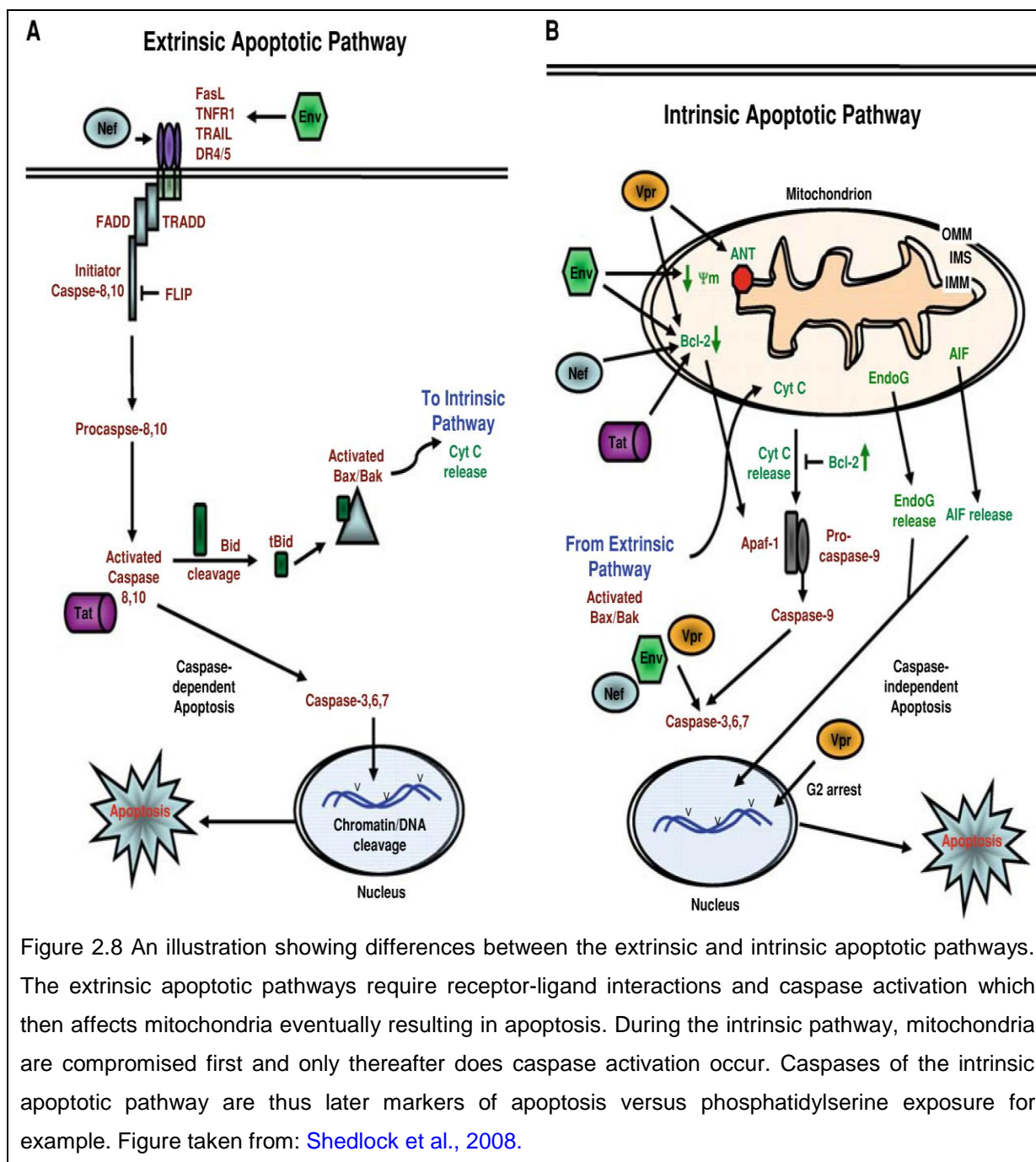
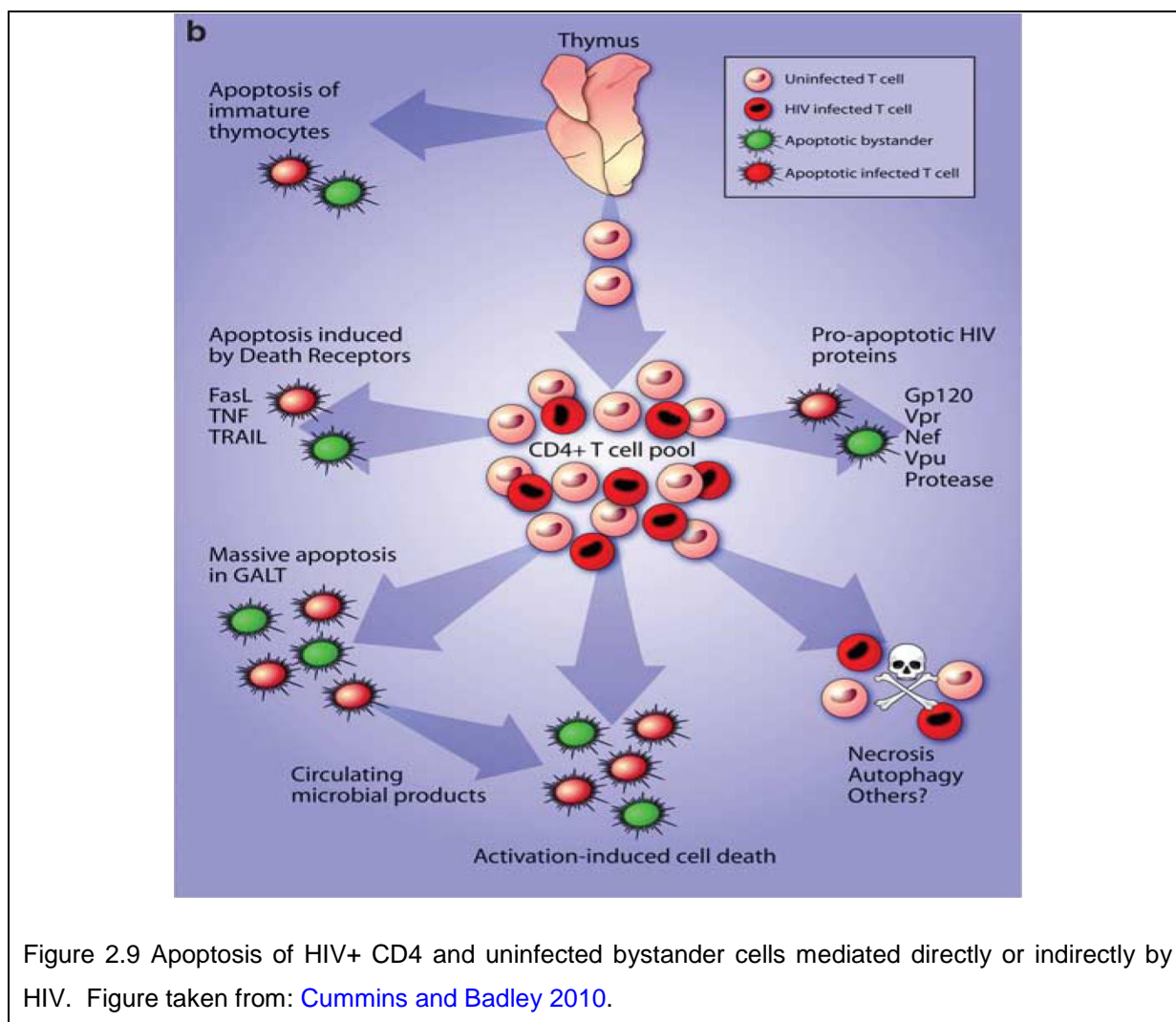


Figure 2.8 An illustration showing differences between the extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathways require receptor-ligand interactions and caspase activation which then affects mitochondria eventually resulting in apoptosis. During the intrinsic pathway, mitochondria are compromised first and only thereafter does caspase activation occur. Caspases of the intrinsic apoptotic pathway are thus later markers of apoptosis versus phosphatidylserine exposure for example. Figure taken from: [Shedlock et al., 2008](#).

During HIV infection not all the CD4+ cells are infected with virus. The numbers of apoptotic cells are usually more than the percentage of infected cells. This is largely due to the apoptosis of uninfected bystander cells (Figure 2.9, [Selliah and Finkel 2001](#)). In an attempt to better understand **HIV-specific apoptosis** and the mechanisms leading to this form of cell death in uninfected and HIV-infected T lymphocytes *in vitro*, [Herbein et al \(1998\)](#) isolated peripheral blood lymphocytes (PBLs) and monocyte-derived macrophages (MDMs) from a healthy individual and subjected these cells to *in vitro* HIV infection. An HIV reporter virus expressing green fluorescent protein (GFP) was then used to distinguish the uninfected and infected cell populations within PBLs and MDMs respectively. After selecting only the

infected cell population (cells expressing GFP) CD4 and CD8 apoptosis was detected within the PBL population. When PBLs were examined higher levels of apoptosis was measured in the infected CD4 cell population than in the CD8 cells. When a mixed population of cells (i.e. the PBLs together with the MDMs) was examined apoptosis was found to occur primarily in uninfected bystander cells (cells not expressing GFP). MDMs added to the infected CD4 cells undergoing apoptosis did not affect the apoptosis pattern of these cells. The authors concluded that the apoptosis of uninfected T cells *in vitro* was dependent on the presence of monocytes/macrophages. The apoptosis of uninfected bystander cells has subsequently been identified by researchers as a viral mechanism for removing potentially immunogenic cells (Holm and Gabuzda 2005). Despite the apoptosis of uninfected bystander cells there is ample evidence which shows that the PBMCs and T cells of HIV+ individuals still experience a greater percentage of apoptosis (Herbein et al., 1998; Meyaard et al., 1992) compared to their uninfected counterparts.

While cell death is believed to occur primarily in CD4 and bystander cells (Herbein et al., 1998), the apoptosis of both CD4 and CD8 cells isolated from HIV-infected individuals has been reported (Cotton et al., 1997; Gougeon et al., 1996; Meyaard et al., 1992). Apoptosis of CD4 and CD8 cells were also observed following *in vitro* HIV infection of primary T cells (Holm and Gabuzda 2005). According to Holm and Gabuzda (2005), CD8 apoptosis does occur but is dependent on the presence of virion-exposed CD4 cells which release a soluble factor(s) for apoptosis to proceed in the CD8 cells. Not only can apoptosis occur in both T cell subsets but cells isolated from infected patients can experience more CD8 than CD4 apoptosis (Lewis et al., 1994; Meyaard et al., 1992). The percentage PBMCs undergoing apoptosis was measured for HIV- and HIV+ individuals (Sections 5.2.4 and 5.3.2) as well as the apoptotic levels in both subsets of T cells (Sections 5.2.5. and 5.3.3.). With a hyperactivated immune state, increased apoptosis and a subsequent rise in ROS experienced during HIV infection, the host is placed under constant **oxidative stress** (Repetto et al., 1996; Pace and Leaf 1995).



2.7.2 Oxidative Stress

Oxidative stress according to [Hulgan et al \(2003\)](#) refers to the *in vivo* production of reactive free radicals that damage cells and tissues. ROS are produced during metabolism and have a functional role in the immune system ([Eruslanov and Kusmartsev 2010](#)). However, when the production of ROS and other oxidants are elevated and a concurrent decrease in the anti-oxidant defence system occurs; oxidative stress persists ([Mollace et al., 2001](#); [Thérond et al., 2000](#); [Repetto et al., 1996](#); [Schwarz, 1996](#); [Pace and Leaf 1995](#)). The radicals produced alter the structure and functioning of lipids, proteins, nucleic acids, etc ([Thérond et al., 2000](#)). Various factors as shown in Figure 2.10 contribute to the development of oxidative stress including: infection, exposure to toxins, inflammation, stress, exercise, alcohol, smoking, diets consumed, preparation of foods at high temperatures, etc ([Gitto et al., 2002](#); [Kohen and Nyska 2002](#)).

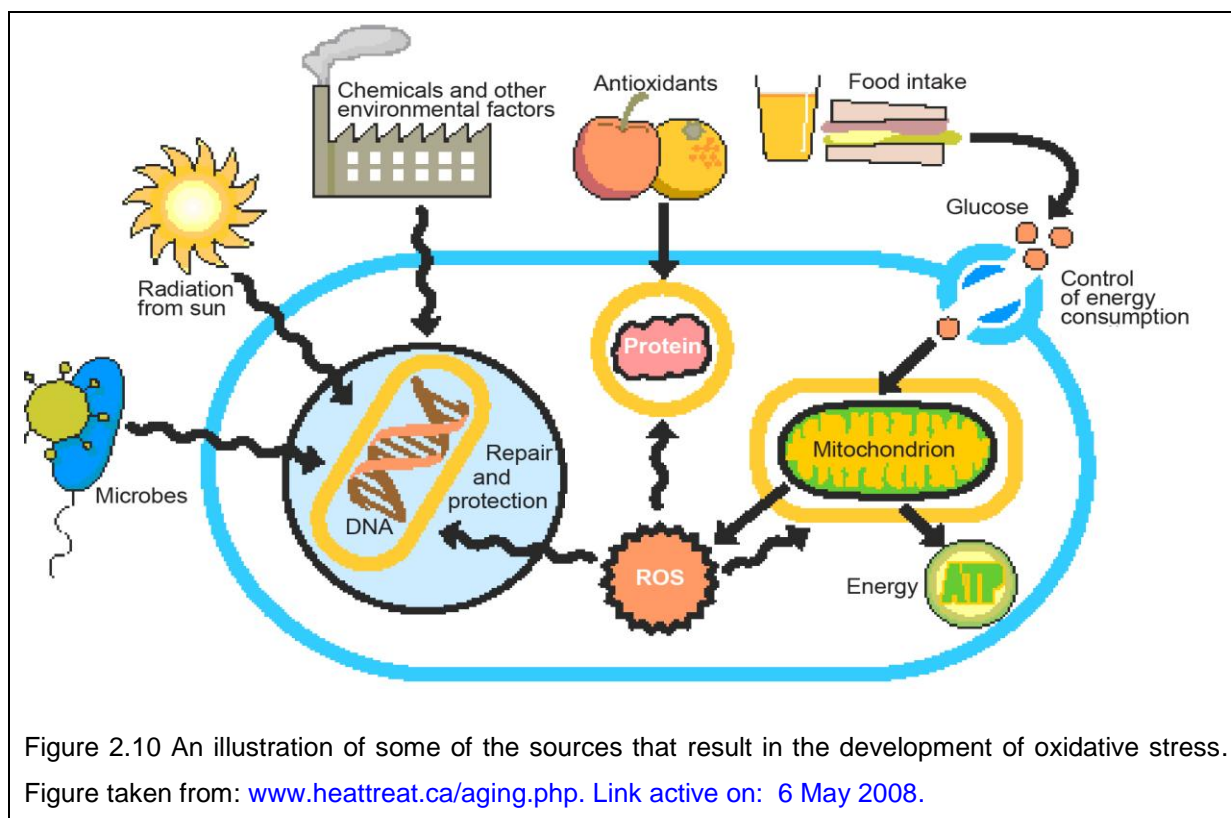


Figure 2.10 An illustration of some of the sources that result in the development of oxidative stress. Figure taken from: www.heattreat.ca/aging.php. Link active on: 6 May 2008.

Bulk of the ROS produced in living systems can be found in mitochondria where adenosine triphosphate (ATP) production primarily occurs through electron transfer processes. During this process some electrons leak and contribute to the oxidative stress signal. As confirmation that HIV+ individuals are under oxidative stress Pace and Leaf (1995) showed increased amounts of hydroperoxides to be present in the serum of HIV+ individuals. Hydroperoxides are produced during asymptomatic HIV infection (Mollace et al., 2002) and signal oxidative damage to membranes as well as changes in membrane fluidity (Repetto et al., 1996; Pace and Leaf 1995). These changes detected by the hydroperoxides ultimately results in cell death measured as apoptosis. Hydroperoxides are therefore not only markers of oxidative stress but of early apoptosis as well. Hydroperoxides have been shown to induce apoptosis by causing the release of pro-apoptotic factors (Bayir and Kagan 2008). The *in vitro* exposure of glutathione peroxidase (which protects against oxidative stress) deficient T cells to hydroperoxides also resulted in apoptosis (Sandstrom et al., 1994). As an indirect measure of oxidative stress various literature report on disturbed glutathione metabolism during HIV infection and similar disease models (Aukrust et al., 2003; Aukrust et al., 1995; Roederer et al., 1991). Decreases in antioxidant reserves have previously been measured in SIV-infected monkeys while an increase in oxidative stress was measured in the brain and CSF of HIV+ individuals with dementia (Turchan et al., 2003). Gil et al (2003) showed that HIV-induced metabolic events lead to an increase in oxidative stress whilst studies by Lane and Provost-Craig (2000); Hommes et al (1991) and Hommes et al (1990)

recorded an increase in resting energy expenditure in clinically stable HIV+ individuals. Resting energy expenditure is associated with increased oxygen consumption and therefore oxidative stress in these individuals. Studies by [Wanchu et al \(2009\)](#) and [DobMeyer et al \(1997\)](#) also confirmed an increase in oxidative stress during HIV/AIDS.

Oxidative stress has been implicated in various pathologies and viral diseases ([Peterhans, 1997](#)). Its role in HIV infection is of particular concern since it contributes to disease progression (favouring Th2 cytokine responses), compromises the functioning of the immune system and prevents DNA repair mechanisms from functioning optimally ([Deresz et al., 2007](#); [Baruchel and Wainberg 1992](#)). In addition, ROS are involved in metabolic regulation ([Peterhans, 1997](#)) and are of relevance since HIV makes use of the host biosynthetic machinery to survive. HIV's influence on both the metabolic and immune systems is investigated in Chapters 4 and 5 respectively.

2.7.3 Dysregulation in cytokine production

The elevated levels of ROS produced during HIV infection initiates a range of deleterious reactions within the host system such as a **dysregulation in cytokine production** often in favour of the Th2 cytokine profile. TNF- α , IL-1, IL-2, IFN- γ , IL-6 and IL-10 represent some of the typical cytokines studied in HIV research. Measuring these cytokines gives an indication of the degree of immune activation, extent of the immune response and disease progression. TNF- α for example is one of the first cytokines produced by T cells during infection ([Aukrust et al., 2005](#)). Elevated amounts of this cytokine and IFN- γ occur during HIV infection. These cytokines tend to increase the production of ROS ([Baier-Bitterlich et al., 1997](#); [Baruchel and Wainberg 1992](#)) and are associated with HIV-induced apoptosis. TNF- α , IL-1 and IL-2 are associated with HIV-induced oxidative stress whereas TNF- α , IL-1 and IL-6 are indicative of an activated immune state. The levels of TNF- α and Fas are also elevated during HIV infection ([Gil et al., 2003](#)) and HIV-induced apoptosis. Elevated TNF- α levels have been shown to contribute to an increase in oxidative stress by increasing the metabolic rate in HIV-infected patients ([Glade, 2000](#)). Just as TNF- α causes an increase in oxidative stress so does oxidative stress influence the secretion of TNF- α and the percentage apoptosis. In previous reports progression to HIV disease has predominantly been associated with a Th2 phenotype i.e. decrease in IL-2 and IL-12 production and an increase in IL-4, IL-6, IL-10 and/or IL-13 ([Clerici and Shearer 1993](#)). The production of Th2 cytokines is associated with CD4 cell loss, apoptosis and the development of AIDS ([Clerici et al., 1997](#)). Contrary findings disputing the Th1 \rightarrow Th2 shift during HIV infection have however been published ([Sarih et al., 1996](#); [Graziosi et al., 1994](#); [Maggi et al., 1994](#)) and is mainly attributed to the different experimental approaches utilized (i.e. measuring cytokine changes

in unfractionated versus purified cell populations, in the presence or absence of stimulants, at the primary cell versus clonal level, etc). Disagreement of the Th1→Th2 shift by [Sarih et al \(1996\)](#) was linked to the fact that levels of key cytokines linked to non-progression (IL-2) and disease progression (IL-4) respectively were in contrast to that measured by [Clerici and colleagues](#). [Maggi et al \(1994\)](#) detected low levels of IL-4 in their model. IL-4 is a Th2 cytokine. To explain the low levels of IL-4 [Maggi and colleagues](#) showed HIV to preferentially infect and deplete the CD4 Th2 cell population and subsequently the levels of type 2 cytokines. Cytokine detection is thus influenced by a change in the composition of the cells ([Graziosi et al., 1994](#)).

The role of IFN- γ and TNF- α as key mediators in the activation, inflammatory, oxidative and apoptotic processes of HIV+ cells was previously mentioned. Although IFN- γ does not have as much of an effect on metabolic processes (deduced from the available literature), the detection of this cytokine is important because of its role as a “counter” cytokine to TNF- α which usually amplifies inflammatory reactions. In addition to providing information on immune function and disease pathogenesis IFN- γ production should indicate whether the same cells that produce TNF- α following mitogenic or antigenic stimulation can elicit an antiviral immune response possibly through a CMI response (CD8 cells producing IFN- γ).

Despite the fact that HIV induces such a range of cytokine changes there has still been a tendency in the literature to measure one analyte at a time using the standard ELISA ([Elshal and McCoy 2006](#)). The drawbacks of the cytokine ELISA assay is that it requires large volumes of sample, has a limited detection range and is not very sensitive or specific ([Elshal and McCoy 2006](#); [Morgan et al., 2004](#)). The analysis time for obtaining a measurement is long and has subsequently caused the use of cytokines in the diagnosis and prognosis of disease to be neglected ([Tang et al., 2011](#); [Tang et al., 2008](#)). Recent advances in the field of immunology, particularly the development of CBA kits and multi-parametric flow cytometers which allows for measuring an increased number of cytokines per sample is improving the situation. The principles on which CBAs are based is similar to that of the standard ELISA but have the added advantage of allowing the researcher to detect and quantify more than one analyte in a smaller volume of sample. Similar to the metabonomics approach employed in Chapter 4 for profiling metabolic changes, the measurement of a number of cytokines allows for a cytokinomics analysis of the immune data. [Clerici \(2010\)](#) recently introduced the concept of cytokinomics which he defined as the systematic study of cytokine production and the interactive effects of these molecules in a biological system. If the definitions of other “omics” are considered these usually refer to measuring all the

analyte within a particular system. Strictly speaking cytokinomics should then refer to the measurement and statistical analysis of all cytokine within the “cytokinome”. As part of the immune data which forms part of Chapter 5 seven cytokines were measured and although this is not “all” cytokines these molecules are produced by the Th1/Th2 and Th17 system of cells and as such qualifies to be assessed through a cytokinomics approach. The definition was further adapted here to allow for investigations into the role of these molecules as biomarkers of HIV/AIDS. More background on CBAs and the concept of cytokinomics is supplied in Section 5.2.6.2.1. With the advent of faster analysis times and more information from limited samples the potential clinical usefulness of measuring an array of cytokines to probe immune dysfunction and immune-based diseases has gained popularity (Salem et al., 2009; Wong et al., 2008). Measuring changes in the cytokine profile during HIV infection (Keating et al., 2011; Rahman et al., 2011; Tang et al., 2011; Nixon and Landay 2010; Roberts et al., 2010; Tang et al., 2008) and other pathological states such as tuberculosis (Frahm et al., 2011; Hussain et al., 2002) has thus grown and shown promise for possible use in predicting disease progression. Cytokines measured one at a time during ELISA assays are mostly analyzed using univariate statistics. Similarly, the data derived from measuring a multitude of cytokines using CBA technology and flow cytometry have been analyzed using univariate statistical approaches as well as correlation analysis. Multi- instead of univariate approaches should be utilized for analyzing these data. Multivariate statistical approaches for this work refers to the analysis of more than one variable at a time and may assist in evaluating the effect of multiple cytokines on one another in the asymptomatic model of HIV infection presented here since cytokines do not function in isolation. Only recently did Roberts et al (2010) use multivariate statistics to show that the plasma cytokine profile can predict progression to disease in an acute model of HIV infection. The effect of HIV on the Th1/Th2/Th17 cytokine profile has however not been shown before using multivariate approaches as is done in this study on the serum of clinically stable patients.

2.7.3.1 Cytokines and metabolic changes

In addition to their immunological role cytokines mediate several **metabolic changes** (Salas-Salvadó and García-Lorda 2001, Lane and Provost-Craig 2000; Hommes et al., 1990) and induces a hypermetabolic state in the host. IL-1, IL-6 and TNF- α for example are linked to tissue wasting processes (Al-Harathi and Landay 2002; Hommes et al., 1991). During HIV infection cytokines such as IL-1, IL-6, TNF- α and IFN- γ induce immune responses which stimulate leptin production and decrease lipoprotein lipase activity as well as glucose uptake via glucose transporter type 4 (Glut4). An increase in hepatic lipid synthesis and triglyceride content occurs. There is also inhibition of adipocyte differentiation

and an increase in glycogenolysis, lipolysis, proteolysis and apoptosis (Slama et al., 2009; Faintuch et al., 2006; Salas-Salvadó and García-Lorda 2001; Glade, 2000). TNF- α for one has been shown to interfere especially with glucose and fatty acid metabolism (Cossarizza et al., 2002) and is associated with the development of lipodystrophy and other changes in lipid metabolism (Gougeon and Piacentini 2009).

The general functioning of the immune system is not that simple though. Whether the immune system gets to interact with the cell and how this happens is largely determined by metabolic processes (Newell et al., 2006). In an article by Newell et al (2006) this was explained by using the deprivation of glucose from cells as an example. Low levels of glucose decreased the cell surface expression of Fas which in turn decreased the visibility of the cells to the immune system i.e. those cells expressing Fas L could not bind and so apoptosis was inhibited. In the review by Slama et al (2009) cytokine changes were linked to the metabolic syndrome (a combination of separate but interconnected metabolic changes/risk factors, Slama et al., 2009; Alberti et al., 2006; Powderly, 2004) and the immune and metabolic systems described as sharing common pathways. Cytokines therefore not only regulate the immune system but also influences host metabolism (Matarese and La Cava 2004).

2.7.4 HIV-specific immune responses to *in vitro* peptide stimulation

In addition to measuring secreted cytokine profiles intracellular cytokine staining in response to *in vitro* HIV peptide stimulation is routinely done (Jansen et al., 2006; Kaushik et al., 2005). Viral peptides when used as stimulants *in vitro* triggers cytokine production by memory cells. Whilst the levels of secreted cytokine in HIV-infected serum provide information on the virus' effect on the immune system, the stimulation of infected cells with HIV antigen can be used to characterize HIV-specific cytokine responses *in vitro*. For example, cellular immune responses detected *in vitro* in long term nonprogressors (LTNPs) were shown to be associated with slow progression to disease (Rosenberg et al., 1997). HIV-specific CD8 T cell responses control HIV infection and were found to be reduced in those individuals progressing to disease (Gougeon and Piacentini 2009; references within [Betts et al., 2006]). Stimulating cells with peptides *in vitro* and measuring the associated cytokine responses therefore present as useful prognostic indicators. For example, a loss of IL2+ and IL2+IFN- γ + Gag-specific T cells was shown to be associated with HIV/AIDS disease progression (Jansen et al., 2006).

A common peptide used for the *in vitro* stimulation of HIV-infected cells is Gag and more recently R7V which is derived from β 2m, a protein incorporated by HIV during budding (Bremnaes and Meyer 2009). Gag is one of the most conserved of the HIV-1 proteins and is

favoured since it together with the Nef proteins has the highest epitope density. Due to this property some of the strongest responses have been directed and elicited against regions within this protein. Gag proteins are also preferred since HIV-specific CD4 T cells target multiple regions of this protein (and that of Nef) whilst other regions are targeted infrequently or not at all (Kaushik et al., 2005; Venturini et al., 2002). Some of the latest developments related to measuring HIV-specific T cell responses have been to investigate not only the effects of single peptides but that of peptide pools as well. With peptide pools a comprehensive assessment of immune system functioning is expected since responses will be elicited to all possible epitopes contained in the peptide pool (Betts et al., 2001).

The immunological consequences of HIV outlined above (immune activation, apoptosis, immunodeficiency, elevated ROS and alterations in cytokine production) ultimately affect mitochondria and enhances metabolic imbalances (i.e. hydroperoxide molecules signalling oxidative membrane damage, intrinsic apoptotic pathways being activated during infection and cytokines inducing or augmenting apoptosis). Such links will be explored in subsequent chapters to assist in characterizing the metabolic and immune profiles of HIV-infected individuals and further establishing links between the two systems.

2.8 Host Metabolism

Metabolism refers to all chemical processes occurring in a living system. It is subdivided into catabolic processes where molecules are broken down to release energy and anabolic processes where molecules are synthesized following energy consumption (Voet et al., 1999). Metabolic pathways comprise a series of interconnected enzymatic reactions of which the reactants, intermediates and products are termed metabolites (Voet et al., 1999). This complex, interconnected nature of metabolic pathways is shown in Figure 2.11. Metabolites are small molecules which participate in metabolic reactions and that are required for the maintenance, growth and functioning of the cell (Pendyala et al., 2007). These molecules are present at low concentrations and have different physio-chemical properties. As a result, various techniques have been developed to facilitate their detection. Still, there is no single instrument that can detect and analyze all metabolites at one time (Dettmer et al., 2007).

The host metabolism is dynamic and influenced by both endogenous (such as pathogenic invasions, inborn metabolic disorders) and exogenous factors (such as nutritional intake/toxins and stresses from the environment). The response of cells to such stress factors generally results in an adjustment of their extra-cellular environment in order to maintain homeostasis. This metabolic change is usually characteristic of the nature of the toxic insult or disease process, precedes protein and genetic changes and is representative

of the organism's phenotype (Serkova and Niemann 2006). In this thesis the metabolic and immune responses of individuals in response to HIV infection were investigated.

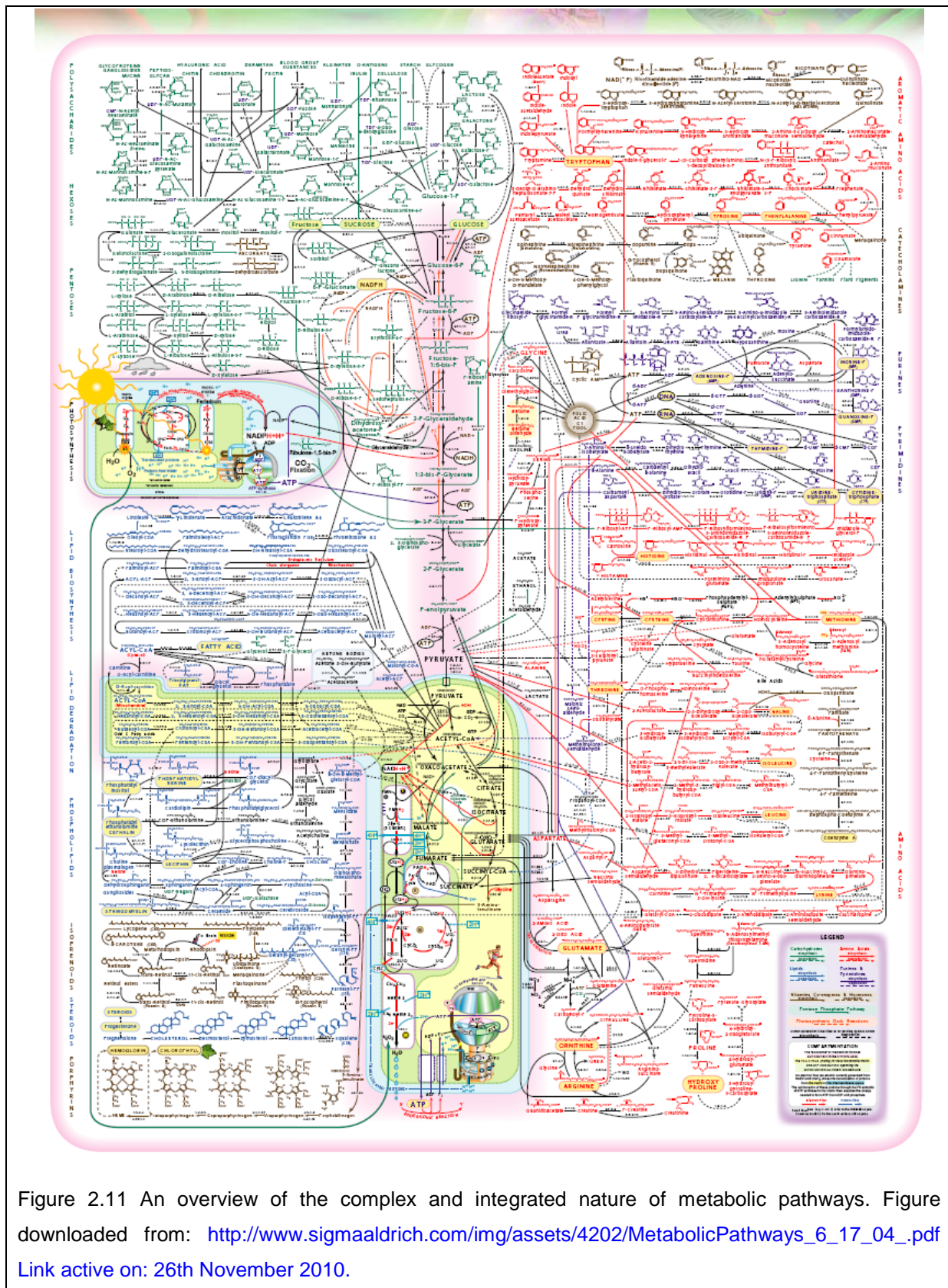


Figure 2.11 An overview of the complex and integrated nature of metabolic pathways. Figure downloaded from: http://www.sigmaaldrich.com/img/assets/4202/MetabolicPathways_6_17_04_.pdf Link active on: 26th November 2010.

2.8.1 HIV and other virus-induced metabolic changes

Due to the chronic asymptomatic stage of HIV infection, immune responses to HIV and/or the administration of ART, metabolic complications are expected in the infected individuals. Because more than one metabolic change can be induced at a time the term metabolic syndrome has been defined to include several of these separate but interconnected changes ([Slama et al., 2009](#); [Alberti et al., 2006](#); [Powderly, 2004](#)).

HIV-induced metabolic changes were recognized during the early stages of AIDS research, before the implementation of HAART and were shown to be prevalent in asymptomatic individuals with “normal” weight and CD4 counts ([Martin and Emery 2009](#); [Slama et al., 2009](#)). A key risk factor found to be associated with the development of the metabolic syndrome during HIV infection was viral load ([Slama et al., 2009](#)). In 1990 and 1991, [Hommes et al](#) produced the first publications which reported on the effect of HIV on the metabolism of seemingly “healthy” infected individuals. The authors showed metabolic abnormalities even whilst CD4 counts were still high. In these studies as well as that of [Lane and Provost-Craig \(2000\)](#) the clinically stable HIV+ individuals were shown through calorimetric experiments to have higher rates of resting energy expenditure. This is in keeping with the high energy demands of infected cells. These individuals also had high fat oxidation rates and this led the authors to speculate that the greater amount of energy lost versus that taken in would make the affected individual prone to catabolic processes. Hypermetabolism was therefore recognized as a characteristic of the asymptomatic phase of infection. Similar studies are referenced by [Salas-Salvadó and García-Lorda \(2001\)](#) and also reflect high resting energy expenditure. Subsequent to the work of [Hommes et al](#), [Pascal et al \(1991\)](#) showed through the use of positron emission tomography and magnetic resonance imaging (MRI), increased cerebral metabolic rates for glucose in the brains of asymptomatic HIV+ patients. By doing this study the authors showed metabolic alterations in the brain ahead of structural changes. Detections such as these provide information on disease progression and could serve as a guide for the implementation of corrective therapy prior to the development of clinical symptoms.

Other HIV-induced metabolic changes that have been identified in clinically stable patients include: changes in body composition, fat distribution, changes in lipid, glucose, energy and protein metabolism ([Martin and Emery 2009](#); [Salas-Salvado and Garcia-Lorda 2001](#)). Changes in body composition are largely attributed to an increase in the catabolic state of the host ([Powderly, 2004](#)). Other metabolic changes not often mentioned in the literature includes: bone loss as well as liver disease. An article by [Safrin and Grunfeld \(1999\)](#) summarises and compares some basic HIV-induced metabolic changes which most

researchers believe are a direct consequence of chronic HIV infection and the associated immune responses (Slama et al., 2009; Salas-Salvado and Garcia-Lorda 2001).

In results produced by Hattingh et al (2009) using conventional techniques HIV was shown to elevate serum protein and triglyceride levels as well as lower serum albumin and cholesterol in a group of infected South African women. The effects of viral infection on the metabolism is not unique to immunodeficiency viruses but have also been documented for other viral models. Infection of human fibroblasts with the human cytomegalovirus (HCMV) resulted in an increase in glycolysis, Krebs cycle intermediates and pyrimidine nucleotide biosynthesis (Munger et al., 2006). These authors measured an increase in fatty acid catabolism due to HCMV infection. These findings largely co-incide with that obtained for HIV infection models also discussed in this section.

With the advancements that have been made in terms of technologies for detecting metabolic change, virus and ART-induced metabolic changes are now being investigated using “omics” approaches (Ghannoum et al., 2011; Hollenbaugh et al., 2011; Williams et al., 2011; Pendyala et al., 2009; Philippeos et al., 2009; Wikoff et al., 2008; Hewer et al., 2006; and in this thesis) largely because of the sensitivity, specificity, reproducibility and most of all high-throughput capabilities associated with these techniques (Wikoff et al., 2008).

2.8.2 Detecting HIV-induced metabolic changes

Research to date has focused on proteins as biomarkers for HIV infection (Pendyala and Fox 2010). The number of immunological, protein and macromolecular markers with which to characterize disease progression has therefore increased (reviewed by Kanekar, 2010). Using a proteomics-based MS approach Pendyala et al (2009) was able to show several proteins associated with immune system function to be upregulated in the CSF of SIV-infected primates. In a study investigating the co-epidemic of substance abuse and SIV infection, Pendyala et al (2011) measured an increase in glutathione-S-transferase as a compensatory response to the high level of oxidative stress experienced during infection and methamphetamine use. Laspur et al (2007) made use of proteomics to obtain the CSF protein profiles of HIV-infected individuals experiencing cognitive impairment. Protein markers associated with HIV-induced dementia were also identified when the serum and CSF of HIV-infected individuals were analyzed through matrix-assisted laser desorption/ionization (MALDI)-MS (Berger et al., 2005). In this particular study of Berger the intensity of the detected proteins was found to correlate with the degree of dementia and therefore had prognostic value. Although proteomic technologies have significantly contributed to an understanding of the general as well as specific pathological

consequences of HIV infection, the application of metabonomics to viral infections and HIV-infected biofluid in particular, is limited ([Pendyala and Fox 2010](#)).

Metabonomics refers to the study of metabolites and how these molecules change in response to stimuli. Using this approach changes in metabolite levels are detected through analytical instrumentation, the data analyzed through multivariate statistics and molecules of statistical significance interpreted in a biological context. The primary objective of these types of analysis is to identify biomarkers for use in disease diagnosis, prognosis and treatment-success monitoring. Up to now MS-based metabonomics investigations have only been presented in the form of posters ([Cassol et al., 2011](#)) or articles where the focus was mainly directed to the effects of ART. The limited application of MS to the study of HIV-infected biofluid is also apparent from the lack of reference to any low molecular weight molecules in the review of [Kanekar \(2010\)](#).

In those cases where metabonomics has been applied to the study of HIV-infected biofluid, NMR spectroscopy has mainly been utilized. In one of the first metabonomics articles published by this laboratory, [Hewer et al \(2006\)](#) used NMR spectroscopy together with pattern recognition analysis to distinguish between HIV-, HIV+ and HIV+/AIDS patients on ART. The existing knowledge that antiretroviral medication used to treat HIV infection can cause metabolic change led the authors to investigate whether metabonomics can distinguish uninfected and infected sera as well as indicate ART-induced metabolic changes. A comparative study using 300 and 600 MHz NMR instruments ([Philippeos et al., 2009](#)) demonstrated that data pre-treatment and the statistical evaluation method had an impact on data interpretation. Regions of the NMR spectra that showed significant differences ($p < 0.05$) for uninfected and infected individuals were mainly lipids, including low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL). These observations concurred with the irregularities of lipodystrophy and hyperlipidaemia common in HIV/AIDS patients on ART ([Calza et al., 2003](#)). The work of [Hewer](#) and [Philippeos](#) therefore showed that metabolic changes induced by HIV and/or ART can be revealed by metabonomics data which had been generated through NMR. Taking the chemometric analysis of NMR data a step further, [Maher et al \(2011\)](#) showed that it was possible to correlate plasma and CSF metabolite data of HIV-infected individuals to magnetic resonance spectroscopy (MRS)-derived brain metabolic data. Such co-analysis allows for the retrieval of biological information that would otherwise be unavailable if the respective biofluids were analyzed independently or with only one type of technique.

In the literature there are a number of studies which have utilized techniques such as NMR to study the various cytopathic effects of HIV but did not necessarily employ metabolomics approaches for analysis of the data (i.e. measuring stimuli-induced metabolite changes and analyzing it through multivariate statistical approaches). For example, in the work of [Apostolov et al \(1989\)](#), *in vitro* HIV-induced cytopathic effects were linked to changes in the levels of oleic and stearic acid. Oleic acid is an unsaturated fatty acid and high amounts of it lead to an increase in membrane fluidity, cell fusion and subsequent syncytia formation. Because syncytia formation entails fusion of the membranes of immune cells to form one large cell body, the virus can infect this cell mass killing many immune cells at once. To reverse the *in vitro* effects of oleic acid, saturated fatty acids such as stearic acid are added to the media of infected cells to decrease membrane fluidity, cell fusion, syncytia formation, the infection and subsequent death of immune cells. Using NMR, structural and metabolic changes were also detected in chronically infected cell lines as well as cell lines infected with HIV *in vitro* ([Luciani et al., 1991](#)). A change in the membrane structure of the cells was evident by a decrease in fatty acid signals during the first 30-60 minutes of *in vitro* infection and co-occurred with virus internalization and uncoating. The fatty acid signal later increased (after two hours) as is known to occur during HIV infection and decreased again after a few days when budding occurred. An alteration in phospholipid synthesis was also observed.

In addition to NMR another widely used technique for metabolomics is MS. In two recent articles the application of LC-MS and GC-MS to the analysis of HIV-infected saliva ([Ghannoum et al., 2011](#)) and the application of GC-MS to the analysis of the organic acid metabolome of HIV-infected sera ([Williams et al., 2011](#)) was reported. The application of MS to the study of biofluid metabolomics has been limited but where utilized the data seemed promising for HIV-specific biomarker discovery. Employing a global MS metabolomics approach; [Wikoff et al \(2008\)](#) investigated the metabolic profile of CSF of SIV-infected monkeys before and after infection with the aim of identifying biomarkers associated with neuroAIDS complications. The results showed that the carnitines, acyl-carnitines, fatty acids and phospholipids were primarily affected following SIV infection with most molecules being elevated in concentration. The increase in fatty acids (e.g. palmitic acid) and lysophospholipids was associated with an increase in phospholipase activity and thus lipid breakdown processes. Although the authors concluded that the identified metabolites did not share any structural or chemical characteristics that could be related to a single biochemical mechanism underlying their increase, they did suggest the existence of a biochemical relationship between these molecules through the fatty acid oxidation pathway.

2.8.3 HIV and mitochondria

In addition to the afore-mentioned metabolic changes HIV also impacts on the metabolic signature of the host through its effect on mitochondria. These are ominous, diverse intracellular organelles essential for cellular energy production, maintaining the redox potential of cells, calcium (Ca^{2+}) storage, heat production, radical production, apoptosis, oxidation of fatty acids, etc (Mazat et al., 2001). Mitochondria have been dubbed the “metabolic hub” of cells. When the structure and function of these organelles are affected, changes occur in the concentration of the molecules which participate in the Krebs cycle and electron transport chain (Pieczenik and Neustadt 2007) simply because these are key processes associated with mitochondria (see Figure 2.12). Because of the role of these organelles in producing ATP, energy metabolism is ultimately affected.

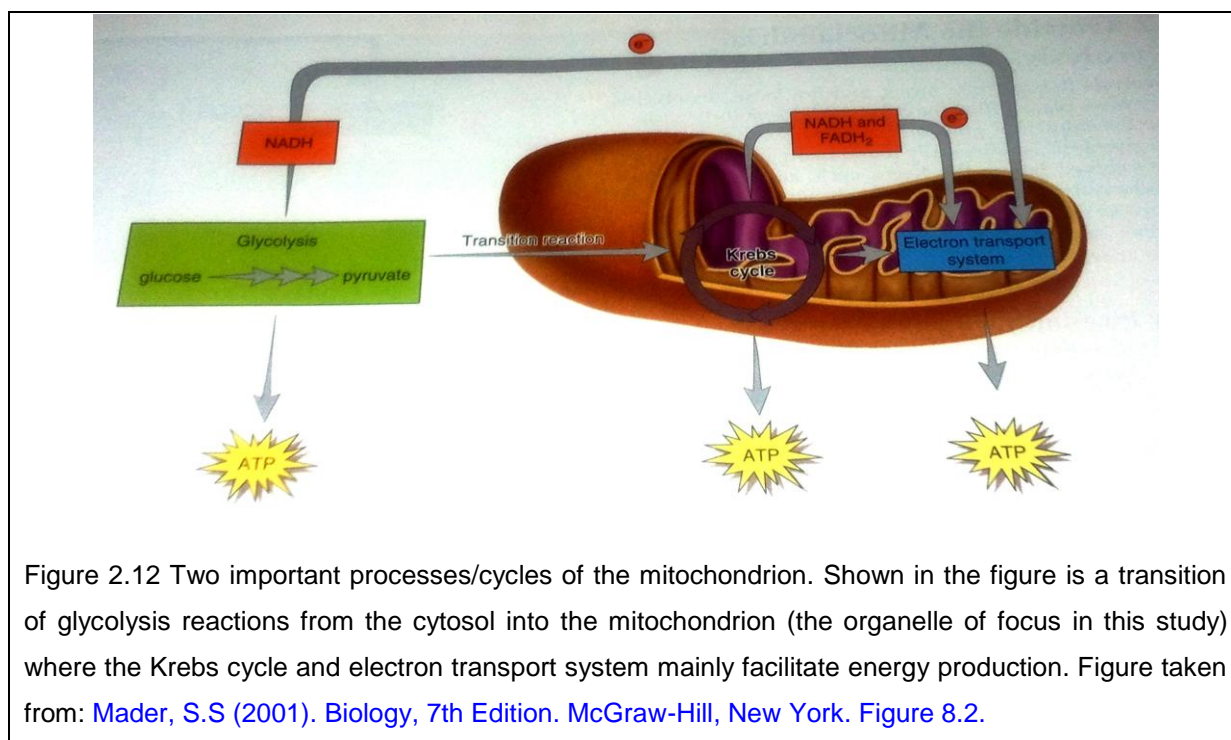


Figure 2.12 Two important processes/cycles of the mitochondrion. Shown in the figure is a transition of glycolysis reactions from the cytosol into the mitochondrion (the organelle of focus in this study) where the Krebs cycle and electron transport system mainly facilitate energy production. Figure taken from: Mader, S.S (2001). *Biology, 7th Edition*. McGraw-Hill, New York. Figure 8.2.

In addition to indirectly affecting mitochondria through immunological responses (outlined in Section 2.7.1 through to 2.7.3), the literature documents HIV to have direct effects on mitochondria which further contributes to the metabolic failure of these organelles (Crain et al., 2010; Polo et al., 2003; Macho et al., 1995). It is known that mitochondria play a central role in the apoptotic death process (Tolomeo et al., 2003) and that HIV acts on the regulation of pathways associated with apoptosis (Pinti et al., 2010). For example, whole virus as well as viral proteins augment the apoptotic pathway (Cossarizza et al., 2002) by activating the immune system (Ross, 2001), ultimately affecting mitochondrial membrane potential which in turn causes the release of cytochrome c, apoptosis inducing factors and a range of metabolic intermediates (Lemasters et al., 1998). HIV proteins contribute to destabilizing the

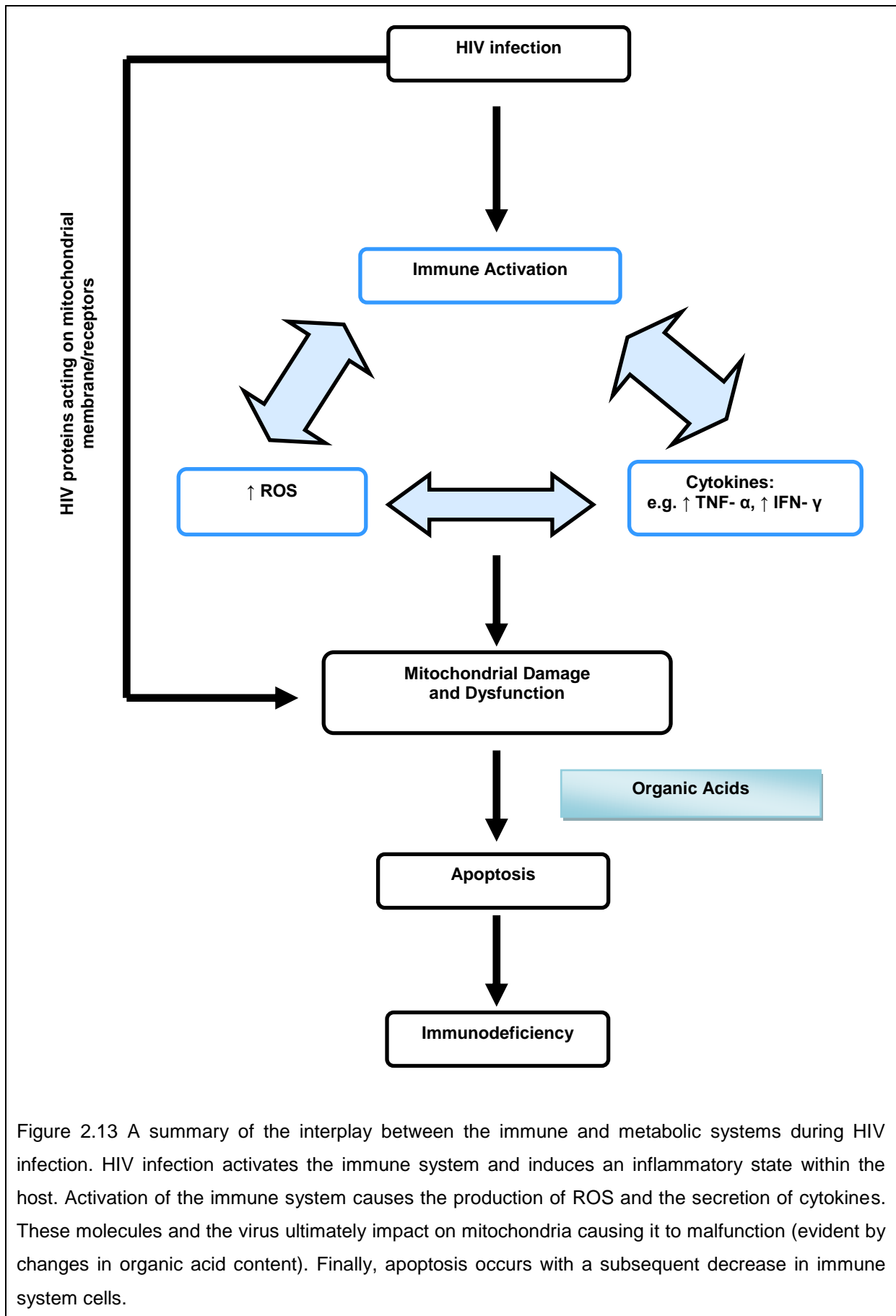
mitochondrial membrane potential by directly acting on the membrane and receptors of these organelles (Shedlock et al., 2008; Boya et al., 2004). A common example in the literature is the interaction between Vpr and adenine nucleotide translocase (a protein of the mitochondrial permeability transition pore complex). Mitochondrial dysfunction due to the destructive action of viral protein products is also reviewed by Cummings and Badley (2010). Products of the *env*, *nef*, *tat*, and *vpr* HIV genes in particular affect mitochondria by exhibiting pro-apoptotic activity. The p75 subunit (NDUFS1) of complex I of the mitochondrial respiratory chain (MRC) was shown to be susceptible to caspase cleavage resulting in the disruption of mitochondrial function during apoptosis (Ricci et al., 2004). Complex I activity was furthermore found to be impaired due to downregulation of the NDUFA6 subunit following HIV infection (Ladha et al., 2005). In a study done by Míro et al (2004) a decrease in the activity of MRC complex II, III and IV was observed. Coupled to lowering the activities of the MRC complexes other effects such as lowered mitochondrial DNA (mtDNA) content and increased lipid peroxidation of PBMC membranes was noted (Míro et al., 2004). It might be argued that the decrease in mtDNA is due to a decrease in CD4 T cells but this was found by Côté et al (2002) to not be the case. Since hydroperoxides are indicators of oxidative damage to membranes their role as markers of mitochondrial dysfunction can also be imagined.

Various markers with which to define the functional status of mitochondria exist. In the past HIV-induced mitochondrial dysfunction was mostly detected through the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Del Llano et al., 1993) as well as apoptosis assays (Shedlock et al., 2008). Using flow cytometry, Macho et al (1995) confirmed that mitochondrial dysfunction was evident in the T cells of HIV-infected individuals after measuring a decrease in mitochondrial membrane potential and a rise in superoxide anion production in these cells. Although flow cytometry is a useful technique for measuring metabolic changes it does become difficult to analyze a cascade or profile of metabolites using this technique since the enzymatic changes produce numerous products (Whitmore et al., 2007; Krylov et al., 2000). Alterations to the structure and function of these organelles are also assessed through a decrease in mtDNA content (Crain et al., 2010; Míro et al., 2004), alterations in metabolic processes (studied using MTT) and intermediates such as the organic acids (Hoffmann and Feyh 2005) amongst others.

2.8.4 Organic Acids; markers of mitochondrial dysfunction

The body largely depends on oxidation processes for its energy supply. This occurs mainly through the breakdown of fats, carbohydrates and amino acids in mitochondria and peroxisomes, with organic acids forming as intermediates. Organic acids, also known as

carboxylic acids (Zhang et al., 2007; Meletis, 2006), are established biomarkers of mitochondrial dysfunction. These molecules are components of biochemical pathways of intermediary metabolism, can represent exogenous compounds (Hoffmann and Feyh 2005) and have been well characterized in urine using GC-MS (Jellum, 1981). Because organic acids are intermediates, their concentrations in biofluids are relatively low. Defective enzymes can however cause the levels of these molecules to increase. To illustrate how a change in organic acid levels comes about the fatty acid oxidation pathway can be used as an example. Briefly, the transportation of fatty acids to mitochondria is usually facilitated through carnitines. If the carnitine pool is low or mitochondrial function disrupted, beta (β)-oxidation of the fatty acids cannot occur. This causes molecules such as adipic acid, suberic acid and ethylmalonate to accumulate through alternative routes such as the omega (ω)-oxidation pathway (<http://www.metamatrix.com/files/learning-center/leifm/book-Laboratory-Evaluations-in-Molecular-Medicine.pdf>). In the case of energy production processes the levels of pyruvate and lactate increase following inhibition of pyruvate dehydrogenase activity causing less fatty acid to be synthesized. In Section 2.8.3, the metabolic failure of mitochondria is referenced to be associated with changes in energy metabolism. With less fatty acid being oxidized it is clear where some of the ATP depletion stems from. Other pathways in which these molecules participate include: carbohydrate metabolism, amino acid catabolism, detoxification processes, neurotransmitter metabolism and dysbiosis (imbalance in gut microflora, <http://www.metamatrix.com/files/learning-center/leifm/book-Laboratory-Evaluations-in-Molecular-Medicine.pdf>). It was previously highlighted that HIV impacts negatively on mitochondria (references under Section 2.8.3) and that organic acids are markers of mitochondrial dysfunction (Hoffmann and Feyh 2005). These molecules therefore represented an appropriate component of the metabolome for investigating metabolic and immune changes linked to the disruption of mitochondrial structure and function during HIV infection. This thesis presents the use of MS-metabonomics as a detection mechanism for organic acid changes as indicators of HIV-induced mitochondrial dysfunction (Chapter 4). Although in its infancy, investigating virus-induced metabolic changes using metabonomic approaches certainly holds promise for providing information on viral-host interactions, mechanisms of viral infection (e.g. detection of fatty acids which facilitate viral infection and viral spread) and HIV/AIDS pathogenesis. Because HIV is known for its effect on the immune system and its effect on the metabolic system is increasingly being investigated, we also report on associated immune parameters linked to mitochondrial dysfunction in Chapter 5. Figure 2.13 provides a schematic summary of the interplay between the immune and metabolic systems (mitochondria) during HIV infection as expected for this report.



2.9 Rationale, Research Questions/Objectives and Hypothesis

The purpose of this chapter thus far was to provide a brief background to HIV and to highlight some of the immunological and metabolic consequences which follow infection. Several issues requiring research, in particular, the complications associated with HIV infection were raised. Although much has been learnt from the immune system there is a need to identify biomarkers to facilitate the detection, prognosis and monitoring of HIV infection. Biomarker identification can inform on the history of HIV infection, guide the development of therapeutics against those molecules found to be associated with HIV/AIDS disease progression and ultimately have use in HIV/AIDS management strategies (Touloumi and Hatzakis 2000). The metabolic system represents an avenue for such investigations as it too is affected by HIV. Whilst there has been favouritism toward studying the immune system (often one analyte at a time) and assessing HIV-induced metabolic changes through conventional methodology (e.g. MTT); metabonomics-based analyses have lagged behind. Infact, better-suited biomarkers may be obtained by analyzing biochemical pathways in concert to the immune system and applying multivariate statistical approaches for data analysis. In a recent publication by Roberts et al (2010) multivariate statistics was applied to the analysis of inflammatory cytokines in plasma derived from an acute model of HIV infection. The work presented in this thesis does the same for serum Th1/Th2/Th17 cytokine changes during chronic HIV infection and in addition correlates HIV-induced immune changes with virus-associated metabolic changes. The complexity of the immune and metabolic systems also lends itself more freely to multi- instead of univariate statistical approaches since HIV induces a wide array of immune changes and enzymatic changes result in the formation of numerous metabolic products.

The **objectives** of this work were therefore to **characterize the (a) metabolic and (b) immune profiles of biofluid which had been collected from HIV- and clinically stable treatment naive HIV+ patients**. To achieve this, MS-based metabonomics and flow cytometry were employed for the former and latter respectively. For the purposes of this project key metabolic and immune changes associated with HIV infection were of interest. Metabolic and immune processes were therefore measured in context to **natural HIV infection** (patients not given anything) and were primarily investigated *in vitro*.

a. Metabolic Profiles

HIV induces mitochondrial dysfunction and thus metabolic changes in its host. Such changes qualify for detection through metabonomics which is the study of how metabolites change in response to stimuli. Organic acids are markers of mitochondrial dysfunction. The

profile of these molecules has mainly been investigated in urine (Barshop, 2004; Duez et al., 1996; Tanaka et al., 1980). Since our analysis focused on HIV's effect on mitochondria, biofluids representative of or close to target cells commonly infected by the virus were used i.e. serum and PBMCs. By investigating the organic acid profile in these biofluids we attempt to answer the following questions:

- 1) Is extraction of these molecules possible from serum and cells infected with HIV?
- 2) Does the organic acid profile of HIV- and HIV+ individuals differ?
- 3) Do the measured profiles provide information on the use of organic acids as reliable indicators of HIV-induced mitochondrial dysfunction?

The likelihood of sera and PBMCs having similar metabolites is high because of the related source of these materials (i.e. blood). Differences may however be apparent in the concentration of the metabolites where those occurring at very low levels do not necessarily reflect as peaks on GC chromatograms. Differences in the metabolic profiles of these biofluids can also be expected if one considers that the biofluids may reflect different sensitivities toward the HIV stimulus. This raised an additional question for investigation;

- 4) How does the organic acid profile in the different biofluids compare?

Metabonomics-based approaches are relatively new thus there is no standard way of executing such an experiment. Different software programmes have been developed to facilitate data processing, data analysis and metabolite identification. These programmes run on different algorithms and address different needs of the respective scientist/projects. For this thesis the impact of three software programmes as well as multi- and univariate statistics on data processing and analysis was investigated. With this the goal was to comment on the following:

- 5) Does the data generated from the different software differ substantially?
- 6) Is one software better suited than another?

b. Immune Profiles

Immune changes associated with HIV infection that were considered in addition to the obvious clinical markers (such as CD4 count) included; redox status, apoptotic and cytokine profiles of the biofluids as well as CD4 and CD8 cell frequencies.

Hydroperoxides are species which signal oxidative damage to membranes and are representative of various biochemical pathways. We wanted to report on whether these molecules

- 1) Can be detected and show significant differences when profiled in HIV- and HIV+ serum?

In terms of the apoptotic profiles of HIV+ cells, there has been controversy as to the cells predominantly undergoing apoptosis. While some studies have shown apoptosis to occur in both CD4 and CD8 subsets (Cotton et al., 1997; Gougeon et al., 1996; Meyaard et al., 1992) there have been reports confirming the phenomenon to predominantly occur in either CD4 cells (Herbein et al., 1998) or CD8 cells (Lewis et al., 1994). The aim of the current work was not only to show differences in apoptosis between the experimental groups but also to determine:

- 2) Which subset of (immune system) cells was undergoing apoptosis?

Infection with HIV results in the loss of cell numbers and a loss in immune cell function. As a result, cells show reduced proliferation and a decrease in Th1 cytokine production (Clerici and Shearer 1993). Mitogens serve as general stimulants and in some assays as positive controls for proliferation and/or cytokine production (O'Neil-Andersen and Lawrence 2002; Pala et al., 2000) and can inform on cell functionality while antigens stimulate pathogen-specific responses which can be used to deduce disease progression (Jansen et al., 2006). In addition, treating infected cells with antigenic peptides may stimulate favourable, protective responses against HIV *in vitro* mainly because memory B cells will be activated to produce antibody against cell-free virus and memory T cells activated to bring about cellular responses against cell-associated virus. Here, cells were exposed to peptides based on β 2m and Gag. The goal was to answer the following:

- 3) Are the cells of clinically stable HIV-infected patients still functional when treated with mitogen and antigen *in vitro*? Loss of cellular function has been reported to occur even during the asymptomatic stages of infection (Sarih et al., 1996).
- 4) Are the HIV-specific immune responses (as detected by single and pooled peptides) more prominent than a non-specific response (memory versus no memory)? According to Betts et al (2001), cells respond better to peptide pools as antigens *in vitro* since more epitopes are displayed and more information on the overall immune response can be extracted.

- 5) Is the detectable *in vitro* response (cytokine production) anti- or proinflammatory i.e. IFN- γ or TNF- α and how does the result contribute to understanding HIV/AIDS pathogenesis? IFN- γ is representative of an anti-inflammatory, antiviral cytokine whilst TNF- α is a pro-inflammatory cytokine (Reeves and Todd 1996). The latter cytokine can however portray bi-functional activity i.e. it has the ability to increase and decrease the survival of HIV through different mechanisms.

Cytokines have routinely been quantified using the ELISA. When using this assay one cytokine is usually measured at a time and the data thereof analyzed using univariate statistics. Analyzing more than one cytokine at a time is becoming more routine as shown by recent investigations (Keating et al., 2011; Tang et al., 2011; Nixon and Landay 2010; Roberts et al., 2010; Tang et al., 2008). The data related to multiple cytokines are however also analyzed using univariate statistics. The measurement of a number of immune parameters at once, also called multiplexing has been largely facilitated through CBA technology and flow cytometry. The data matrices associated with such analysis is complex but provides more information and can better evaluate the effects of several cytokines within a system by using multivariate statistical analysis. The goal was to answer the following:

- 6) Which cytokine profile is observed; Th1, Th2 or Th17 and what does this mean in terms of disease pathogenesis?
- 7) How does uni- and multivariate analysis of the cytokine data compare? Are new conclusions reached with the latter?
- 8) Does the Th1/Th2/Th17 cytokines allow for discrimination between HIV- and HIV+ groups?

An understanding of the sole metabolic effects of HIV during the asymptomatic stage of infection is limited. Although there is literature available of the metabolic and immunological effects of HIV, the effects of HIV on metabolism measured concurrent to immune changes is scarce. When metabolic and immunological changes are reported, the results for the two systems are usually done in isolation. By concurrently assessing the metabolic and immunological status of HIV-infected biofluid in one study (data presented in subsequent chapters), the **hypothesis** that HIV disrupts the function of the metabolic and immune systems, primarily through its effect on mitochondria, is tested. Since these systems are linked to each other through these organelles, mitochondrial dysfunction should then be visible by modifications in the processes of both systems and be detectable through MS-based metabolomics and flow cytometry respectively. In addition to facilitating hypothesis-testing as was subsequently done for this project, metabolomics approaches are also

important for generating new hypotheses from the experimental data of such investigations (Kell, 2004). The approach used here is different from previous investigations in that a range of metabolic and immune parameters were assessed allowing for the extraction of more biological information in comparison to detecting and statistically analyzing only one molecule.

Based on the detected differences between the HIV- and HIV+ groups, the **third objective** of this study was to **identify** those molecules affected by HIV (metabolic and immunological) which could possibly translate into HIV-specific biomarkers with which to diagnose, prognose and monitor disease. Biomarkers provide biological information about the site of infection and/or mechanism of disease and can contribute to unravelling the history and pathogenesis of AIDS. These markers could also serve as endpoints for clinical studies and assist in identifying those patients at risk of disease (Touloumi and Hatzakis 2000).

Although MS metabonomics has been applied to the SIV model (Wikoff et al., 2008), the saliva of HIV+ individuals (Ghannoum et al., 2011) as well as CD4 and macrophage cells which had been subjected to *in vitro* HIV infection (Hollenbaugh et al., 2011), the approach has not been applied to blood-based biofluids of chronically infected, treatment naive individuals. Animal and invertebrate models have been successfully utilized in metabonomics-based studies and a summary of this is provided by Kamleh et al (2009). Animal and cell culture-based models are easier to work with since there is greater control of the variables under such conditions (Kamleh et al., 2009). Although the monkey model allows for the execution of controlled experiments, the animals usually die too early, making biomarker discovery problematic (Pendyala et al., 2007). Investigating HIV-induced changes in biofluid obtained non-invasively from humans may be a means to address this problem and these materials should be more representative of the *in vivo* situation.

The **fourth objective** was to establish a biochemical link between the metabolic (organic acids) and immune parameters measured (i.e. how does the detected metabolites relate to changes in ROS, apoptosis and cytokines). Sections 2.7 and 2.8 provide a brief summary of some of the immune and metabolic changes associated with HIV infection. The affected molecules once identified will be interpreted and linked based on the available literature.

Compared to other methods, MS-based metabonomics is relatively new and unexplored in HIV research so as a **fifth and final objective, the usefulness of MS and to a lesser extent flow cytometry as alternative methodologies with which to measure HIV-**

induced changes will be commented on. The techniques currently in use for confirming HIV infection and HIV-induced changes are briefly reviewed below followed by an overview of the “newer” technologies for addressing the research questions and objectives of this thesis.

2.10 Current tools for measuring HIV infection, HIV-induced changes and disease progression

Diagnostic tests for HIV were developed in the 1980s following confirmation that HIV was the causative agent of AIDS. Presently, infection is diagnosed by measuring antibody production, HIV antigen levels and viral nucleic acids (Fearon, 2005; Luft et al., 2004). The most basic diagnosis which detects viral antibody in host biofluid employs enzyme immunoassays (EIAs). These assays are based on the principle that an immobilised antigen binds to HIV antibodies in a patients’ blood/biofluid. Upon complexing to an enzyme-labelled anti-human immunoglobulin G (IgG), antibody is detected via a colorimetric reaction where colour development equates to antibody concentration. These assays generally lack specificity and the results have to be confirmed using either Western blot techniques or polymerase chain reaction (PCR) methods.

During western blot analysis, antibody in a patient sample is allowed to react with HIV peptides that had been separated via electrophoresis and blotted onto a membrane whilst PCR methodologies amplify viral nucleic acid levels. Although Western blots and PCR reactions are sensitive, these assays are labour intensive, costly and non-specific. Other approaches include direct determination of virus by conducting cell culture assays (e.g. plaque assays) and determining p24 antigen levels. The p24 antigen is however not useful for diagnostic purposes as it is not consistently detected in all HIV seropositive patients (Fearon, 2005).

Drawbacks associated with current diagnostics (Zhang and Versalovic 2002) have led to the search for alternative options with which to rapidly and more sensitively detect HIV-induced changes and monitor infection. In many instances HIV infection goes unnoticed because of the similarity in symptoms to that of the “normal flu” caused by the influenza virus. The different diagnostic tests available are not effective for detecting the different strains of HIV and may therefore not be applicable as a diagnostic test in some countries where an alternative subtype may prevail. In addition to diagnostic hindrances, the CD4 T cell count used to monitor disease progression in HIV-infected individuals is unreliable as it shows significant laboratory and physiological variation (Touloumi and Hatzakis 2000). The value of this parameter is influenced by factors such as time of sample collection, co-infection with other pathogens, exercise etc (Gupta and Gupta 2004; Touloumi and Hatzakis 2000; CDC, 1993). High costs associated with determining CD4 counts hinder the use of this

parameter. There are however other soluble immune-based markers such as β 2m, serum neopterin, HIV p24 antigen, etc that are associated with HIV infection. It is only that these markers are less preferred because of their limited sensitivity, specificity and predictive power (Gupta and Gupta 2004). Of the current available techniques and/or markers known, the majority are associated with immune or virological changes. In most of the literature on HIV biomarkers (often proteins) only one analyte is detected or analyzed at one time through conventional biochemical or clinical techniques (Hattingh et al., 2009; Lindon and Nicholson 2008; Del Llano et al., 1993). These techniques are not always sensitive, may be subject to interference, costly to execute and laborious to perform. Screening more than one molecule at a time using more sensitive analytical instrumentation may be more advantageous for clarifying HIV-induced events and associated infection mechanisms.

Assessing changes representative of immunological and metabolic function can be troublesome since some molecules are short-lived (e.g. ROS produced during HIV infection, Baruchel and Wainberg 1992). To measure the production of ROS complicated and time-consuming methodologies such as electron spin resonance and spin trapping are usually employed. These techniques present with technical difficulties, low sensitivity and produce a lot of noise (Freinbichler et al., 2008). The accuracy, reliability and rapid analysis of individual cells offered by flow cytometry has now become the favoured alternative for indirectly measuring oxidative stress parameters. With this technique probes such as 2'-7'-dichlorodihydrofluorescein diacetate upon interacting with radicals are converted into a fluorescent molecule where the fluorescence is directly proportional to the levels of ROS (Eruslanov and Kusmartsev 2009; Sarkar et al., 2006). Another compound capable of converting to a fluorescent or coloured product for cytometric or spectrophotometric determination is N, N-diethyl-para-phenyldiamine (DEPPD) sulphate.

In the study done by Hattingh et al (2009) methodologies used for measuring metabolic changes in a group of HIV-infected women in South Africa were primarily based on enzymatic colorimetric principles. As additional indicators of HIV's metabolic effect; changes in body composition are recorded by using dual energy X-ray absorptiometry (DEXA), computer tomography (CT) scanning and MRI. These techniques are expensive and cumbersome to execute (Gkrania-Klotsas and Klotsas 2007). DEXA costs are in the range of \$125 whilst CT and MRI scanning costs range between \$500-1000 per analysis (Safrin and Grunfeld 1999). These techniques usually require an expert to assist with the interpretation of the data and offer limited advantages over conventional tests performed in the clinic (Wohl et al., 2006).

Technological advances allow researchers and scientists to better respond to health challenges such as HIV/AIDS. Recent advances have led to the development of analytical tools which detect and record a large number of parameters off samples. Consequently, metabolic and immune changes are now being measured using rapid, more sensitive, digital, multiplex, user-friendly technologies. As a result, an increased amount of data is obtained requiring sophisticated software and statistical approaches for analysis and interpretation. Due to the countless effects of HIV on the metabolic system, the different types of metabolites and their differing physiochemical properties, particular advances in the field of metabolism has seen the introduction of metabonomics which utilizes techniques such as NMR spectroscopy and MS. Similarly, because of the ample immune changes which occur in response to HIV infection, the field of immunology has seen the introduction of multi-parametric flow cytometers. Other techniques are available with which to measure and characterize HIV-induced metabolic changes but for this study MS-based metabonomics was primarily used and complimented with other types of spectroscopy and flow cytometry for immune-based analysis. The application of MS to the study of infectious disease metabolites is relatively new and especially unexplored in HIV research.

2.10.1 Metabonomics

Metabolism describes all biochemical processes which occur in and that sustain living systems. It is a dynamic process and is influenced by both endogenous and exogenous factors. To measure holistic metabolic changes in plants, the terms metabolomics and metabonomics were introduced by Oliver Fiehn in the beginning of the 2000s (Fiehn, 2002). In the literature metabolomics and metabonomics are used interchangeably (Xu et al., 2009) with the former most often used for plant, *in vitro* and microbial studies whilst the latter is used for animal and human-based studies (Mamas et al., 2011; Lindon and Nicholson 2008; Nicholson et al., 2007). There has however been clarity regarding the use of these terms i.e. metabolomics is aimed at the comprehensive analysis of the metabolome under a set of conditions whereas metabonomics is aimed at measuring the fingerprint of biochemical perturbation as is caused by disease, drugs and toxins (Goodacre et al., 2004; Lindon et al., 2003; Nicholson et al., 1999). Metabonomics therefore measures the metabolic responses of living systems to biological stimuli (Kamleh et al., 2009) such as HIV. Chemical responses are therefore linked to biological processes (Nicholson and Lindon 2008). The term metabonomics applies to this work and will be exclusively used from here on. Publications using the term metabolomics were referenced as such. During metabonomics investigations metabolic data is collected through spectroscopic techniques, analyzed using statistical approaches and interpreted in the context of biological pathways (Wishart, 2005; Griffin and Shockcor 2004). Metabolic changes are important to measure since these changes usually

reflect protein and genetic changes thus representing the organism's phenotype (observable characteristic, [Serkova and Niemann 2006](#)).

2.10.1.1 A Brief History on Metabonomics and its Applications

The concept and history of metabolism dates back to as early as the 1500s where diagnostic charts were constructed to relate urine colour, taste and smell to medical conditions (mentioned in [Cassiday, 2009](#); [Nicholson and Lindon 2008](#)). During these earlier times ants were used as “tools” (similar to our modern use of NMR and MS) to indicate metabolic abnormality. If the urinary glucose content of patients was high the ants were attracted to the biofluid and indicative of diabetes. In 1614, Santorio Sanctorius earned the title of father of metabolic studies following his work on excretory biofluid determinations. In 1971 the first MS-based metabolic experiment was done with the first metabolomics paper published by [Pauling et al](#) in that year. The terms: metabolome and metabonomics were officially used for the first time in 1998 with metabonomics finding a common niche in journal titles as of the year 2000. Still, metabonomics-based studies only really gained popularity as of 2004. Although the methodology has been in existence for long (mainly used by chemists and clinical chemists) it has only recently gained interest in the life sciences and infectious diseases fields following the introduction of chemometrics which assists in simplifying the complex data sets yielded. Chemometrics is the application of statistical analysis to (bio) chemical data. Because the application of metabonomics is so new proposals/plans to standardize this type of research have been put into place and are still ongoing ([Fiehn et al., 2007](#); [Goodacre et al., 2007](#); [Griffin et al., 2007](#); [van der Werf et al., 2007](#)). Standardization will involve setting up standard protocols/guidelines for the design and execution of metabonomics investigations as well as the analysis and organization of metabonomics data. Coupled to chemometrics, technological advancements have enabled the development of better suited analytical techniques and software programmes for such complex analysis. In the past, metabonomic studies were mainly used for diagnosing inborn errors of metabolism and for evaluating drug toxicity. Its applications have since increased and include but are not limited to: environmental studies, plant metabolomics, organism-plant relationships, forensic analysis, mechanisms of bacterial and viral infection, disease diagnosis, gene function, intervention monitoring, nutrition research, pharmacometabolomics, micrometabolomics, clinical metabolomics, structural studies etc ([Koal and Deigner 2010](#); [Xia et al., 2009](#); [Lindon and Nicholson 2008](#); [Taylor et al., 1996](#)).

2.10.1.2 MS Metabonomics Workflow

A typical MS metabonomics workflow comprises sample collection, stopping the sample's metabolic activity and extracting the molecules of interest. Halting the metabolic

activity of the sample ensures inactivation of the metabolome and maintains sample integrity. It also reduces variation in the physiochemical properties and concentrations of the metabolites (Álvarez-Sánchez et al., 2010). During the extraction process the sample may also be spiked with an internal standard which has similar properties as the molecules to be extracted. This is a compound of known concentration and can be used to quantify extracted metabolites. Internal standards detect technical variation within datasets and are used for the normalization of MS data and removal of systematic bias (Engelen et al., 2010). Bias occurs when there is “masking” of the true biological effect of a stimulus, e.g. HIV, by confounding factors such as age, gender etc (Issaq et al., 2009). Finally, internal standards also serve as a retention index reference for the identification of unknown molecules. The extraction of metabolites from cells/tissue ensures permeabilization of membranes and the release of metabolites from the sample. It also removes interferences and makes metabolites compatible with the analytical technique to be used. The extracted sample is then concentrated through drying, derivatized (if needed), subjected to chromatographic separation and introduced into the mass spectrometer for ionization. Ionized molecules are separated based on their mass to charge ratio followed by analyte detection. The intensity of the analyte’s ion signal is recorded and eventually processed into a spectrum (Álvarez-Sánchez et al., 2010). The peaks representing metabolites, are then deconvoluted (i.e. overlapping peaks are separated into individual spectra/components), aligned and the data standardized. The associated data is analyzed through statistics followed by biological interpretations. This simplified workflow of MS-based metabonomics experiments is shown in Figure 2.14.

2.10.1.3 Sample Choices for Metabonomics-based Analysis

The choice of sample to use for metabonomics investigations is largely dependent on the biological question being addressed and the instrumentation that is available. Samples are also chosen according to their ease of access. Those that can be obtained non-invasively are usually the first choice and are most often chosen in such a way so as to represent the *in vivo* state of the individual. Various biofluid types have been used for metabonomic analysis and include but are not limited to: liver extracts, blood plasma, urine, whole blood, serum, tissue, cell pellets including brain and spinal cord extracts (Dettmer et al., 2007, Saghatelian et al., 2004), bronchoalveolar lavage fluid, dialysis fluid, CSF, seminal fluid, cyst fluid, amniotic fluid, cell media, synovial fluid, digestive fluids, blister fluids, lung aspirates etc (Lenz and Wilson 2007; Nicholson et al., 2007). The analysis of volatile compounds has also been done on breast milk, saliva, feces, hair and breath (Saric et al., 2008, Dettmer et al., 2007; Inagaki et al., 2007; Mills and Walker 2000). It is clear that a range of samples can be

analyzed and that this range will continue to grow as analytical methods develop. Reasons for the biofluids used in this study are provided in Section 3.4.

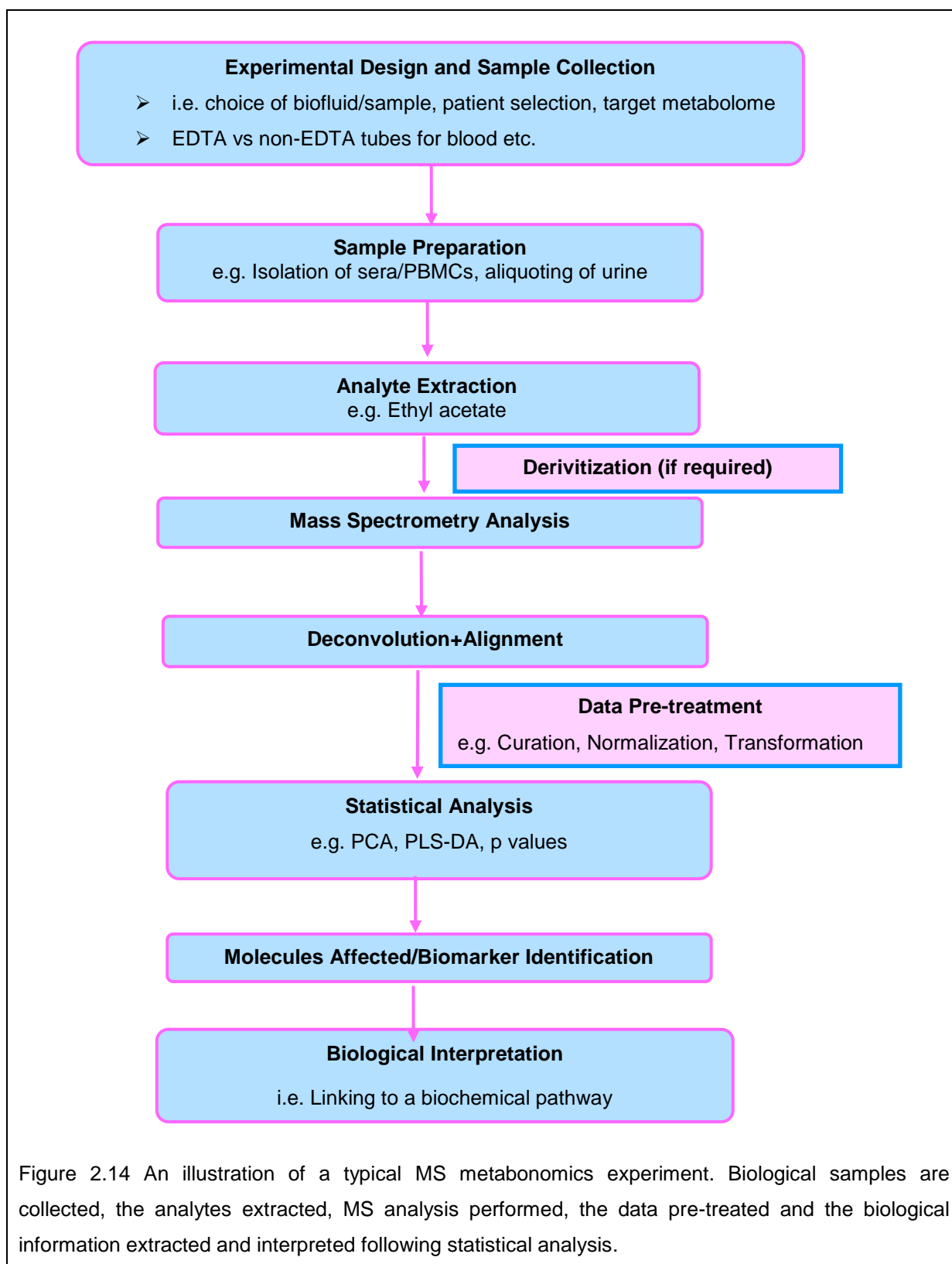


Figure 2.14 An illustration of a typical MS metabolomics experiment. Biological samples are collected, the analytes extracted, MS analysis performed, the data pre-treated and the biological information extracted and interpreted following statistical analysis.

By analyzing the metabolic state of various biofluid types, dominant metabolites or biomarkers are extracted which provides biological information about the site of infection and/or mechanism of the particular medical condition being studied. Because of the inherent biological variation amongst individuals, i.e. different lifestyles, environments etc, the number of metabolites extracted cannot be predicted nor will it remain constant within an individual over time. Metabonomics accounts for these differences by giving a global picture of all factors influencing the metabolome ([Nicholson and Lindon 2008](#)).

2.10.1.4 Commonly used Metabonomics-based techniques

Various platforms have been developed with which to analyze metabolites but the diversity and different concentrations of these small molecules hinders the ability to detect every single metabolite within a sample. In a feature article by [Blow \(2008\)](#) it was reported that with the present tools available only 10-15 % of the metabolome is ever analyzed at a time. Incorporating metabonomic (in conjunction with genomic, proteomic and transcriptomic) techniques and a combination of biofluid types to study a biological problem should provide complementary information to provide a clearer picture of metabolic changes experienced ([Xia et al., 2009](#)).

Commonly employed platforms for metabonomic analysis include MS and NMR spectroscopy. MS is an analytical tool for the identification of molecules based on mass. It has four main processes associated with it based on its components: sample introduction, ionization, filtering of ions and their subsequent detection ([Lawson and FitzGerald 2002](#)). During MS experiments the sample is caused to disintegrate producing fragment ions. These ions are primarily produced in the gas phase either through the addition or subtraction of electrons and/or protons, accelerated to a specific velocity, projected into an analyzer and detected. The process happens under a vacuum which functions to minimize collisions between the ions and air molecules and also to carry away neutral species.

Both MS and NMR spectroscopy require low sample volumes and thus have lower run times. The advantage however of choosing MS over NMR is that the technique can be coupled to separatory techniques such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) prior to MS analysis, making it more sensitive. MS is more selective and offers a wide dynamic range for analyte detection ([Dettmer et al., 2007](#)). Because of the ability to ionize in positive or negative mode, added information about the properties of the molecules is obtained when using MS. Although MS is more selective than NMR, it is also prone to matrix effects (increase or decreases in analyte ion intensities) and can be insensitive to some analyte classes ([Goodacre et al., 2004](#)). MS requires the sample to be modified to make it compatible with the instrument

whereas NMR does not. During MS the sample is destroyed and cannot be used for further analysis. This is not the case with NMR analysis. Although the principles of MS and NMR differ the data obtained from the two techniques are usually complementary (Koal and Deigner 2010). The choice of technique is therefore dependent on sample type and the research question being addressed. Although instrumentation for metabonomics analysis is expensive (Dunn et al., 2011), the costs associated with running a sample is rather feasible and allows for high-throughput analysis.

Mass spectrometers detect only charged ions and the data yielded is dependent on the mass and charge of the ion. The ions produced are represented as peaks in chromatograms and are used for determining the molecular mass of samples. When MS is used, metabolites are primarily indexed through their mass to charge (m/z) ratio, intensity of ion and retention time (Dettmer et al., 2007). Of particular interest to our analysis was the utilization of GC-MS for the analysis of organic acids. Organic acids are established biomarkers of mitochondrial dysfunction and inborn errors of metabolism. Although studied for these purposes, the profile of these molecules in HIV-infected biomaterial despite the virus' effect on mitochondria has not been previously determined.

2.10.1.5 Gas Chromatography-Mass Spectrometry

GC-MS is an analytical tool comprising of two interfaces: gas chromatography, which separates semi-volatile to volatile organic compounds and MS which identifies these separated compounds based on mass and charge ratios. During an analysis, sample is injected into the gas chromatograph where it is transported by a "carrier" gas to the separating column. The analytes within the mixture then interacts with the stationary phase exiting from the column at different times. The higher the temperature, the less the sample interacts with the stationary phase and the faster the molecule elutes (Guiochon and Guillemin 1990). Upon exiting the GC column these analytes enter the ionization chamber of the mass spectrometer where they are bombarded with electrons to form ionized fragments. These fragments which are characteristic of a particular molecule (Pasikanti et al., 2008) are accelerated and separated on the basis of their m/z ratio. Fragmented molecules enter the mass detector and the information is recorded. As output, the MS computer records spectra which show the abundance of each ionized mass fragment. This principle of GC-MS analysis (and LC-MS) is depicted in Figure 2.15.

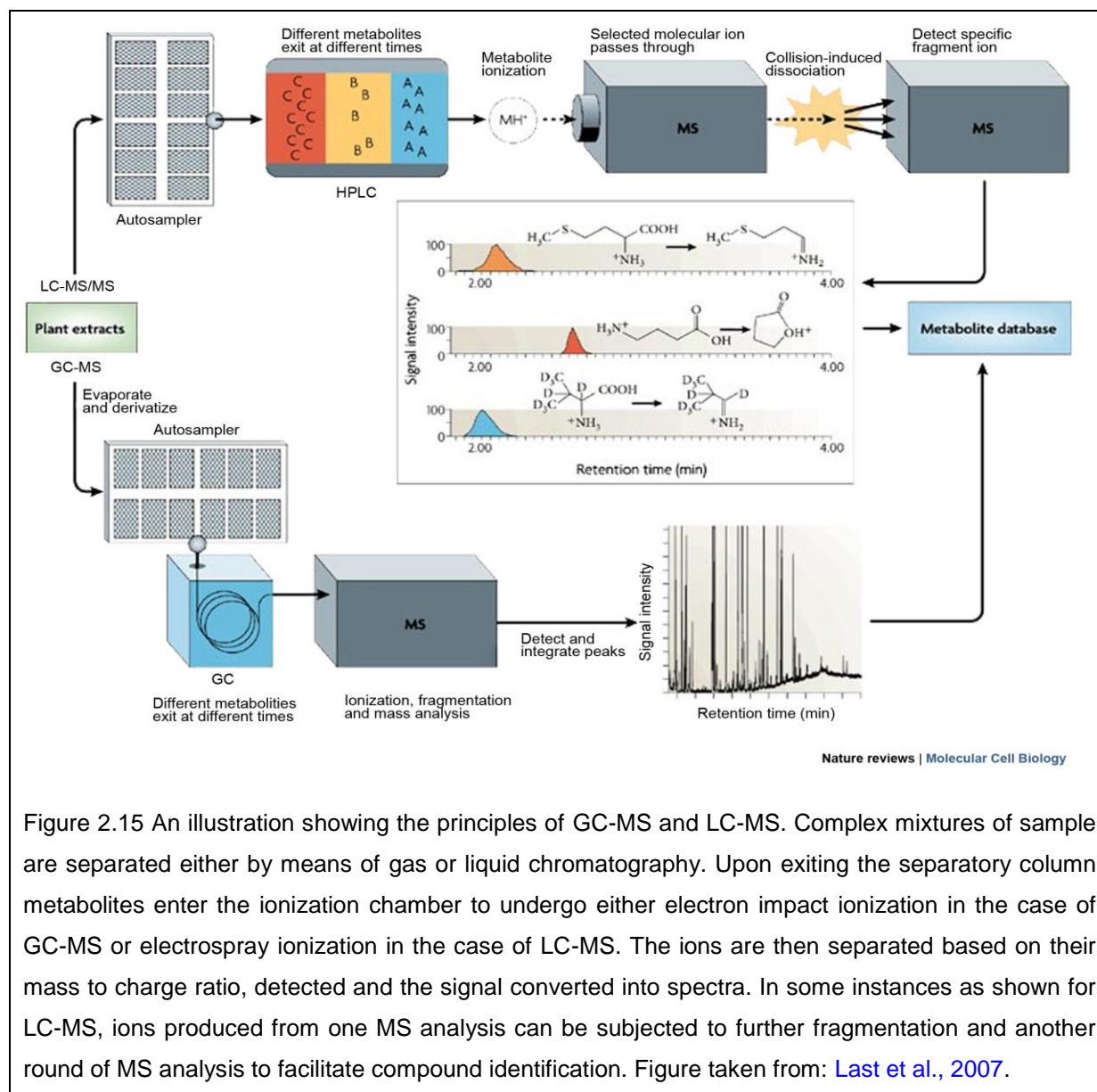


Figure 2.15 An illustration showing the principles of GC-MS and LC-MS. Complex mixtures of sample are separated either by means of gas or liquid chromatography. Upon exiting the separatory column metabolites enter the ionization chamber to undergo either electron impact ionization in the case of GC-MS or electrospray ionization in the case of LC-MS. The ions are then separated based on their mass to charge ratio, detected and the signal converted into spectra. In some instances as shown for LC-MS, ions produced from one MS analysis can be subjected to further fragmentation and another round of MS analysis to facilitate compound identification. Figure taken from: [Last et al., 2007](#).

2.10.1.6 Advantages of GC-MS

GC-MS was the technique of choice here for several reasons: it is regarded by some as the gold standard for metabolite analysis ([Jellum et al., 1989](#)); it is superior over other methods in the quality of data it produces ([Meletis, 2006](#)), has increased sensitivity, peak resolution and is reproducible ([Pasinkati et al., 2008](#)). There is also an increased amount of databases and protocols that exists for use with this technique. Finally, previous use of this technique to profile and detect pathological conditions and to characterize infectious diseases ([Jellum et al., 1989](#)) made it a favourable system to choose.

2.10.1.7 Disadvantages of GC-MS

The disadvantage of GC-MS analysis is that it requires the samples to be thermally stable and volatile. Most metabolites in nature are polar and non-volatile ([Pasikanti et al., 2008](#)). To increase the polarity, stability and volatility of metabolites, derivatization at the polar functional group is employed. Functional groups containing active hydrogen atoms are most often trimethylsilylated ([Álvarez-Sánchez et al., 2010](#)). Silyl derivatives are more stable and volatile. Various derivatization reagents are available and are summarized by [Dettmer et al \(2007\)](#). The disadvantage of derivatized products is that they are sensitive to moisture and conversion reactions can take place producing artefacts that may hinder data interpretation.

2.10.1.8 General Limitations of Metabonomics Research

Limitations of metabonomics research includes the lack of universally accepted standard protocols for executing a metabonomics-based experiment, capturing of data and reporting of results even though the Metabolomics Society does have a working-group on standardization of the generation and reporting of metabolic data. In terms of metabolite detection, these molecules are present at different concentrations or dynamic ranges ([Lu et al., 2008](#)) which further complicate data analysis. The execution of experiments by different groups is done using different instruments from different suppliers. Authors report their metabolic findings in different ways, within institutions and laboratories and across multiple publications. Limited explanations of the statistical analysis are supplied ([Goodacre et al., 2007](#)). This lack of standardization has been recognized and is being attended to by societies such as The Metabolomics Standards Initiative (MSI). Coupled to the lack of standardization, derivatized GC-MS samples often present with multiple peaks for a particular metabolite and complicates data interpretation. The origin of multiple peaks is attributed to decomposition reactions which occur during the derivatization process and the heating of some of the instrument's parts ([Xu et al., 2009](#)). Deconvolution is the term given when large numbers of overlapping peaks with similar retention times are separated into individual chemical peaks ([Chen et al., 2009](#); [Goodacre et al., 2007](#); [Katajamaa and Orešič 2007](#)). The conversion of chromatographic-MS datasets into data usable for statistics and the identification of metabolites is complex ([Sansone et al., 2007](#); [Broeckling et al., 2006](#)). Another factor which has hindered the growth of metabonomics has been the lack of electronic databases for compound identification ([Psychogios et al., 2011](#)). For most of the metabolites that have been detected and identified, the information is dispersed making retrieval of biological information difficult ([Psychogios et al., 2011](#)). Most available libraries are still incomplete ([Styczynski et al., 2007](#)) as are the databases which house the associated biological information ([Dunn et al., 2011](#)). Many of the available libraries are also

based on synthetic chemicals and not innate, metabolically relevant molecules (Psychogios et al., 2011). There are many enzymes without assigned biological functions just as there are many unidentified products (Fiehn et al., 2011).

2.10.1.9 Software for the generation of data matrices and for data analysis

Chromatographic separation of analytes usually produces chromatograms as output while MS analyses produce spectra. To detect changes in metabolites and enable a comparison of two or more samples, alignment of the data is often required. Various software programmes functioning on different algorithms have been developed for the deconvolution and alignment of chromatographic data. The development of spectrometers each having their own data file formats have further contributed to slowing the development of universal metabolomics software (Dettmer et al., 2007). As a result, the automated mass spectral deconvolution and identification system (AMDIS) software is the most commonly used for deconvolution purposes whilst the choice of alignment and statistical software varies and is chosen based on the needs of the researcher. MetAlign for example filters out noise that is produced during spectrometry, does a baseline correction and chromatographic alignment of TICs whereas MatLab allows for in-house software development and offers multivariate statistical functions (Dettmer et al., 2007). XCMS which allows for peak picking, non-linear retention alignment as well as relative quantification is used particularly during non-targeted metabolomic analysis (Dettmer et al., 2007; Smith et al., 2006). XCMS², an upgrade to XCMS, has the added advantage of searching MS/MS spectra against the METLIN database to help with the identification of metabolites (Benton et al., 2008). MetaboliteDetector allows for the automated analysis of targeted and non-targeted chromatographic data (Hiller et al., 2009) and is to an extent similar to MET-IDEA which semi-quantifies chromatographic data following the extraction of ion abundances that are associated with metabolite peaks (Broeckling et al., 2006). In comparison to MET-IDEA where the full spectrum is utilized, MetaboliteDetector performs single ion chromatogram peak detections. An online programme called SpectConnect (<http://spectconnect.mit.edu/>) registers GC-MS spectra that are unidentified but conserved amongst samples. By extracting conserved spectra the investigator is assured that “real” compounds and not noise are detected (Blow, 2008). Other software programmes include MZmine, MetaboAnalyst, MetaboMiner and so on. After processing of the chromatographic data with any of these programmes the data is exported into a user-friendly format for statistical analysis, most often with the variables arranged in columns and the samples arranged in rows.

2.10.1.10 Multivariate statistical options for data analysis

A primary aim of metabonomics is to classify samples and to identify those variables or features responsible for the classification. The number of variables generated is generally larger than the number of cases analyzed. Visual inspection of the data is therefore impossible and so statistics has to be employed to extract biological information (Goodacre et al., 2007). Following MS analysis, data pre-treatment (removal of duplicate peaks, variable selection etc) and standardization of the data is done. Normalization and transformation particularly help to reduce variability in the data by making the scales of the cases/samples and metabolites comparable. Metabolites occur at different concentrations. To account for this wide dynamic range transformation is employed. Transformation (through the log function) reduces high-intensity values and also keeps low-intensity values and in essence ensures that abundant molecules do not dominate when subsequent statistical analyses are performed. Following standardization of the data, multivariate statistical analysis facilitates with information recovery (Lindon and Nicholson 2008). An array of statistical methods is available and is selected according to the aim of the particular study. Commonly used statistical analysis includes the use of classification models such as principal component analysis (PCA), hierarchical clustering analysis (HCA) and independent component analysis (ICA). These are all unsupervised methods as no prior information about the molecules' class is made known. This is in contrast to supervised methods where prior information about the molecules' class is made known. Supervised methods are thus used for biomarker discovery and for building models from which the class of a new set of samples can be predicted from an initial modelled data set. Partial least squares discriminant analysis (PLS-DA) or soft-independent methods of class analogy (SIMCA) are representative of supervised methods. Some of the above-mentioned statistical methods were used in this project and are elaborated on in Chapter 3 (Section 3.7).

2.10.1.11 Identification and Biological Interpretation of Important Molecules

Following the identification of molecules which differ significantly between the groups, metabolite names are assigned. For this purpose online library sources such as the Wiley, National Institute of Standards and Technology (NIST) 05 and 08 libraries, the Agilent Fiehn GC-MS Metabolomics Library or Human Metabolite Library (HML) etc are used. Mathematical models and in-house libraries can also be developed to help identify metabolites. Following identification, there is consultation of metabolome databases to determine the biochemical pathway and enzymes affected/involved and to assist in the biological interpretation of the information. These databases include for example: The

Human Metabolome Database (HMDB) and the most recently introduced; Serum Metabolome Database (SMD). Other databases commonly consulted includes: KEGG, METLIN, The Golm Metabolome Database, PubChem and The Madisa Metabolomics Consortium Database to name but a few ([Psychogios et al., 2011](#); [Wishart et al., 2009](#); [Xia et al., 2009](#); [Wishart et al., 2007](#); [Smith et al., 2005](#)). These databases provide structural information and physio-chemico properties about the metabolites; reflect clinical data relating to the metabolites, shows biological or biomedical data such as metabolite-disease associations, biofluid concentrations etc. Reviewing all the available software programmes, databases, statistical tools and libraries available is beyond the scope of this thesis but a few have been mentioned nevertheless to show how these utilities have been developed and are being upgraded based on the scientist's needs and to address current metabonomics problems.

2.10.2 Spectroscopy

To measure some of the well-known immunological changes reviewed in this chapter, spectroscopy and flow cytometry represents standard techniques that were employed. To determine the redox status of a system the amount of ROS has to be quantified. ROS production is quick and short-lived making the detection of these molecules difficult. Oxidative status is therefore mainly measured using indirect assays. Currently, no ideal method for measuring oxidative stress exists. Although various markers of oxidative stress can be measured using more sensitive techniques such as flow cytometry, an assay based on spectroscopic principles was chosen since the detected hydroperoxide molecules would be representative of changes in all biochemical pathways.

Spectroscopy measures the interaction of electromagnetic radiation with matter. This technique was mainly used by synthetic chemists to characterize and analyze newly synthesized compounds. During the 1600s various scientists were involved in the development of this technique but it was only in the 1930s that the first colorimeter or spectrophotometer was developed ([Thomas, 1991](#)). Spectroscopy is based on the principle that an atom is at its lowest energy level when at the ground state. When energy is absorbed the atom is excited to a higher energy level and upon its return to the ground state emits electromagnetic radiation ([Wilson and Walker 2000](#)). The light emitted is characteristic of the element under study and can be easily detected. This process therefore comprises of atom formation, excitation and emission. Spectroscopy differs from spectrometry in that the particles are quantified by light instead of mass and charge properties.

For the determination of oxidative stress in this project; the conversion of DEPPD sulphate into a coloured radical cation was measured following the decomposition of serum

hydroperoxides (products of oxidation) into alkoxy and peroxy radicals (Hayashi et al., 2007; Verde et al., 2002).

2.10.3 Flow Cytometry

Flow cytometry is one of the standard techniques used for studying the immune system. It measures the multi-parametric physical characteristics of single cells, allows for cells to be examined, counted and sorted into distinct populations. This technique was chosen for the measurement of immunological parameters affected by HIV mainly because of its sensitivity over other available methods.

The Fluorescence Activated Cell Sorter (FACS) was invented in the 1960s by Bill Bonner, Richard Sweet, Russ Hulet, Lee Herzenberg and many others. The first commercial machines were introduced in the early 1970s by Becton Dickinson Immunocytometry Systems. Since the development of the first machines advancements have been made in developing the hardware, software as well as dye reagents (Tung et al., 2007). The arrival of the FACS signalled an era where the analysis and sorting of live cells could be done. However, the use of the FACS and the concept of flow cytometry only really developed following the production of hybridomas which allowed for the production of monoclonal antibodies which could be coupled to fluorochromes (fluorescent dyes) and used as labelling reagents (Herzenberg et al., 2002). Information regarding cell size, granularity, complexity and fluorescence intensity is therefore obtained. Based on the technological advances which have been made, the numbers of parameters that can be measured have increased as well as the speed of sorting. This is evident in articles which have measured 17 and more parameters in order to unravel processes linked to immune system function (Perfetto et al., 2004). Articles reporting on measuring an array of cytokines to probe immune dysfunction and immune-based diseases have also gained popularity (Salem et al., 2009; Wong et al., 2008). As part of a typical analysis, cells are usually labelled with antibodies which are conjugated to fluorochromes. When multiple fluorochromes are used an overlap in the emission spectra of the fluorochromes exists and compensation therefore has to be done (Tung et al., 2007). Compensation is the mathematical elimination of the spectral overlap between different fluorochromes (Baumgarth and Roederer 2000). Characterization of cells is also done in the absence of stains using only the light scattering properties of the cells. Based on these physical properties different cell types are sorted from their complex mixtures into purified populations (Ibrahim and van den Engh 2003).

During cytometric analysis a suspension of cells is passed through a stream and intercepted by a laser source. The fluorescent tag attached to the particle of interest (usually a cell) is excited as it gains energy and upon returning to a lower energy level emits photons

at a specific wavelength. The fluorescence intensity or number of “events”, terms most often used for describing flow cytometry data, is usually representative of the number of fluorochrome that binds to the cell. The fluorescent signal is collected by photodetectors, processed by the electronics, digitized and stored on the computer. When sorting is required a transducer/nozzle (found after the flow cell) vibrates causing the stream to break up into little droplets. An electric charge (i.e. positive, negative or neutral) is applied to each of the droplets using a voltage pulse. These droplets are then deflected from the main stream by an electric field and collected based on differences in charge (Ibrahim and van den Engh 2003). The working of a flow cytometer and the principle of cell sorting is shown in Figure 2.16 below.

Having supplied background information on the influence of HIV on the immune and metabolic systems, what these systems entail and ways of measuring the induced changes, some information relating to the experimental design and some practical aspects which guided this work is provided next (Chapter 3).

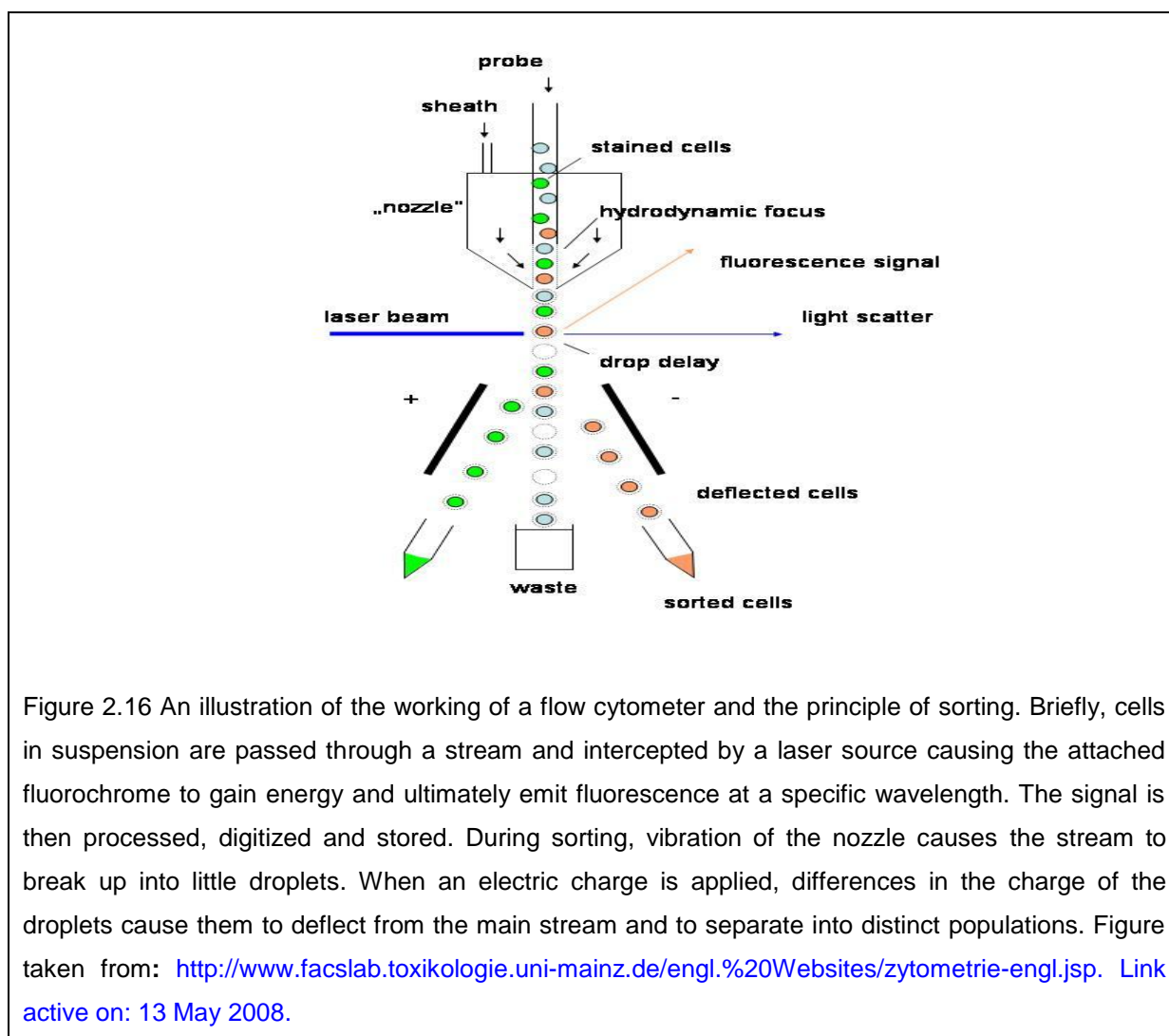


Figure 2.16 An illustration of the working of a flow cytometer and the principle of sorting. Briefly, cells in suspension are passed through a stream and intercepted by a laser source causing the attached fluorochrome to gain energy and ultimately emit fluorescence at a specific wavelength. The signal is then processed, digitized and stored. During sorting, vibration of the nozzle causes the stream to break up into little droplets. When an electric charge is applied, differences in the charge of the droplets cause them to deflect from the main stream and to separate into distinct populations. Figure taken from: <http://www.facslab.toxikologie.uni-mainz.de/engl.%20Websites/zytometrie-engl.jsp>. Link active on: 13 May 2008.

CHAPTER 3

EXPERIMENTAL DESIGN & PRACTICAL CONSIDERATIONS

3. Design and Practical Considerations

3.1 Ethics Approval

Ethics approval for the use of human samples in this research was obtained from the Faculties of (1) Natural and Agricultural Sciences and (2) Health Sciences of the University of Pretoria with protocol numbers E080-506-019 and 163/2008, respectively. Blood and urine were collected from HIV- and HIV+ individuals following written informed consent.

3.2 Selection of Biochemical/Metabolic Pathway for MS analysis

Firstly, consideration was given to the section of the metabolome to be investigated. A key decision concerned metabolic changes which occur at the mitochondrial level but that could be detected in the sera, PBMCs and urine following HIV infection. Organic acids fit these criteria and were analyzed using a semi-targeted metabolomics approach. These molecules are often used as indicators of a variety of mitochondrial disorders ([Hoffmann and Feyh 2005](#)). Because mitochondrial dysfunction is commonly detected by measuring apoptosis of immune cells, this assay was also included as part of this study.

3.3 Selection of Immune Parameters

Obvious aspects of HIV infection (such as CD4 count) were considered in sample selection. Because the metabolic parameters under investigation were related to HIV's effect on mitochondria, immune effects indicative of mitochondrial dysfunction were selected for investigation; primarily the virus' effect on apoptosis and cytokine production. HIV's effect on the oxidative status of the host, the functional role of oxidative stress in the immune system as well as the importance of ROS in metabolic processes made it a natural choice for inclusion in this study. The immunology studied was therefore not new but was measured to compliment GC-MS findings.

ROS: These molecules have been implicated in various pathological conditions and viral diseases including AIDS. For this project, a colorimetric assay which detects hydroperoxides was chosen since it allowed for the measurement of oxidative species from various

biochemical pathways giving a representation of the total oxidative state of the individual. The principle and execution of this assay is elaborated on in Chapter 5.2.3.

Apoptosis: The annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit (BD Biosciences, California, USA) was chosen since it allowed for the determination of apoptosis and necrosis and would thus provide information about the mode of cell death induced by the virus. This assay would also allow for a distinction to be made between early and late apoptotic events.

Considering that the patient group was still relatively healthy (see Section 3.6), an increase in hydroperoxides which are produced during the early asymptomatic phase of infection was expected. Based on the health status of the patients, cells were also expected to undergo early apoptosis.

Intracellular Cytokines: Resting cells produce minimal or no cytokines (O'Neil-Andersen and Lawrence 2002) thus phytohemagglutinin-p (PHA-P, a general stimulant for inducing cell proliferation) and phorbol 12-myristate 13-acetate-ionomycin calcium salt (PMA-ionomycin) were used to induce cytokine production in the cells.

R7V and Gag were chosen as representative antigens to induce HIV-specific cytokine responses. Briefly, these peptides are representative of a single epitope or pooled peptides respectively. R7V is representative of an epitope derived from the host whilst Gag is virus-derived. Immune responses to peptide stimulation have also been documented to have prognostic value. Reasons for particularly measuring IFN- γ and TNF- α is highlighted in Section 2.7.3. These cytokines were chosen for their role in oxidative stress, apoptosis and metabolism. They are also representative of cytokines produced by cells of the Th1/Th2 lineage and will allow for commentary on disease pathogenesis based on their anti- and pro-inflammatory roles.

Secreted Cytokine: (IFN- γ): Because intracellular IFN- γ was produced in higher quantities compared to TNF- α , secreted levels of this cytokine were measured using the ELISA. This assay which measures one cytokine at a time allowed for the detection of cytokine that may have leaked from the cells as a result of continuous HIV-induced immune activation and prior to GolgiPlug having an effect. The data was analyzed using univariate statistics.

CBA: To measure multiple cytokines in sera in context to natural HIV infection (no *in vitro* stimulant), CBA technology and flow cytometry was used and the data analyzed through multivariate statistics.

3.4 Biofluid Selection

Next, the biofluid to be analyzed was considered. Immune changes were measured in sera and PBMC samples whilst metabolic changes were assessed in sera, PBMC and urine samples of HIV- and HIV+ individuals, respectively. Blood-based samples and urine are preferred over other biofluids since these have traditionally been used for the diagnosis and prognosis of many diseases. Blood bathes and transports molecules across the entire body (Psychogios et al., 2011; German et al., 2005). It therefore contains various secreted, excreted and discarded molecules making it a useful indicator of immune and metabolic function. Serum is the clear liquid that separates from blood when allowed to clot (Psychogios et al., 2011; Issaq et al., 2009). Since HIV primarily infects immune cells, serum was chosen for analysis because of its homeostatic regulation and close proximity to the cells that are commonly infected. The metabolic profile of PBMCs was assessed because of the improved capabilities of MS technology to detect endogenous molecules present at low concentrations (Go et al., 2006). In addition, PBMCs allow for the measurement of corresponding cellular immune changes in response to HIV infection. There is a lack of information on organic acid content in biofluids other than urine (Hoffmann and Feyh 2005). By utilizing these biofluids this study therefore represents one of the first reports for organic acid content in HIV-infected blood products. Metabolic changes in urine were investigated because this still represents the most non-invasive biofluid type with which to study the metabolome (Zhang et al., 2007) and metabolite changes are more obvious in this sample type (Hoffmann and Feyh 2005). By analyzing the urinary organic acid profile an indication of those nutrients that are depleted at a cellular level is obtained (Meletis, 2006) since urine contains excreted metabolites discarded from the body as a result of catabolic processes. Because the clinical course of HIV infection varies amongst individuals, differences in the metabolic and immune profiles may present at the different stages of infection. In the same manner, because of the compartmentalized nature of eukaryotic systems, different metabolic changes may present in different localities – thus biofluids representative of different compartments were analyzed.

3.5 Analysis Techniques

The primary aim of this project was to characterize the metabolic and immune profiles of HIV-infected individuals using MS for the former and flow cytometry for the latter. The inability of flow cytometry to detect and measure a vast amount of metabolic products was discussed in Section 2.8.3. Metabonomics, which represents the global analysis of all metabolites in response to stimuli, was reviewed for the analysis of HIV-induced metabolic changes with MS as the primary analysis technique. With this technique the detection of

hundreds of metabolites was anticipated. Additional reasons for the utilization of MS, particularly GC-MS were supplied in Section 2.10.1.4 and 2.10.1.6. Flow cytometry was primarily chosen for the measurement of HIV-induced immune changes since it is one of the standard techniques for assessing immunological changes. This technique was also chosen due to its sensitivity and availability.

3.6 Sample Selection

The study population consisted of South Africans. It was therefore assumed that all HIV+ individuals were infected with HIV-1 subtype C. Nested PCR employing subtype C specific primers (Yagyu et al., 2005) was used to confirm this for a few of the samples before starting the project (detail in Section 3 of the Appendix). Blood samples were obtained from HIV+ donors attending the Fountain of Hope Clinic and the Steve Biko Academic Hospital (Pretoria, South Africa). Blood as well as urine were collected from HIV+ donors attending the King's Hope Development Foundation and Mooiplaas Clinics in Diepsloot, Johannesburg.

The participating donors were recruited from a cohort of individuals who were aware of their HIV status (being HIV positive), had not been diagnosed as having AIDS (CD4 counts above 200 cells/ μ l blood) and were not on retroviral therapy. In a few cases, CD4 counts of < 200 cells/ μ l were detected after the study was initiated. These cases were retained in the experimental groups since they were still healthy and ART naive. Viral load measurements were determined by means of the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test (Roche Molecular Systems, Inc Pleasanton, CA). This parameter was not part of the inclusion criteria since practices at the source clinics were such that viral load was only determined when testing for HIV infection for the first time and directly before patient treatment was initiated. The viral load measurements had a wide range (2328-10 000 000 copies/ml plasma) for those cases for which it was obtained. Some of the participating HIV+ donors comprised of LTNPs. Healthy control donors were recruited at the campus of the University of Pretoria and had no known metabolic or other medical condition at the time of blood collection. The HIV negative status of these samples was confirmed with VISITECT[®] HIV 1/2 rapid tests (Omega Diagnostics Limited, Scotland, UK). Immunophenotyping experiments also confirmed that the healthy controls had a significantly higher percentage CD4 cells than their HIV+ counterparts, which was to be expected because of their HIV-negative status. The samples utilized in this study were fairly well-matched in terms of gender and age. A brief overview of the patient profiles is provided under Section 4.3.2 and summarized in Table 4.1 through to Table 4.3.

Samples were collected as per their availability and batch analysis anticipated as part of our MS experimental design. For ease of metabolite extraction approximately twenty blood samples (10 HIV+, 10 HIV-) were collected at a time. Metabolites were extracted and the samples stored until analyzed. This was repeated 3-4 times with approximately a four month interval between the respective analyses as per sample availability. In the case of the urine samples, only two batches of samples were analyzed. For the number of cases within each batch please refer to Table 4.1 through to Table 4.3.

3.7 Statistical Methods

Statistics allows for the identification of metabolites that are statistically significant between experimental groups. To assist with the large data matrices expected from organic acid profiling (Chapter 4) and CBA analysis (Section 5.2.6.2.1 and 5.3.4.2), multivariate approaches were employed to assist with data reduction and the classification of HIV- and HIV+ groups. **Multivariate statistics** is a statistical approach which allows for the simultaneous observation and analysis of more than one variable at a time. It is best suited for the analysis of large, complex datasets but univariate analysis can also be applied to measure the significance of an analyte when it stands on its own. Multivariate approaches include both the use of unsupervised and supervised methods. Unsupervised approaches are used when no prior information about the molecules that is being classified, is known whilst with supervised methods prior information about the molecules is known. Whereas unsupervised approaches primarily classify groups, supervised methods are used for biomarker discovery and for building models from which the class of a new set of samples can be predicted from an initial modelled data set. The variables/identified biomarkers thus give biological meaning for why a particular classification pattern is obtained ([Goodacre et al., 2004](#)). Described below are representative examples of multi- and univariate analysis that were of importance to this study. Statistical methods used to analyze metabonomics data, CBA data and other data are discussed briefly in this sequence.

Metabonomics data: Principal component analysis is the most commonly used pattern recognition approach and gives an overview of the samples in the data table, its groupings, trends and identifies outliers ([Trygg et al., 2007](#); [Trygg and Lundstedt 2007](#)). PCA explains variances in the data by using the least number of principal components (PCs). PC1 therefore describes the largest variation followed by PC2 etc. Those components which do not explain a lot of the variation are ignored since they primarily explain noise ([Wibom et al., 2006](#)). The PCs are uncorrelated and is a linear combination of the original variables ([Lindon and Nicholson 2008](#)). When the data matrix is converted to PCs the outcome is two data matrices called scores and loadings ([Lindon and Nicholson 2008](#)). Scores basically describe

similarities and differences between samples whilst the loading plots describe similarities and differences between variables/metabolites (Trygg et al., 2007). Those samples that are similar therefore cluster together whilst those that differ cluster separately. Variables of importance are identified by calculating the PCA modelling power of each variable.

PLS-DA on the other hand is based on multivariate regression principles (with more than one predictor variable expected). This analysis shows relationships between the data matrix (X) and a response (Y) and shares commonality with PCA in that PCs are extracted to reduce the dimension of the data matrix (Wibom et al., 2006). PLS-DA can be used in an explorative or predictive context where the data is merely analyzed to see whether it contains information or classified as belonging to a control or experimental group, respectively. The PLS-DA approach is however better-suited for biomarker discovery instead of classification of groups since it overfits the data. Even then, validation of PLS-DA models are still needed (Westerhuis et al., 2008). Validation of discriminations obtained with PLS-DA models are inferred by the Q^2 value i.e. the prediction error measure. An optimal Q^2 value of 1 is favourable but is rarely obtained due to inherent biological variability amongst individuals (Westerhuis et al., 2008). As such, no defined value is given for this parameter but its value is compared between groups within an analysis (e.g. -0.2 would be less favourable than 0.4). Variations which exist within a particular group or class can however be revealed with a large degree of certainty using this approach. Variables of importance are identified by calculating the variables important in projection (VIP) value for each variable.

As a representation of univariate analysis, effect sizes (ES) are applied to compliment p-values of the t-test. This parameter is especially useful in cases where small sample sizes are used. Small sample sizes are associated with low statistical power and in such a case ES are best used to indicate practical significance. In addition, ES give an indication of the magnitude of the effect (Nakagawa and Cuthill 2007). Effect size is defined by the equation: $ES = \frac{|\bar{X}_1 - \bar{X}_2|}{S_{max}}$, where \bar{X}_1 = mean of the respective variables of the control group, \bar{X}_2 = mean of the respective variables of the HIV-infected group, and S_{max} = maximum standard deviation of the two groups. According to Cohen's 1992 rule; effects are classified as small, medium and large when values are 0.2, 0.5 and 0.8 respectively (Thalheimer and Cook 2002).

CBA data: For the analysis of the CBA data (Section 5.3.4.2), linear discriminant analysis (LDA) was applied in order to classify a case as HIV- or HIV+. Stepwise discriminant analysis was used to select the best set of cytokines for the classification. LDA is similar to

PCA but where the dependant variable is a numerical value in PCA, it is categorical in the case of LDA. This statistical method primarily maximizes between-class variation compared to within-class variation to bring about an improved separation of experimental groups. Logistic regression is a model for the probability that an individual case belongs to a particular group. It makes use of a predictor variable which can be numerical or categorical. In the present study a stepwise analysis was used to select the cytokines which would result in the best classification of the HIV- and HIV+ groups. Comparison of the mean log-transformed cytokine concentrations of the two groups (HIV- and HIV+) was done by means of analysis of variance (ANOVA) *F*-tests. This was done in order to establish whether or not there were significant differences between the groups. Only cases where the significance level (p-value) was less than 0.05 were considered.

Other data: Where immune parameters in Chapter 5 were compared between unpaired HIV- and HIV+ samples (e.g. apoptosis data) the nonparametric **Mann-Whitney test** was used. In the case of intracellular cytokine detection where a paired assessment of untreated to treated cells was made, the nonparametric **Wilcoxon signed-rank test** was used. **Unpaired tests** compare the means/medians of groups of samples which have been independently collected whereas **paired tests** compare samples within a population before and after a particular treatment have been applied. The reason for the use of **nonparametric tests** was based on the expectation that the data would not necessarily follow a normal distribution. Indeed, the data generated consisted mostly of positively skewed data for which any normality test, e.g. Shapiro-Wilk, will reject normality supporting the use of nonparametric analysis. Deviation from normality was also informally inspected and inferred from the asymmetrical distribution of the data in the box plots. Most of the data in subsequent chapters are shown in the form of **box plots** as these show various characteristics about the data (e.g. mean/median, high and low value etc).

This chapter provided a brief background to the experimental design and highlighted why certain samples, assays, reagents, statistics, etc, were chosen over others. Subsequent chapters will now elaborate on exactly how the metabonomic (Chapter 4) and immunological assays (Chapter 5) were done, the findings that were obtained and the interpretation thereof. Figure 3.1 serves as an overview of all the analysis performed in this project.

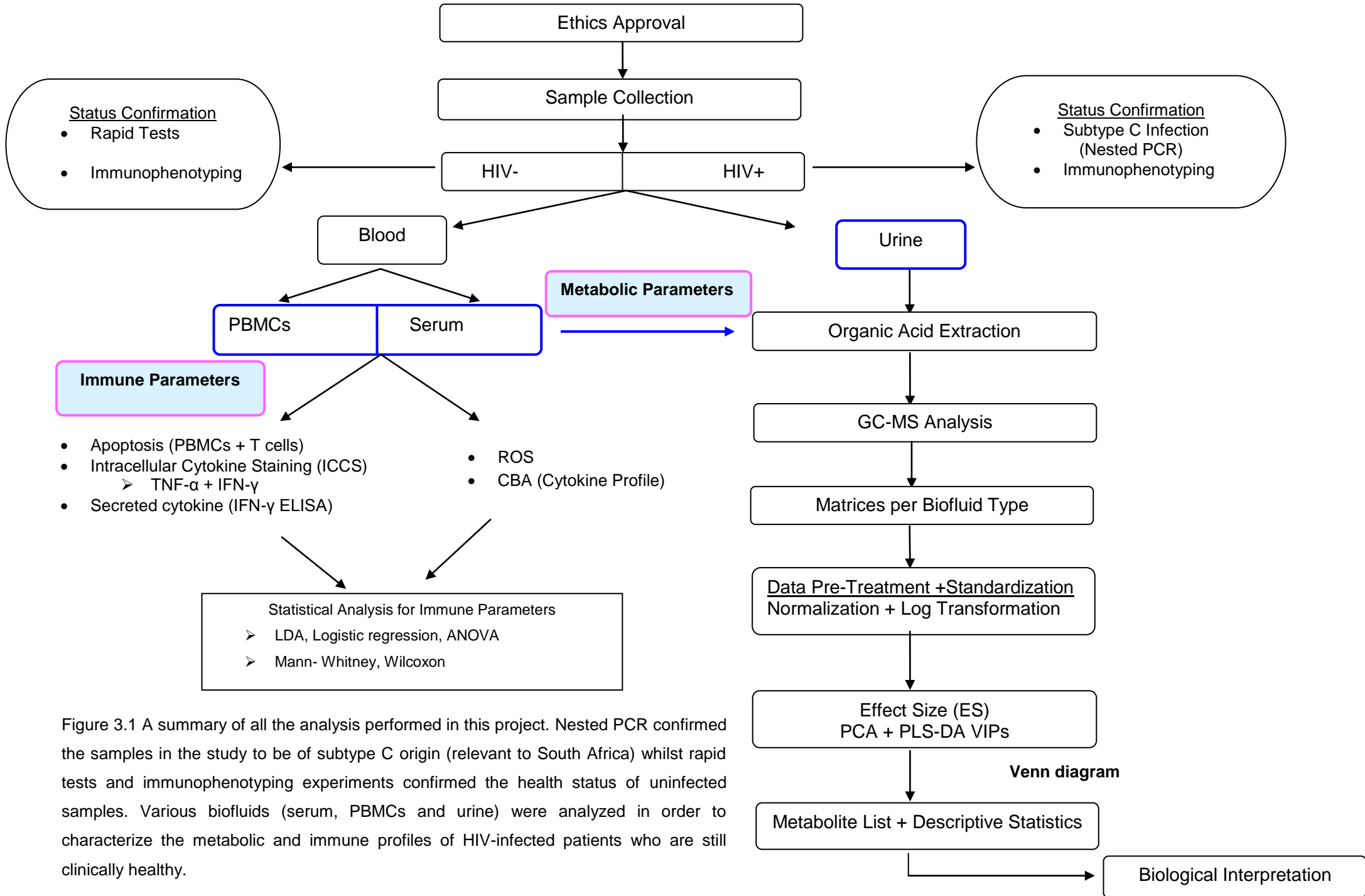


Figure 3.1 A summary of all the analysis performed in this project. Nested PCR confirmed the samples in the study to be of subtype C origin (relevant to South Africa) whilst rapid tests and immunophenotyping experiments confirmed the health status of uninfected samples. Various biofluids (serum, PBMCs and urine) were analyzed in order to characterize the metabolic and immune profiles of HIV-infected patients who are still clinically healthy.

CHAPTER 4

METABONOMICS PROFILE OF HIV INFECTED BIOFLUID

4. Summary

Background: HIV and the immunological changes it induces have a negative effect on mitochondria. Mitochondria are central to various biochemical pathways but disruptive effects due to HIV results in the suboptimal functioning of these organelles and leads to an accumulation or depletion of metabolites representative of HIV-induced mitochondrial dysfunction. Organic acids are known markers of mitochondrial dysfunction but have not been investigated as indicators of HIV-induced mitochondrial damage. A GC-MS metabonomics approach incorporating the use of various software programmes and statistical analysis was employed to determine whether organic acids could be used to confirm the virus' effect on mitochondria.

Methods and Results: The organic acid profile of sera, PBMC lysates and urine from HIV+ individuals not on antiretroviral treatment was investigated using GC-MS to gain insight into virus-induced mitochondrial dysfunction. Data was collected in batches (± 20 samples per analysis); GC-MS peaks were deconvoluted and aligned followed by pre-treatment and statistical analysis of the data matrices. MET-IDEA proved to be the most appropriate software for the analysis of the data. Classification of HIV- and HIV+ groups based on endogenous metabolites was assessed using multivariate statistics. PCA showed a partial separation of the HIV- and HIV+ groups whilst more defined separations were observed following PLS-DA. The PCA separation profiles improved when cases in the advanced stage of the disease were included as part of the analysis. The metabolites detected in the various biofluid types differed in identity and type but overlapped in terms of the biological information retrieved. Significant differences in the metabolites of HIV- and HIV+ individuals clearly indicated an effect of HIV infection on the host metabolism.

Discussion and Conclusion: Metabonomics-based analysis yield large, complex data matrices. The characteristics of the data as well as the aim of the research determine which analysis software and statistical methods to apply. The overlap in the organic acid profiles for some batches suggests the metabolic state of clinically stable HIV+ individuals to be similar to that of HIV- individuals. If individuals with AIDS are included in the analysis the metabolic state is markedly deteriorated and reflects an improved separation between the two groups, implying a prognostic application for metabonomics. Despite the overlap in organic acid profiles, biological information with which to probe early metabolic changes

could still be extracted. Some of the main molecules affected by HIV were identified and related to disrupted mitochondrial metabolism, changes in lipid, sugar, energy and neurometabolism as well as oxidative stress, all of which are known aberrations caused by HIV infection. Altogether, the results showed that GC-MS detects minor metabolic changes during early infection, before the development of AIDS and the administration of retroviral therapy. Based on the metabolic profiles observed, the types of molecules identified and the increase in the levels of these molecules with viral load, the technique may potentially be used for disease monitoring and provides insight into virus-host interactions and mechanisms of viral infection (i.e. HIV interacts with mitochondria to bring about an increase in organic acids, particularly fatty acids and other oxidative stress markers. These molecules are indicative of oxidative damage but can also increase membrane permeability, fusion between cells and as such the ability of the virus to rapidly infect and kill immune cells).

Some of the main findings related to this section of the thesis were accepted for publication in an international peer-reviewed journal: [Aurelia Williams](#), Gerhard Koekemoer, Zander Lindeque, Carolus Reinecke and Debra Meyer (2011). Qualitative serum organic acid profiles of asymptomatic HIV-infected individuals not on antiretroviral treatment. *Metabolomics*. In Press. DOI: 10.1007/s11306-011-0376-2.

4.1 Introduction

HIV is parasitic and dependent on the host infrastructure for energy and macromolecular precursors ([Munger et al., 2006](#)). As a result, HIV-infected individuals suffer from various metabolic complications (most of which were highlighted in Section 2.8.1). Despite this fact, metabolic complications due to HIV infection have been largely neglected. If addressed; insensitive, conventional methodologies are employed and most often the focus is not on the key metabolic effects of the virus but on the effects of the antiretroviral drugs. In Section 2.7 through to 2.8 some sequential effects of HIV on the immune and metabolic systems were highlighted. Because of the effect of HIV on the immune system, immunopathogenic events associated with HIV infection were explained first to show how these changes link to metabolic imbalances. The immunological changes ranged from the activation of the immune system, to the increased production of ROS and thus oxidative stress, apoptosis as well as an alteration in cytokine production and secretion. The development of such immune responses was explained to impact on mitochondria and to subsequently augment metabolic imbalances (Section 2.7.4).

Mitochondria drive metabolic processes ([Shedlock et al., 2008](#)) and together with the Krebs cycle serves as the “hub” of cellular metabolism. These organelles are the primary site for ATP production as per the Krebs cycle and electron transport processes (see Figure

2.12). Mitochondria therefore have an important role in energy metabolism. If compromised, as occurs during HIV infection, energy processes are affected such that ATP is depleted and the body left with an increased requirement for energy. Mitochondrial dysfunction drives the body to obtain its energy resources from stored fat reserves. The production of various metabolites that can be measured through analytical techniques is also initiated to compensate for the energy requirements.

Some ways in which mitochondrial function is assessed was provided in Section 2.8.3. Of these, flow cytometry is representative of a sensitive technique which can be utilized for this purpose. Enzymes however catalyze more than one biochemical reaction producing many products in turn. Flow cytometry is incapable of measuring the vast number of metabolic products produced by the enzymes. Metabonomics in turn allows for the measurement of hundreds of metabolites and was therefore considered. Metabonomics and chemometrics (statistical analysis applied to biological data) are new tools applied to the study of infectious diseases. These tools allow for the classification of control versus experimental groups and enable the extraction of metabolites which differentiate these groups from each other.

Of the examples which have been listed for assessing mitochondrial function (Section 2.8.3) organic acids are representative metabolic intermediates which have previously been used for probing the functional status of mitochondria. In a more recent investigation these molecules proved valuable in highlighting perturbations due to an array of related disorders linked to mitochondria ([Reinecke et al., 2011](#)). Despite this relationship that exists between organic acids and mitochondria, as well as HIV and mitochondria, there has been no research done to profile these molecules following HIV-induced mitochondrial failure. Assaying these molecules to detect differences in the serum, cellular and urinary organic acid metabolome of clinically stable HIV-infected individuals (not on antiretroviral treatment) was thus anticipated through a metabonomics approach.

The mass spectrometric analysis of chronically infected blood-based biofluids has not been done previously. An overview of the concept of metabonomics i.e. its history, workflow, sample/biofluid types, instrumentation, software and statistical tools that are commonly used was therefore briefly introduced in Section 2.10.1 and its subsections. In subsequent sections of this chapter, the organic acid profile of three main biofluid types (serum, PBMCs and urine) was analyzed through the use of three software programmes (AMDIS, SpectConnect and MET-IDEA) and a combination of uni- and multivariate statistics (PCA, PLS-DA, ES, t-tests). The basics of how samples for metabonomics experiments are chosen were provided in Section 2.10.1.3 whilst reasons for particularly choosing serum, PBMCs

and urine were highlighted in Section 3.4. Urine is obtainable through non-invasive procedures and has become the standard biofluid for organic acid testing. It has been reported to contain a profile of approximately 500 organic acids (Jellum, 1981). This estimate is applicable to other biofluids. There is however a difference in the concentration of the metabolites with it being generally lower and undetected in other biofluid types. As a result, these metabolites may not reflect on chromatograms (Jellum, 1981). Biofluid types such as serum thus appear to have less complex profiles than does urine. To assist with analyzing and comparing such complex profiles between samples, software packages are used.

AMDIS is a software package utilized for the deconvolution of GC-MS data. It is freely accessible and compatible with various file formats (Styczynski et al., 2007). This software was initially developed for detecting potential warfare chemicals and pesticides in complex mixtures of sample and has become progressively applicable in the clinical environment (Chen et al., 2009). Despite its numerous drawbacks AMDIS is still the most utilized deconvolution software package. This software uses the spectra of pure reference compounds to characterize complex and sometimes co-eluting chromatographic peaks (Dunn et al., 2005). A key disadvantage of AMDIS is that it only identifies metabolites based on the library that is attached to it. If a particular variable occurs in the raw spectra but is not listed in the library, it is simply ignored in the identification process (Chen et al., 2009). AMDIS yields a high level of false positives and data matrices with many missing values (Behrends et al., 2011). Missing values occur when metabolites are identified in some samples and not in others. When these values are incorporated into the data subsequent statistical analysis and the interpretation thereof is influenced. The missing values may be corrected for by substituting it with zeros but there is the risk that the distribution of the variables is no longer accurately described (Behrends et al., 2011). Deconvolution also leads to a large number of overlapping peaks with similar retention times (Katajamaa and Orešič 2007). Although the most utilized, AMDIS is not the only usable software. Many other software programmes functioning on different algorithms have therefore been developed.

SpectConnect for example, is an online, web-based programme (<http://spectconnect.mit.edu/>) that has been designed to extract unidentified, conserved components across samples. It does this by comparing every spectrum in each sample to the spectra of every other sample. If a peak is “real” the mass spectrum in the different samples will be similar to each other (Styczynski et al., 2007). Through the use of the algorithm inconsistent signals are removed from the data matrix (Barupal et al., 2010) and spectra which occur as a result of artefacts eliminated. The user is thus assured that “real” peaks instead of noise are detected. No reference library is required but one can be

uploaded with the data files in order to identify the extracted components. This software is therefore specifically aimed at biomarker discovery instead of identifying all the detected metabolites of samples. Its use is however dependent on the deconvolution output files obtained from AMDIS i.e. following the deconvolution of a chromatogram with AMDIS; an *.Elu file is generated and uploaded onto the SpectConnect webpage for processing. SpectConnect generates an output file which is downloaded by the user following an email notification. SpectConnect is also unable to resolve peaks with similar MS spectra and that are close in time (Usaite et al., 2009).

MET-IDEA on the other hand processes chromatographic MS data and allows for the extraction of quantitative ion abundances. A representative sample (single sample or pooled mixture of several samples) is chosen to serve as an input list which guides the ion extraction process. This input list is composed of a series of ion/retention time pairs (IRt) with each IRt being unique and characteristic of a specific compound. IRt lists can be manually generated within MET-IDEA, imported as text files from metabolite databases or by use of AMDIS. When AMDIS is used, deconvolution is performed and the output thereof (*.elu and *.fin files) used in MET-IDEA. From the output MET-IDEA accesses the “model” ions (as recognized by AMDIS and listed in the *.elu output file). If no model ion is available the software selects abundant ions that meet a certain set of criteria. The criteria are specified by the user and include a low mass cut-off and an exclusion list for common background ions originating from contamination, column materials, derivatization reagents, and solvent clusters. The low mass cut-off eliminates ions below a certain value from consideration. If on the exclusion list, the particular ion is not considered as a quantifier ion. If multiple model ions are identified MET-IDEA selects the more abundant of the ions for quantification. When no models are reported MET-IDEA selects a non-model ion from the raw mass spectrum and where these do not match the specified criteria, a value of “-1” is reported. Once IRt pairs in a given file are located, retention time values are compared to that in the first reference file and a correction for retention time made on a file-by-file basis. The correction is made using a fixed value (based on the average deviation of experimental retention values from the expected values) or linear correction. Where the linear correction is applied the change in retention time is regressed against the compound retention time. Ion abundance data is then extracted from the net.cdf files on the basis of several extraction parameters of which the main one is “average peak width” (width of an average peak at its base). Peak area is used for quantification and is calculated as the sum of selected ion intensity values for all scans within the peak range. In Section 2.10.1.8; derivatized GC-MS data was referenced as presenting multiple peaks for a particular metabolite. This problem presents when two components are listed with either identical or nearly identical retention

times. In such a case MET-IDEA selects the more abundant component and discards the lesser. In the case of non-identical retention times MET-IDEA extracts model ion values for both peaks and allows the user to perform a correlation-based redundancy analysis. Those peaks that are truly independent will have a low r^2 value whereas improperly deconvoluted peaks (those artificially separated into two peaks) will possess high r^2 values (Broeckling et al., 2006).

According to van der Werf et al (2007); metabonomics is about extracting biologically relevant information and not about generating data. When metabonomics is used to classify samples no biochemical information is gained. There is thus a need to identify molecules that differentiate sample groups (Dettmer et al., 2007). For this reason various statistical analyses as described in Chapter 3 (Section 3.7) were incorporated to analyze metabonomics-based data.

Background knowledge of the effects of HIV infection, metabonomics and chemometrics was used to guide the mass spectrometric assessment of organic acids as indicators of HIV-induced mitochondrial and metabolic complications in the biofluids of clinically stable HIV+ patients. Although an MS-based metabonomics approach was applied to the saliva of HIV+ individuals (Ghannoum et al., 2011), to CD4 and macrophage cells infected with HIV *in vitro* (Hollenbaugh et al., 2011) and the CSF of SIV-infected primates (Wikoff et al., 2008) the approach has not been applied to chronically infected blood-based biofluids and the urine of treatment naive HIV+ individuals. Following GC-MS analysis, MET-IDEA was found to be the most suitable software package for data analysis. The metabolic profiles of HIV- and HIV+ groups overlapped implying that the profiles of the two groups were relatively similar. When cases in the advanced stage of the disease were included as part of the analysis, an improved separation between the groups was observed. Metabolites relevant to the asymptomatic phase of infection were identified and were representative of disrupted mitochondrial metabolism, changes in lipid, sugar, energy and neurometabolism as well as oxidative stress. Biochemical relationships were established between the metabolites detected in the three biofluid types even though differences in the type and identity of the metabolites was evident for these biofluids.

This work is one of very few worldwide addressing HIV/AIDS metabolic influences using metabonomics and is novel as it represents the first of its kind attempting to link HIV-induced metabolic and immune changes using the sensitive analytical technology applied here.

4.2 Materials and Methods

4.2.1 Sample Collection and Preparation

The success of any metabonomics experiment is largely determined by good experimental design and the availability of high quality samples (Wishart, 2007). These aspects were addressed in Chapter 3 (Section 3). Following the design of experiments, samples were selected (Section 3.6), collected and processed accordingly.

4.2.2 Serum Isolation

Venous blood collection was performed at room temperature. For serum isolation, samples were collected in non-ethylenediaminetetraacetic acid (EDTA) vacutainers (Greiner Bio-One GmbH, Kremsmünster) and allowed to clot. Serum was separated after centrifugation at 1610 $\times g$ for 10 minutes. Approximately 1.5 ml of serum was obtained from 4 ml of blood sample using these conditions, of which 1 ml was used for organic acid extraction. The remaining serum was aliquoted and stored at -70°C for subsequent determination of the oxidative and secreted cytokine profiles of the samples (detail in Chapter 5).

4.2.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Venous blood was collected in EDTA vacutainers (Greiner Bio-One GmbH, Kremsmünster) from HIV- and HIV+ donors on several occasions. The blood was allowed to stand in a biological safety cabinet to allow plasma to settle out. The top plasma layer was transferred to an Eppendorf tube and this was clarified by centrifugation at 800 $\times g$, 30 minutes. Clarified plasma was stored as 1 ml aliquots at -70°C . After collecting plasma the remaining cells in the tube were used for the isolation of PBMCs. Following a 1:1 dilution of the blood cells with warm Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Chemical Company, St. Louis, MO); PBMCs were isolated by density gradient centrifugation (1912 $\times g$ for 30 minutes) using Ficoll-hypaque (Sigma Chemical Company, St. Louis, MO). The recovered buffy coat was washed with plain RPMI media (1028 $\times g$ for 10 minutes at room temperature) and lysed with 5 ml of Ammonium Chloride Potassium (ACK) buffer (0.15M NH_4Cl , 0.010M KHCO_3 and 0.0001M EDTA; pH 7.2) for 5 minutes in order to lyse contaminating red blood cells. ACK was removed by centrifugation (306 $\times g$ for 10 minutes at room temperature) and the PBMCs resuspended in 10 % RPMI i.e. RPMI 1640 + 500 μl antibiotic antimycotic and 500 μl of 1 % Gentamycin Sulphate + 10 % (v/v) Fetal Calf Serum (FCS); Sigma Chemical Company, St. Louis, MO or plain RPMI 1640 (in the case of MS analysis). Cell viability and concentration was determined using Trypan Blue (Sigma Chemical Company, St. Louis, MO). HIV- and HIV+ cells (having the same cell

concentration) were thus compared for their responses in the respective analysis. For MS, apoptosis and cytokine analysis, the cell concentration was adjusted to 5×10^6 , 1×10^6 and 2×10^7 cells/ml respectively.

4.2.4 Urine Preparation

Urine samples from HIV- and HIV+ individuals were collected at room temperature. Each sample was aliquoted, frozen as soon as possible and stored at $-20\text{ }^{\circ}\text{C}$ until used (Tanaka et al., 1980). An aliquot of each urine sample was transported on ice to a pathology lab where the level of creatinine in each of the samples was determined (Tanaka et al., 1980). The volume of urine used for organic acid extraction was based on urinary creatinine content (see guide below). Creatinine content was taken relative to body weight since its production is dependent on muscle mass. Creatinine is associated with renal function and served as a guide to compensate/standardize between cases where urine may be diluted or concentrated.

| | | |
|------------------------------------|--------|------------------|
| Creatinine > 100 mg % / 8.8 mmol/l | —————> | Use 0.5 ml urine |
| Creatinine < 100 mg % / 8.8 mmol/l | —————> | Use 1 ml urine |
| Creatinine < 5 mg % / 0.44 mmol/l | —————> | Use 2 ml urine |
| Creatinine < 2 mg % / 0.18 mmol/l | —————> | Use 3 ml urine |

After determining the amount of urine to use the metabolites of interest were extracted as described below.

4.2.5 Organic Acid Extraction

4.2.5.1 Serum and Cells

The flow diagram from the point of collecting serum, cells and urine to identification of the respective organic acid profiles is shown in Figure 4.1. Organic acids were isolated from HIV- and HIV+ biofluid by solvent extraction prior to GC-MS analysis according to standard operational procedures used in The Laboratory for Metabolic Disorders at the North-West University (NWU), Potchefstroom. The extraction of metabolites allows for the sample to be in a format that is compatible with the analytical instrument (Dettmer et al., 2007). All reagents used for organic acid extractions were purchased from Merck unless stated otherwise. Extractions were carried out in Kimax glass tubes which were purchased from LASEC, South Africa. Briefly, 1 ml of serum and 2 ml of cells at 5×10^6 cells/ml (serum-free media) was transferred to a glass tube respectively. The tubes containing cells were

centrifuged at 1118 \times g for 10 minutes to obtain a pellet. A thin film of media was left so that the immediate cellular environment of the cells was not disrupted (Munger et al., 2006). To each sample (1 ml serum and the PBMC pellet respectively) 100 μ l of the internal standard, 3-phenylbutyric acid (Sigma-Aldrich, St Louis, MO, USA) was added to a final concentration of 52.5 mg/l. The pH of the respective samples were then adjusted to 1 using 5M HCl. Ethyl acetate (6 ml) was added to each sample followed by shaking for 30 minutes. Centrifugation at 1118 \times g for 3 minutes at room temperature facilitated phase separation and the resulting organic phase was transferred into a clean glass tube. Diethyl ether (3 ml) was added to the remaining (lower) aqueous phase and the samples subjected to shaking for 10 minutes. Phase separation was once again initiated through centrifugation (1118 \times g for 3 minutes at room temperature) and the resulting organic phase added to the previous extract. Anhydrous sodium sulphate (Na_2SO_4) was added to the extracted samples and the mixture vortexed and centrifuged. The extracted organic phase of each sample was transferred to a clean glass tube and evaporated to dryness under nitrogen gas (40 $^\circ\text{C}$) to pre-concentrate the analytes. All extracts were stored at 4 $^\circ\text{C}$ until further analysis. When water is removed, most metabolites are stable and extracts can therefore be stored for long periods (Villas-Bôas et al., 2005; Tanaka et al., 1980).

4.2.5.2 Urine

The extraction of organic acids from urine was performed similarly to that of serum and cells with slight modifications. After having determined the volume of urine to use; the volume of internal standard added in μ l was 5 \times creatinine mg % with the rest of the extraction performed as outlined before (Section 4.2.5.1).

4.2.6 GC-MS analysis

All MS analysis was performed at the Centre for Human Metabonomics, NWU, (Potchefstroom Campus) according to the protocol established in that laboratory (Reinecke et al., 2011). Calibration of the instrument was done by running standards through the instrument and comparing the spectra to that contained in an online library. Prior to GC-MS analysis all samples were once again evaporated to dryness under nitrogen gas (40 $^\circ\text{C}$) to remove possible traces of water that may have formed as a result of storage. The dried serum and cell lysate residues were derivatized with 40 μ l N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA, Supelco Analytical, USA), 8 μ l trimethylchlorosilane (TMCS, Sigma-Aldrich) and 8 μ l pyridine (Sigma-Aldrich, Suh et al., 1997; Duez et al., 1996; Mardens et al., 1992). In the case of the urine samples the volume of BSTFA and TMCS in μ l was 2 \times creatinine mg % and 0.4 \times creatinine mg % respectively. The sera, PBMC and urine extracts were then heated at 70 $^\circ\text{C}$ for 45 minutes, injected into the chromatograph and

analyzed using an Agilent 7890A/5975B inert XL GC-MSD system equipped with a DB-1MS capillary column (30 m x 0.25 mm x 0.25 μ m). The temperature program was started at 60 °C for 2 minutes, increasing at 4 °C /min to 120 °C, and then at 6 °C /min to 285 °C and kept for 2 minutes. Samples (1 μ l) were injected in splitless mode with the injector temperature set to 280 °C. The carrier gas was helium (17.73 psi) and electron impact (EI) ionization was applied at 70 eV (electron volt). MS acquisition was performed in scan mode.

As explained under Section 3.6, samples were collected and analyzed as per their availability. MS data was thus processed and analyzed in batches. As a result, sera and PBMC data are reported for 3 and 4 batches respectively whilst the urine data is reported for 2 batches of sample only.

To compare samples and compensate for small drifts in retention time across sample runs, various software programmes were used for the processing and alignment of the data. Each software programme presented with advantages and disadvantages. Processing of the data was done using AMDIS as well as the fully- and semi- automated software programmes, SpectConnect and MET-IDEA respectively.

4.2.7 Peak Deconvolution, Alignment and Identification

4.2.7.1 AMDIS and in-house library

Processing of the total ion chromatograms (TICs) was done using AMDIS (version 2.66, from the National Institute of Standards and Technology) which contained an in-house library of organic acids. Macros were written for the software to generate output matrices in Microsoft Excel. The concentrations of the identified organic acids were determined relative to the internal standard and were reported as mg/L for serum and PBMC lysates. For the urine samples, the concentrations of organic acids were reported as mg/g creatinine or mmol/mol creatinine. These concentrations were determined by taking the area of the organic acid / area of internal standard multiplied by a constant (262.5 or 180 in the case of urine samples depending on whether concentration was to be expressed as mg/g or mmol/mol creatinine respectively). In the case of the sera the constant was 52.5 and reflects the final concentration of the internal standard used. The data matrices obtained from this software were however not subjected to statistical processing and the reason for this is explained under the results and discussion section.

4.2.7.2 AMDIS and SpectConnect

Peak deconvolution was done for each sample using AMDIS. A simple analysis was done with the following deconvolution settings: component width of 20, adjacent peak subtraction of one whilst the resolution, sensitivity and shape requirements were all set to low. The resulting *.elu files generated were saved for use with SpectConnect. In SpectConnect the criteria for peak matching, error in retention time, and the amount of times a peak should be repeated to be regarded as conserved, was specified. The NIST 08 mass spectral library was uploaded with the data files. An output file from SpectConnect was downloaded following an email notification. The output was obtained in a matrix suitable for statistical analysis. As with the data obtained through AMDIS, the data matrices of the AMDIS-SpectConnect analysis were not subjected to statistical analysis. An explanation for this is provided in the results and discussion section as well.

4.2.7.3 AMDIS/MET-IDEA/NIST 08

Based on the difficulties experienced with using AMDIS and the in-house library as well as AMDIS and SpectConnect; peak deconvolution, alignment and analysis was also attempted with AMDIS and MET-IDEA. A representative sample (having the highest number of components) from each batch was deconvoluted in AMDIS with deconvolution settings as specified in Section 4.2.7.2. The output from AMDIS i.e. the *.elu file was uploaded into MET-IDEA as an IRt pair to collect values for ions which are selective for a given metabolite (Broeckling et al., 2006). Data extraction in MET-IDEA was performed using default values for GC coupled to a quadrupole MS instrument. The following ion channels were omitted to limit the extraction of false positive peaks: TIC 73 and 147 m/z. Following deconvolution, peaks were identified in AMDIS by running a search against the NIST 08 mass spectral library. The data matrices were exported/copied into Microsoft Excel into a format suitable for further statistical processing.

4.2.8 Data pre-processing

The data matrices obtained following analysis with the respective software programmes were arranged with all the variables/metabolites in columns and the cases/experimental samples in rows. As explained above, only the data matrices obtained with MET-IDEA were subjected to further statistical processing. MET-IDEA generated data matrices were inspected for deconvolution errors by examining the peaks and using the correlation function in MET-IDEA. Deconvolution errors occur when a single compound peak is detected as two separate peaks (Xu et al., 2009). These replicate variables were removed by manual curation and the number of features that remained, reported on in the results and discussion.

4.2.9 Standardization of Data

As part of data pre-processing normalization and transformation was applied to remove non-biological variation between samples and to stabilize variance across peaks thus making the scales of the data sets comparable. Normalization can be done relative to molecular components such as internal standards or via the regression approach (Redestig et al., 2009; Sysi-Aho et al., 2007). Because there was some variability in the signal of the internal standard normalization was done through the latter approach whilst transformation of the data was done using a shifted log function. Variability in the intensity values of the internal standard may be due to certain molecules featuring in that part of the spectrum. For example, a triacylglycerol (TAG) standard was subjected to suppression effects due to different TAG species occurring/masking the region of the spectrum where this standard is known to elute. Variability in intensity signals is thus not necessarily due to sample extraction only (Sysi-Aho et al., 2007). If this is the case normalization cannot be done relative to the IS (Redestig et al., 2009) but regression approaches are applied instead. Regression approaches assumes that variation in one measurement (analyte) is dependent on variation measured in another (such as the internal standard, Callister et al., 2006). By applying such an approach correlated variances between the analytes and internal standard were removed (Sysi-Aho et al., 2007). Transformation reduces high-intensity values and keeps low-intensity values. Its use thus ensures that high intensity peaks would not be dominant during subsequent statistical analysis (Hrydziuszko and Viant 2011). The precision of the GC-MS approach used here was also assessed by determining the relative standard deviation (RSD) of the internal standard's intensity signal for each batch.

4.2.10 Variable Selection

The internal standard which was used as a reference for indexing unknown molecules was removed from the data matrices prior to statistical analysis. Blood transports molecules across the entire body whilst urine mainly contains excreted metabolites discarded from the body as a result of catabolic processes. These biofluids contain endogenous molecules from various biochemical processes as well exogenous molecules taken up from various sources. To accurately account for HIV's influence on the metabolome, only those molecules known to occur endogenously were subjected to further statistical processing. Variables were thus classified as endogenous, exogenous and unclassified (if no biological information about it was available). Classification of the variables was facilitated through consultation of various electronic (the HMDB, PubChem, Google) and other published resources (scientific articles). Subsequently, unclassified and

exogenous molecules were excluded from further analysis leaving only the endogenous molecules for further statistical processing.

All pre-processing and subsequent statistical analysis was done through the statistical computing package R (<http://www.r-project.org>) and STATISTICA (version 10, StatSoft®). Following standardization of the data, scatter plots of the mean log relative intensity of all identified endogenous metabolites for the respective batches was generated. Multi- as well as univariate statistical analysis was performed after the log-scaled data was centred (scaling technique which measures the change in a metabolite's intensity around zero and not the mean).

4.2.11 Statistical Analysis

4.2.11.1 Classification of experimental groups (PCA and PLS-DA)

Principal component analysis (Trygg et al., 2007) and PLS-DA (Barker and Rayens 2003) which are described under Section 3.7 were performed as part of multivariate analysis. Because the study was explorative the PLS-DA model was not validated but used only for the selection of significant metabolites contributing to the differentiation of the two groups.

4.2.11.2 Identification of Molecules affected by HIV

(PCA VIPs, PLS-DA VIPs, ES and p-values)

The classification of samples alone does not provide useful biological information. Variables of importance contributing to the separation of samples into their respective groups (HIV- and HIV+) has to be extracted and identified so that biochemical knowledge can be gained and the results interpreted in context to a biochemical pathway (Dettmer et al., 2007). For this reason, the measure of modelling power associated with each variable in the PCA analysis was used to rank the metabolites in order of importance. A ranked list of metabolites expressed as variables important in projection was also obtained following PLS-DA.

Owing to the small sample size in each of the batches and low statistical power associated with it, ES (Section 3.7) were calculated to assist with the selection of important molecules contributing to the organic acid profile observed. ES were calculated from the normalized, log transformed data. The larger the ES, the greater the contribution of the molecule to the profile observed. For this study, highly ranked PCA markers, PLS-DA VIPs as well as molecules with $ES \geq 0.8$ were used to identify the most prominent molecules in

the chemical profiles of patients and controls. To discover any significant differences between the means and medians of HIV- and HIV+ groups, p values were calculated using t-tests and nonparametric Mann-Whitney tests.

4.2.11.3 Venn diagram of common metabolites in VIP and ES lists

Venn diagrams are commonly used to show relationships between sets of data. Those molecules having high PCA and PLS-DA VIP rankings as well as large ES (i.e. ≥ 0.8) were presented in Venn diagrams to identify important metabolites (within a batch) that were common to the three statistical lists. The identified metabolites pooled from the different batches were chosen as important for the particular biofluid type analyzed.

4.2.11.4 Venn diagram of common metabolites in different biofluid types

A Venn diagram was constructed to show the relationship/commonality between the metabolites of the respective biofluid types analyzed.

4.2.12 Database Consultation and Retrieval of Biological Information

Molecules found to be significant were categorized according to biological function and interpreted in context to HIV. The overall workflow (as shown in Figure 4.1) was similar to that of [Almstetter et al \(2009\)](#) where significant molecules were identified following PCA analysis unlike other cases where significant molecules are first extracted and followed by classification or pattern recognition analysis ([Hewer et al., 2006](#)).

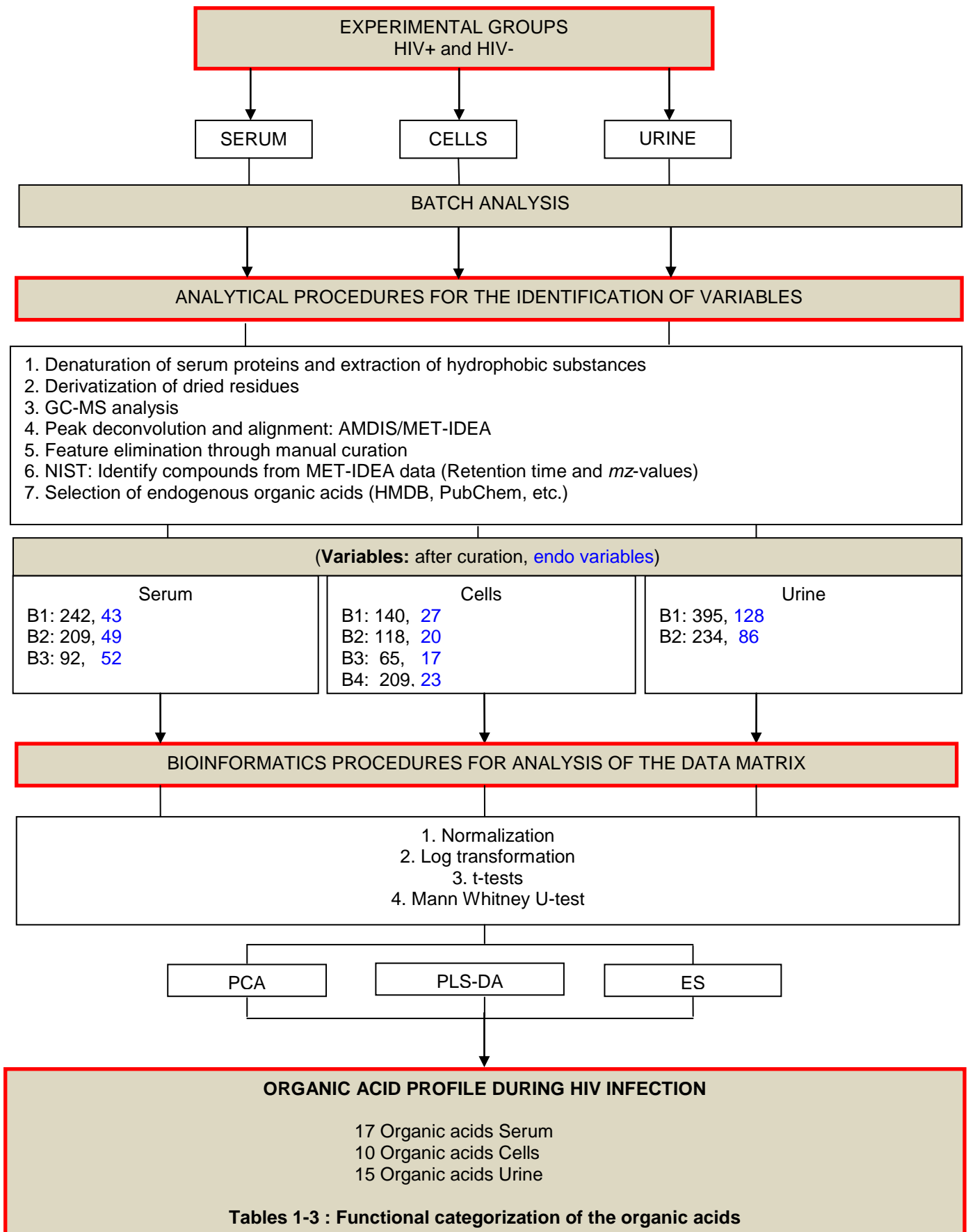


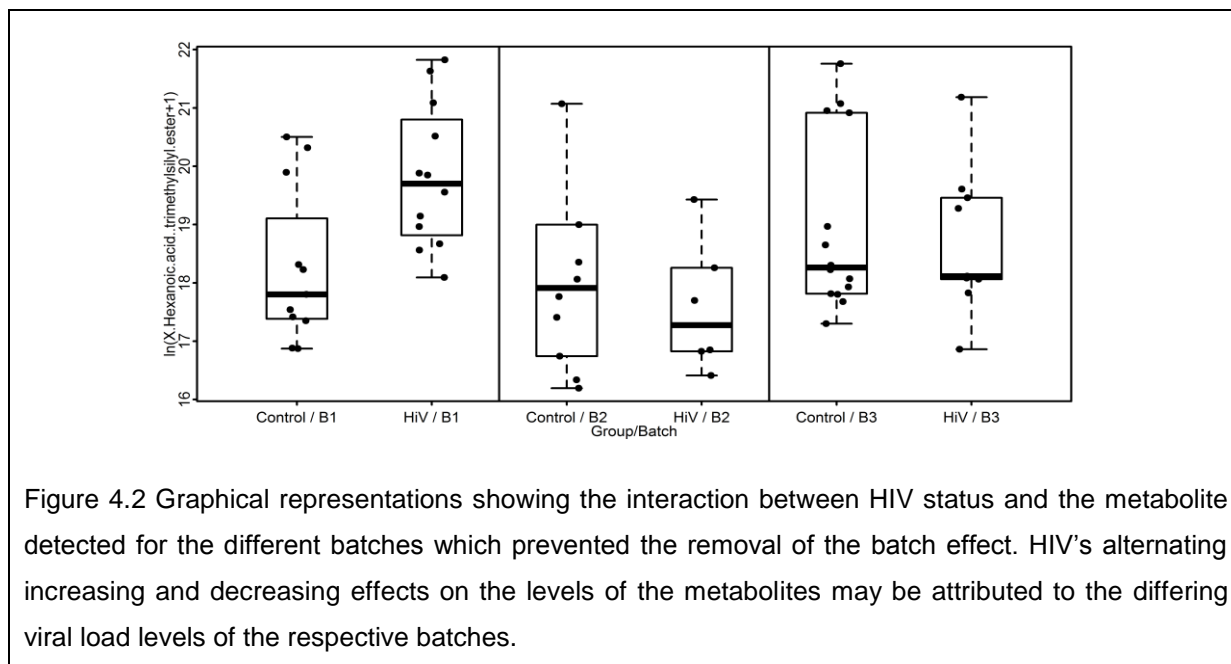
Figure 4.1 Simplified workflow of the metabolomics approach employed in this project.

4.3 Results and Discussion

Metabonomics approaches yield large sets of data that require pre-processing in order to generate a matrix of operational size suitable for multivariate analysis. The approach utilized here (Figure 4.1) primarily consisted of selecting the experimental groups, extracting and identifying the target molecules and analyzing these through statistics in order to identify possible indicators associated with HIV-induced mitochondrial failure.

4.3.1 Batch Analysis

The experiments were largely influenced by sample availability and the clinical profile of the experimental subjects. Sample collection was done at different times. Approximately 20 blood samples (10 HIV+, 10 HIV-) were processed and analyzed at a time for ease of extraction and to minimize the chance for errors associated with processing large numbers of samples at once. Working with ± 20 samples was thus reasonable for extraction purposes. Processing was done in batches to compensate for batch effects. If data is analyzed without removing or compensating for batch effects this may mask possible biological differences that are primarily investigated during metabonomics experiments. Batch effects can be avoided or corrected but in the case of experiments like those presented here where experiments are dependent on the availability of samples, batch effects are sometimes unavoidable (Luo et al., 2010). While statistical power increases with an increase in sample size (Chadeau-Hyam et al., 2010) the discriminatory power of multivariate analysis models decrease (Underwood et al., 2006). There is thus a trade-off between sample size and the amount of data collected (Underwood et al., 2006). Although large sample sizes are advantageous for eliminating batch effects (Luo et al., 2010) and for determining significance through t-tests, smaller sample sizes used here still allowed for a comprehensive metabolic characterization of the samples. To illustrate the batch effect phenomenon, hexanoic acid (Figure 4.2) was used to show that the batch effect could not be removed by batch centering and scaling. This was due to an interaction between HIV status as well as the particular variable shown for the three batches (alternating increasing and decreasing profile during HIV infection). The presentation of score plots in batches is now common especially where stimuli have minimal effects on the metabolome (Ametaj et al., 2010; Redestig et al., 2009; Bijlsma et al., 2006; Hendrawati et al., 2006). Intermediate effects of the stimuli are thus compared to drastic effects when data is presented in this manner.



4.3.2 Profile of the experimental groups

General as well as clinical information of the participating donors is summarized in Tables 4.1 through to 4.3. We attempted to obtain all three biofluid types from the same individual but obtaining samples from individuals on a voluntary basis linked to the investigation of such a sensitive topic, like HIV, complicates sample collection. Consequently the biofluid (e.g. urine and/or blood) collected from individuals varied in this investigation. This resulted in slight differences in the CD4 count, viral load etc for those experimental groups who provided serum, PBMCs and urine. In some cases only one of the biofluid types was collected from a patient for one of many reasons (vein collapsing, blood flow stopped, patient not willing to supply urine etc). Overall more women than men voluntarily participated in this study. This concurs with the HIV infection pattern reported for sub-Saharan Africa where HIV incidence is generally higher in women (UNAIDS Report, 2002 and 2009). The age group primarily infected was within South Africa's working class population (25-65 years, <http://www.statssa.gov.za/keyindicators/keyindicators.asp>) and therefore holds numerous socio-economic implications for the country (i.e. early deaths, less productivity, less economic gain etc). The ages of the HIV- donors between batches and biofluid types were comparable. This was also observed for the HIV+ donors. The HIV- donors were generally younger than their infected counterparts since the group mainly comprised of students. Some demographic information for uninfected controls who donated urine was not documented by the healthcare provider/nurse collecting the sample and as such could not be documented here.

As explained in Section 2.6, the clinical course of HIV comprises of primary infection, asymptomatic infection, the symptomatic stage, AIDS and finally death. HIV+ individuals experiencing chronic infection were included in this study and fell within the WHO clinical stage 2 phase. Some individuals with low CD4 counts and high viral loads were retained in the experimental groups for reasons explained in Section 3.6. CD4 counts of the HIV+ cases between the batches and biofluid types were comparable (± 300 cells/ μ l blood) whereas viral load was not. The mean viral load between the batches and biofluid types ranged between 73 000– 2 300 000 RNA copies/ml plasma. Mean viral load measurements were therefore affected by the inclusion of individuals advancing to AIDS. These values can only be interpreted qualitatively, but suggests that the disease probably progressed to a somewhat more advanced stage for some individuals than others.

Table 4.1 **General and clinical information of the participating donors who provided serum**

| Serum Samples | | |
|--------------------------------------|------------------|------------------------------|
| | HIV Status | |
| Batch 1 | HIV- n=11 | HIV+ n=12 |
| % Females | 90.91 | 100.00 |
| % Males | 9.09 | 0.00 |
| Mean Age \pm SD | 31.9 \pm 12.62 | 33.4 \pm 7.86 |
| Mean CD count (cells/ μ l blood) | Not Determined | 376.5 |
| Viral Load (copies/ml, range) | Not Applicable | 381 542 (77 641-951 995) |
| Batch 2 | HIV- n=10 | HIV+ n=6 |
| % Females | 60.00 | 50.00 |
| % Males | 40.00 | 50.00 |
| Mean Age \pm SD | 25.9 \pm 7.29 | 34.8 \pm 8.93 |
| Mean CD count (cells/ μ l blood) | Not Determined | 342.2 |
| Viral Load (copies/ml, range) | Not Applicable | 72 740 (2328-178 260) |
| Batch 3 | HIV- n=14 | HIV+ n=9 |
| % Females | 42.86 | 55.56 |
| % Males | 57.14 | 44.44 |
| Mean Age \pm SD | 21 \pm 2.39 | 38 \pm 12.76 |
| Mean CD count (cells/ μ l blood) | Not Determined | 287.1 |
| Viral Load (copies/ml, range) | Not Applicable | 1 662 006 (32 665-8 841 057) |

Table 4.2 **General and clinical information of the participating donors who provided blood for the isolation of PBMCs**

| PBMC Lysates | | |
|--------------------------------------|------------------|---------------------------|
| | HIV Status | |
| Batch 1 | HIV- n=7 | HIV+ n=9 |
| % Females | 85.71 | 88.89 |
| % Males | 14.29 | 11.11 |
| Mean Age \pm SD | 31.1 \pm 13.93 | 32.2 \pm 11.09 |
| Mean CD count (cells/ μ l blood) | Not Determined | 295.9 |
| Viral Load (copies/ml, range) | Not Applicable | 86 154 (65 831 - 114 990) |

| Batch 2 | HIV- _{n=11} | HIV+ _{n=6} |
|--------------------------------|----------------------|------------------------------|
| % Females | 63.64 | 50.00 |
| % Males | 36.36 | 50.00 |
| Mean Age ± SD | 25.73 ± 6.94 | 34.8 ± 8.93 |
| Mean CD count (cells/μl blood) | Not Determined | 342.2 |
| Viral Load (copies/ml, range) | Not Applicable | 72 740 (2328-178 260) |
| Batch 3 | HIV- _{n=10} | HIV+ _{n=8} |
| % Females | 40.00 | 50.00 |
| % Males | 60.00 | 50.00 |
| Mean Age ± SD | 21.2 ± 2.82 | 39.5 ± 12.76 |
| Mean CD count (cells/μl blood) | Not Determined | 308.6 |
| Viral Load (copies/ml, range) | Not Applicable | 1 901 285 (32 665-8 841 057) |
| Batch 4 | HIV- _{n=10} | HIV+ _{n=10} |
| % Females | 70.00 | 60.00 |
| % Males | 30.00 | 40.00 |
| Mean Age ± SD | 25.4 ± 4.00 | 40.8 ± 8.93 |
| Mean CD count (cells/μl blood) | Not Determined | 347.8 |
| Viral Load (copies/ml, mean) | Not Applicable | 1 025 248 |

Table 4.3 General and clinical information of the participating donors who provided urine

| Urine Samples | | |
|----------------------------------|----------------------|-------------------------------|
| | HIV Status | |
| Batch 1 | HIV- _{n=16} | HIV+ _{n=16} |
| % Females | 56.25 | 62.50 |
| % Males | 43.75 | 37.50 |
| Mean Age ± SD | 22.5 ± 2.39 | 37.81 ± 11.73 |
| Mean CD 4 count (cells/μl blood) | Not Determined | 335.6 |
| Viral Load (copies/ml, range) | Not Applicable | 2 301 459 (32 665-10 000 000) |
| Batch 2 | HIV- _{n=11} | HIV+ _{n=12} |
| % Females | Not Available | 66.67 |
| % Males | Not Available | 33.33 |
| Mean Age ± SD | Not Available | 38.67 ± 10.27 |
| Mean CD 4 count (cells/μl blood) | Not Determined | 358.3 |
| Viral Load (copies/ml, range) | Not Applicable | 383 752 (50 288- 1 025 248) |

4.3.3 Data Generation

4.3.3.1 GC-MS analysis and total ion chromatograms (TICs)

The isolation of organic acids, derivatization and GC-MS analysis thereof was done according to the protocol standardized in the laboratory of [Reinecke et al \(2011\)](#). GC-MS analysis yielded chromatograms as outputs which were deconvoluted using the AMDIS software package. Representative TICs are displayed below to show differences in the metabolic profiles of HIV- and HIV+ biofluids (Figure 4.3) as well as differences between the respective biofluid types (Figure 4.3 through to Figure 4.5). In Figure 4.3 it is evident that serum has a complex metabolic profile and that there are differences in the organic acid pattern of HIV- (top) and HIV+ serum (bottom). For an example of an area where differences

were observed the reader is referred to the red rectangle in Figure 4.3. For the representative chromatograms shown, components as well as targets were identified (see red arrows in figure). The number of components represents the total number of peaks detected whereas targets represent the number of peaks identified based on the co-use of a library with AMDIS. Depending on the software, protocol and library used; the number of components and targets identified will vary. The chromatograms also reflect small and overlapping peaks which hampers visual comparison of the experimental groups.

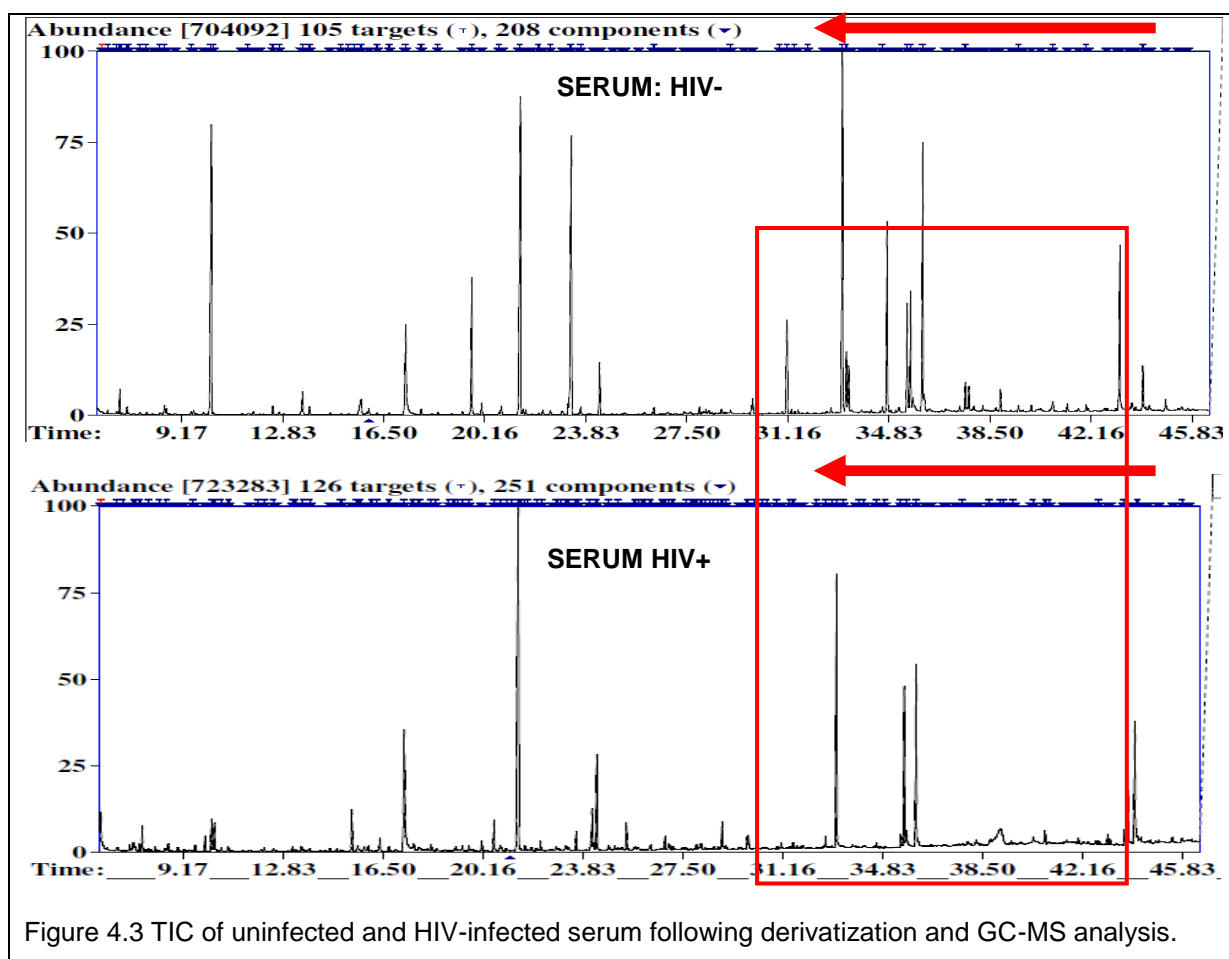
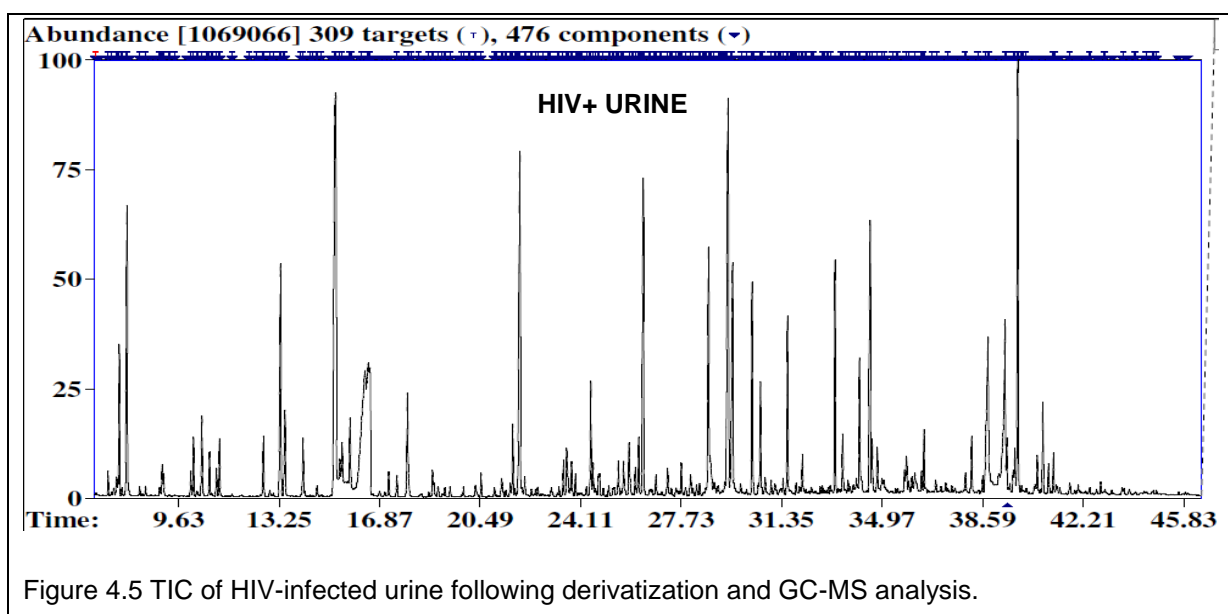
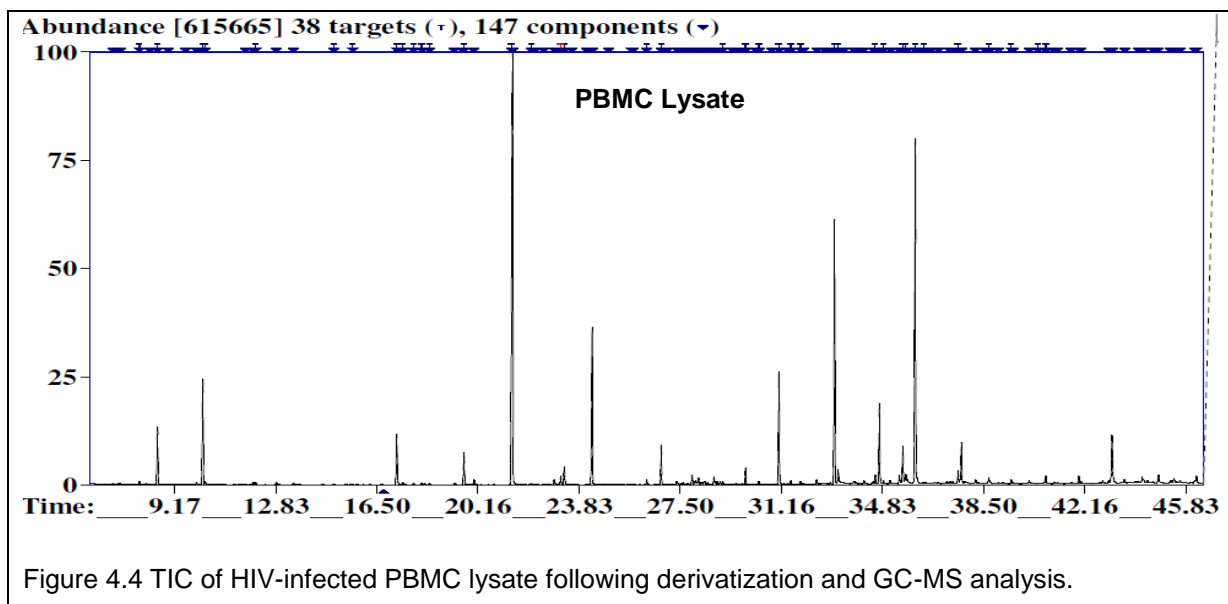


Figure 4.3 TIC of uninfected and HIV-infected serum following derivatization and GC-MS analysis.

Compared to serum (Figure 4.3), PBMC lysates (Figure 4.4) had fewer peaks and thus a less complex metabolic profile. This was observed for both uninfected and infected cells and so only a representative TIC is shown.

Urine presented with the most components (Figure 4.5) and was the most complex of the biofluid types analyzed in this study. This is in keeping with the estimated profile of 500 molecules expected from this biofluid (Jellum, 1981). According to Jellum (1981), different biofluids have the same metabolic profile but the molecules differ in concentration. Where concentrations are lower than the detection limit of the instrument peaks for a particular

metabolite may not be reflected. These biofluids can however differ in their response to stimuli yielding different metabolic profiles in turn. Differences in the complexity of serum, cells and urine were observed in Figure 4.3 through to Figure 4.5.



4.3.3.2 Comparison of Software Programmes

Although there were differences observed in the number and intensity of the peaks (Figure 4.3-4.5), chromatograms cannot be accurately distinguished by just using the naked eye especially if large numbers of samples are to be compared at once. For this reason, various software programmes capable of performing peak deconvolution, identification and alignment of the samples were used. To compliment the software, multivariate statistics for

the identification of significantly altered metabolites was also incorporated into the analysis. In this project three software packages were used for processing of the data.

Being the standard deconvolution package, AMDIS was used first together with an in-house library of organic acids (NWU) to facilitate peak identification. When performing an analysis the AMDIS interface (Figure 4.6) shows the TIC as well as the library and extracted spectra. Below the TIC, to the right, peaks are identified based on the inclusion of a library. This information, together with intensity values, retention time and m/z values are then converted into a report and exported to Microsoft Excel through the use of macros. Although AMDIS was able to identify peaks and quantify it relative to the internal standard (Table 4.4.), a large number of peaks remained unidentified, thus the question marks before the given names. For most of the metabolites detected the concentrations were low or listed as zero, implying that the metabolite was not present or that it was below the detection limit of the instrument. In addition to technical issues such as the detection limit contributing zero values; the occurrence of the missing values may also be representative of a true biological difference ([Hrydziuszko and Viant 2011](#)). When an individual is healthy, MS analysis of the biofluid may show some metabolites to be present at low concentrations. During infection and disease, metabolites are further diluted by the increase of other metabolites, driving the metabolites which are at a low concentration to undetectable concentrations ([Pendyala et al., 2009](#)).

Table 4.4 is representative of data from one sample which was analyzed using AMDIS and the in-house library. To enable a comparison to be made between a number of samples; “R” (a statistical computing programme) was used to align the datasets and found to yield unfavourable outputs. When “R” was used more missing values were introduced into the data matrices for those cases where a particular metabolite was absent. The programme subsequently filled these missing values with zeros resulting in an increase in the size of the data matrix (Table 4.5). In a recent publication by [Hrydziuszko and Viant \(2011\)](#) the nature of missing values was investigated as these are usually neglected in metabonomics data processing steps. Researchers handle these missing values differently by either omitting those variables which contain missing values; they use statistical analysis capable of handling missing values or make use of algorithms to estimate a value in place of the zero. All of these methods ultimately affect statistical outcomes. In cases where sample numbers are limited, removal of missing values contributes to lowering the power of subsequent statistical tests applied ([Hrydziuszko and Viant 2011](#)). There is also bias in that high intensity peaks are kept in the dataset (missing values are usually associated with low peak intensities). [Hrydziuszko and Viant \(2011\)](#) found that missing values were not random and that it does contain biological information. Depending on how the occurrence of missing

values is addressed (disregarded, algorithms etc) this will largely affect multivariate analysis. Because the incorporation of zeros runs the risk that the distribution of the variables are no longer accurately described (Behrends et al., 2011), further statistical processing of data obtained through AMDIS and the in-house library as well as “R” was not carried out.

An automated software programme, SpectConnect, was then used. This web-based programme was found to have a user-friendly interface (Figure 4.7). This software primarily identifies “real” peaks but there is no guarantee that the “real” peak, upon identification, will be of endogenous origin. Upon uploading the in-house and NIST libraries respectively with the data files, majority of the conserved metabolites that were identified were found to be of exogenous origin. Since part of the aim of this work was to relate the classification of the experimental groups back to metabolic aberrations, the detection of a large number of exogenous molecules would mean that it would be difficult to link the metabolites back to an enzymatic/biochemical pathway. Analysis of the deconvoluted chromatograms with SpectConnect showed that the final ion abundances for some metabolites differed after comparison to ion abundances measured with AMDIS. In some cases SpectConnect would not have ion abundances for a sample that AMDIS had a value for. Based on this, additional software was tried.

The co-use of AMDIS and MET-IDEA (for deconvoluting, aligning and extracting ion abundances from peaks) yielded better quality data when compared to matrices derived from AMDIS and the in-house library i.e. there were less zeros in the data matrices leading to a better distribution of the variables (Table 4.6). Zeros are usually assigned when a metabolite might be present, but below the detection limit of the instrument, when the software fails to detect peaks, when features do not match tolerance criteria set by the user and when improper deconvolution occurs (Almstetter et al., 2009; Bijlsma et al., 2006). Missing values are also as a result of true biological differences (Hrydziuszko and Viant 2011). Compared to SpectConnect which only detects conserved metabolites, MET-IDEA detected all possible metabolites based on the reference file which was used as the IRT input list. The use of a reference file is a limiting factor during MET-IDEA analysis in that the analysis is largely based on the detected metabolites contained in this particular sample.

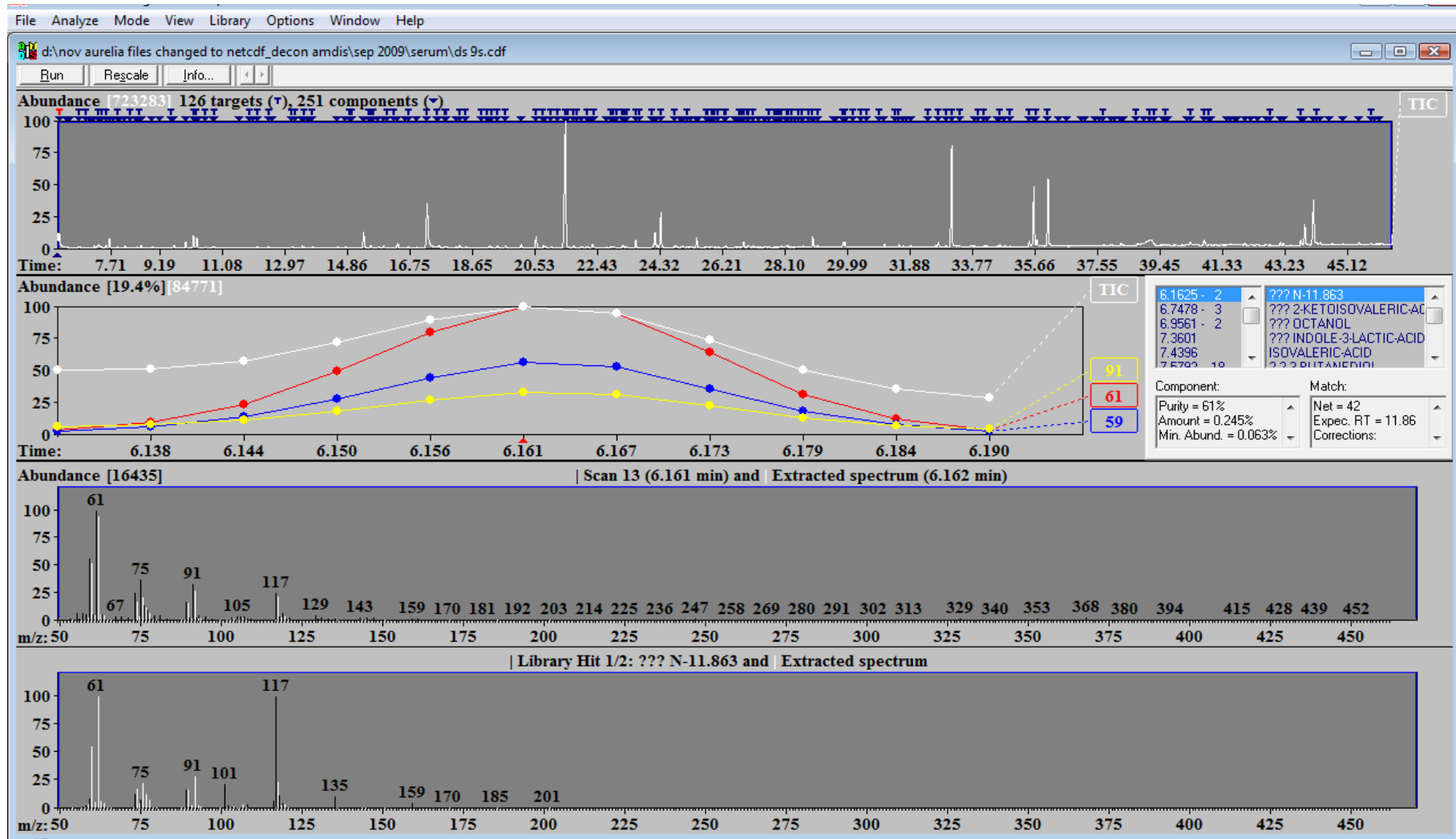


Figure 4.6 AMDIS user interface.

Table 4.4 Table showing a representative output obtained from AMDIS linked to an in-house library. Not all the identified molecules are shown, only a small insert to highlight disadvantages associated with the use of AMDIS

| D:\NOV AURELIA FILES CHANGED TO NETCDF DECON AMDIS\SEP | | | |
|--|-----------|-----------|-------|
| Name | Retention | Area | Conc. |
| ??? N-11.863 | 6.16 | 24457861 | 1.84 |
| ??? 2-KETOISOVALERIC-ACID-MO | 6.75 | 6043348 | 0.45 |
| ??? OCTANOL | 6.96 | 891710 | 0.07 |
| ??? INDOLE-3-LACTIC-ACID | 7.36 | 19815227 | 1.49 |
| ISOVALERIC-ACID | 7.44 | 1755540 | 0.13 |
| ? 2,3-BUTANEDIOL | 7.58 | 7815184 | 0.59 |
| ??? SHIKIMIC-ACID | 7.59 | 157102 | 0.01 |
| 1,2-DIHYDROXYETHANE | 7.92 | 2449056 | 0.18 |
| ??? METHYLCROTONIC-ACID | 8.30 | 343002 | 0.03 |
| BORIC-ACID | 8.57 | 880984 | 0.07 |
| PHENOL | 9.54 | 218493 | 0.02 |
| LACTIC-ACID | 10.22 | 41303823 | 3.10 |
| ? DECAMETHYLTETRASILOXANE | 10.29 | 139636 | 0.01 |
| CAPROIC-ACID | 10.32 | 34041937 | 2.56 |
| GLYCOLIC-ACID | 10.58 | 4203397 | 0.32 |
| ?? OCTAMETHYLTRISILOXANE | 10.85 | 2807740 | 0.21 |
| 3-HYDROXYPYRIDINE | 11.92 | 618861 | 0.05 |
| OXALIC-ACID | 12.15 | 4690495 | 0.35 |
| 2-HYDROXYBUTYRIC-ACID | 12.49 | 3797420 | 0.29 |
| ?? 3-METHYLVALERIC-ACID | 13.19 | 6633249 | 0.50 |
| ? 2,3-DIHYDROXYBUTANE | 13.29 | 2372250 | 0.18 |
| 3-HYDROXYISOBUTYRIC-ACID | 13.57 | 2020817 | 0.15 |
| 2-HYDROXY-ISO-VALERIC-ACID | 13.82 | 3193681 | 0.24 |
| DODECAMETHYLPENTASILOXANE | 14.96 | 2175230 | 0.16 |
| ??? 3-METHYL-2-HEXENOIC-ACID | 15.03 | 1150470 | 0.09 |
| ??? HEPTANOIC-ACID | 15.53 | 680176 | 0.05 |
| PYRUVIC-ACID | 15.58 | 6855866 | 0.52 |
| BENZOIC-ACID | 15.68 | 1196111 | 0.09 |
| ? RIBOSE | 16.09 | 699295 | 0.05 |
| ??? METHYLMALONIC-ACID | 16.30 | 663755 | 0.05 |
| OCTANOIC-ACID | 16.72 | 5866123 | 0.44 |
| PHOSPHORIC-ACID | 17.28 | 247794012 | 18.62 |
| 2-HYDROXYHEXANOIC-ACID | 17.60 | 1552158 | 0.12 |
| ??? MALEIC-ACID | 17.83 | 5123187 | 0.38 |
| GLYCEROL | 17.88 | 1929674 | 0.14 |
| SUCCINIC-ACID | 18.27 | 7952502 | 0.60 |
| 1,2-DIHYDROXYBENZENE | 18.45 | 1525910 | 0.11 |
| URACIL | 18.92 | 1271111 | 0.10 |
| ??? ITACONIC-ACID | 19.11 | 441337 | 0.03 |
| ??? CITRACONIC-ACID | 19.25 | 1964931 | 0.15 |
| FUMARIC-ACID | 19.42 | 6920266 | 0.52 |
| NONANOIC-ACID | 19.66 | 7003535 | 0.53 |
| ??? 3-HYDROXYADIPYLLACTONE | 20.59 | 43200623 | 3.25 |
| ? GLUTARIC-ACID | 20.81 | 5188442 | 0.39 |
| ?? 3-HYDROXYPYRUVIC-ACID | 21.04 | 1783272 | 0.13 |
| ??? 4-KETOVALERIC-ACID | 21.22 | 1041704 | 0.08 |
| ? PHENYLBUTYRIC-ACID | 21.47 | 503544028 | 37.83 |
| 3-PHENYLBUTYRIC-ACID-(IS) | 21.48 | 698839598 | 52.50 |
| ??? DIHYDROCODEINE | 21.55 | 1093133 | 0.08 |

Welcome to SpectConnect!
Analyze your GC/MS data in three steps...

Step 1: Extract putative components using AMDIS
[Click here to show more detailed instructions on how to use AMDIS.](#)

Step 2: Use SpectConnect to find conserved components and biomarkers
Fill in the fields below and click the submit button.

Number of conditions: 1 Maximum number of samples per condition: 3

Condition 1 name: CONDITION1

.ELU file #1:

.ELU file #2:

.ELU file #3:

.MSL library file for target identifications (optional):

Email address to receive notification/results (optional):

Step 3: Analyze the results that you receive from SpectConnect.
Click [here](#) for some tips on interpreting the results that SpectConnect gives you.

Figure 4.7 SpectConnect Online User Interface.

If an additional metabolite was present in another sample which was not chosen as the reference input, the metabolite is automatically excluded from the analysis. This ultimately means that some metabolites will always be omitted or lost from the analysis. Since many researchers have recognized this limitation the concept of quality control (QC) samples has been introduced to avoid the loss of metabolites following alignment of samples with software programmes such as MET-IDEA. QC samples comprise of a pool of all samples used in the study (Bijlsma et al., 2006) or a pool of representative samples from each condition being investigated (e.g. HIV- and HIV+; Dunn et al., 2011). QC samples assists with conditioning the instrument, combining batches of sample (batch correction) and enables one to analyze the variability between batches of sample. Data quality is assessed through the use of QC samples which also corrects for drifts in signal and retention time. As mentioned previously, the mass spectrometric analysis of organic acids in HIV-infected biofluid has not been done previously and was planned as per the standard protocols of the Centre for Human Metabonomics at the North-West University (Potchefstroom). Analysis of organic acids at this Centre is done primarily through AMDIS and an in-house library. Problems such as metabolite loss after choosing a reference sample for alignment during MET-IDEA analysis was thus not envisioned and QC samples not prepared. Limited starting sample meant that these pooled samples could also not be prepared afterward. Obtaining blood from the same individual at a later stage was also impractical. In cases where pooled

samples are not prepared, a reference sample having the most components/metabolites is usually recommended as an alternative which was the route chosen for the data presented in this thesis.

4.3.4 Data pre-processing

4.3.4.1 Number of detected features

In the case of the sera samples; the co-use of AMDIS and MET-IDEA yielded 282 components for batch one, 237 for batch two and 104 for batch three. This is in the range of an anticipated 300 organic acids or related compounds believed to be amongst the total set of 4229 compounds recently reported for the human serum metabolome ([Psychogios et al., 2011](#)). In the case of the cell lysates; AMDIS/MET-IDEA yielded 160 components for batch one, 147 for batch two, 81 for batch three and 253 for batch four. For the urine samples, batch one and two had 476 and 274 components respectively.

4.3.4.2 Manual curation

Because the deconvolution process leads to errors i.e. a single compound peak is detected as two separate peaks ([Xu et al., 2009](#)) an inspection for deconvolution errors was done and manual curation of duplicated peaks performed. The three batches of sera therefore remained with 242, 209 and 92 features whilst the four batches of cell lysate remained with 140, 118, 65 and 209 features respectively. Manual curation reduced the features detected in urine to 395 and 234 respectively. These numbers are considerably smaller than the 3687 compounds identified in the global metabolomics analysis of CSF from SIV+ rhesus macaques ([Wikoff et al., 2008](#)) but in line with the selective metabolome analysis presented here.

Fewer variables were identified for batch three of the serum and cells as well as one batch of urine sample. There are a number of reasons why such a result would be obtained. Firstly, it may be that the batch of samples had less of the metabolites to begin with. Slight changes in the extraction procedure (for example the temperature of laboratory) may have contributed to the result obtained. Finally, a technical problem with the instrument may have occurred during the analysis. [Psychogios et al \(2011\)](#) suggests differences in the detection and concentration ($\pm 50\%$ variation) of metabolites to: contaminants, volatility/stability of metabolite, sample collection and preservation effects, small sample sizes, technical problems with separation and/or extraction, age, gender, genetic background, health status etc. Variable selection (Section 4.3.4.4 below) finally resulted in the number of endogenous variables to be comparable between the batches. These batches were therefore not excluded from further analysis.

4.3.4.3 Quality of the extraction and analysis procedure

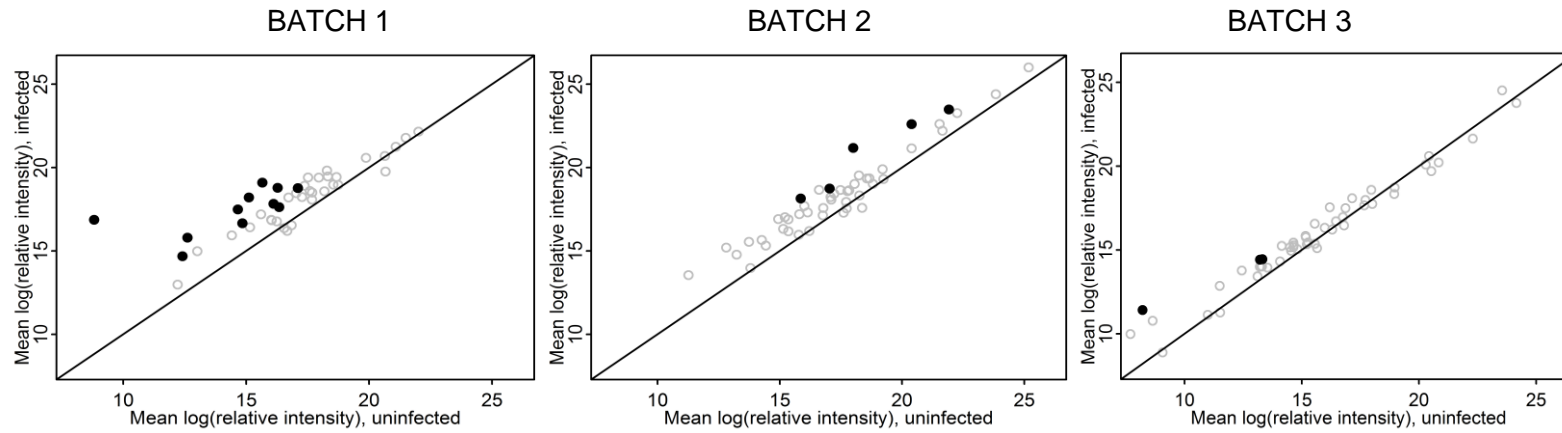
There is a degree of variability associated with GC-MS analysis (Dunn et al., 2011) making it impossible to get high reproducibility and accuracy for all metabolites detected. A RSD or coefficient of variation (CV) within 30 % is usually accepted in metabonomics investigations due to the variability associated with chemical derivatization commonly employed during GC-MS. For the change in the intensity signal of the internal standard the calculated RSD was within the 30 % range for serum and urine but very large in the case of the cells (> 50 %). The larger degree of metabolic heterogeneity observed for cell-based samples is similar to that observed by Hrydziuszko and Viant (2011). Precision is compromised by pipetting errors, nonreproducible extraction and derivatization (Jiye et al., 2005). PBMCs comprise of a mixture of immune cells (Schulze-Bergkamen et al., 2005). The different cell subsets within the PBMC mixture may therefore have contributed to the metabolic profile measured. High RSDs calculated for the cell extracts may also be because of the use of a standard cell concentration during the extraction of metabolites. In the analysis of Munger et al (2006) normalization of the metabolite signals was done relative to the protein content of the cells instead of cell concentration. This was because HCMV-infected cells increased 2-fold in a volume. In the current work presented, the extraction of metabolites was done immediately after cell isolation thus there was no time for cell numbers to increase drastically. Extraction was therefore performed for a fixed concentration of cells each time but improved precisions by standardizing extractions according to protein content may provide clarity on this aspect.

4.3.4.4 Variable selection

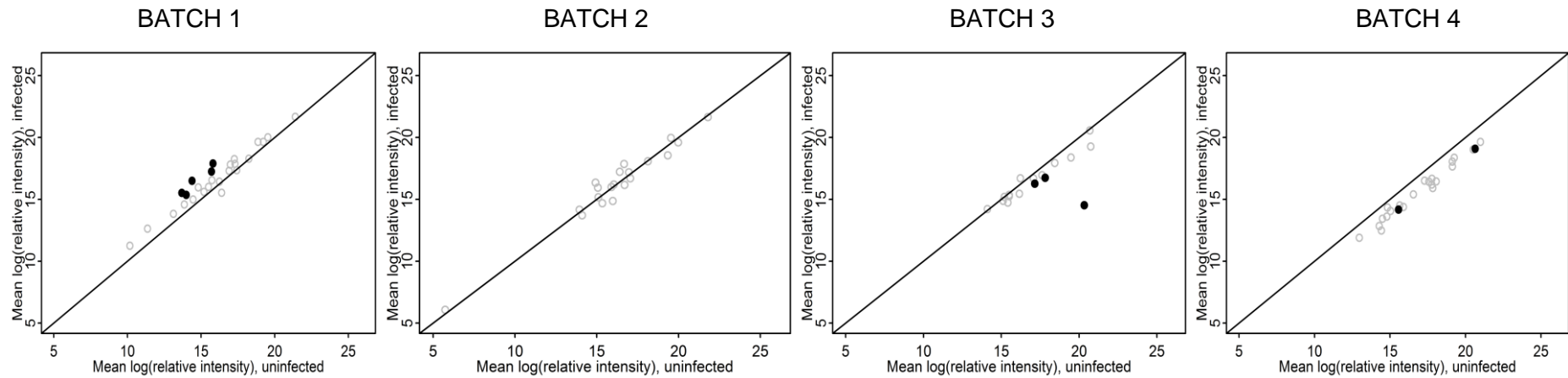
Following manual curation and removal of the internal standard from the data matrix classification of the features as endogenous, exogenous and unclassified further reduced the size of the data matrices. Batch 1-3 of the sera remained with 43, 49, and 52 of the endogenous molecules respectively whilst batch 1-4 of the cell lysates remained with 27, 20, 17 and 23 endogenous molecules respectively. The two batches of urine samples remained with 128 and 86 molecules respectively. Although the data presented in this thesis is representative of endogenous molecules, it is recognized that the metabolic profile may change should the unclassified molecules be included as part of the analysis or later be assigned biochemical functions. The final outcome was matrices for each batch of samples analyzed, each comprising controls and HIV+ cases, and a list of known compounds (variables/metabolites) expressed as numerical values for the respective model ions. These matrices were regarded as suitable for identifying indicators/markers that were differentially present in the HIV+ individuals and uninfected controls. Scatter plots of the mean log

(relative intensity) of all identified endogenous metabolites for the respective batches is shown in Figure 4.8 a-c. Most of the identified organic acids increased in relative intensity in the HIV+ cases compared to the controls. A similar observation was made by [Wikoff et al \(2008\)](#) for features which had a p value less than or equal to 0.01, indicating that most of the metabolite concentrations increased significantly during SIV-induced encephalitis as well.

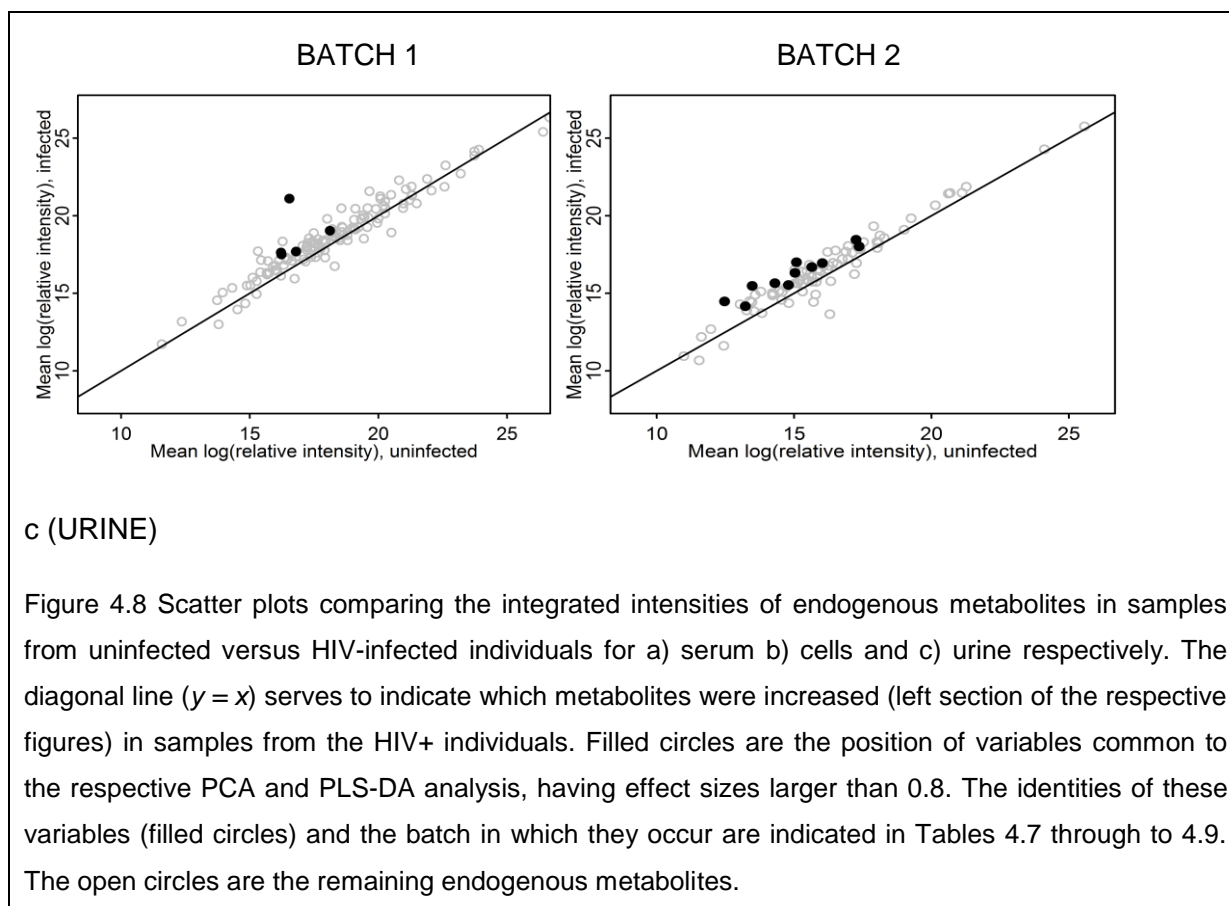
For batch three and four of the cell extracts; nonanoic, lauric and pyroglutamic acid levels as well as elaidic acid and tyramine levels decreased (filled circles in Figure 4.8b, two plots to the right). The interpretation of these metabolites in context to HIV are elaborated on in Section 4.3.6.



a (SERA)



b (CELLS)



4.3.5 Statistical Analysis

4.3.5.1 Classification of experimental groups (PCA and PLS-DA)

Sera: The outcome of the multivariate analysis for **sera** is shown in Figure 4.9a. The unsupervised PCA indicated sufficient variation between the experimental groups from batch one to achieve a complete separation between data from controls and HIV individuals (Figure 4.9a, left panel). Such a separation was not observed for batch two and three respectively (plot in the middle and to the right). These observations suggest the possibility that individuals from batch one were at a more advanced stage of disease than those represented by batch two (this is also supported by viremia data, Table 4.1). [Slama et al \(2009\)](#) documented viral load to be a risk factor for the development of metabolic complications. Batch three included individuals with an even higher viral load than batch one but a complete separation was not observed and may be explained by the low number of metabolites initially detected for this batch. Endogenous metabolites influenced by viral load and having the potential to contribute to the separation profile were therefore less since a low number of metabolites were extracted for this batch to begin with. For each of the batches a list of variables (representing compounds) and their respective PCA modelling power as well as PLS-DA VIP values were used to identify important variables contributing

to the organic acid profiles of the experimental groups. Classification profiles were finally only interpreted from PCA plots whilst both PCA modelling power and PLS-DA VIPs were used together with ES determinations to extract biologically relevant metabolites.

Cells: Multivariate analysis of the **PBMC lysates** (Figure 4.10a) showed a similar trend to that of the sera where samples having higher viral loads caused improved separation profiles to be observed (viral load for batch 3>batch 4>batch 1>batch 2). Although more metabolites can be extracted from a higher concentration of PBMCs a concentration of 5×10^6 cells/ml was utilized here so that cells needed for complementary assays (e.g. apoptosis) would be left over. Limited biological material thus determined the maximum cell concentration that could be used for MS extractions in this study. The possibility of extracting more metabolites and biological information from a higher cell concentration is noted.

Urine (Figure 4.11a) showed a different trend to the sera and cell extracts with viral load not contributing much to the separation of the groups (viral load for b1>b2). Sera and cells utilized for the extraction of organic acids was derived from blood. Blood and urine do not necessarily make use of the same substrate during enzymatic reactions. As a result, metabolic changes in these biofluids do not occur to the same extent when a particular defect occurs ([Mazat et al., 2001](#)). That the urine profiles show more overlap than separation implies that sera and cells are more sensitive to the effects of HIV infection and the subsequent enzymatic reactions which alter the organic acid profile. According to [Mazat et al \(2001\)](#), there is a threshold at which metabolic flux is affected. Only once this threshold is reached do phenotypic changes become visible. This threshold seems to have been reached for the metabolome of sera and cells but not urine.

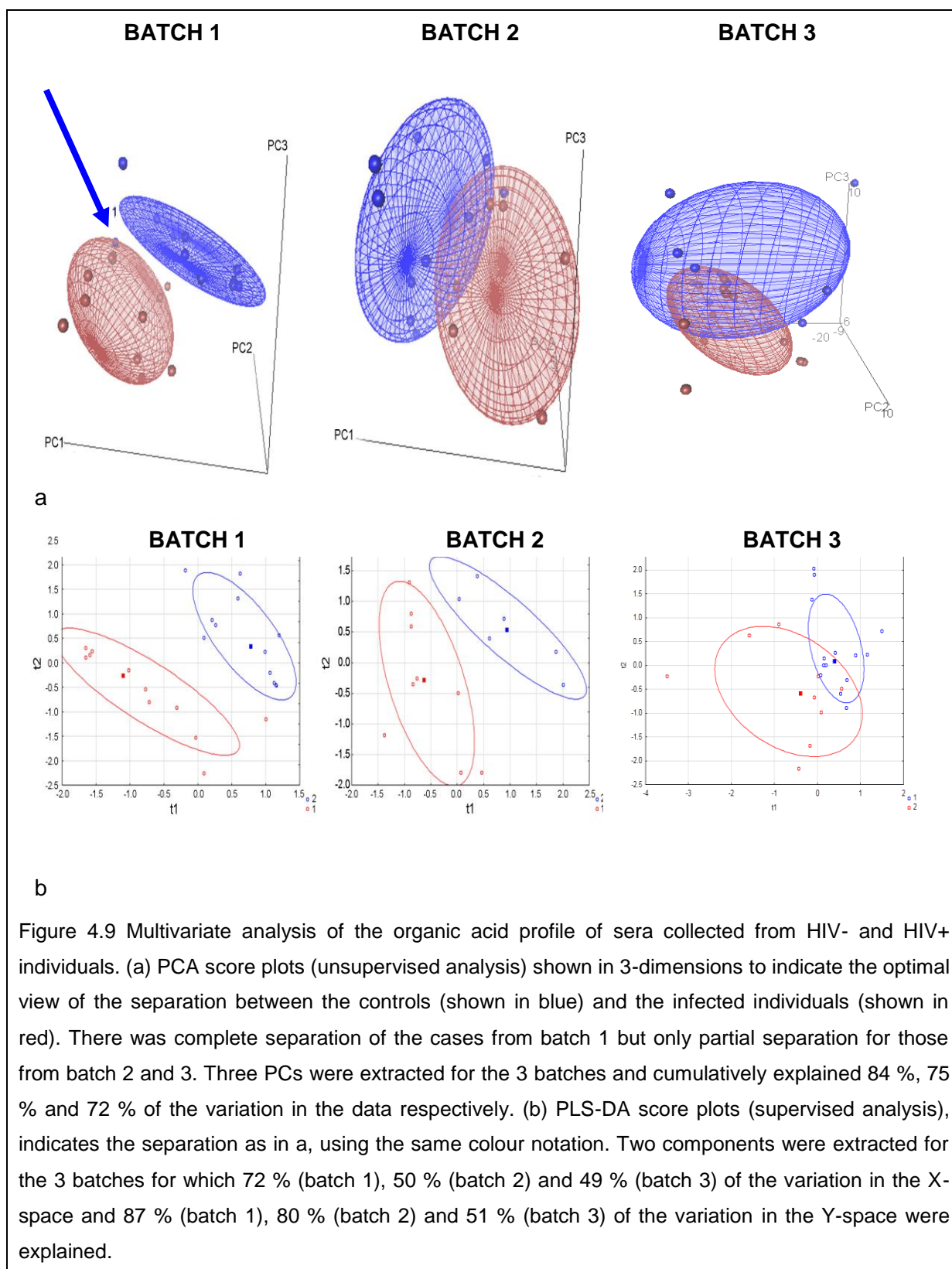
The improved separation profiles obtained with higher viral loads in sera and cells (Figure 4.9a-4.10a) as well as the alternating increase and decrease of molecules as noted in Figure 4.2 despite matched CD4 counts, implies that viral load which was not part of the experimental design may be responsible for the nature and magnitude of the metabolic response observed. The metabolic profiles measured for sera and cells was bias in terms of viral load yielding clearer separation profiles where samples with higher viral loads were included. For those individuals where the organic acid profile of controls and HIV-infected individuals overlapped, in addition to being metabolically similar as a result of the clinically stable condition of the HIV patients, the overlap can also be explained by the sensitivity of MS. This technique can detect minor changes in metabolite profiles not necessarily detected by instruments with a lower sensitivity (e.g. NMR) that usually shows clear defined separations ([Philippeos et al., 2009](#)). In addition, during asymptomatic infection, 1 CD4 cell

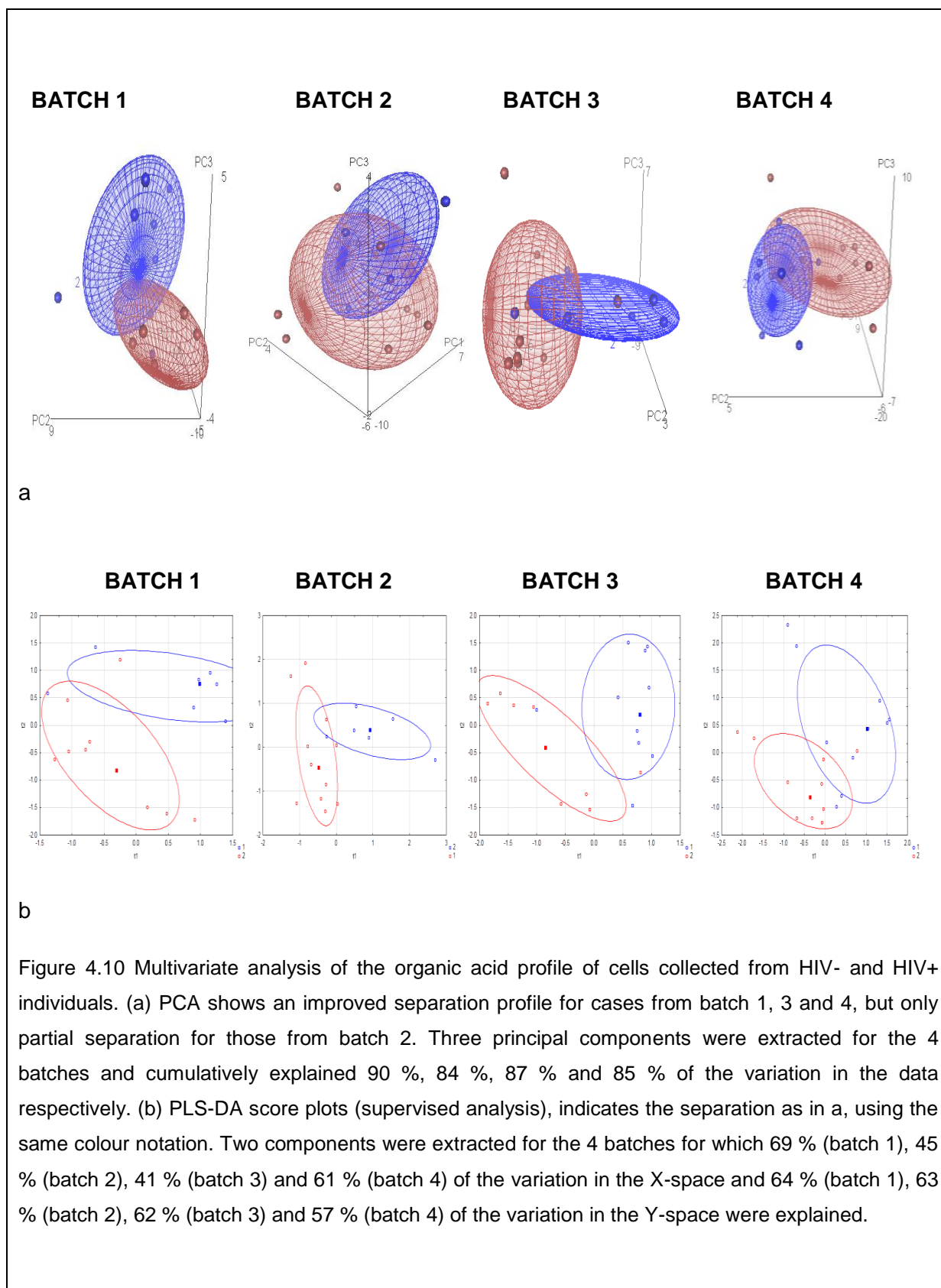
in 50 000 PBMCs are infected (Weber, 2001). This implies that in 5 million cells as used here, approximately 100 CD4 cells are potentially infected. According to Rosenberg and Fauci (1991) a small percentage of PBMCs actively express HIV i.e. virus is found in 1 in 10 000 - 1 in 100 000 PBMCs. The greater percentage of PBMCs not infected therefore masks the “HIV stress” in the minority of infected cells thus obscuring the difference in metabolic profile between the two groups and yielding overlapping profiles. PBMCs comprise of a mixture of immune cells (Schulze-Bergkamen et al., 2005). Each of the cell subsets within the PBMC mixture may be affected to a different degree by HIV and impacted on the metabolic profile measured.

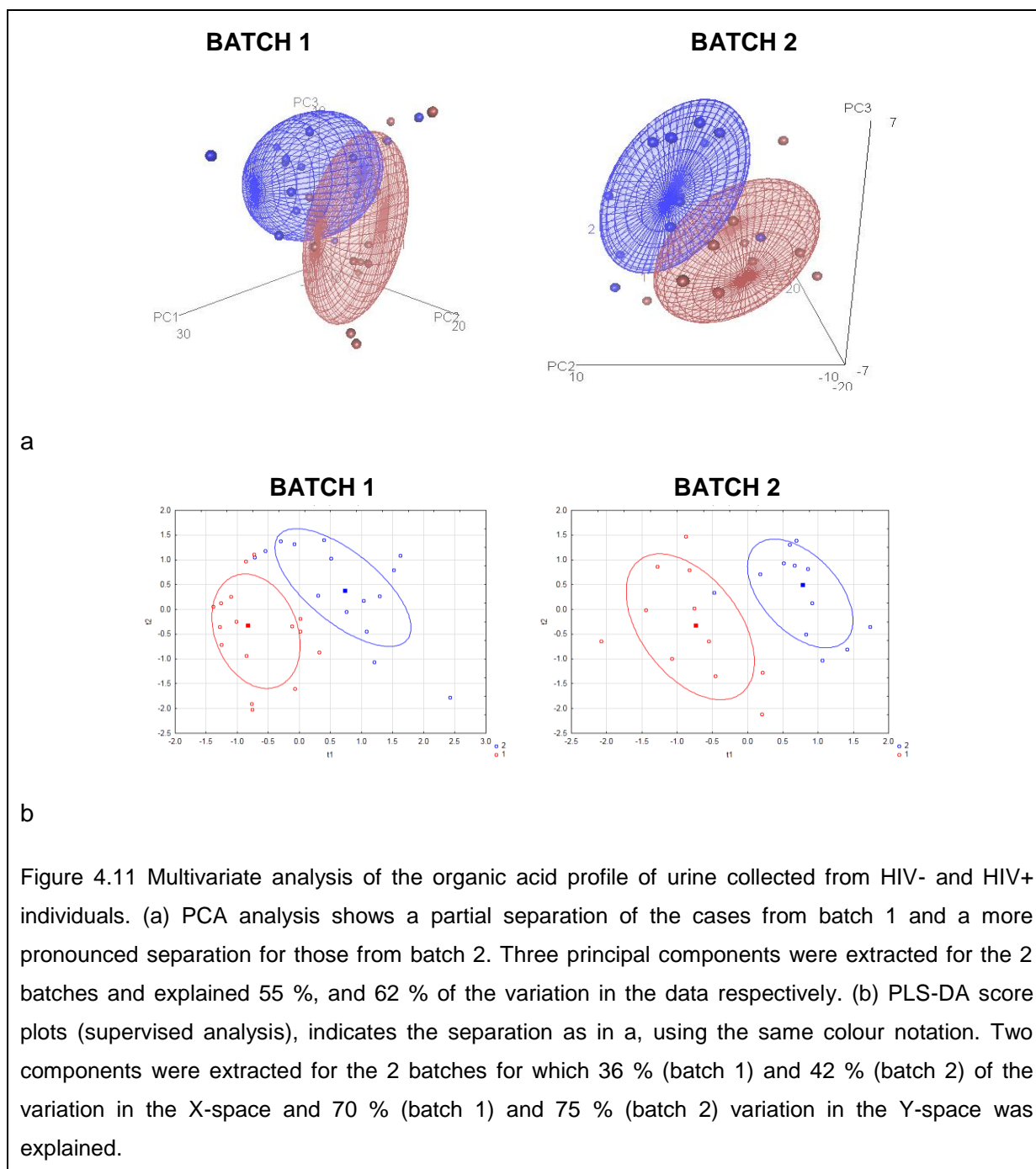
An interesting observation was made for one of the uninfected control samples following PCA analysis (blue arrow in Figure 4.9a, batch 1). This sample clustered with the HIV+ samples and is unique in that antibody testing for HIV has repeatedly (> 3 years) shown the individual to be HIV- even after repeated exposure to the virus through sexual contact with her HIV seropositive partner. Confirmation of the patient’s HIV- status was also done in our laboratory using a rapid test. At this time it can only be speculated that exposure to HIV without the development of infection and/or disease may also trigger metabolic changes. This is not impossible since SIV-infected monkeys have previously been shown to present with metabolic changes even before seroconversion (Eck et al., 1991). In the latter study virus was however detected even though seroconversion had not taken place. In support of the speculation made, Rowland-Jones et al (1995) as well as Clerici and Shearer (1993) reported on immunological changes in HIV-exposed but uninfected individuals. Exposure-induced metabolic changes are thus not impossible and would therefore require further investigation. The use of systems biology approaches in facilitating our understanding of HIV-induced changes in exposed individuals was recently reviewed (Burgener et al., 2010). The finding obtained is therefore important as it suggests metabonomics (which forms part of the systems biology approach) to be sensitive enough to detect metabolic changes following exposure.

The results in Figure 4.9b to Figure 4.11b shows an improved separation of the HIV- and HIV+ cases for all batches of samples analyzed. A larger percentage of the variance is explained in the Y-space. The profiles observed in Figures 4.9 through to 4.11 suggests a degree of heterogeneity within the classes (HIV- and HIV+) despite the fact that individuals having moderate to high CD4 counts were chosen. This criterion may not have been strict enough resulting in a CD4 count range that was still too broad for such a targeted analysis. Viral load was not part of the selection criteria but where this parameter was known and found to vary; organic acid profiles were found to be largely affected. Stricter criteria set on CD4 counts, viral load and other demographic particulars is expected to give a better

indication of the metabolic profiles during the different stages of infection but may limit sample numbers as groups with these characteristics may be difficult to source.







4.3.5.2 Identification of Molecules Affected by HIV Infection

➤ Venn diagram of common metabolites in PCA VIP, PLS-DA VIP and ES lists

To identify molecules affected by HIV within each batch of the respective biofluid types, all variables in the first half of the PCA and PLS-DA lists, having an ES value greater than 0.8 were selected. From this analysis Venn diagrams were constructed to show metabolites that were common between the three statistical methods (PCA VIP, PLS-DA VIP and ES) used for metabolite selection. Batch 1-3 for the sera had 12, 7 and 3 metabolites (Figure 4.12). Venn diagrams showing similar information for PBMC lysates and urine are shown in

the Appendix (Figure A1-A2). Standard t-tests and the nonparametric Mann-Whitney U-tests were used to test the equality of group means and medians of significantly altered metabolites. Only those metabolites that showed statistically significant values and which were present in human metabolome databases were finally taken into account to characterize HIV-induced mitochondrial dysfunction through organic acid profiling. Seventeen significant metabolites were finally identified from sera while 10 and 15 significant metabolites were identified from the cell lysates and urine respectively. The molecules were categorized according to biological function and interpreted in context to HIV. The identified metabolites are listed in Tables 4.7-4.9 and shows analytical information (feature names, chemical names and molecular formulae) as well as statistical information for each metabolite. Areas in the tables which are shaded in red highlights those molecules for which no particular association to HIV could be made. A representative spectrum of one metabolite from each biofluid type is shown in Figure 4.13 a-c.

4.3.6 Interpretation of the identified metabolites

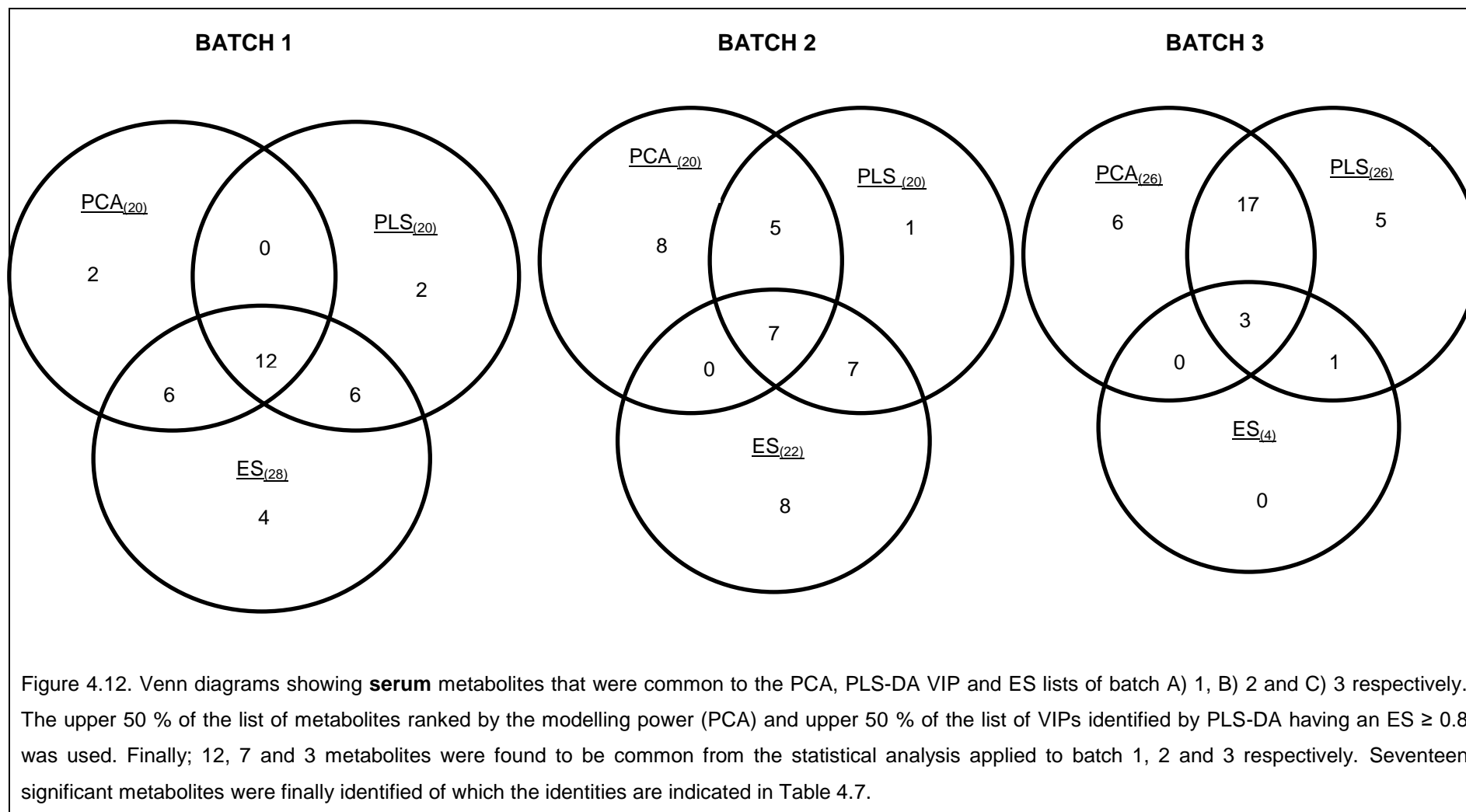
The overall objective of this part of the thesis was to apply GC-MS metabonomics to the analysis of organic acid profiles in biofluid of asymptomatic HIV-infected individuals. The use of deconvolution and alignment software packages as well as statistical analysis facilitated with reaching this objective. Various metabolites altered as a result of HIV infection was identified (Table 4.7-4.9) and classified into the following categories:

- [i] Markers of mitochondrial dysfunction
- [ii] Fatty acids, other lipids and metabolites involved in lipid metabolism
- [iii] Neurological/Oxidative stress metabolites
- [iv] Other metabolites of the human metabolome database.

The role of these molecules in HIV infection is elaborated on below.

[i] Markers of mitochondrial dysfunction

Several metabolic parameters associated with mitochondrial dysfunction were identified and included succinic, fumaric, adipic and suberic acid. Of these metabolites succinic acid was a common metabolite between serum and cell lysate. Succinic and fumaric acid are intermediates of the Krebs cycle. [Barshop \(2004\)](#) noted increased urinary fumaric and malic acid in a metabolomics investigation of patients with mitochondrial disorders. In a recent investigation [Reinecke et al \(2011\)](#) found succinic, fumaric and malic acids to be elevated in the urine of patients suffering from a dysfunctional mitochondrial respiratory chain. The order



of significance of elevated tricarboxylic acid cycle metabolites in the study of these authors was found to be succinate > fumarate > malate. The respiratory chain is important for energy production. Any defect therefore impacts several metabolic and other biochemical processes (Reinecke et al., 2009). Components of the respiratory chain have also been shown to be impaired during HIV infection (Ladha et al., 2005; Ricci et al., 2004). The fumarate intermediate (salt/ester of fumaric acid) in the Krebs cycle is generally used by cells to produce ATP from food sources. Elevated levels of succinic and fumaric acid indicate impaired functioning of the Krebs cycle and an increased demand for energy by infected cells. The increase in resting energy expenditure and therefore oxygen consumption as measured in clinically stable patients (Lane and Provost-Craig, 2000; Hommes et al., 1990) is supportive of this demand for energy. The majority of the body's energy is obtained from oxidation/catabolic processes. Adipic and suberic acid was significantly higher in HIV-infected individuals than in controls due to disrupted mitochondrial function which in turn limits (β)-oxidation of fatty acids causing these molecules to accumulate through the (ω)-oxidation pathway instead. Impaired β -fatty acid oxidation contributes to ATP depletion which is compensated for by the alternative oxidative ω pathways and an increase in Krebs cycle intermediates such as fumarate. Reduced ATP production by mitochondria triggers glycolysis (Hofhaus et al., 1996) where glucose is converted to pyruvate and the released energy is used to form ATP. The increase in these Krebs cycle intermediates may also be due to an impaired flow of electrons past coenzyme Q10 (CoQ10) which subsequently lowers electrotransfer flavoprotein but increases electrotransfer flavoprotein ubiquinone oxidoreductase activity. Fibroblasts infected with HCMV were shown to contain an increase in metabolites involved in glycolysis, the Krebs cycle and pyrimidine synthesis (Munger et al., 2006). It appears that the activation of processes such as glycolysis is a normal feature associated with viral infection. An increase and decrease in glycolytic intermediates was detected following the *in vitro* infection of primary CD4 cells and a macrophage cell line with HIV, respectively (Hollenbaugh et al., 2011).

Increased cerebral metabolic rates for glucose in the brains of asymptomatic HIV+ patients were reported early on in AIDS research (Pascal et al., 1991). Insulin resistance (IR) which leads to type 2 diabetes mellitus is a metabolic complication associated with HIV infection and more especially with the use of HAART (Rao et al., 2010; Jevtović, 2009). HIV infection as well as the therapeutics used to treat it therefore disrupts glucose/sugar metabolism (Dubé, 2000). An association between elevated urinary adipic and suberic acid as well as diabetes (Niwa et al., 1981) and glutaric aciduria type 1 exists. Metabolic stress (induced by HIV in this case) is also associated with a decrease in glucose levels (Schulze-Bergkamen et al., 2005). Elevated levels of adipic acid could just be an indication of the

development of a sugar disorder in infected patients. In the study of [Reinecke et al \(2011\)](#) adipic and suberic acid were included as indicators of complex I deficiency in patients with clinical defects of the respiratory chain. It would appear that deficiencies in mitochondrial enzymes are reflected in the increased profile of these related markers in HIV+ individuals.

[ii] Fatty acids, other lipids and metabolites involved in lipid metabolism

Alterations in fatty acid metabolism were noticed and described in the early phases of AIDS research. Hypertriglyceridemia was reported as early as 1989 ([Grunfeld et al., 1989](#)) while an increase in free fatty acids was reported in the early nineties ([Grunfeld et al., 1992](#)). In the data presented in this thesis an increased number of fatty acids and/or metabolites which partake in lipid metabolism were found to be altered following HIV infection (Table 4.7-4.9). Azelaic acid (a second metabolite found to be common between sera and cell lysates) as well as capric acid is characterized as endogenous metabolites but can also have exogenous origins. Immunocompromised patients are burdened with opportunistic infections (OIs) such as *Candida albicans* (yeast infection). Yeast present on the skin causes an increase in nonanoic acid which is finally degraded to azelaic acid. The increased degradation of nonanoic acid explains the low levels measured for this metabolite in the cells (Figure 4.8b, batch 3) and the consequent rise in azelaic acid. Antibiotics are used to treat OIs but can also augment the yeast infections. According to the Human Metabolome Database, azelaic acid is a new topical drug for treating hyperpigmentary disorders and is also known to treat OIs during HIV/AIDS, as is capric acid. Self-medication (rather than HIV infection) may therefore be a source of azelaic and capric acid.

Alterations in fatty acid metabolism were also an important finding in the study of [Wikoff et al \(2008\)](#). In the SIV-infected model used by these authors the increase in fatty acids was attributed to an increase in brain phospholipase expression. In the present study phospholipase activity was not measured. It is however noteworthy that stearic acid (also reported by [Wikoff et al., 2008](#)), vaccenic acid and arachidonic acid were detected. These molecules are constituents of phospholipids. Vaccenic acid is a structural component of the cardiolipins (bisphosphatidyl glycerol), which are important components of the inner mitochondrial membrane. This metabolite increases in individuals with mental disorders and suggests neurological complications to be associated with HIV infection. Arachidonic acid is a membrane glycerophospholipid and serves as a substrate for phospholipase A2 (PLA2, [Sandstrom et al., 1994](#)). Adequate levels of this metabolite are usually required for proper neurological function. A disruption in arachidonic acid metabolism is thus associated with neurological dysfunction. [Basselin et al \(2010\)](#) found an increase in arachidonic acid metabolites and PLA2 activity in the brains of HIV-infected rats. Increased arachidonic acid

can be detrimental to the immunocompromised and was shown by [Scorrano et al \(2000\)](#) to increase mitochondrial permeability, the release of cytochrome c and cell death when added to isolated mitochondria and intact cells. Arachidonic acid as measured in the current study is thus in agreement with the increased levels measured in the SIV and rat models.

Research on oleamide was stimulated by the discovery that it induces satiety in humans and decreased body weight in experimental animals ([Rodriguez de Fonseca et al., 2001](#)). It has since been considered as a pharmacological or nutritional means of addressing the prevalence of obesity which is so prominent in developed/rich societies. Oleamide is an amide of oleic acid and modulates lipid metabolism ([Rodriguez de Fonseca et al., 2001](#)) by stimulating lipolysis. This ultimately causes a rise in triglycerides and free fatty acids. Free fatty acids further imply lipid breakdown *via* phospholipases. The concentration of oleamide was elevated in HIV+ individuals who are susceptible to wasting ([Grunfeld and Kotler 1992](#)). Wasting in the absence of other identifiable cases of weight loss was classified by the CDC as a criterion for the development of AIDS. It is however premature to emphasize this observation prior to further verification.

HIV and HAART disrupt the lipoprotein metabolic pathway by increasing LDL cholesterol levels and slightly lowering high density lipoprotein (HDL) cholesterol levels ([Worm and Lundgren 2011](#)) thus increasing the risk for cardiovascular and other metabolic complications. Lauric acid was present at low levels in cell lysate (Figure 4.8b, batch 3). It is usually associated with increased HDL cholesterol. HDL facilitates the transportation of lipid molecules within blood and particularly carries cholesterol to the liver to be re-used or excreted. High levels of HDL cholesterol are associated with a reduced cardiovascular risk. Since less of the lauric acid was present, it implies lowered HDL cholesterol and an increased risk for heart diseases as is known to occur during HIV infection ([Worm and Lundgren 2011](#)). The risk for this metabolic disorder coincides with the elevated viral load measured where this metabolite was detected (Table 4.2). Elaidic acid is associated with increased VLDL and decreased HDL cholesterol. Elaidic acid was present at low levels in the cell lysate following HIV infection (Figure 4.8, batch 4) implying less VLDL and elevated HDL. The elevated HDL associated with the low levels of elaidic acid may therefore be representative of a counter response to low lauric acid and low HDL (which is associated with increased cardiovascular risk). Dietary elaidic acid was shown to influence membrane permeability and is believed to have a role in membrane function ([Decker and Mertz 1967](#)). Mitochondrial dysfunction is associated with changes in membrane permeability making the detection of this molecule relevant.

Alpha glyceryl palmitate and stearate comprise of glycerol linked to a fatty acid and serve as intermediates in triglyceride metabolism. HIV-induced lipodystrophy is associated with elevated triglyceride content, free fatty acids, LDL cholesterol, glycerol and signals lipolysis. Lipodystrophy is also associated with insulin resistance. The increased disruption in lipid metabolism suggests the risk for cardiovascular disease in the experimental group used here. Behenic acid, a cholesterol raising fatty acid was also found to be increased. Although cholesterol increased (Figure 4.8a, batch 1 and Table 4.7) several authors have shown this molecule to be lowered following HIV infection ([Powderly, 2004](#); [Wanke, 1999](#)). According to [Hattingh et al \(2009\)](#) a decrease in cholesterol levels is not necessarily visible during the earlier stages of HIV infection. The elevated cholesterol measured is thus representative of the asymptomatic patients used in this study.

When the body is unable to make energy due to impaired β -oxidation processes or dysfunctional mitochondria; 3-methyladipic, 3-methylglutaconic and 3-hydroxy-3-methylglutaric acids build up, although the significance of 3-methylglutaconic acid is still controversial. 3-hydroxy-3-methylglutaric also accumulates due to decreased mitochondrial lyase activity (3-hydroxy-3 methyl glutaryl-CoA) and/or a decreased synthesis of CoQ10. It is a hypolipidemic agent increasing fatty acid content, a feature synonymous with HIV infection.

The metabolite, 2-indolecarboxylic acid is an inhibitor of lipid peroxidation. Alterations to fatty acid and/or lipid metabolism and increased apoptosis due to lipid peroxidation of cell membranes are features associated with HIV infection. Fatty acids such as *cis*-paranic acid are indicators of the degree of oxidative damage to PBMC membranes ([Míro et al., 2004](#)). The increase in 2-indolecarboxylic acid therefore serves as a counter molecule possibly produced by the host in response to the increase in fatty acids, lipid peroxidation and apoptosis which occurs during HIV infection.

The metabolite, 3-heptenedioic acid, 4-trimethylsilyloxy-, bis(trimethylsilyl) ester was also detected and found to be elevated during HIV infection. No literature was found for this metabolite but based on its nomenclature this metabolite is a hydroxy fatty acid and in addition to signalling a disruption in lipid metabolism it also provides information about the oxidative status of the host system. [Christeff et al \(1991\)](#) noted variations in the lipid profiles of individuals during the different stages of HIV infection. Other studies reporting on lipid changes during HIV infection have been published ([Haughey et al., 2004](#); [Sacktor et al., 2004](#)). Although the study by [Sacktor](#) is not metabolomics orientated it employed MS. Increases and decreases in polyunsaturated fatty acids during HIV infection have been reported elsewhere as well ([Woods et al., 2009](#)). The above literature (although not the only) was referenced in support of HIV's role in disrupting lipid metabolism. Numerous other

reports exist but a selected few were chosen to highlight the relevance of the detected molecules.

[iii] Neurological/Oxidative stress metabolites

An increase in neurological and oxidative stress markers was observed in HIV+ individuals compared to controls. This further supports the detection of the hydroxy fatty acids as noted above in ii. In animal studies, pyroglutamic acid which is a derivative of glutamic acid was found to inhibit mitochondrial complex I and III activity (Silva et al., 2001). Its detection here shows that this metabolite is linked to HIV-induced mitochondrial dysfunction. Pyroglutamic acid was found to be a common metabolite between serum and cells. This molecule is an intermediate in the γ -glutamyl cycle and is indicative of glutathione and γ -glutamylcysteine synthetase deficiency (Larsson et al. 1985; Jellum et al., 1970) and therefore oxidative stress. As mentioned in Section 2.7.1; HIV infection causes activation of the immune system and induces apoptosis. During the apoptotic process there is a subsequent rise in ROS which places the host under oxidative stress. Oxidative stress is involved in the pathogenesis of HIV infection. That molecules relevant to oxidative stress are detected is confirmatory of the flow cytometry findings of Macho et al (1995). Pyroglutamic acid impairs brain energy production and contributes to the development of neuropathologies (Silva et al., 2001). In batch 3 of the cell extracts (Figure 4.8b); pyroglutamic acid was lowered highlighting an alteration in the levels of glutathione and thus oxidative stress. This batch of cells had the highest viral load (Table 4.2) for this specific biofluid. Because the host is able to alter its metabolism in order to deal with infection (Beisel, 1972), the decreased pyroglutamic acid (elevated in all other batches and associated with oxidative stress) is most likely representative of a protective response against the oxidative signal. In a model representing SIV infection and substance abuse, Pendyala et al (2011) measured an increase in glutathione-S-transferase which served as a compensatory response to the high level of oxidative stress in that system.

An oxysterol in the form of 7-ketocholesterol was also detected. Oxysterols are produced under conditions of high oxidative stress, are indicative of cholesterol oxidation (Iuliano et al., 2003) and cause cell death. Nonenzymatic production of 7-ketocholesterol was reported to arise via a free radical-mediated mechanism operating under conditions of oxidative stress (Bjorkhem and Diczfalusy 2002). *In vitro*, 7-ketocholesterol has wide-ranging effects and can induce apoptosis in cells by decreasing mitochondrial membrane potential, increasing cyt c release, caspase activation, increasing ROS and decreasing glutathione. The oxidation of LDL also produces hydroperoxides and oxysterols. The formation of 7-ketocholesterol by a mechanism involving free radicals concurs with its production from experimentally oxidized

LDL. Whether this mechanism relates to previous observations of a marked difference in LDL between controls and ART-treated patients (Philippeos et al., 2009) requires further investigation.

Quinolinic acid is a tryptophan metabolite and was increased during SIV infection (Wikoff et al., 2008). In the current study presented it was the only metabolite found to be common between serum and urine. It is generally raised during chronic inflammation and neurodegeneration. It has been shown to be involved in neurodegenerative processes of the brain during AIDS, to induce lipid peroxidation, free radical production and cell death in cells (Guillemin et al., 2005; Wiley, 1995). An additional marker associated with neurodegeneration and/or neurometabolic processes, was also detected i.e. 2-hydroxyglutaric acid. This metabolite has been shown to induce oxidative stress in the brain of rats (Latini, 2003). The detection of stress-related markers in clinically stable individuals is indicative of persistent infection and activation of the immune system. The detection of markers associated with neurodegeneration indicates the possible development of AIDS-related dementia. These stress markers were primarily detected in batch 1, 2 and 3 of the respective biofluids (Table 4.7-4.9) where individuals with low CD4 counts and high viral loads were included as part of the analysis. This indicates that the detection of these markers may be due to the inclusion of these few cases. Metabolic changes detected in HIV-infected samples thus suggest metabonomics to be an early detection method for AIDS and AIDS-related dementia. This reveals the potential of MS-based metabonomics as a prognostic tool for the early detection of metabolic change prior to the development of clinical symptoms in these individuals.

[iv] Other metabolites of the human metabolome database

The detection and identification of 4-hydroxybenzaldehyde does not seem to have a clear link to HIV infection. Its presence was however noted in a metabolomics investigation testing for biomarkers of prostate cancer (Sreekumar et al., 2009). Tyramine is derived from tyrosine and acts as a neurotransmitter being associated with a sudden rise in blood pressure. In this study, tyramine was one of five metabolites that decreased following HIV infection and thus signalled a low risk for hypertensive crisis. Oxidative phosphorylation is a process which primarily takes place in mitochondria. During this process, carbon fuels are oxidized to yield energy with a subsequent transfer of electrons from NADH or FADH₂ to O₂ which ultimately causes a proton gradient to develop and the phosphorylation of ADP to ATP. When there is defective oxidative phosphorylation, as a compensatory mechanism, ATP is produced by the action of neurotransmitter molecules (such as tyramine) instead (Reinecke et al., 2011; Korzeniewski, 2001). The metabolite, 4-hydroxyphenylacetic acid is a

breakdown product of tyramine and is associated with increased bacterial growth. The Great Plains Laboratory, Inc lists this metabolite as an indicator of celiac disease. This disease is characterized by damage to the lining of the small intestine which in turn limits the absorption of foods and nutrients causing an individual to be malnourished. Investigations into the role of this molecule can help further understand malnutrition in context to HIV/AIDS, HIV-induced weight loss and wasting. Succinylacetone is a metabolite of tyrosine and was found to be elevated during HIV infection. In a recent metabolomics-based investigation utilizing saliva from HIV-infected individuals; tyrosine levels were found to be unchanged between treatment-naive and treated HIV patients but elevated in HIV patients compared to uninfected controls. The authors also showed tyramine levels to be low upon comparing treatment naive and treatment experienced HIV patients (Ghannoum et al., 2011). 3-Hydroxysebacic acid was elevated in HIV patients compared to controls. This metabolite is generally elevated in individuals suffering from Zellweger syndrome and in infants who are malnourished and suffering from glycogen storage disease. The Zellweger syndrome is characterized by impaired peroxisome function and thus impaired peroxisomal β -oxidation (Bennett et al., 1992). As a result very long and branched chain fatty acids cannot be degraded. This syndrome leads to impaired brain development. The detection of 3-hydroxysebacic acid is in accordance with the disrupted fatty acid/lipid metabolism mentioned earlier as well as the development of neurological pathologies associated with HIV-associated dementia. Based on its detection in malnourished infants, this metabolite may also add to our understanding of HIV-associated malnourishment.

Mandelic acid is an antimicrobial agent which decreases inflammation and was found to increase during HIV infection. This molecule is generally high in individuals suffering from phenylketonuria (a genetic disorder which arises due to a mutation in the phenylalanine hydroxylase gene) which if left untreated can cause severe problems with brain development. Once again, a molecule potentially signalling HIV-induced neurological complications was detected.

Indole acetic acid is a catabolic product of tryptophan metabolism (a metabolite which usually declines during HIV infection, Murray, 2003). In humans an increase in indole acetic acid is mostly linked to the action of bacteria in the gut, the decarboxylation of tryptamine or the oxidative deamination of tryptophan. Changes in the levels of indole acetic acid therefore indicates defective tryptophan metabolism. In a recent study, indole acetic acid was increased in the saliva of treatment naive HIV patients whereas tyramine was decreased (Ghannoum et al., 2011). Such changes in the levels of these metabolites were also observed in the present study described.

Glycine serves as a precursor to purines and is involved in DNA, phospholipid and collagen synthesis. It is also characterized as an inhibitory neurotransmitter, decreasing the probability for cells to fire action potentials. Glyceric acid on the other hand is produced from the oxidation of glycerol. Glyceric acid is elevated when glycerate kinase levels are low. Its accumulation is associated with neurological impairment, a feature known to occur when HIV associated dementia develops. Orotic acid on the other hand is involved in the synthesis of pyrimidines. A mutation in uridine monophosphate (UMP) synthase inhibits the conversion of orotic acid into UMPs, which is the base from which all other pyrimidines are synthesized. Orotic acid was shown to increase when arginine levels were low (arginine is important for immune system function) and to possess antiviral effects ([Hoffmann et al., 2011](#)). An increase in this metabolite may therefore be compensatory for the lowered immune response commonly observed in immunocompromised individuals. There was no information for pregna-3,5-dien-20-ol, O-trimethylsilyl and methylcitric acid, tetrakis (trimethylsilyl).deriv which could be explained in context to HIV.

Having identified metabolites significantly altered as a result of HIV infection, representative spectra of metabolites from each biofluid type was extracted. The extracted spectra (Figure 4.13 a-c) complimented the spectra contained in the NIST library (shown as an insert).

The CD4 count is currently regarded as one of the most important prognostic markers for HIV infection, even in the light of its many disadvantages. No appropriate correlation was done on the CD4 count and the important metabolites identified in this study but the categories of metabolites disclosed by this investigation proved to be sufficiently relevant and significant to be taken into account in studies on HIV infection. It can hardly be expected that a small number of metabolite biomarkers could account for all the cellular aberrations caused by HIV infection. This concurs with the divergent rather than convergent association between the metabolites presented in this thesis (Table 4.7-4.9) and is in agreement with data reported by [Wikoff et al \(2008\)](#) for SIV-infected rhesus macaques. It is, however, noteworthy that most metabolites indicative of mitochondrial dysfunction were present in those batches which had the highest values for the HIV-RNA in plasma (Tables 4.1-4.3 and Tables 4.7-4.9). This may indicate that for the chronic phase of the disease the organic acid profile develops from mild organic acidemia in the early stages to more advanced organic acidemia, including markers of mitochondrial dysfunction in the later stages. Whether this is a consistent characteristic of disease progression — from chronic HIV infection to full-blown AIDS — will have to be addressed by more detailed investigations.

Table 4.7 Summary of metabolites from serum identified as being indicators of HIV infection in this metabonomics study

| Features from MET-IDEA/NIST 08 Library | Common and alternative names | Formula | Batch | ES | t-test | Mann-Whitney |
|--|--|--|-------|------|----------|--------------|
| Indicators of mitochondrial dysfunction | | | | | | |
| Butanedioic acid, bis(trimethylsilyl) ester | Succinic acid / 1,4-Butanedioic acid | C ₄ H ₆ O ₄ | 1 | 2.45 | < 0.0001 | < 0.0001 |
| 2-Butenedioic acid (E)-, bis(trimethylsilyl) ester | Fumaric acid / trans-1,2-Ethylenedicarboxylic acid | C ₄ H ₄ O ₄ | 1 | 1.12 | 0.0043 | 0.0056 |
| Hexanedioic acid, bis(trimethylsilyl) ester | Adipic acid / 1,6-Hexanedioic acid | C ₆ H ₁₀ O ₄ | 1 | 2.13 | < 0.0001 | 0.0001 |
| Octanedioic acid, bis(trimethylsilyl) ester | Suberic acid / 1,6-Hexanedicarboxylic acid | C ₈ H ₁₄ O ₄ | 1 | 1.98 | < 0.0001 | 0.0001 |
| Fatty acids, other lipids and lipid metabolism | | | | | | |
| Azelaic acid, bis(trimethylsilyl) ester | Azelaic acid / Nonanedioic acid | C ₉ H ₁₆ O ₄ | 1 | 1.72 | < 0.0001 | 0.0001 |
| Decanoic acid, trimethylsilyl ester | Capric acid / Decoic acid | C ₁₀ H ₂₀ O ₂ | 1 | 2.49 | < 0.0001 | < 0.0001 |
| 11-trans-Octadecenoic acid, trimethylsilyl ester | Vaccenic acid / 11-Octadecenoic acid | C ₁₈ H ₃₄ O ₂ | 2 | 0.88 | 0.0187 | 0.0312 |
| Oleamide, N-trimethylsilyl- | Oleamide / Oleoylethanolamide | C ₁₈ H ₃₅ NO | 1 | 1.01 | 0.0074 | 0.0106 |
| Octadecanoic acid, trimethylsilyl ester | Stearic Acid / Octadecanoic acid | C ₁₈ H ₃₆ O ₂ | 2 | 1.79 | 0.0003 | 0.0001 |
| Arachidonic acid, trimethylsilyl ester | Arachidonic Acid / 5,8,11,14-Eicosatetraenoic acid | C ₂₀ H ₃₂ O ₂ | 2 | 1.28 | 0.0098 | 0.0001 |
| Neurological/Oxidative stress metabolites | | | | | | |
| N,O-Bis-(trimethylsilyl)-2-pyrrolidone carboxylic acid | Pyroglutamic acid / 5-oxoproline | C ₅ H ₇ NO ₃ | 1 | 2.08 | < 0.0001 | < 0.0001 |
| >Quinolinic acid, bis(trimethylsilyl) ester | Quinolinic acid | C ₇ H ₅ NO ₄ | 3 | 1.04 | 0.0112 | 0.0069 |



| | | | | | | |
|--|--|--|---|------|--------|--------|
| Cholest-5-en-7-one, 3-[(trimethylsilyl)oxy]- | 7-ketocholesterol / 3b-Hydroxycholest-5-en-7-one | C ₂₇ H ₄₄ O ₂ | 1 | 0.95 | 0.0136 | 0.0129 |
|--|--|--|---|------|--------|--------|

Other metabolites recognized as part of the human metabolome

| | | | | | | |
|---|---|--|---|------|--------|---------|
| Benzaldehyde, 4-[(trimethylsilyl)oxy]- | 4-hydroxybenzaldehyde | C ₇ H ₆ O ₂ | 2 | 1.12 | 0.0345 | 0.0312 |
| >Benzeneacetic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 4-Hydroxyphenylacetic acid | C ₈ H ₈ O ₃ | 3 | 0.92 | 0.0201 | 0.0230 |
| >Benzeneacetic acid, à-[(trimethylsilyl)oxy]-, trimethylsilyl ester | Mandelic acid | C ₈ H ₈ O ₃ | 3 | 1.04 | 0.0016 | <0.0001 |
| 1H-Indole-3-acetic acid, 1-(trimethylsilyl)-, trimethylsilyl ester | Indole acetic acid / Indole-3-acetic acid | C ₁₀ H ₉ NO ₂ | 2 | 1.33 | 0.0131 | 0.0159 |

Table 4.8 Summary of metabolites from cell lysates identified as being indicators of HIV infection in this metabolomics study. Areas shaded in red highlights those molecule(s) for which no particular association to HIV could be made

| Features from MET-IDEA/NIST 08 Library | Common and alternative names | Formula | Batch | ES | t-test | Mann-Whitney |
|---|--------------------------------------|--|-------|------|--------|--------------|
| Indicators of mitochondrial dysfunction | | | | | | |
| >Butanedioic acid, bis(trimethylsilyl) ester | Succinic acid / 1,4-Butanedioic acid | C ₄ H ₆ O ₄ | 1 | 1.48 | 0.0032 | 0.0164 |
| Fatty acids, other lipids and lipid metabolism | | | | | | |
| >Azelaic acid, bis(trimethylsilyl) ester | Azelaic acid / Nonanedioic acid | C ₉ H ₁₆ O ₄ | 1 | 1.36 | 0.0151 | 0.0164 |
| >Nonanoic acid, trimethylsilyl ester | Nonanoic acid/Pelargonic acid | C ₉ H ₁₈ O ₂ | 3 | 1.58 | 0.0027 | 0.0062 |
| >Dodecanoic acid, trimethylsilyl ester | Lauric acid | C ₁₂ H ₂₄ O ₂ | 3 | 0.82 | 0.0216 | 0.0117 |
| >trans-9-Octadecenoic acid, trimethylsilyl ester | Elaidic acid | C ₁₈ H ₃₄ O ₂ | 4 | 1.13 | 0.0171 | 0.0185 |
| >Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester | α-Glyceryl palmitate | C ₁₉ H ₃₈ O ₄ | 1 | 1.09 | 0.0243 | 0.0418 |
| >Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester | α-Glyceryl stearate | C ₂₁ H ₄₂ O ₄ | 1 | 1.44 | 0.0043 | 0.0079 |
| >Docosanoic acid, trimethylsilyl ester | Docosanoic acid/ Behenic acid | C ₂₂ H ₄₄ O ₂ | 1 | 0.96 | 0.0308 | 0.0549 |
| Neurological/Oxidative stress metabolites | | | | | | |
| >L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester | Pyroglutamic acid / 5-oxoproline | C ₅ H ₇ NO ₃ | 3 | 1.39 | 0.0004 | 0.0009 |
| Other metabolites recognized as part of the human metabolome | | | | | | |
| >Tyramine, trimethylsilyl ether | Tyramine | C ₈ H ₁₁ NO | 4 | 1.05 | 0.0231 | 0.0433 |

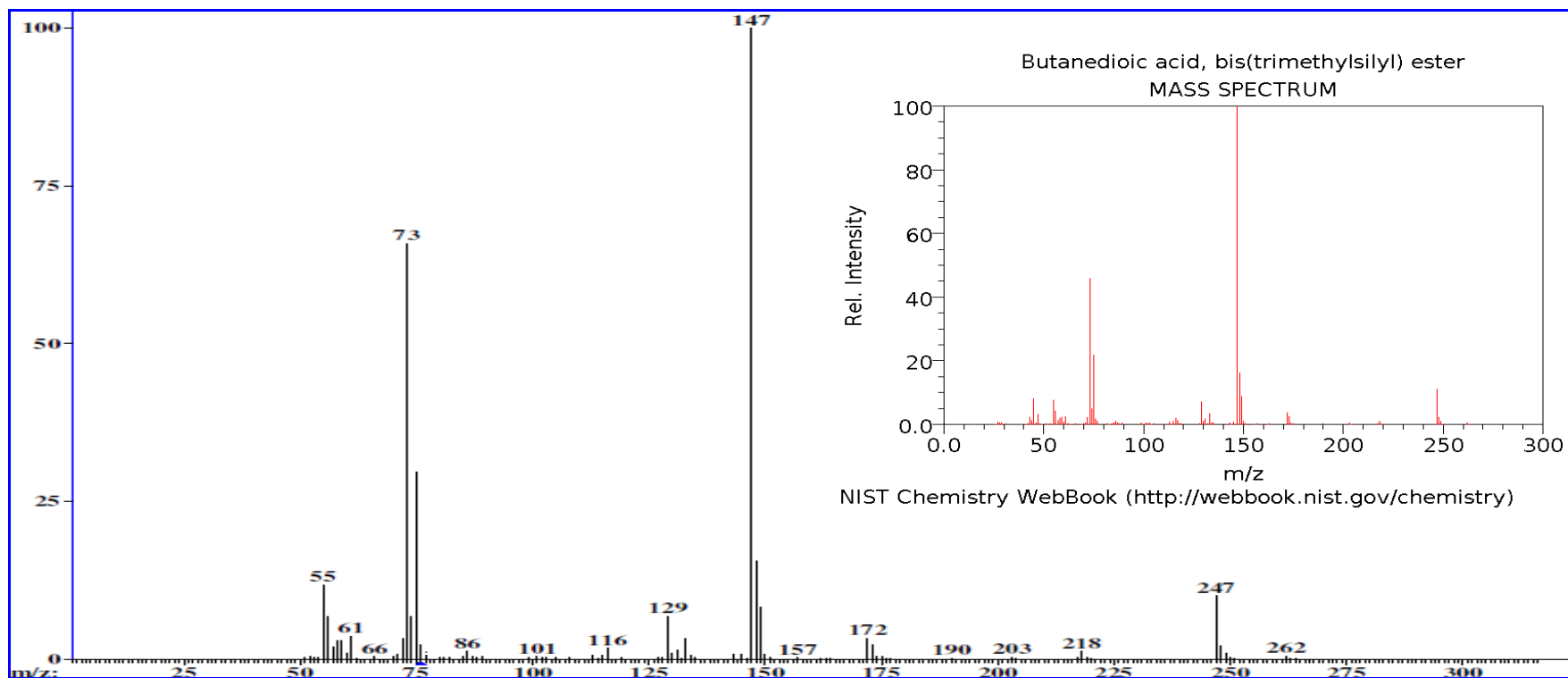
Table 4.9 Summary of metabolites from urine identified as being indicators of HIV infection in this metabolomics study. Areas shaded in red highlights those molecule(s) for which no particular association to HIV could be made

| Features from MET-IDEA/NIST 08 Library | Common and alternative names | Formula | Batch | ES | t-test | Mann-Whitney |
|--|---------------------------------|---|-------|------|--------|--------------|
| Fatty acids, other lipids and lipid metabolism | | | | | | |
| >2-Pentenedioic acid, 3-methyl-, bis(trimethylsilyl) ester | 3-Methylglutaconic acid | C ₆ H ₈ O ₄ | 2 | 0.81 | 0.0646 | 0.0317 |
| >Pentanedioic acid, 3-methyl-3-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester | 3-Hydroxy-3-methylglutaric acid | C ₆ H ₁₀ O ₅ | 2 | 0.85 | 0.0427 | 0.0374 |
| >Hexanedioic acid, 3-methyl-, bis(trimethylsilyl) ester | 3-Methyladipic acid | C ₇ H ₁₂ O ₄ | 2 | 1.49 | 0.0010 | 0.0017 |
| >1H-Indole-2-carboxylic acid, 1-(trimethylsilyl)-, trimethylsilyl ester | 2-Indolecarboxylic acid | C ₉ H ₇ NO ₂ | 1 | 1.41 | 0.0002 | 0.0002 |
| >3-Heptenedioic acid, 4-trimethylsilyloxy-, bis(trimethylsilyl) ester | [REDACTED] | | 1 | 1.03 | 0.0050 | 0.0039 |
| Neurological/Oxidative stress metabolites | | | | | | |
| >Pentanedioic acid, 2-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester | 2-Hydroxyglutaric acid | C ₅ H ₈ O ₅ | 2 | 0.88 | 0.0156 | 0.0317 |
| >Quinolinic acid, bis(trimethylsilyl) ester | Quinolinic acid | C ₇ H ₅ NO ₄ | 2 | 1.48 | 0.0009 | 0.0028 |
| Other metabolites recognized as part of the human metabolome | | | | | | |
| >Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester | Glycine | C ₂ H ₅ NO ₂ | 2 | 1.29 | 0.0016 | 0.0028 |



| | | | | | | |
|---|-----------------------|-------------------|----------|----------------|--------------------|--------------------|
| >Propanoic acid, 2,3-bis(trimethylsilyloxy)-, trimethylsilyl ester | Glyceric acid | $C_3H_6O_4$ | 2 | 1.13 | 0.0066 | 0.0129 |
| >4-Pyrimidinecarboxylic acid, bis(trimethylsiloxy)-, trimethylsilyl ester | 2,6- Orotic acid | $C_5H_4N_2O_4$ | 2 | 1.33 | 0.0020 | 0.0007 |
| >4,6-Dioxoheptanoic acid, tris-(O-trimethylsilyl)- | Succinylacetone | $C_7H_{10}O_4$ | 1 | 1.04 | 0.0016 | 0.0009 |
| >3-Trimethylsiloxysebacic acid, bis(trimethylsilyl)- ester | 3-Hydroxysebacic acid | $C_{10}H_{18}O_5$ | 1 (2) | 0.83 (1.03) | 0.0141 (0.0099) | 0.0211 (0.0374) |
| >Pregna-3,5-dien-20-ol, O-trimethylsilyl | | | 1 | 0.91 | 0.0041 | 0.0034 |
| >Methylcitric acid, tetrakis(trimethylsilyl) deriv. | | | 2 | 1.40 | 0.0029 | 0.0022 |

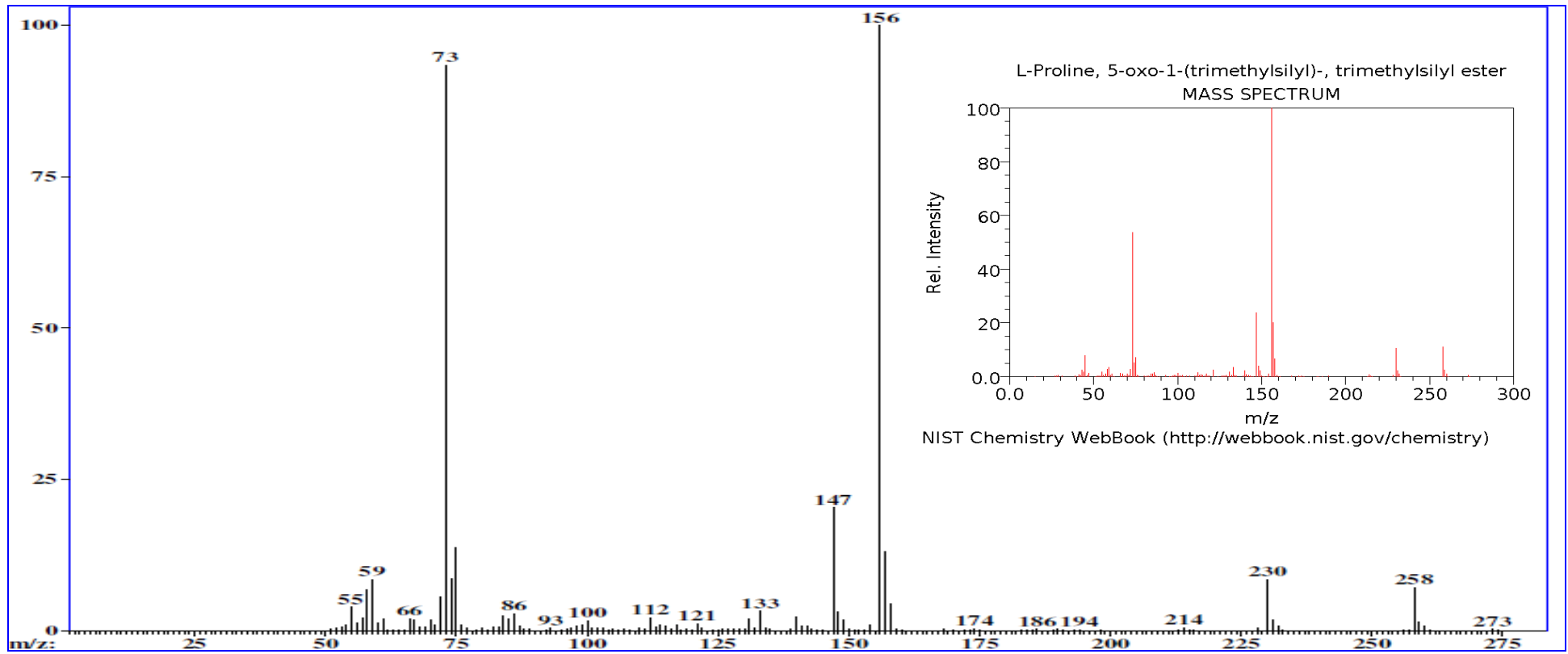
Extracted spectrum (18.273 min)



a

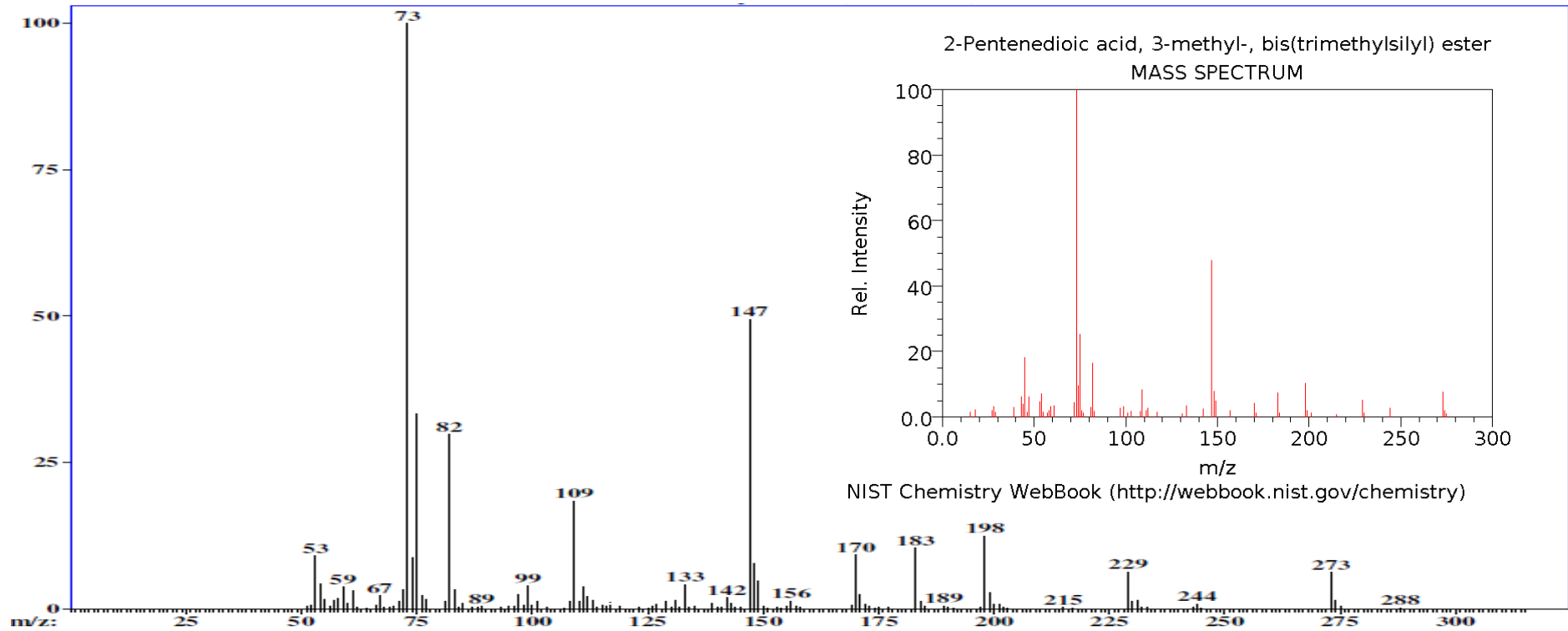
Figure 4.13 Representative spectra of metabolites following derivatization, electron impact GC-MS analysis, deconvolution and identification through the NIST 08 library. Representative spectra were taken from a) sera b) PBMC lysate and c) urine respectively and represent: butanedioic acid, bis(trimethylsilyl) ester, L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester and 2-Pentenedioic acid, 3-methyl-, bis(trimethylsilyl) ester, respectively.

Extracted spectrum (23.332 min)



b

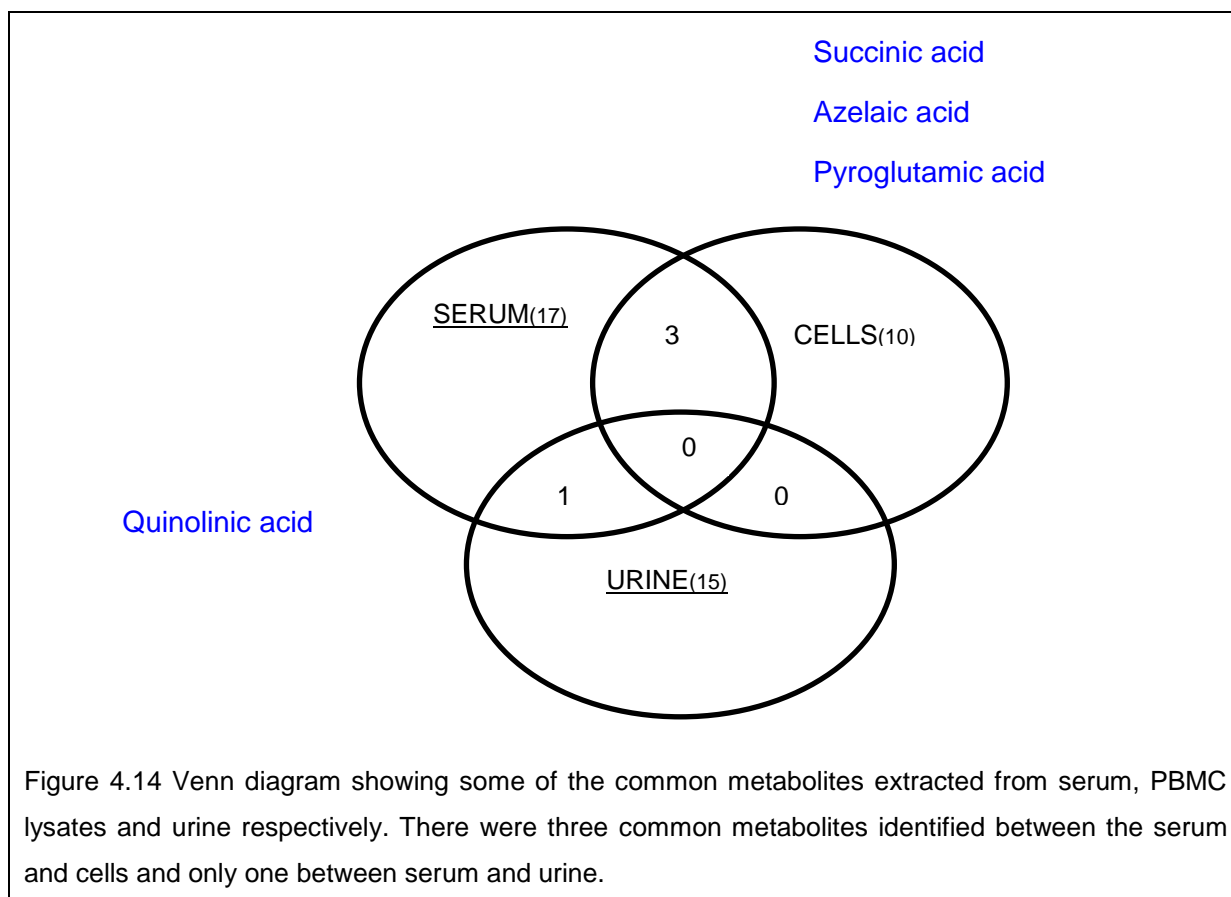
Extracted spectrum (22.083 min)



C

4.3.6.1 Venn diagram of common metabolites in the different biofluid types

Organic acid extractions from sera, cells and urine; GC-MS analysis and a metabonomics approach led to the identification of metabolites altered as a result of HIV infection. These metabolites differed in identity and intensity between the respective biofluid types, shared no physio-chemical characteristics but could be related to mitochondrial dysfunction which ultimately impacted on energy, lipid, sugar, oxidative and neurological processes implying sharing of a common biochemical pathway. Using a Venn diagram (Figure 4.14), three metabolites were found to be common between serum and cells (i.e. succinic acid, azelaic acid and pyroglutamic acid). Only one metabolite (quinolinic acid) was found to be common between serum and urine. That only a small number of metabolites were common between the biofluid types is in keeping with the fact that the different biofluids do not necessarily make use of the same substrate during enzymatic reactions and thus respond differently when a stimuli presents. The greater number of common molecules shared between serum and cells is explained by the fact that these fluids are derived from a common source i.e. blood which is under homeostatic control ([Wishart, 2007](#)). Because of this characteristic of blood the metabolic profile of these biofluid types stays relatively constant. The composition of urine however varies a lot depending on food, water intake, physiological conditions, age, gender, environment etc ([Álvarez-Sánchez et al., 2010](#); [Wishart, 2007](#)). Urine, despite having the most complex metabolic profile and therefore the most number of peaks (Figure 4.5) did not necessarily yield more information in terms of the number of significant metabolites identified, compared to serum and cells in this study (Table 4.7 and 4.8 compared to 4.9). For a targeted organic acid analysis of HIV-infected biofluid, blood-based samples seem to be the more favourable to use.



4.4 Conclusion

Mitochondrial dysfunction is one of the pathological consequences of HIV infection. Using GC–MS, mitochondrial and thus metabolic change detectable in the sera, cellular and urinary organic acid metabolome of HIV-infected individuals was successfully profiled in these biofluids. To facilitate the alignment and comparison between large sample numbers and experimental groups, good quality data is needed. In this case MET-IDEA provided such datasets. PCA score plots showed that HIV has a moderate effect on the organic acid metabolome during early infection yielding overlapping metabolic profiles. The metabolic overlap was interpreted as being due to a masking of the metabolic change by the high number of uninfected cells which are in the vicinity of the infected ones. Instrument sensitivity could also contribute to the overlap. The metabolic profiles of the experimental groups were more distinct and better separated if viral loads were higher. The extraction of organic acids from HIV-infected blood-based biofluids is possible and is even more informative than extractions from urine for this pathological condition using the approach outlined in this chapter. Urine had the most peaks following GC-MS analysis but did not necessarily have the most biologically relevant information in the end. In fact, the numbers of significantly altered metabolites were eventually similar for the three biofluid types analyzed. Using uni- and multivariate statistical methods, metabolites which differed significantly

between HIV- and HIV+ individuals were identified. No single biomarker associated with HIV-induced mitochondrial dysfunction was identified, instead a range of different molecules were found to be significantly altered. The detected metabolites were confirmatory of mitochondrial dysfunction, changes in lipid, sugar, energy and neurometabolism as well as oxidative stress, all of which are known aberrations of HIV infection. The disruption in lipid metabolism and the detection of stress markers mainly signalled risks for the development of cardiovascular and neurological complications. As a consequence of disrupted mitochondrial function, metabolites reflecting disrupted energy metabolism and produced as a compensatory mechanism in response to reduced ATP levels were also detected. The molecules detected impacted on common biological processes. This profile thus gives information on the well-being of the patients in the respective study groups and indicates that HIV-induced mitochondrial and therefore metabolic dysfunction can be detected early on during the infection stage. These observations were obtained by analyzing HIV+ individuals who, according to their CD4 counts, were in the chronic, asymptomatic phase (WHO stage 2) of the disease. This is in agreement with literature in Section 2.8.1 where metabolic changes were documented in asymptomatic individuals having high CD4 counts. These individuals were not on ART at the time their blood was collected. This study therefore demonstrated that a metabolomics investigation can disclose information on the markers that define the asymptomatic stage of HIV infection and may be developed into a method for monitoring more advanced stages of the disease and potentially also the response of infected individuals to ART. The inclusion of individuals with low CD4 counts and high viral load support the possibility that MS metabolomics will be invaluable to the study of virus-induced metabolic changes (better separation between the infected and uninfected groups).

CHAPTER 5

IMMUNOLOGICAL PROFILE OF HIV INFECTED INDIVIDUALS

5. Summary

Background: HIV infections in Sub-Saharan Africa are predominantly of subtype C origin. Based on the location of our research project the assumption was that the patients involved would most likely be infected by HIV-1 subtype C. During HIV infection the immune system is activated and produces an increase in oxidative species and soluble factors. By interacting with each other these immune molecules as well as HIV ultimately affect metabolic processes, particularly through mitochondria. It becomes important to measure the oxidative status of the host since downward metabolic processes which drive energy production, the immune response and the survival of cells is dependent on this redox state ([Pace and Leaf 1995](#)).

Based on the types of molecules detected in Chapter 4 (fatty acids, oxidative stress markers etc) several immune parameters which could further characterize the experimental groups and compliment metabolites representative of mitochondrial dysfunction were measured. As an example; fatty acids are indicators of the peroxidation of PBMC membranes, and therefore the apoptotic process. These molecules can also serve as immunomodulators ([Christeff et al., 1991](#)). As such; the oxidative, apoptotic and cytokine profile of sera and cells was measured, not to study something new about HIV's effect on the immune system but to link the detected immune parameters to the metabolic changes that were measured in Chapter 4.

Methods and Results: To determine which subtype of HIV the samples were infected with, nested PCR was employed. This assay confirmed the samples to be infected with HIV-1 subtype C (more detail in the appendix, Section 3). As a representation of the immunopathogenic events associated with HIV infection; the oxidative, apoptotic and cytokine profiles of HIV- and HIV+ biofluid was measured using spectroscopy and flow cytometry. ROS production and the percentage apoptosis was significantly higher ($p=0.004$ and $p<0.0001$) in the HIV+ samples. Higher levels of apoptosis occurred in the CD8 cells. After treating HIV+ cells with R7V and the pooled Gag peptide, intracellular cytokine staining was performed and the production of IFN- γ and TNF- α measured using flow cytometry. Both IFN- γ and TNF- α were produced. Responses were more elevated in response to Gag than R7V with more IFN- γ generally produced compared to TNF- α . HIV infection alone can cause

activation of the host immune system. This in turn leads to the production and secretion of cytokines (Pala et al., 2000). During the *in vitro* stimulation of cells with mitogen and antigen, protein transport inhibitors such as GolgiPlug are used to keep the produced cytokine intracellular but these can take a while before it begins to work. To ensure that the background levels of cytokine (due to HIV and prior to GolgiPlug having an effect) were accounted for, an ELISA was included to measure secreted IFN- γ from the stimulated cells. The data was analyzed using univariate statistics (the detailed protocol and results is presented in the appendix, Section 2 [ij]). Through the application of a cytokinomics approach (definition modified in Section 2.7.3) endogenous IL-6 and IL-10 was found to discriminate between the uninfected and HIV-infected samples.

Discussion and Conclusion: The high levels of ROS detected confirmed the HIV+ individuals to be under oxidative stress, experiencing damage to membranes and thus apoptosis, as confirmed by flow cytometry. CD8 cells experienced more apoptosis, possibly due to the presence of CD4 cells which is believed to produce a soluble factor which initiates apoptosis in CD8 cells (Holm and Gabuzda 2005). Intracellular IFN- γ and TNF- α production in response to host-derived (virus incorporated) peptide and a viral peptide pool imply recognition of the respective epitopes by infected cells. It also confirmed the cells to be functional and capable of producing cytokine with antiviral activity. The application of a cytokinomics approach not only identified molecules associated with HIV infection but also discriminated asymptomatic individuals from those progressing to AIDS based on increased viral load (confirmatory of organic acid profiles obtained in Chapter 4). In untreated, chronically infected biofluid, HIV therefore increases oxidative stress, lowers T cell numbers through apoptosis and enhances the secretion of Th2 cytokines i.e. IL-6 and IL-10. All of these suggest mitochondrial distress. Having proven this with organic acid analysis using MS metabonomics, the associated immune responses confirmed data collected from the metabolic and immune systems to be in agreement.

5.1 Introduction

HIV/AIDS statistics has reached alarming levels and is of concern in the Sub-Saharan region where most individuals are infected with the pathogenic HIV-1 subtype C strain. The introduction of HAART has reduced HIV-related mortalities prolonging the life span of infected individuals. As a result, the prevalence of HIV infection remains high (Abdool Karim et al., 2007) since more individuals remain burdened with chronic HIV infection. Researchers continue with their efforts to develop better treatment options and a vaccine but even if these attempts are successful complications associated with HIV infection will remain evident for years. Coupled to immunodeficiency, HIV infection causes an activated immune state. This

activated state of the immune system is visible in the elevated production of ROS prior to and during apoptosis as well as in the release of specific cytokines. Cytokines are a group of molecules that have a role in immune function and which mediate metabolic processes (Matarese and La Cava 2004). In addition to directly affecting HIV replication (Kedzierska and Crowe 2001) these molecules initiate a range of deleterious reactions in response to HIV infection which places the host under oxidative stress. Oxidative stress ultimately causes suboptimal functioning of enzyme pathways (Newman et al., 2004), damages biological matter and affects metabolic processes.

All the above immunological events eventually impact on mitochondria contributing to the metabolic imbalances caused by HIV. In most literature this effect on mitochondria is shown through apoptosis which is usually confirmed by more than one assay (e.g. the annexin V affinity assay and the TUNEL assay). Here we investigate the immunological profile of biofluid from HIV-infected individuals and show the detection of the hydroperoxides as a measure of ROS, changes in cytokine and the organic acid profile (Chapter 4) to be confirmatory of mitochondrial dysfunction and HIV-induced apoptosis. Using spectroscopy, it was determined that the HIV+ individuals were under oxidative stress while flow cytometry measured higher apoptosis in the PBMCs and CD8 cells of HIV+ individuals respectively.

Flow cytometry (Section 2.10.3) and ELISAs (or EIAs, Section 2.10) are common tools used for assessing immune function (Ford, 2010). While flow cytometry is able to measure multiple cytokines within single cells, ELISAs only quantify one type of extracellular/secreted protein at a time. Unless purified cell populations are used for ELISA-based assays, the cell source producing and secreting the cytokine cannot be identified. HIV causes a dysregulation in cytokine production and secretion (Landay, 1998). It is thus possible to measure cytokine changes inside and outside of their cellular compartments. To measure the secretion of multiple cytokines but still using flow cytometry, CBA kits have been developed. CBA technology (5.2.6.2.1) works on the same principle as the ELISA assay. The major difference is that capture beads having different fluorescence intensities are coated with several cytokine-specific antibodies allowing multiple cytokines to be detected in a sample (Elshal and McCoy 2006).

In this project intracellular IFN- γ and TNF- α were measured through flow cytometry. Since the response of cells to *in vitro* HIV peptide stimulation has prognostic value (Jansen et al., 2006), representative anti-inflammatory (IFN- γ) and pro-inflammatory (TNF- α) cytokines associated with slow and rapid disease progression, were measured. TNF- α is however also bifunctional by being able to suppress as well as enhance HIV replication and thus affect disease progression (Alfano and Poli 2005). Further reasons for the use of these

cytokines were also highlighted in Sections 2.7.3 and 3.3 respectively. Both IFN- γ and TNF- α are antiviral Th1 type cytokines. A cytokinomics approach employing multivariate analysis of Th1/Th2/Th17 cytokines showed HIV infection to be associated with IL-6 and IL-10 secretion. Cytokinomics was defined by Clerici (2010) as the study of cytokine production and the interactive effects of these molecules in a biological system. For the purposes of this project the definition was extended to include the analysis of a number of secreted cytokines (IL-2, 4, 6, 10, 17, IFN- γ and TNF- α) and their subsequent analysis using multivariate statistics to extract information on cytokine interactions, disease pathogenesis and investigate the potential role of these molecules as biomarkers of HIV/AIDS. Based on the definition of Clerici, the concept does not seem to be limited in terms of the methodology that can be used and as such CBA kits together with flow cytometry as detection method, was used here.

5.2 Materials and Methods

In the sections which follow protocols that were used are explained and preceded by background and/or the principles of the methodologies.

5.2.1 Serum Isolation

Serum was prepared as described in Section 4.2.2. Aliquots were thawed on ice when needed.

5.2.2 Isolation of PBMCs

PBMCs were isolated as described in Section 4.2.3. For the determination of apoptosis and detection of intracellular cytokine, the concentration of the cells was adjusted to 1×10^6 and 2×10^7 cells/ml respectively using 10 % RPMI media.

5.2.3 Reactive Oxygen Species (ROS)

Background: HIV-infected individuals are documented to be under constant oxidative stress. Here the redox status of 95 serum samples (53 HIV- and 42 HIV+) was determined using a colorimetric assay in the 96-well plate format. The principle of the assay is based on the fact that DEPPD sulphate, a compound that when allowed to react with serum or plasma samples, will form a long-lived cation. Under acidic conditions metal ions contained within serum samples are released from their transport proteins. Hydroperoxides (products of oxidation) then decompose into alkoxy and peroxy radicals. N-alkylated p-phenylenediamines are then converted into a coloured radical cation which can be measured spectrophotometrically with the absorbance being proportional to the amount of

hydroperoxyl compounds and thus the oxidative status of the sample ([Hayashi et al., 2007](#); [Verde et al., 2002](#)).

Protocol: Briefly, 140 μ l of 0.1M sodium acetate buffer at pH 4.8 was added to each allocated well. To the buffer, 2.5 μ l of standard or sample was added in duplicate and triplicate respectively. DEPPD (Sigma Chemical Company, St. Louis, MO) and iron sulphate (Lab Chem, Edenvale) dissolved in sodium acetate buffer respectively was prepared in a 1:25 ratio (v/v) in a separate glass beaker and 100 μ l of the reagent mixture added to each allocated well. Colour development was recorded at 546 nm, 25 °C, every 10 minutes using the FL 600 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc., Winooski, VT). A standard curve was plotted and the ROS units of each sample determined. Using Microsoft Office Excel 2007 the average ROS production, standard deviation (SD) and RSD for each sample was calculated following normalization with a sample that was analyzed on each of the respective plates. An unpaired, nonparametric t-test (Mann-Whitney) was performed to test for significant differences ($p < 0.05$) between HIV- and HIV+ samples. Box and whisker plots were plotted using STATISTICA (version 9).

For the remainder of the assays explained below, statistical analysis of the data was done using Microsoft Office Excel 2007 and Graphpad Prism 5 (GraphPad Software, San Diego, CA) unless stated otherwise.

5.2.4 PBMC apoptosis

Background: HIV directly and indirectly (through its proteins, ROS production, induced cytokines etc) affect mitochondria ([Maagaard and Kvale 2009](#)). For an immunological assessment of this change the apoptotic profile of PBMCs was measured by flow cytometry using the annexin V FITC and PI kit from BD Biosciences, California, USA. Apoptosis is characterized by various morphological changes and a loss in membrane integrity. During apoptosis; phosphatidylserine, a negatively charged phospholipid, usually located in the inner leaflet of the plasma membrane moves to the surface of cells. Annexin V is a phospholipid-binding protein and has great affinity for phosphatidylserine. When conjugated to FITC and incubated with cell suspensions, annexin V detects apoptosis. PI, a dye excluded by viable cells and taken up by dead ones, stains the DNA of damaged cells and is used to detect necrotic cells. Cells which stain negative for both annexin V and PI are thus considered to be viable whilst cells staining positive for annexin V-FITC are apoptotic. Those cells staining positive for both annexin V-FITC and PI are representative of damaged and dead cells respectively ([van Engeland et al., 1998](#)).

Protocol: To measure the percentage apoptosis occurring in HIV- and HIV+ PBMCs respectively; 2 ml of cells at 1×10^6 cells/ml was washed with ice cold phosphate buffered saline (PBS, 500 \times g, 5 minutes) and resuspended in 100 μ l binding buffer. Cells were stained with 2 μ l annexin V-FITC and 2 μ l PI solution. Cells and dye were mixed and incubated at 4 $^{\circ}$ C in the dark for 15 minutes. Binding buffer (400 μ l) was then added to the cell-dye mixture. At least 10 000 events were collected on a FACSAria (BD Biosciences, California, USA) within 30 minutes of adding binding buffer. In the literature a minimum of 10 000 events (cells) is commonly recorded for this assay (Potter et al., 1999; Lecoeur et al., 2008). Unstained cells, annexin positive and PI positive controls were prepared and utilized for compensation (mathematical elimination of the spectral overlap between different fluorochromes) as well as quadrant specification. Quadrants are defined through the use of fluorescence minus one (FMO) controls. These are staining controls that include all staining reagents except the one of interest and are used to accurately differentiate between cell populations within a stained sample (Roederer, 2001). Annexin positive cells were obtained by treating 2 ml of cells which were at 1×10^6 cells/ml with 20 μ l of a 1 mg/ml PHA-P solution and incubating it at 37 $^{\circ}$ C for 1 hour. PHA-P treated cells were then exposed to 270 μ l of 37 % formaldehyde for 30 minutes at 37 $^{\circ}$ C. Prior stimulation of cells with stimulants has been shown to elevate apoptosis measurements (Potter et al., 1999). PI positive cells were obtained by exposing the cells to ice cold methanol for 5 minutes. The treated cells for annexin and PI were centrifuged, washed with 2 ml cold PBS (500 \times g, 5 minutes), resuspended in binding buffer, stained with 2 μ l annexin V-FITC and 2 μ l PI respectively for 15 minutes on ice and an additional 400 μ l binding buffer added to allow for FACS acquisition. Data analysis was done using FlowJo version 7.6.1 (Tree Star, Inc. Oregon, USA). Apoptosis was finally reported as the percentage cells that stained positive for annexin-V FITC. A total of 91 samples (55 HIV- and 36 HIV+) were finally analyzed from an initial total of 95 and an unpaired, nonparametric Mann-Whitney test performed to determine significant differences in apoptosis between HIV- and HIV+ samples.

5.2.5 T cell apoptosis

Background: Cells, once differentiated, are identified by the presence of certain markers on their surface. T cells are characterized by the CD3 molecule whereas T helper and cytotoxic cells are characterized by CD4 and CD8 respectively. A fluorescently labelled monoclonal antibody to CD3 was included in the staining panel to aid in the selection of T cells which were of interest because these are commonly infected and affected by HIV infection. If CD3 as a marker was excluded from the staining panel, the final data would include the responses of for example CD8 positive NK cells (Lamoreaux et al., 2006). Measuring specifically those cells which contribute to the apoptotic signal is important for understanding

immune system pathologies (Potter et al., 1999). To confirm which cells contribute most to the apoptotic signal (CD4 or CD8); the apoptosis protocol for PBMCs was modified to allow for the measurement of apoptosis in T cells. Staining for T cells as an additional parameter allowed for commentary on the health status of the individual from who the sample was obtained. Furthermore, the percentage cells having surface CD4 gives an indication of the pathological effects of HIV on the immune system (Gupta and Gupta 2004).

Protocol: T cell apoptosis was measured as per the protocol of Gamberale et al (2003) with slight modifications. Two millilitres of PBMCs at 1×10^6 cells/ml from HIV- ($n = 11$) and HIV+ ($n = 12$) individuals were washed with ice cold PBS (500 \times g, 5 minutes). The cell pellet was resuspended in PBS and surfaced stained with a pre-titrated concentration of Pacific Blue™ Mouse Anti-Human CD3, R-Phycoerythrin (PE)-conjugated Mouse Anti-Human CD4 and CD8 Peridinin Chlorophyll Protein Complex (PerCP, SK1). All fluorochromes were purchased from BD Biosciences, California, USA. After 30 minutes of staining; the cells were washed with PBS and resuspended in 100 μ l binding buffer followed by staining with 2 μ l annexin V-FITC solution. Sample and dye were mixed and incubated in the dark for 15 minutes, on ice. Binding buffer (400 μ l) was then added to the cell-dye mixture and the sample analyzed within 30 minutes of adding the buffer. Unstained cells, CD3, CD4, CD8 (Schweneker et al., 2008) and annexin positive controls were prepared and utilized for compensation purposes. Fluorescence minus one controls were also prepared and 30 000 events collected for each sample on the FACS Aria. The number of events collected was increased since more gates were being applied to select the cells of interest. With each gate the total number of events tends to decrease (Roederer, 2008). To compensate for this, more events are collected to begin with. Data analysis was done using FlowJo version 7.6.1. An unpaired, nonparametric Mann-Whitney test was performed to test for significant differences in T cell apoptosis between HIV- and HIV+ samples. To test whether there was a significant difference in the percentage apoptosis occurring in the CD4 and CD8 cells of HIV- and HIV+ cells respectively; the nonparametric Wilcoxon signed-rank test was done.

5.2.6 Cytokine Production

5.2.6.1 Intracellular Cytokine Staining (ICCS)

Background: Infection with HIV not only results in the loss of cell numbers but also a loss in CD4+ T cell function. This loss of cellular function becomes evident in asymptomatic individuals even before CD4 numbers decline (Sarih et al., 1996). When cell function is reduced, the ability of cells to proliferate and respond to mitogenic and/or antigenic stimulation is lowered or lost (Clerici et al., 1997). To assess cell functionality, the

intracellular production of IFN- γ and TNF- α in response to PHA-P (Sigma Chemical Company, St. Louis, MO), PMA-ionomycin (Sigma Chemical Company, St. Louis, MO), R7V (a β 2m derived peptide) and Gag DU422 was measured in HIV- and HIV+ cells. PHA-P and the PMA-ionomycin mixture are mitogens (chemical substances which stimulate cells to divide) and were used as positive controls to indicate cell proliferation and cytokine production respectively. PMA-ionomycin was prepared by mixing PMA with ionomycin prior to treating the cells. These stimulants were finally at 10 ng/ml and 1 μ M when added to cells. The Gag peptide pool (mixture of peptides, 15 amino acids in length overlapping by 11 amino acids) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 DU422 Gag (15-mer) Peptides - Complete Set.

Protocol: ICCS determinations are based on the protocol of [Jung et al \(1993\)](#) and incorporate modifications from more recent articles referenced herein. To measure intracellular cytokine production, 100 μ l aliquots of PBMCs (isolation described in Section 4.2.3 and 5.2.2) at 2×10^7 cells/ml was stimulated with media only, 2 μ g/ml of PHA-P, 10 ng/ml PMA-ionomycin, 10 μ g/ml R7V and a pool of Gag peptides (final concentration of the individual peptides was 1 μ g/ml) respectively. Cells were finally diluted to a concentration of 1×10^7 cells/ml in media. GolgiPlug (1 μ g/ml, BD Biosciences, California, USA) containing brefeldin A, is a protein transport inhibitor and was added to the cells directly after the various stimulants to prevent the secretion of cytokine from cells. The V-shaped 96-well plate (Nunc™, Roskilde, Denmark) containing samples was incubated at 37°C for 6 hours. According to the literature sufficient IFN- γ and TNF- α is obtained after approximately 5 hours of stimulation ([Mascher et al., 1999](#); [Prussin and Metcalfe 1995](#)). Extensive incubation times are also avoided to maintain live cells and minimize the potential for background/excess proliferation ([Maino and Maecker 2004](#)). After incubation, the cells were placed at 4 °C overnight ([Lamoreaux et al., 2006](#); [Betts et al., 2001](#)) to reduce cell activation processes and therefore cytokine production. The next day the cells were equilibrated to room temperature and mixed gently. The cells were centrifuged at 258 \times g for 10 minutes and the supernatant containing secreted cytokines harvested and stored at -70 °C. This was done to later determine background levels of secreted cytokine which is expected with HIV-induced immune activation and the time lapse before GolgiPlug begins to work. Finally, there was also the uncertainty about whether all cytokine is retained in the event of maximum stimulation. The cell pellet was resuspended in 180 μ l blocking buffer (10 % FBS in PBS) for 20 minutes at 4 °C. Blocking buffer was removed and the pellet washed twice (258 \times g, 5 minutes) with 200 μ l PBS. Next, the cells were stained with a predetermined concentration of Pacific Blue™ Mouse Anti-Human CD3, PE-conjugated Mouse Anti-Human CD4, CD8 PerCp (SK1) and the aqua fluorescent reactive dye (Invitrogen, Eugene, Oregon, USA), in

PBS. The aqua fluorescent reactive dye (also known as amine reactive dye) was incorporated into the staining procedure to assist with the exclusion of dead cells which contribute to background staining and incorrect interpretation of flow cytometry immunophenotyping data (Perfetto et al., 2006). This is especially troublesome when detecting low frequency T cell populations. Since cell functionality was being assessed it made sense to exclude the dead cells and only analyze the live cells. In principle, the aqua fluorescent reactive dye reacts with free amines on the surface of viable cells yielding a low fluorescent signal. When cells are damaged/dead the dye penetrates the plasma membrane and reacts with the free amines inside as well as outside the cell to yield an intense fluorescent signal. Based on the differences in fluorescence intensity, live and dead cells are discriminated (Perfetto et al., 2006). This dye is stable under fixation and permeabilization conditions unlike other viability dyes such as PI which leak out of the cells after such treatments resulting in a loss in fluorescence (Tung et al., 2007; Perfetto et al., 2006). Because the amine reactive dye is sensitive to the proteins contained in serum, PBS (without FBS) was used as the staining buffer. Staining was done in the dark for 20 minutes at room temperature. Cells were then centrifuged at 258 $\times g$ for 5 minutes to remove the stains and washed twice with 200 μl staining buffer (3 % FBS in PBS). Cells were fixed and lysed for 20 minutes at 4 °C using the Cytfix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, California, USA). For subsequent wash steps the centrifugation speed was increased (Sander et al., 1991) i.e. cytofix/cytoperm was removed following centrifugation at 402 $\times g$, 5 minutes and the cells subsequently washed with 200 μl of Perm/Wash™ buffer (BD Biosciences, California, USA). The cell pellet was resuspended in Perm Wash™ buffer containing a predetermined concentration of FITC Mouse Anti-Human IFN- γ and Allophycocyanin (APC)-conjugated anti-Human TNF- α respectively. Staining was done in the dark for 30 minutes. Subsequently, the cells were centrifuged at 402 $\times g$ for 5 minutes to remove the stains and washed twice with 200 μl Perm/Wash™ buffer (402 $\times g$, 5 minutes). Cells were finally resuspended in 150 μl of PBS. A further 150 μl of 6 % formaldehyde (in PBS) was added to the cells resulting in a final concentration of 3 % paraformaldehyde. Resuspending the cells in PBS prior to adding paraformaldehyde minimizes cell clumping. Unstained cells as well as amine, CD3, CD4, CD8, IFN- γ and TNF- α positive controls were prepared and utilized for compensation purposes. The amine control was obtained by treating the cell pellet with 200 μl ice cold methanol. FMO controls were prepared to specify gating regions. At least 30 000 events were acquired for the control and test samples using the FACS Aria (the reasoning for the number of events collected is explained in Section 5.2.5). Data analysis was done using FlowJo version 7.6.1. Finally, the percentage cells which produced cytokine were determined. While cell viability and the percentage surface

molecules were reported on a linear scale, data for the percentage cytokine producing cells was log scaled to allow for visual comparisons. Eleven HIV- and 11 HIV+ samples were analyzed for all of the treatments except Gag where $n_{\text{HIV-}} = 1$ and $n_{\text{HIV+}} = 6$ largely due to limited volumes of the peptide.

5.2.6.2 Secreted cytokines

5.2.6.2.1 Cytometric Bead Array (CBA) assay

Background: To further characterize the immune profile of HIV-infected biofluid, the secretion of a range of cytokines; IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ and IL-17A was measured for selected serum samples (34 HIV- and 25 HIV+) that had also been used for metabonomic analysis. This was done using the human Th1/Th2/Th17 CBA kit from BD Biosciences, California, USA. The CBA assay is similar to the standard ELISA but has the added advantage of quantifying more analytes at a time from limited sample (50 μ l). With CBAs the ligands are captured on spherical beads in suspension whereas a flat surface is used during ELISAs. Another difference is that CBA techniques use fluorescence for detection (flow cytometry) whereas ELISAs are based on a colorimetric change ([Elshal and McCoy 2006](#)).

CBA analysis is based on the fact that beads having different fluorescence intensities are coated with capture antibodies specific to the cytokines to be measured (illustrated in Figure 5.1). On the flow cytometer the respective intensities are representative of a population for a single analyte (in this case a cytokine). When the capture beads for the respective cytokines are mixed, the simultaneous quantification of these molecules within one sample is possible. Upon mixing with PE-conjugated detection antibodies (also called reporter antibodies), standards and/or samples, the capture beads form sandwich complexes which yield a fluorescent signal that is proportional to the concentration of the analyte. CBA analysis is precise and offers a wide detection range. It has been used to measure the concentration of various molecules that are relevant in biology ([Morgan et al., 2004](#)), in a wide variety of biofluid types.

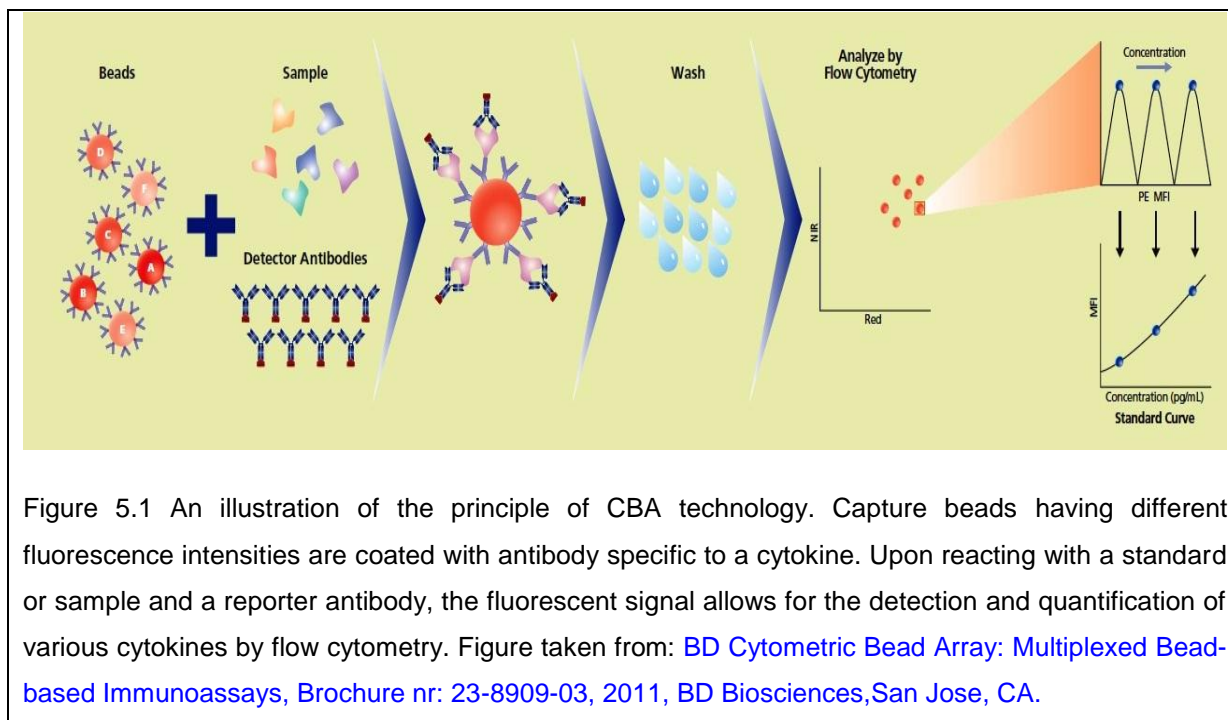


Figure 5.1 An illustration of the principle of CBA technology. Capture beads having different fluorescence intensities are coated with antibody specific to a cytokine. Upon reacting with a standard or sample and a reporter antibody, the fluorescent signal allows for the detection and quantification of various cytokines by flow cytometry. Figure taken from: [BD Cytometric Bead Array: Multiplexed Bead-based Immunoassays, Brochure nr: 23-8909-03, 2011, BD Biosciences, San Jose, CA.](#)

CBA Protocol: For CBA analysis, aliquots of the serum samples were thawed on ice and diluted using assay diluent (1:2 v/v). Cytokine standards were serially diluted (0 -5000 pg/ml) to facilitate the construction of calibration curves necessary for determining the unknown concentrations of test samples. Capture beads coated with antibody specific to IL-2, 4, 6, 10, TNF- α , IFN- γ and IL-17A were pooled (10 μ l per assay tube analyzed). Fifty μ l of capture bead mixture was added to 50 μ l of sample and standard respectively. To these 50 μ l of PE-conjugated detection antibody was added. The mixture was then incubated for 3 hours in the dark with occasional shaking to allow sandwich complexes to form. Following incubation, samples were washed with 1 ml of wash buffer (with centrifugation at 258 \times g, 5 minutes) and the pellet resuspended in 300 μ l wash buffer. Samples (200 μ l) were plated on a PRO-BIND™ 96 well assay plate and analyzed on the FACSArray Bioanalyzer (flow cytometer with plate sampler used for the detection of cell-associated, secreted or cell lysate protein) using FCAP FCS Filter and FCAP Array Software (BD Biosciences, San Jose, CA, USA). With these software packages, debris was filtered from the data and the identification of the bead populations and their mean fluorescence intensities (MFI) automated. The MFI of test samples were fitted into the 5-parameter logistic curve-fitting equation to obtain the concentration of the respective cytokines in the test samples. Three independent experiments were performed.

CBA Data Processing: Data from FCAP Array was exported to Microsoft Office Excel 2007 and standardized using IBM SPSS (version 19.0). Cytokine concentrations of each sample was averaged and a pooled average of the HIV- and HIV+ samples log transformed to make

the scales of the data more comparable. Using the log-transformed cytokine concentrations; LDA, logistic regression and ANOVA was applied to classify the two groups based on endogenous cytokine secretion and to identify cytokines significantly altered by HIV infection (for statistical methods refer to Section 3.7).

Clerici (2010) recently introduced the concept of cytokinomics which refers to the study of cytokine production and the interactive effects of these molecules in a biological system. This definition was modified in 2.7.3 to link the detection of multiple secreted cytokines to multivariate statistical analysis and biomarker discovery in context to HIV/AIDS. This approach in addition to quantifying cytokine levels in HIV- and HIV+ samples, allowed for the discrimination between HIV- and HIV+ samples based on these immune molecules. The association of cytokinomics data to metabonomics data was also evaluated.

For all the cytokine analysis (ICCS and secreted IFN- γ), an unpaired, nonparametric Mann-Whitney test was used to test for significant differences between HIV- and HIV+ samples. Differences between the untreated and treated cells within the study groups (HIV- and HIV+) were analyzed using the nonparametric Wilcoxon signed-rank test. In all the analysis performed a p-value < 0.05 was considered to be statistically significant. Most of the data for this chapter was presented in the form of box plots because these display various characteristics of the data.

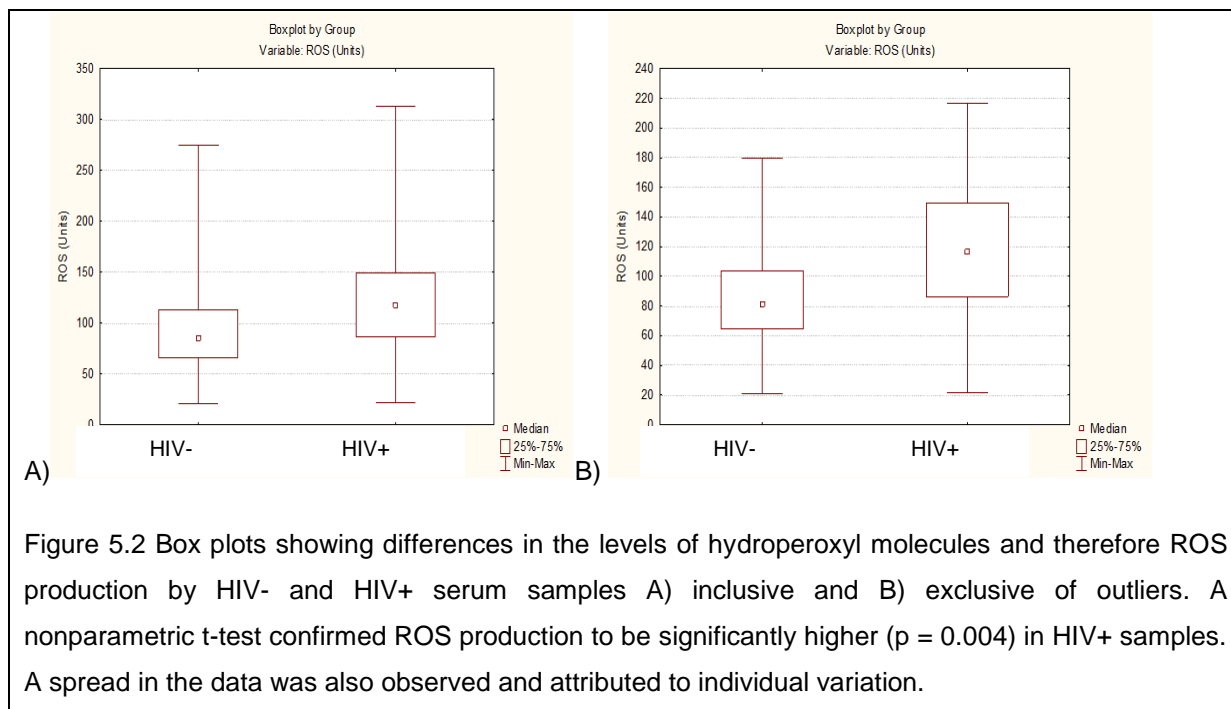
5.3 Results and Discussion

5.3.1 Reactive Oxygen Species (ROS)

The redox status of HIV- and HIV+ serum was determined by spectrophotometry. To avoid batch effects samples were frozen and analyzed on the same day. If analysis was not done this way batch effects occurred (data and explanation provided in the appendix, Section 2a). Figure 5.2 A shows that the amount of ROS measured in the HIV- serum was significantly lower than that of the HIV+ serum samples. Also observed in Figure 5.2 A was a large spread in the data indicating individual variation. In the data related to Figure 5.2 A, four HIV- samples were identified as outliers. These samples had high ROS levels and were primarily obtained from females. There were thus many factors (inflammation due to menstruation, use of contraceptives etc) which contributed to the high ROS measured. In addition, one of the HIV+ samples had extremely high ROS levels and presented as an outlier. Based on the medical records and the CD4 count this individual was clinically stable. Reasons for the higher ROS measured were thus unclear. Five outliers were detected and subsequently removed from the analysis yielding the box plots shown in Figure 5.2 B. Two HIV+ samples had very low ROS levels but did not present as outliers and were kept as part

of the analysis. Medical records indicated that the individuals from whom the samples were collected had been diagnosed with euthyroid syndrome. Thyroid hormones have a role in regulating metabolism. Hyperthyroidism for example induces a hypermetabolic state, excessive electron transport within mitochondria, an increase in ROS and therefore oxidative stress. In contrast; the euthyroid syndrome (as diagnosed for the two cases) causes a decrease in the metabolic activity of cells, a subsequent decrease in radical production by mitochondria and as such serves as a defense against oxidative stress. By decreasing the metabolic activity of the cells, energy is also conserved (Selvaraj et al., 2008; Sarkar et al., 2006). It is also important to highlight that the mean age of the controls was lower than that of the patients and as a result contributed to the low ROS levels detected for this group. Most of the patients who participated in this study were from a poor socio-economic setting/background thus the assumed improved lifestyle conditions of the uninfected controls (healthy diet, less polluted environment etc) served as an additional confounding factor for the differences noted. That some HIV- samples had very high ROS levels while some HIV+ samples had very low ROS levels is also indicative of the various other confounding sources which contribute to the development of oxidative stress (shown in Figure 2.10). Despite these factors there was still a significant elevation ($p = 0.004$) in the amount of ROS measured in HIV+ samples.

Cell membranes are rich in unsaturated fatty acids making them highly susceptible to oxidation (Kohen and Nyska 2002). When oxidative damage to membranes occurs, hydroperoxides are produced and indicate membrane damage to cells as well as changes in membrane fluidity and membrane potential (Repetto et al., 1996; Pace and Leaf 1995). In the work of Bayir and Kagan (2008), hydroperoxides were identified as contributors to lipid peroxidation and mitochondrial dysfunction, early after brain injury. These molecules which signal mitochondrial distress were also found to be elevated early during HIV infection (Mollace et al., 2002; Pace and Leaf 1995) and are indicative of oxidative stress and subsequent apoptosis. Reports of decreases in antioxidant reserves during HIV infection were reported by Wanchu et al (2009), Turchan et al (2003) and Roederer et al (1991). The findings of high ROS during HIV infection is thus in accordance with the literature (Banki et al., 1998) presented here and explained in Section 2.7.2.



5.3.2 PBMC apoptosis

ROS are primarily produced in mitochondria (Kohen and Nyska 2002) with levels peaking prior to and during apoptosis. The indirect detection of ROS through hydroperoxyl molecules was confirmatory of the fact that the immune system was activated. Free radical production triggers apoptosis (Cossarizza et al., 2002) and activated cells are prone to apoptosis (Cossarizza et al., 1997), thus the increased detection of hydroperoxyl molecules and therefore ROS in HIV+ serum meant a corresponding apoptotic profile should be detectable. In addition, fatty acids such as those identified through the metabonomics analysis in Chapter 4 (Table 4.7-4.9) have been previously reported to be indicators of the peroxidation of PBMC membranes, oxidative damage and therefore apoptosis (Míro et al., 2004). Confirmation of the disruption to mitochondrial dysfunction would thus be further validated by an apoptosis profile of HIV+ cells being higher than that of uninfected cells.

Following flow cytometry analysis using annexin V-FITC and PI respectively, FlowJo was used to analyze the data. Shown in Figure 5.3 are dot plots having axes ranging from $0-10^5$. Cells situated closer to zero are unstained and would therefore be observed at the bottom left (BL) quadrant. With the induction of early apoptosis, the cells group in the top left (TL) quadrant and reflect an increase in the annexin V-FITC signal. As apoptosis advances, the signal for both annexin V-FITC and PI increases causing cells to group in the top right (TR) quadrant. When completely damaged, the cells take up PI causing the signal in the PE channel to rise. Necrotic cells are thus visible in the bottom right quadrant (BR). The labels

BL, TL, TR and BR were indicated on only one of the dot plots to minimize cluttering of the data. The gating strategy employed for identifying apoptotic cells (Figure 5.3) entailed selecting out the lymphocytes and quantifying the percentage apoptosis in these cells. Figure 5.4 shows a summary of the viability and PBMC apoptosis profiles for all the samples that were analyzed.

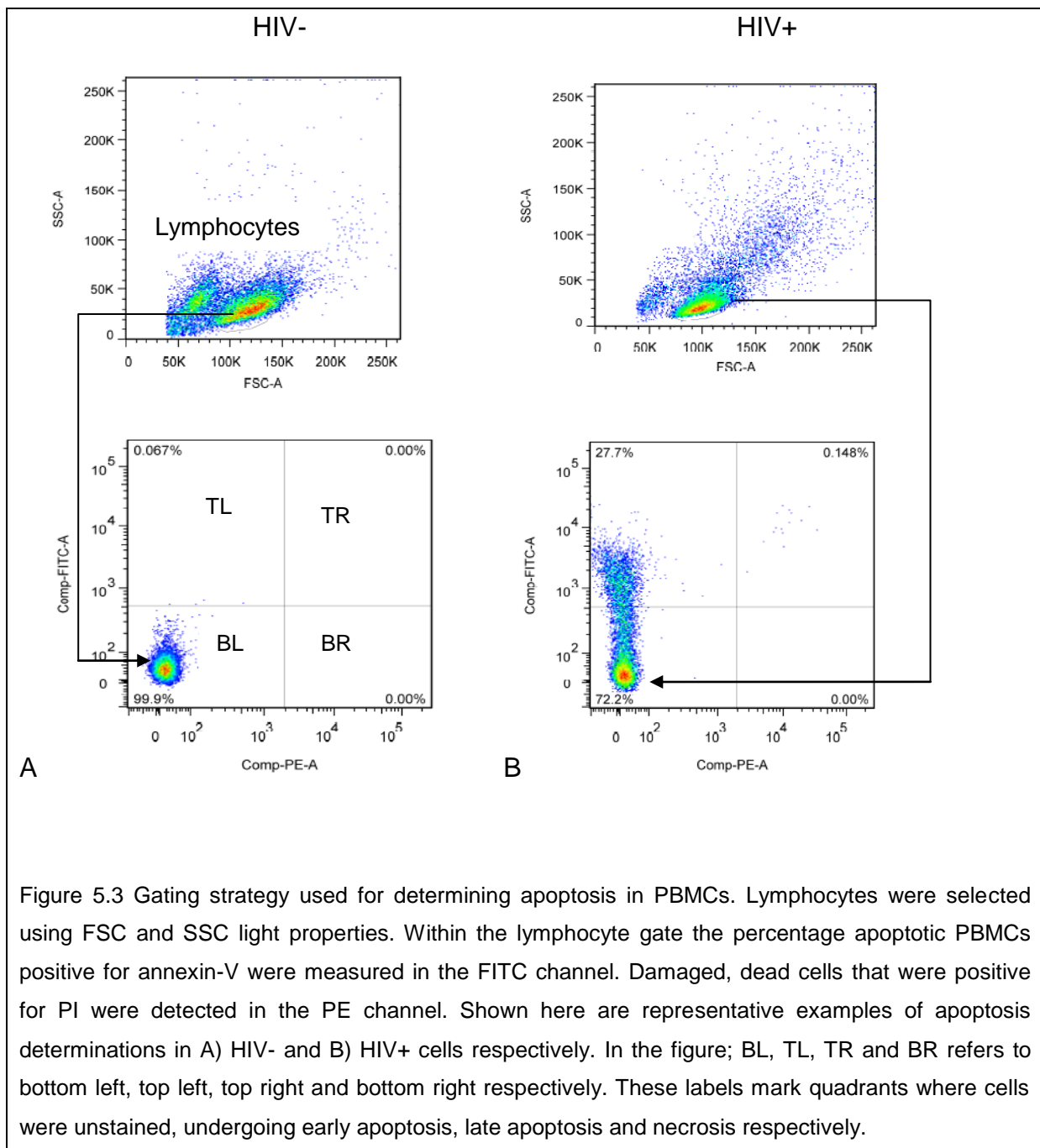


Figure 5.3 Gating strategy used for determining apoptosis in PBMCs. Lymphocytes were selected using FSC and SSC light properties. Within the lymphocyte gate the percentage apoptotic PBMCs positive for annexin-V were measured in the FITC channel. Damaged, dead cells that were positive for PI were detected in the PE channel. Shown here are representative examples of apoptosis determinations in A) HIV- and B) HIV+ cells respectively. In the figure; BL, TL, TR and BR refers to bottom left, top left, top right and bottom right respectively. These labels mark quadrants where cells were unstained, undergoing early apoptosis, late apoptosis and necrosis respectively.

Freshly isolated HIV- and HIV+ PBMCs were found to be viable (Figure 5.4 A) but the viability of the HIV+ PBMCs was significantly lower ($p < 0.0001$) than that of the HIV- PBMCs. This makes sense since these cells are burdened with an additional stressor; the HI virus. Where the viability of cells was lowered, this was mainly as a result of cells experiencing cell death in the form of early apoptosis (which was measured by cells staining positive for annexin V-FITC, Figure 5.4 B). The healthy uninfected cells experienced a degree of spontaneous apoptosis but this was lower compared to the percentage apoptosis experienced by the infected PBMCs. In another study which investigated the role of apoptosis during CD4 depletion, HIV+ samples were found to present with lower viabilities as well (Oyaizu et al., 1993). Similar to the work presented here; PBMCs and T lymphocytes have previously been shown to undergo spontaneous apoptosis with or without any form of stimulation (Cossarizza et al., 2002; Gougeon and Montagnier 1999; Clerici et al., 1997). Spontaneous apoptosis in freshly isolated cells and cells cultured short-term have been reported elsewhere (Potter et al., 1999; Gougeon et al., 1996). This is mainly because the cells are fragile following isolation and during culture. HIV+ cells are more prone to undergoing apoptosis than their uninfected counterparts (Herbein et al., 1998; Meygaard et al., 1992). The findings presented here are in accordance with the degree of oxidative stress experienced as well as the increase in organic acids profiled in HIV+ biofluid (peroxidation of membranes and the increased detection of fatty acids which are indicative of membrane damage and permeability, features synonymous with HIV-induced apoptosis).

No additional assay was done to confirm apoptosis. Since the cells from asymptomatic HIV-infected individuals were mainly undergoing early apoptosis, confirmatory tests through use of the TUNEL assay was not done since DNA fragmentation occurs primarily during late apoptosis. When apoptosis occurs through the intrinsic pathway (as in this case) caspase activation follows after oxidative damage to cell membranes. The detection of hydroperoxides is thus not only relevant as oxidative stress markers but serve as additional indicators of early apoptosis for our study group, mainly because they are produced when there is oxidative damage to membranes and a change in mitochondrial membrane potential, earlier features ultimately associated with apoptosis. Although there is a significant difference in the percentage apoptosis for HIV- and HIV+ cells, the percentages measured here were slightly higher than that in most of the literature reviewed and may be attributed to individual experimental conditions (cell isolation procedures, handling of cells, washing of cells etc) which have an impact on apoptosis detection outcome (Glisic-Milosavljevic et al., 2005). In most of the literature which relates to apoptosis measurements, CD4 counts are not necessarily emphasized. In a study by Sarih et al (1996) the percentage lymphocytes undergoing apoptosis were determined in individuals having low as well as

moderate to high CD4 counts respectively. Those individuals having >500 cells/ μ l of blood experienced approximately 35 % apoptosis. The bulk of the individuals used in the present study had moderate to high CD4 counts, making the measured average of 27 % apoptosis, acceptable.

There is however literature where authors have failed to detect apoptotic lymphocytes in freshly collected blood (Cossarizza et al., 1997). A possible reason for these different findings could be attributed to the use of a different dye and an acute HIV infection model. In most other studies (this one included), investigations were primarily based on chronic HIV infection.

In addition to annexin-V FITC staining which detects early apoptosis, PI (measured in the PE channel) which detects damaged, necrotic cells was included as part of the staining panel (the data and an explanation thereof is provided in the Appendix, Figure A5).

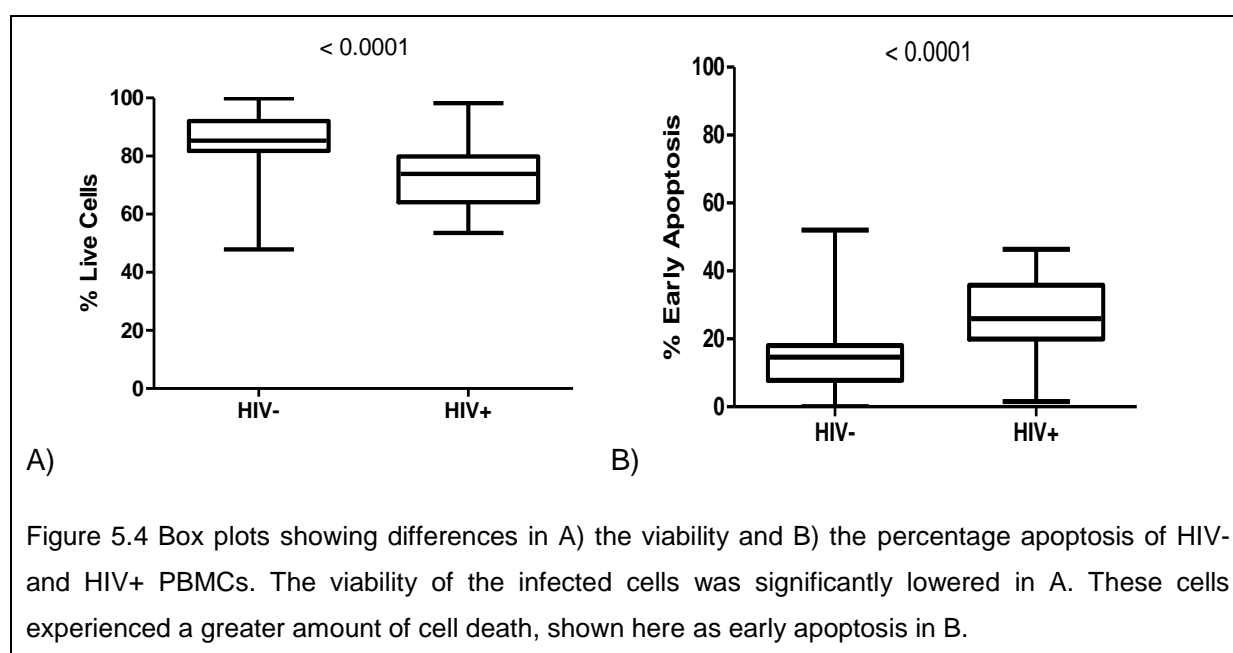


Figure 5.4 Box plots showing differences in A) the viability and B) the percentage apoptosis of HIV- and HIV+ PBMCs. The viability of the infected cells was significantly lowered in A. These cells experienced a greater amount of cell death, shown here as early apoptosis in B.

5.3.3 T cell Apoptosis

PBMCs consist of a mixture of cells. T cells are most commonly affected by HIV infection thus the percentage T cells contributing to the apoptotic signal was determined. Characterizing the contribution of these cells will add to understanding HIV-induced immune pathologies. PBMCs were therefore labelled with surface markers (CD3, CD4 and CD8) followed by annexin-V FITC staining. The data was processed using FlowJo. Similar to the explanation provided in Section 5.3.2, unstained cells group closer to zero whilst stained

cells reflect an increase in fluorescence intensity i.e. move toward 10^5 . Figure 5.5 shows the gating strategy employed i.e. singlets were selected followed by the lymphocytes, T cells and finally measuring the percentage apoptosis in the CD4 cells and CD8 cells respectively. Figure 5.6 shows a summary of T cell apoptosis as detected in the analyzed samples.

A significant amount of apoptosis was seen in the HIV+ CD4 ($p < 0.0001$) and CD8 cells ($p = 0.0002$) compared to the uninfected T cells (Figure 5.6 A and B). A small degree of spontaneous apoptosis was observed in the HIV- T cells. This increase in apoptosis of freshly isolated T cells was also observed by [Herbein et al \(1998\)](#). Spontaneous apoptosis in freshly isolated T cells is attributed to the fragile nature of the cells following isolation and short-term culture as mentioned for PBMCs in an earlier section. Within the infected cells, the percentage cells with CD8 on their surface and positive for apoptosis was more than the CD4 cells ($p = 0.0269$, Figure 5.6 D). Phenotypic data showed a decrease in the CD4: CD8 ratio and is representative of data in the literature. Controversy exists in the relevant literature over whether apoptosis occurs primarily in CD4 or CD8 cells. The greater percentage CD8 cells staining positive for apoptosis as presented here is in agreement with the findings of most researchers ([Cotton et al., 1997](#); [Gougeon et al., 1996](#); [Lewis et al., 1994](#); [Meyaard et al., 1992](#)). Staining with annexin V-FITC showed these cells expressing surface CD8 to be undergoing apoptosis. While the phenotypic and apoptotic data may seem contradictory it is important to note that the two parameters measured are different. Apoptotic cells may still have retained surface expression of CD8 molecules. [Herbein et al \(1998\)](#) failed to show apoptosis of CD8 cells. According to [Holm and Gabuzda \(2005\)](#), apoptosis of CD8 cells is dependent on the presence of CD4 cells that release a soluble factor required for apoptosis to proceed. [Herbein et al \(1998\)](#) may not have observed CD8 apoptosis due to the increase in CD4 cell depletion in their model. The participating donors of this study had moderate to high CD4 counts (although it was still less than that of uninfected controls). Since these individuals were still clinically stable, a higher percentage CD8 apoptosis would be expected.

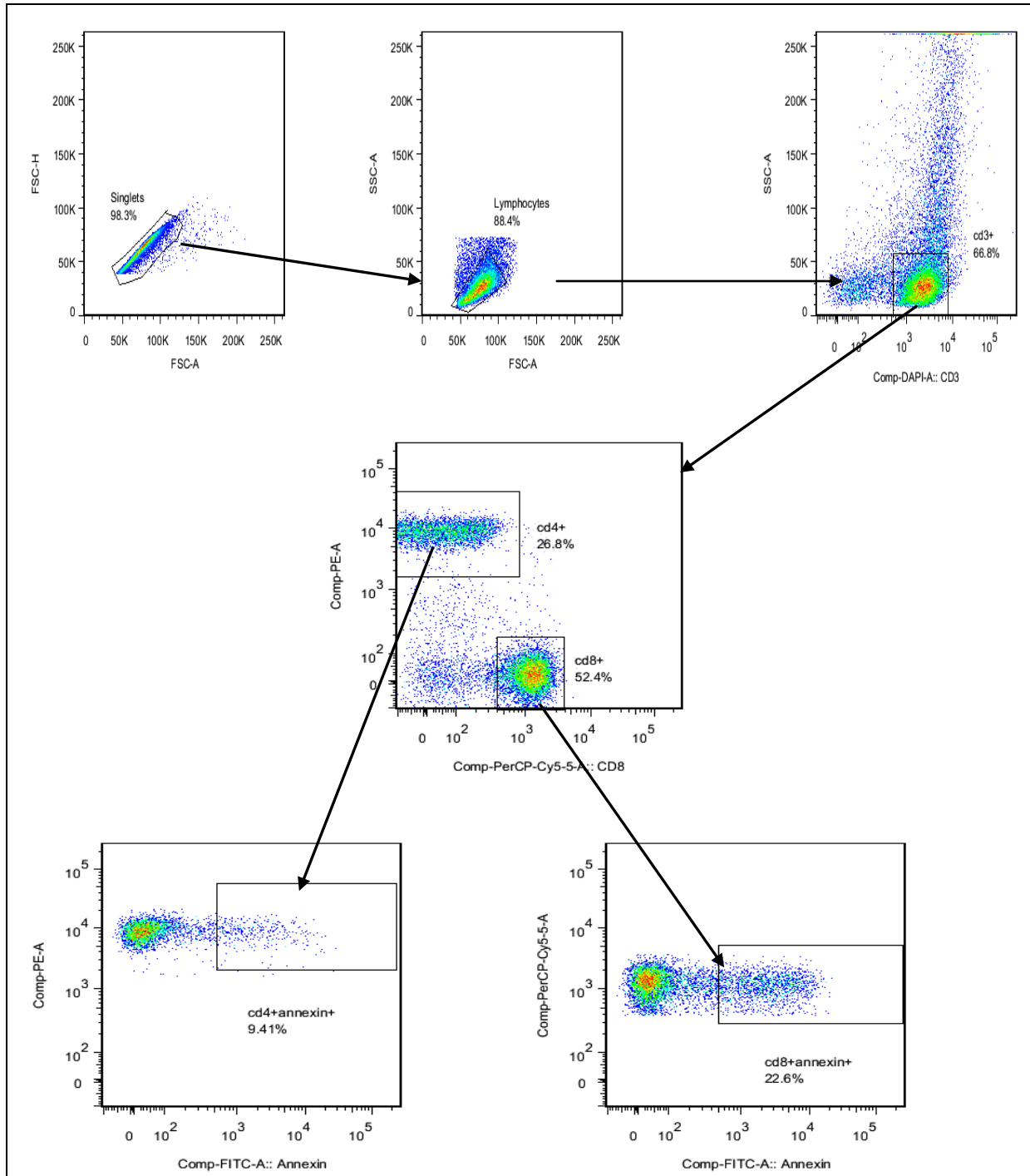


Figure 5.5 Gating strategy used for determining apoptosis in T cells. Singlets were selected followed by the lymphocytes, CD3, CD4 and CD8 cells. Lastly, the percentage apoptotic CD4 and CD8 cells (measured in the PE and PerCP-Cy5.5 channels respectively) positive for annexin-V FITC was determined. Shown here is an example of apoptosis in the T cells of an HIV+ individual. Compared to Figure 5.4 the FSC of these cells are lower. Apoptotic cells shrink thus they become smaller in size. CD8 cells detected in the PerCP-Cy5.5 channel (last dot plot to the right) experienced more apoptosis (detected in the FITC channel) than the CD4 cells (detected in the PE channel, last dot plot to the left).

Still, apoptosis of HIV- CD8 cells is markedly lower than that of the HIV+ CD8 cells. The expression of Bcl-2 (an anti-apoptotic protein) was found to be decreased in CD8 cells and according to [Lecoeur et al \(2008\)](#) explains the higher percentage apoptosis experienced by the cells. To further confirm CD8 apoptosis and clarify which mechanisms lead to the death of these cells, this protein's levels could be determined in future analysis.

For both the PBMC and T cell apoptosis determinations, some HIV+ samples presented with low apoptotic profiles. Some of the samples utilized in the study comprised of LTNPs (patients who complied with the selection criteria of being HIV+ and ART naive). These individuals are known to experience less oxidative stress, mitochondrial damage and subsequently less apoptosis.

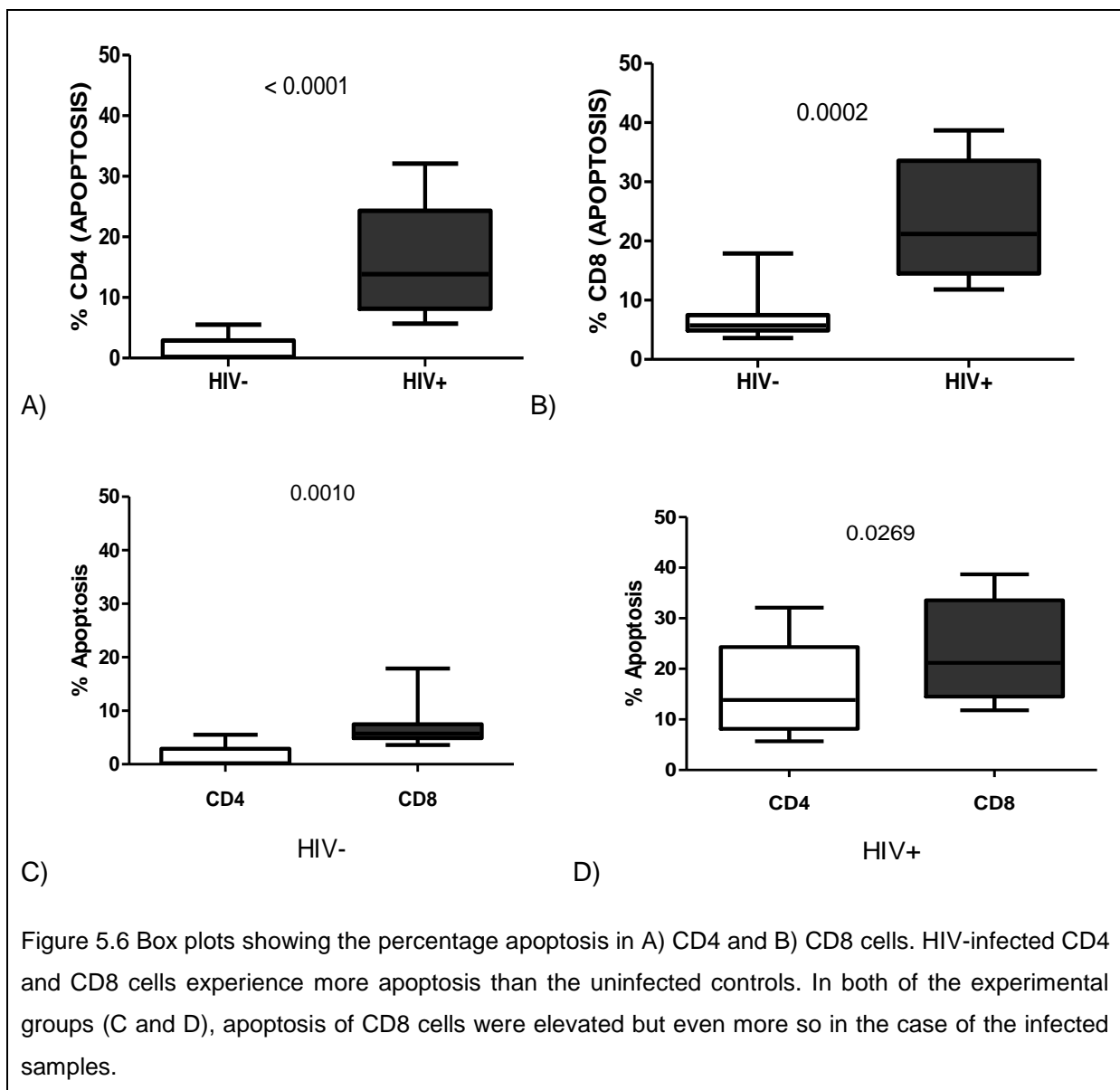


Figure 5.6 Box plots showing the percentage apoptosis in A) CD4 and B) CD8 cells. HIV-infected CD4 and CD8 cells experience more apoptosis than the uninfected controls. In both of the experimental groups (C and D), apoptosis of CD8 cells were elevated but even more so in the case of the infected samples.

5.3.4 Cytokine Production

5.3.4.1 Intracellular

Flow cytometry allows for the detection of cytokines in defined cell populations. Here T cell functionality was determined by measuring the percentage surface molecules on cells and the ability of the cells to produce IFN- γ and TNF- α in response to *in vitro* stimulation with mitogen or virus-derived peptide/antigen. An eight parameter analysis was performed; the size and complexity of the cells were measured, four phenotypic markers and two intracellular cytokines. The data was processed using FlowJo. The concept of unstained cells grouping closer to zero whilst stained cells reflect an increase in fluorescence intensity, i.e. move toward 10^5 , was explained in Section 5.3.2. To determine the phenotype of the cells (surface markers) as well as its functional characteristics (cytokine production) the following gating strategy was used (shown in Figure 5.7). Singlets were selected, followed by gating of the lymphocytes, viable cells, CD3 T cells, CD4 T cells and CD8 T cells respectively. Finally, the percentage CD4 and CD8 cells producing IFN- γ and TNF- α was measured. A summary of the data is shown in Figure 5.8 through to Figure 5.12.

Cell Viability: As previously mentioned (Section 5.2.6.1), an amine-reactive dye was included as part of the staining panel to allow for the exclusion of dead cells and subsequent analysis of viable cells only. HIV+ cells exhibited lower cell viabilities than the HIV- cells (Figure 5.8). This is in keeping with the fact that these cells are burdened with a stressor; HIV. Compared to the untreated HIV- and HIV+ cells respectively, PHA-P (which was used as a positive control for cell proliferation and through this served as a stimulant for cytokine production) significantly lowered the cells' viability ($p=0.0049$ for HIV- and $p=0.0244$ for HIV+). The concentration of PHA-P used was relatively low ($2 \mu\text{g/ml}$) and was in agreement with that used in the literature which is usually in the range of $1-10 \mu\text{g/ml}$ (Pala et al., 2000). Fragile cells (due to culturing) and over proliferation (mitogen stimulated) in a small surface area (96-well plate) may have contributed to cell death. It is possible that PHA-P caused an increase in cell number so much that the growth surface area and nutrient supply of the cells were depleted causing cells to become stressed and die. This finding suggests that the HIV- cells were more highly activated by PHA-P to proliferate than the HIV+ cells. The death of healthy uninfected cells after six hours of mitogenic activation as reported by Baran et al (2001) supports this possibility of cell death due to over activation. PMA-ionomycin and R7V stimulation significantly increased the viability of the HIV- cells ($p=0.0134$ and $p=0.0067$) whereas the Gag peptide pool had no effect irrespective of HIV status. PMA-ionomycin is known to negatively affect cell viability (Baran et al., 2001; Pala et al., 2000) but the viable state of the cells suggests that the combined concentrations of PMA (10 ng/ml) and

ionomycin (1 μM) utilized was non toxic to the cells. PHA-P and PMA-ionomycin treatments between the two experimental groups (HIV- and HIV+) differed significantly ($p= 0.0002$ and 0.0051).

Percentage cells with surface CD3: The percentage HIV+ cells having CD3 on their surface (Figure 5.9) was lowered following PHA-P and PMA-ionomycin treatments ($p=0.0420$ and $p=0.0098$). Polyclonal activation with mitogens such as PHA-P and PMA-ionomycin induce changes in the phenotype of membranes, the expression of receptors and membrane molecules (Biselli et al., 1992). The mitogens used here are particularly well-known for reducing the expression of surface molecules. This occurs primarily through the activation of protein kinase C (PKC) which translocates from the cytoplasm to the plasma membrane inducing phosphorylation of CD4 serine molecules as well as the dissociation of the CD4-tyrosine kinase $p56^{\text{lck}}$ complex. Subsequently, there is an increased association of CD4 with coat proteins which ultimately decrease surface CD4 molecules through internalization/endocytosis. Surface CD3 is downregulated similarly (Jason and Inge 2000) and therefore explains the result obtained. CD8 is not usually endocytosed but can become protoplasmic following crosslinking and similar to CD4, can then be internalized (Anderson and Coleclough 1993; Jason and Inge 2000). Ionomycin primarily causes an increase in Ca^{2+} influx which leads to the formation of new gene products. These new products possess the ability to remove surface molecules from cells (Anderson and Coleclough 1993).

The percentage HIV- and to a lesser extent HIV+ cells displaying surface CD3 increased after cells were treated with the Gag peptide pool (Figure 5.9). Recombinant and synthetic Gag peptides were shown to increase the proliferation of healthy uninfected lymphocytes and purified CD3 cells *in vitro* (Nair et al., 1988) and may explain the observed findings. Although the increase in surface CD3 in response to Gag (HIV- cells) seems significant (Figure 5.9), the data is to be interpreted with caution since a p-value could not be calculated (response measured in one HIV- case). Treating the cells with R7V had no effect on the percentage cells presenting surface CD3. This observation is in agreement with data collected by Bremnaes (2010) who showed that R7V was unable to induce proliferation in HIV- and HIV+ cells *in vitro*.

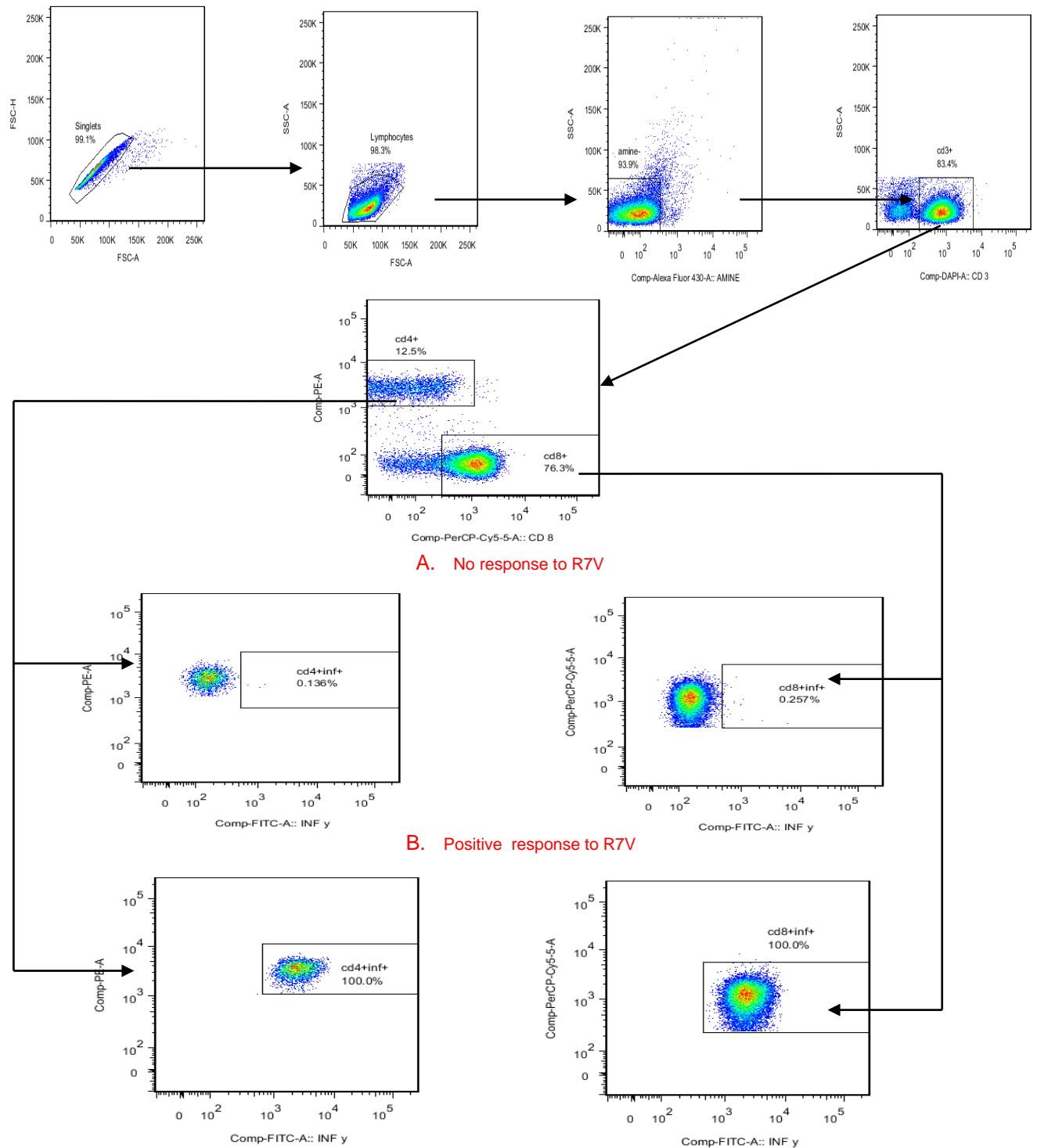
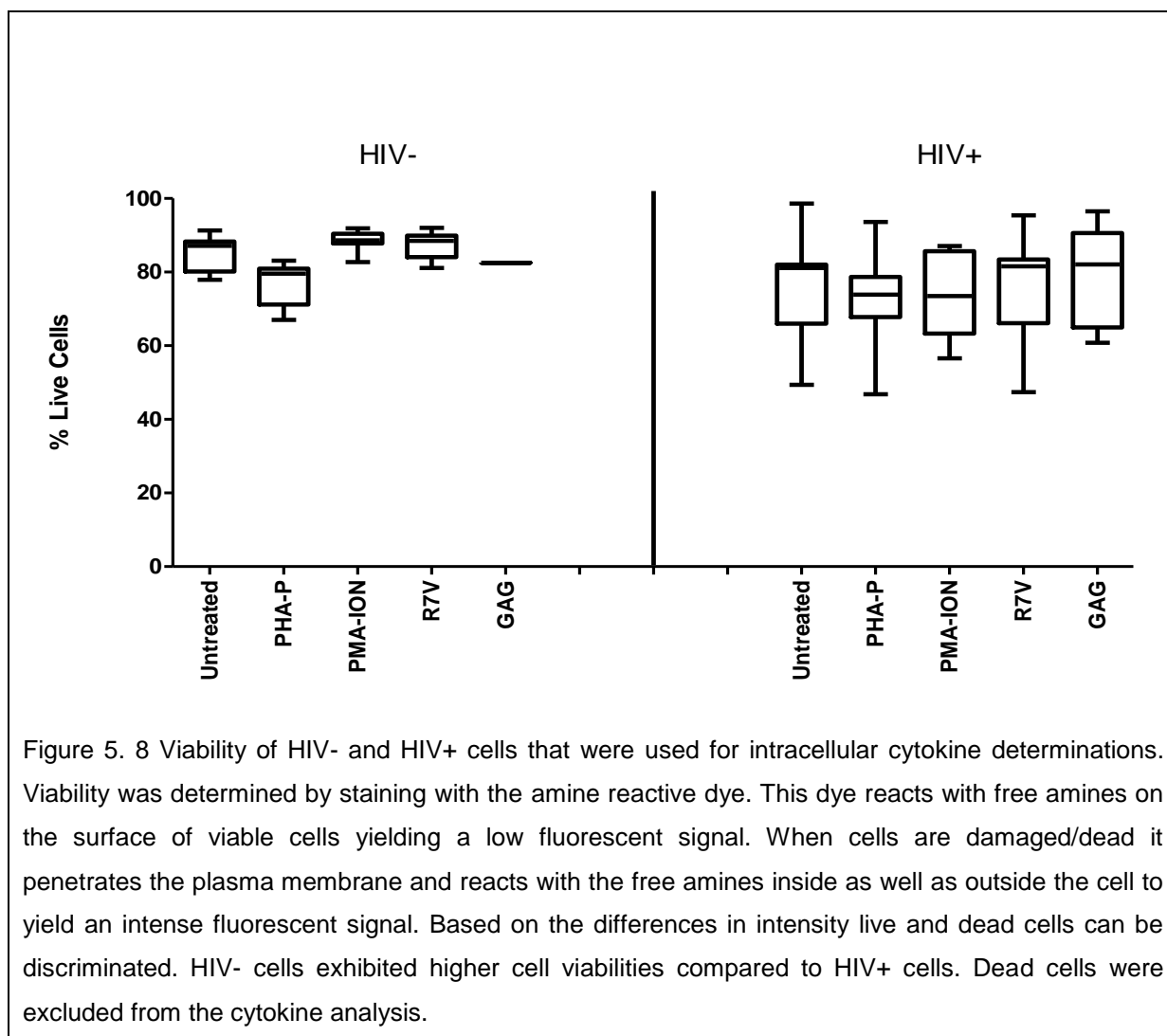
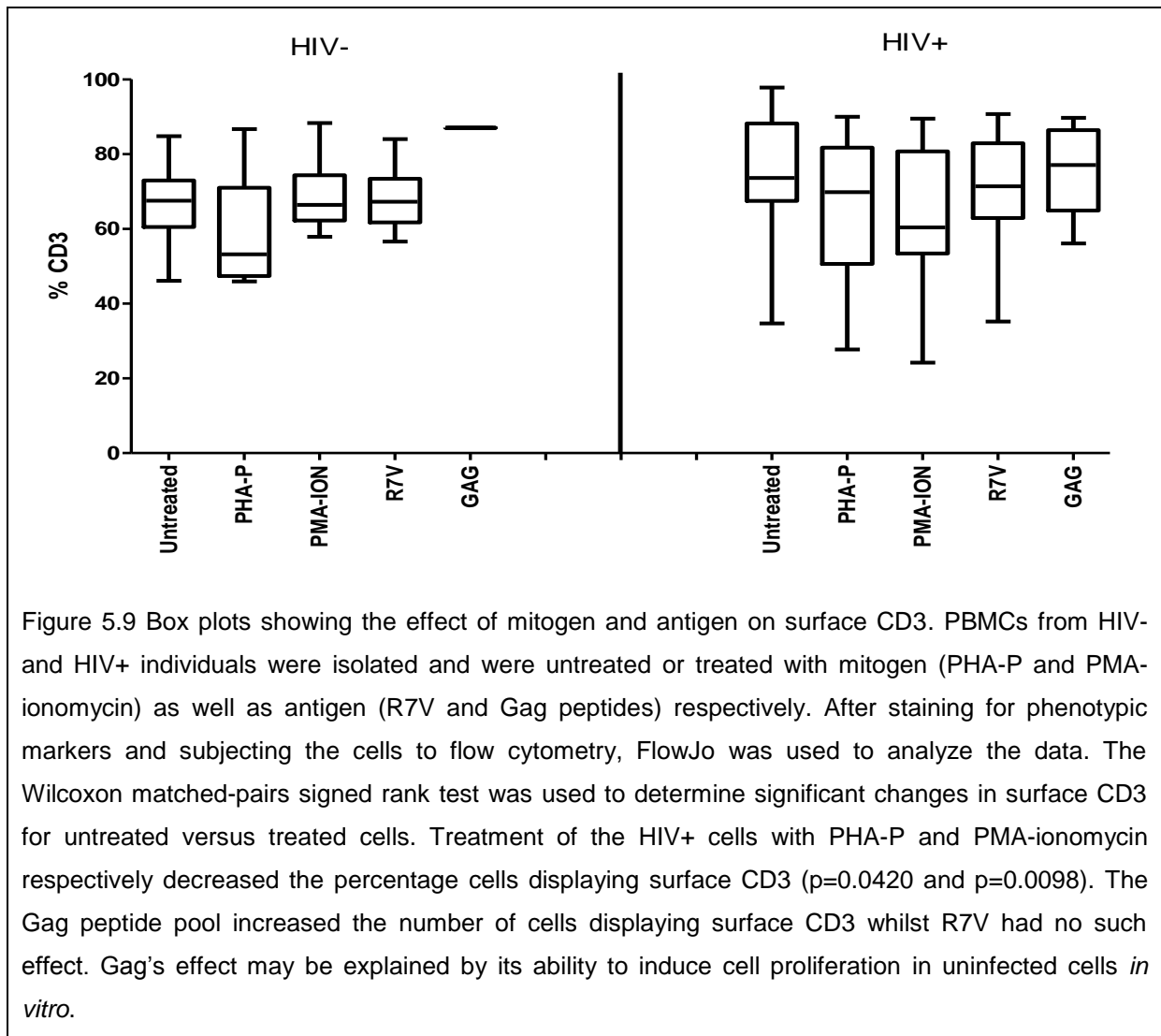


Figure 5.7 Gating strategy used for determining the percentage T cells producing intracellular cytokine, IFN- γ . Singlets were selected then the lymphocytes. Dead cells were then excluded by selecting those cells that were negative for the amine dye. The T cells (CD3+ labelled with Pacific Blue and detected in the 4',6-diamidino-2-phenylindole (DAPI) channel) were selected followed by the CD4 and CD8 cells which were detected in the PE and PerCPCy5.5A channels respectively. The percentage CD4 and CD8 cells producing IFN- γ were determined. IFN- γ detection was facilitated through FITC. Shown is an example of the percentage HIV+ T cells which A) failed to produce IFN- γ and a sample which B) produced IFN- γ in response to R7V treatment. Note that in A, the cells are closer to zero whilst in B the cells are further along the axis ($> 10^3$) indicating a positive response.

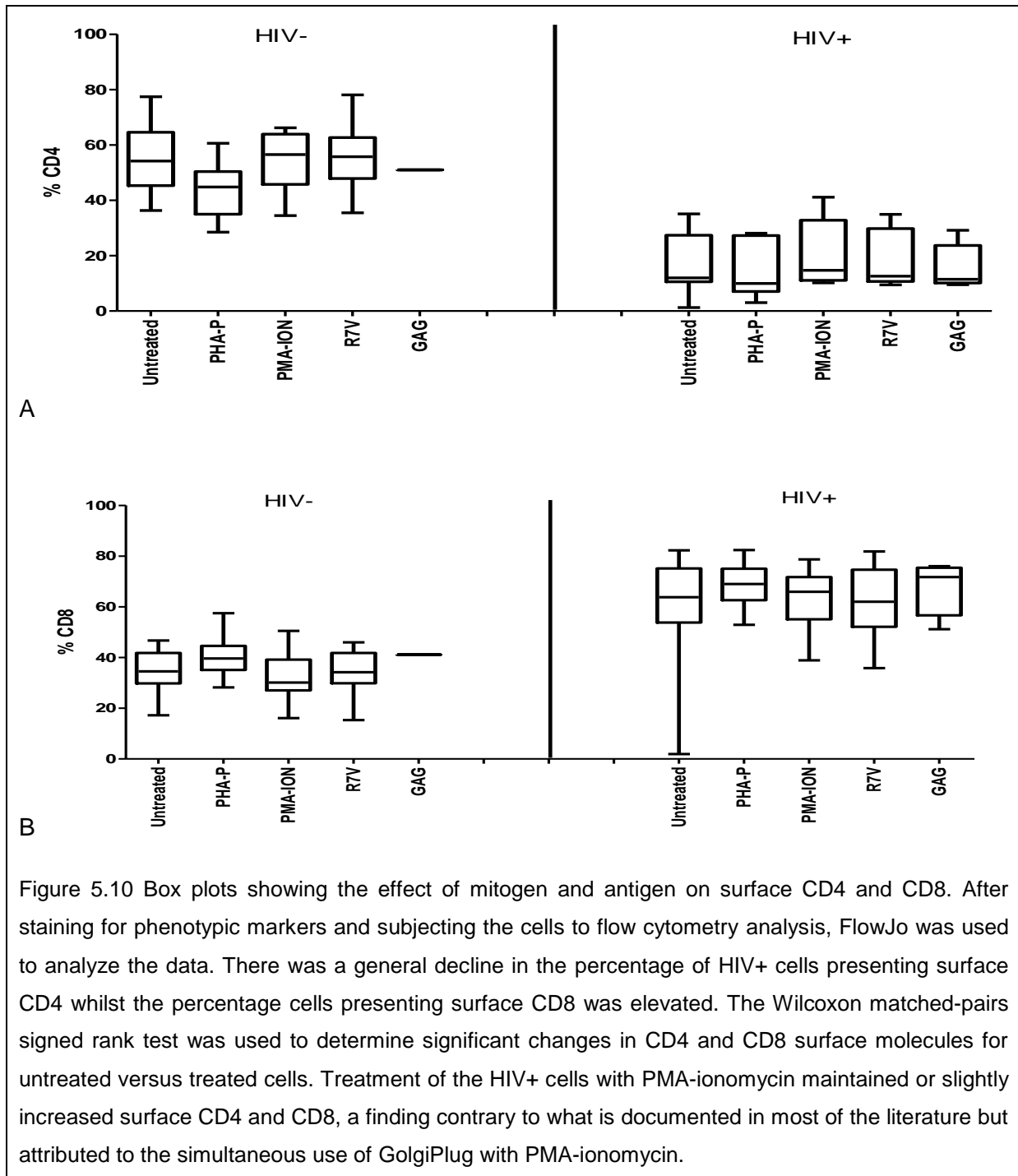


Percentage cells with surface CD4 and CD8: Compared to the uninfected cells, the HIV+ cells showed a severe depletion of CD4 surface molecules (Figure 5.10 A) but an elevation in CD8 surface molecules (Figure 5.10 B). Consequently, all treatments applied to the two groups had a significant effect on the percentage cells presenting surface CD4 and CD8. That HIV depletes CD4 cells ultimately causing immunodeficiency is well known. Concurrent to this, CD8 cells are activated to assist with the inhibition of viral replication mainly by lysing virally infected cells (Goepfert, 2003). In the case of PMA-ionomycin treated HIV+ cells, the mitogen maintained or significantly increased the percentage cells presenting surface CD4 and CD8. The concentration of PMA and ionomycin used was 10 ng/ml and 1 μ M respectively. In the literature concentrations in the range of 1-100 ng/ml PMA are usually used for cytokine production (O'Neil Andersen and Lawrence 2002; Pala et al., 2000; Sander et al., 1991; Weyand et al., 1987).



Although the high concentrations induce cytokine production, it negatively affects cell viability and the presentation of surface molecules (Baran et al., 2001). The lower, non-toxic concentration used here (10 ng/ml) was sufficient for stimulating cytokine production and still proved beneficial in maintaining cell viability and cell surface markers. To detect intracellular cytokine production, the secretion of protein from the cell had to be inhibited. This was done by using GolgiPlug which contains brefeldin A. Under normal conditions, Arf1 which is a GTPase, recruits coat proteins onto Golgi membranes to form transport vesicles. Following the addition of GolgiPlug, these coat proteins dissociate from the Golgi causing a redistribution of Golgi enzymes to the endoplasmic reticulum (ER). Fusion of the Golgi and ER compartments follow with a subsequent block in ER-to-Golgi transport (Nebenführ et al., 2002). Stimulation of the cells with mitogen activates PKC, causes phosphorylation of CD4 serine molecules and an increased association of CD4 with coat proteins which ultimately decrease surface CD4 molecules. The fact that surface CD4 and CD8 were not decreased

can be explained by the fact that GolgiPlug which was added with the mitogen during the stimulation protocol led to the impaired formation of clathrin coated vesicles (coat proteins) reducing internalization and thus endocytic processes. [Anderson and Coleclough \(1993\)](#) reported of two other studies where PMA failed to reduce the percentage of cells presenting surface molecules. The Gag peptide pool increased only minimally the percentage cells displaying surface CD8. All the other stimulants did not modify HIV's effect on cell surface CD4 and CD8.

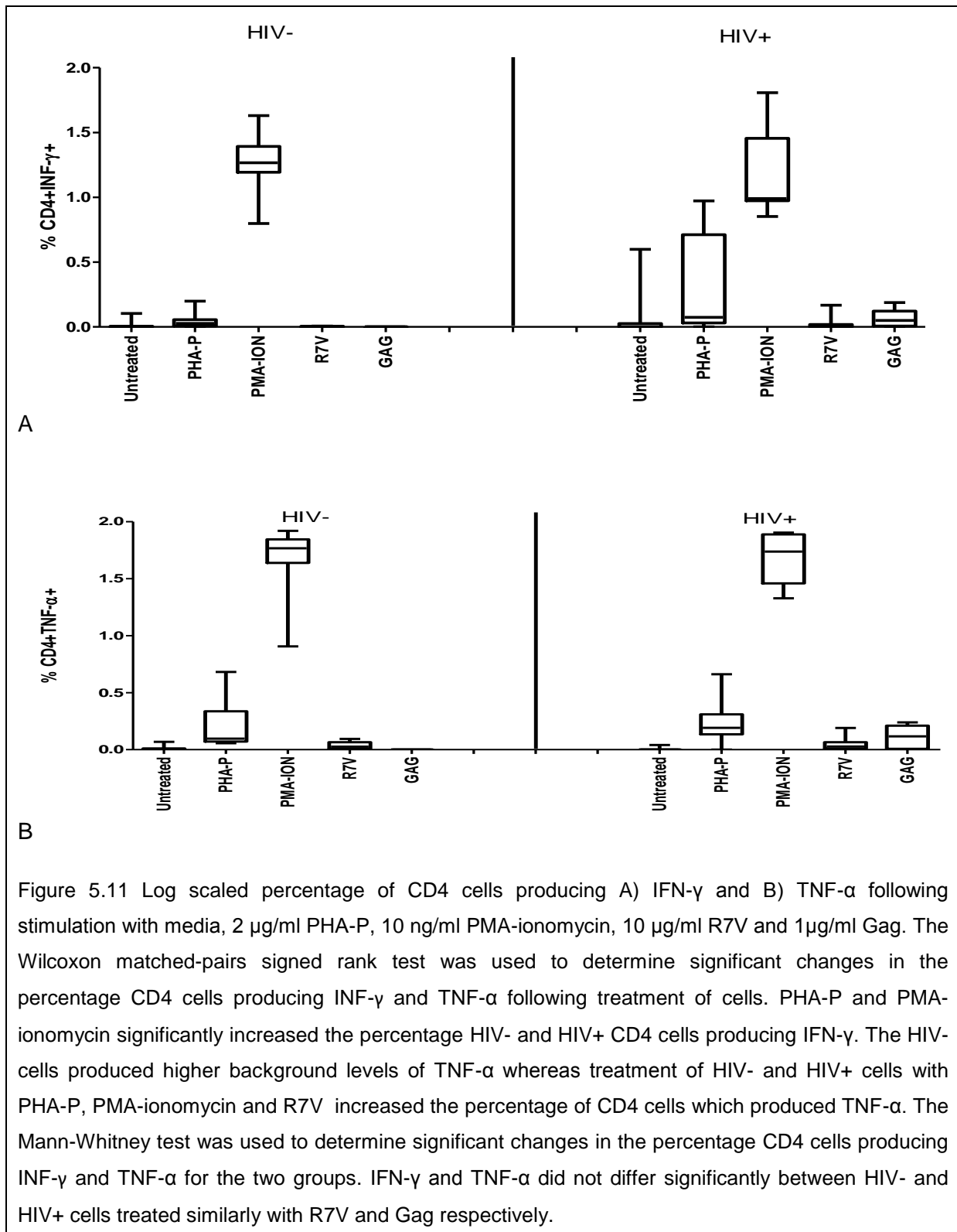


IFN- γ producing CD4 cells: The intracellular accumulation of cytokine was successfully achieved through the use of GolgiPlug which contains brefeldin A. In the data obtained, the percentage CD4 cells which produced IFN- γ (irrespective of treatment) did not differ significantly between HIV- and HIV+ PBMCs (Figure 5.11 A). When the untreated controls of the HIV- and HIV+ cells were compared to the respective treatments applied (Figure 5.11 A); **PHA-P** ($p=0.0488$ for HIV- and $p=0.0059$ for HIV+) and **PMA-ionomycin** ($p=0.0025$ for HIV- and $p=0.001$ for HIV+) significantly increased the percentage CD4 cells producing IFN- γ in both sample groups. These mitogens are normally used as positive controls for the production of cytokines. The positive response of especially the HIV+ cells to mitogen stimulation suggests that cell functionality had not been lost.

The response of HIV- CD4 and CD8 cells to the Gag peptide pool was measured in only one case thus a p -value could not be calculated to determine whether the response of these uninfected Gag-treated cells compared to untreated cells was significant. This note is valid for responses measured in Figure 5.11 through to Figure 5.12. When untreated HIV+ cells were compared to cells treated with the Gag peptide pool, a large percentage CD4 cells producing IFN- γ was observed (Figure 5.11 A, HIV+) but the response was not statistically significant ($p=0.875$).

TNF- α producing CD4 cells: Prior to any stimulation, the percentage CD4 cells which produced TNF- α was significantly higher in HIV- than HIV+ cells ($p=0.0461$, Figure 5.11 B). According to the literature, TNF- α levels are usually higher in HIV+ cells (Gil et al., 2003) even when unstimulated because viral infection in these cells already serves as a source of antigenic stimulation (Moss et al., 2000). The fact that the HIV- cells produce higher background levels of TNF- α implies that there are other factors at play contributing to the increase of this cytokine. In comparison to the untreated controls of both HIV- and HIV+ cells; **PHA-P** ($p=0.002$ for HIV- and HIV+), **PMA-ionomycin** ($p=0.0005$ for HIV- and $p=0.001$ for HIV+) and **R7V** ($p=0.0342$ for HIV- and $p=0.0039$ for HIV+) significantly increased the percentage CD4 cells which produced TNF- α (Figure 5.11 B). When untreated HIV+ cells were compared to cells treated with the Gag peptide pool, a large percentage CD4 cells producing TNF- α were measured (Figure 5.11 B, HIV+) but was also found to not be significant ($p=0.125$). Once again the response of the cells to PHA-P, PMA-ionomycin and R7V were indicative of functional cells. Such a response was expected of the HIV+ cells given the clinically stable condition of the patient group selected (HIV+ART- with moderate to high CD4 counts). Although there have been reports on the loss of cell functionality even whilst CD4 counts are still high (Clerici and Shearer 1993) this was not reflected in the data obtained here.

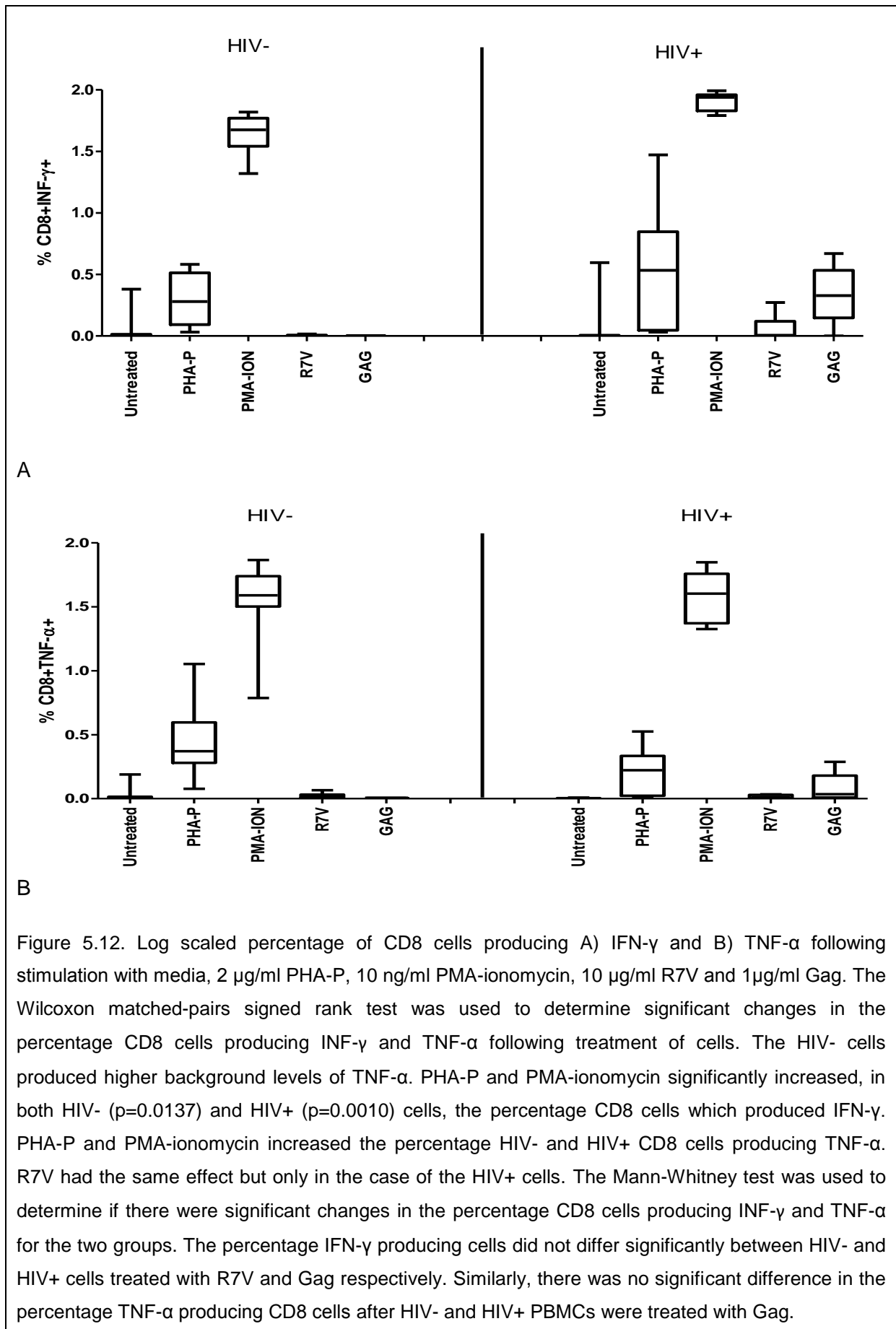
HIV-specific INF- γ and TNF- α production in CD4 cells: INF- γ and TNF- α produced by the HIV+ CD4 cells following treatment with R7V and Gag respectively did not reach statistical significance when compared to the HIV- cells treated similarly.



IFN- γ producing CD8 cells: In comparison to the untreated cells; PHA-P significantly increased, in both HIV- ($p=0.0137$) and HIV+ ($p=0.0010$) cells, the percentage CD8 cells which produced IFN- γ (Figure 5.12 A). This was also observed following stimulation with PMA-ionomycin ($p=0.0005$ and $p=0.0010$). The effect of PMA-ionomycin was more pronounced in the infected cells ($p=0.0002$) and once again signals that the cells had retained a functional state. When inspected visually, the Gag peptide pool increases the percentage IFN- γ producing CD8 cells (Figure 5.12 A, HIV+) but not to significant levels ($p=0.2500$).

TNF- α producing CD8 cells: Prior to any stimulation; the percentage CD8 cells which produced TNF- α was significantly higher in HIV- than HIV+ cells ($p=0.0245$, Figure 5.12 B). This is consistent with the high background TNF- α detected in HIV- CD4 cells. Compared to the untreated control; the percentage HIV- CD8 cells which produced TNF- α was increased following PHA-P and PMA-ionomycin stimulation ($p=0.0010$ and $p=0.0005$). In the case of the HIV+ cells, both mitogens (PHA-P and PMA-ionomycin) as well as R7V increased the percentage CD8 cells which produced TNF- α ($p=0.0010$, $p=0.0010$ and 0.0195). Similar to the observation made for IFN- γ producing CD8 cells, the percentage TNF- α producing CD8 cells (Figure 5.12 B, HIV+) also increased when HIV+ PBMCs were treated with Gag but the increase was not significant ($p=0.1250$).

HIV-specific INF- γ and TNF- α in CD8 cells: Stimulation of the HIV+ cells with R7V and Gag respectively induced IFN- γ production in the CD8 cells although not to significant levels when compared to HIV- cells treated similarly (Figure 5.12 A). Similarly, the stimulation of HIV+ cells with R7V and Gag induced TNF- α production in the CD8 cells but not to significant levels when compared to HIV- cells treated the same way (Figure 5.12 B).



General commentary on cytokine production in T cells: Overall, the response of the T cells to PMA-ionomycin was greater than that measured for PHA-P. Similar findings for these stimulants were obtained by [Baran et al \(2001\)](#). Treatment of the HIV+ cells with R7V and Gag resulted in both IFN- γ and TNF- α production implying recognition of the epitopes/peptides.

Visual inspection of Figure 5.11 and Figure 5.12 respectively, showed that the Gag peptide pool elicited stronger responses in the HIV+ cells, visible by the higher percentage of IFN- γ and TNF- α producing T cells (Figure 5.11 A and Figure 5.12 A). Measuring HIV-specific T cell responses to single epitopes like R7V is not as effective as exposure to the pooled Gag peptides. Exposing the cells to peptide pools yields responses to all potential epitopes displayed and allows for larger HIV-specific responses to be detected ([Betts et al., 2001](#)).

Short, envelope-derived peptides with cytotoxic lymphocyte (CTL) epitopes stimulate the production of IFN- γ when added to PBMCs. Host-derived peptides such as R7V that are incorporated into the envelope of HIV have not yet been implicated in antiviral/CTL responses but elevated amounts of antibody directed to this peptide have been found in LTNPs ([Sanchez et al., 2008](#); [Gal ea et al., 1996](#)). β 2m epitopes like R7V have been implicated as inducing a strong immune response early in infection of LTNPs ([Margolick et al., 2010](#)). [Bremnaes and Meyer \(2009\)](#) reviewed all available R7V data and designed experiments to detect antibody responses to the epitope in HIV-1 subtype C infected samples. The outcome differed from that of earlier work in that R7V antibodies was very low in LTNPs if samples were collected in later stages of infection (> 5 years). This was supported by [Margolick et al \(2010\)](#) who demonstrated that R7V antibodies were only high in LTNPs whose blood was collected early in infection (1 year or less) and that these high levels signified progression to disease rather than the opposite. Based on how the immune system functions, early infection is characterized by antigen exposure (viremia) which in turn activates antibody and cellular immune responses. PBMCs used here were from LTNPs not necessarily in the early stage of infection which may explain the low HIV-specific responses that were measured.

In Figures 5.11 and 5.12 some HIV- samples are shown to have recognized the R7V epitope and thus elicited a response in the form of cytokine production. R7V is believed to be immunogenic only once incorporated into the envelope of the virus. Recognition of the R7V epitope suggests that the HIV- donors might be infected with other enveloped viruses displaying R7V-like epitopes ([Sanchez et al., 2008](#)).

TNF- α despite also being a Th1 cytokine is associated with a hypermetabolic state, the wasting syndrome of HIV+ individuals and HIV disease progression. Here the percentage CD4 and CD8 cells which produce TNF- α following R7V and Gag treatment respectively (Figure 5.11 B and Figure 5.12 B) is not necessarily a bad finding. TNF- α is bi-functional in that it increases and inhibits the survival of HIV through the activation of the transcription factor, nuclear factor-kappa beta (NF- κ β) or CMI responses respectively (Imperiali et al., 2001; Kaushal Sharma et al., 2003; Reeves and Todd 1996). TNF- α levels detected here were very low (Figure 5.10 B and Figure 5.12 B). Low levels of the cytokine have been shown to inhibit HIV disease progression (Than et al., 1997) and may be linked to the fact that this cytokine activates CMI responses (Reeves and Todd 1996). Hepatitis C virus (HCV) infected cells treated with R7V have previously been shown to produce and secrete IFN- γ (Bain et al., 2004). These authors also detected secreted TNF- α following treatment of cells with R7V. In addition to TNF- α ; IL-2, IL-4, IL-5 and IL-10 were also secreted (Bain et al., 2004). Secretion of both IFN- γ and TNF- α implies production of the cytokines and thus supports the findings of this thesis i.e. treatment of HIV+ PBMCs with R7V can induce intracellular IFN- γ and TNF- α production.

A unique case was identified during the project and evaluated separately. This patient (DS 50) had 100 % of its T cells producing IFN- γ following stimulation with R7V (see Appendix, Section 2e). Additional data on secreted IFN- γ is also discussed under Section 2i of the appendix and shows how the inclusion of this patient's sample in the ELISA assay influenced the data.

Overall, the intracellular cytokine staining data confirmed that the HIV+ cells were still functional and although not always significant the response to single and pooled peptide suggested an ability to initiate CMI responses (IFN- γ and TNF- α producing CD4 and CD8 cells) which are necessary for viral control. HIV- cells generally produced less cytokine compared to the HIV+ cells. This is consistent with the fact that HIV- cells are less activated than HIV+ cells (Graziosi et al., 1994).

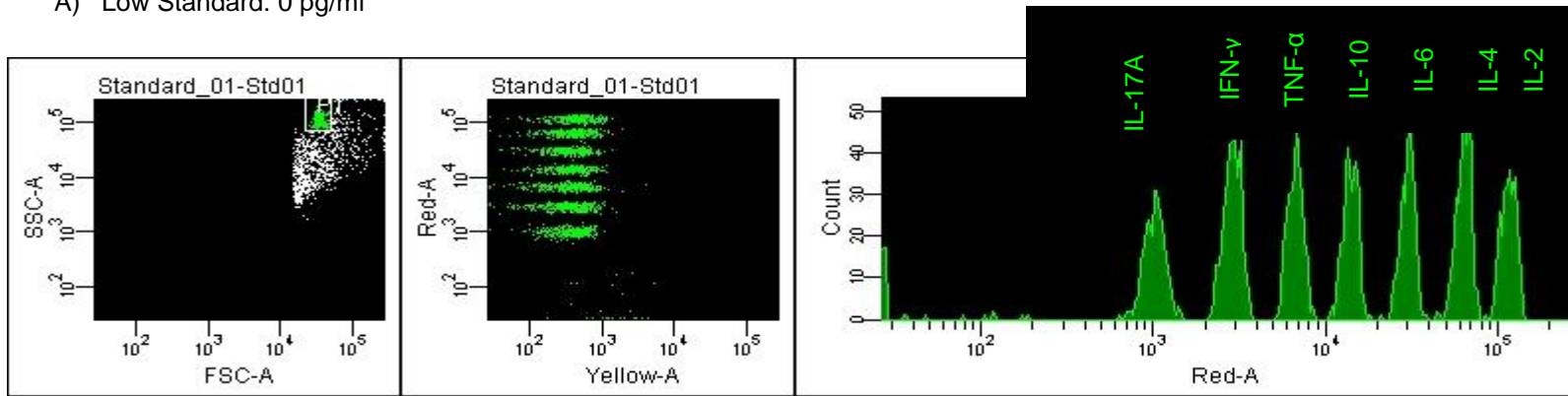
5.3.4.2 CBA: analysis of endogenous cytokine secreted into sera during HIV infection

The immune system is complex with many cytokines forming part of this network. HIV causes a dysregulation in various immune processes and ultimately an imbalance in cytokine production and secretion. Since part of the objective of this project was to measure the immune profile of HIV-infected individuals and to provide commentary on how the measured profiles impact on disease pathogenesis, a cytokinomics approach employing CBA technology and multivariate analysis respectively, was used to measure HIV-induced

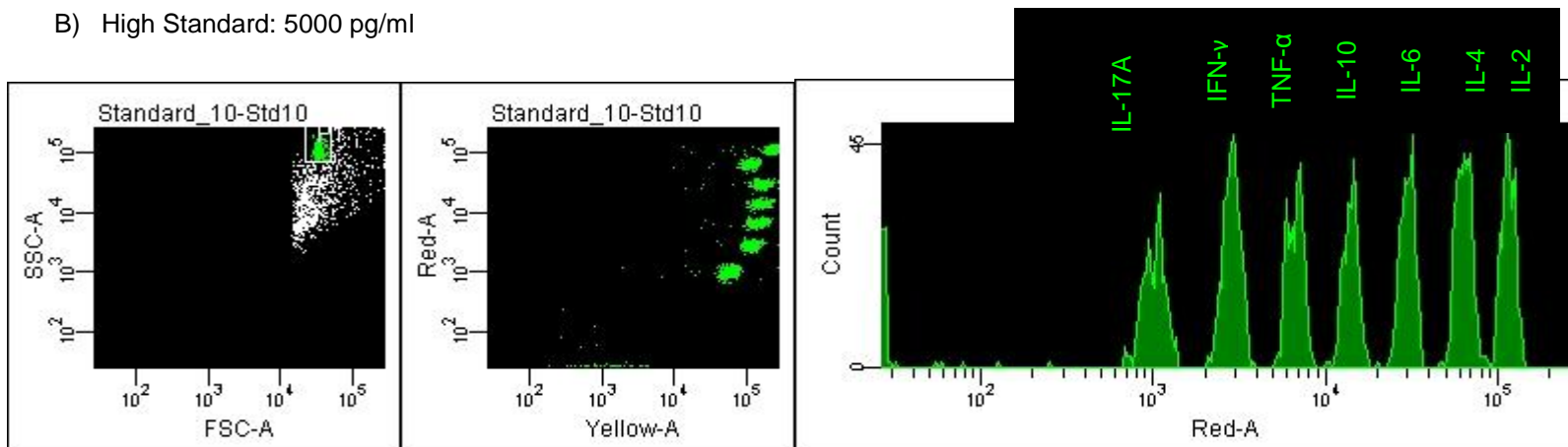
Th1/Th2/Th17 cytokine changes in the serum of treatment naive HIV+ individuals compared to uninfected controls. In addition to assessing HIV's influence on the cytokine profile we also wanted to clarify the role of cytokine changes during chronic infection, address the lack of multivariate analysis being applied to this highly networked/complex system and investigate the potential of cytokines as markers of HIV infection and disease progression in these individuals. As stated before (Section 2.7.3), the long analysis times associated with doing an ELISA caused cytokines to be overlooked as diagnostic and prognostic markers. The development of multi-parametric flow cytometers and multiplex kits has now changed this. Cytokines assayed here were representative of endogenous/"natural" cytokine secreted in response to HIV infection unlike the intracellular detection of IFN- γ and TNF- α where cells required prior activation and a protein transport inhibitor to trap cytokine.

The participating donors for this experiment were well-matched in terms of gender but not age. HIV+ individuals were characterized as being asymptomatic, experiencing chronic HIV infection. Following CBA analysis, the data was analyzed using IBM SPSS (version 19.0) and interpreted. Figure 5.13 A shows representative plots obtained following CBA analysis and is representative of capture beads which reacted with 0 pg/ml of the standard. Figure 5.13 B is an example of capture beads which reacted with 5000 pg/ml of a standard. In Figure 5.13 A and B respectively, the beads were selected based on FSC and SSC properties and based on their different fluorescence intensities were representative of the respective cytokines. Figure 5.13 A shows that capture beads which react with a standard having no analyte does not result in much of a shift in terms of fluorescence (beads are more to the left of the dot plot between 10^2 and 10^3). Figure 5.13 B shows that capture beads which react with a standard having the analyte present at high concentrations does cause a shift in the MFI (beads now to the right of the dot plot, in the range of 10^5). The shifts in MFI is also shown as histograms (labels for the various cytokines have been added and apply to the dot plots as well). Representative plots for an HIV+ sample is shown in Figure 5.13 C. The dot plot primarily shows that IL-6 undergoes a large shift in MFI and translates to increased IL-6 concentrations being calculated for the particular sample from the respective standard curve (Figure A9). All samples tested were analyzed in this manner.

A) Low Standard: 0 pg/ml



B) High Standard: 5000 pg/ml



C) Representative Sample

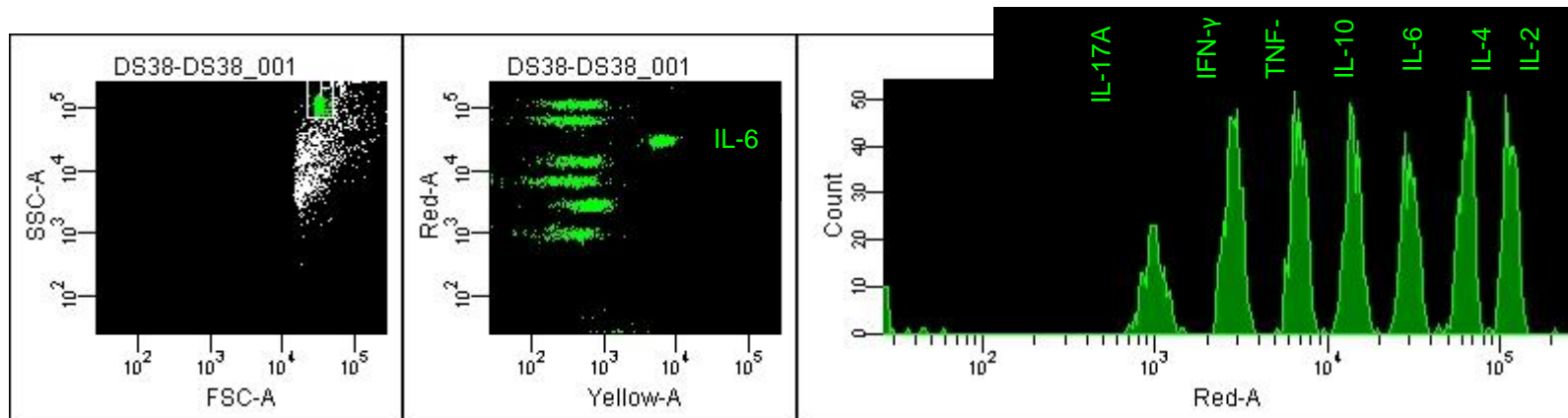


Figure 5.13. Representative plots obtained following CBA analysis. Beads were selected based on FSC and SSC properties. Shown in (A and B respectively) are bead populations which reacted with assay diluent (0 pg/ml) as well as a high concentration standard (5000 pg/ml). A bead population which reacted with HIV+ serum is shown in C and indicates a shift in the IL-6 cytokine profile. This shift is also shown in the form of a histogram and based on the mean fluorescence intensity, the concentration of cytokine was determined off a standard curve.

Because cytokines form part of a network the immune profile obtained would be better understood if all or most variables contributing to it are analyzed simultaneously. Variables may not be of importance/significance when analyzed in isolation but this may change when analyzed in combination with other variables (Philippeos et al., 2009). Therefore, multivariate statistics where more than one variable is measured and analyzed at one time was employed. Table 5.1 shows that LDA was able to correctly classify 74.6 % of cases i.e. 91.2 % of HIV- and 52 % of HIV+. Also shown is the percentage of misclassified cases (8.8 % HIV- and 48 % HIV+). IL-6 was identified as the best discriminatory parameter of infection. The probability that an individual case belongs to a particular group was assessed through logistic regression. Table 5.2 shows that this analysis correctly classified 71.2 % of the cases i.e. 91.2 % HIV- and 44 % HIV+. IL-6 was once again identified as the discriminatory cytokine. Although the overall percentage of correctly classified cases is similar between LDA and logistic regression, the statistical methods applied for classification of the samples had a high percentage HIV+ samples that were incorrectly classified (48 and 56 % respectively). This suggests that the probability of obtaining false positives with these tests for the experimental groups tested is low but that there is still concern for the number of incorrectly classified HIV+ samples. The reason for the high number of misclassified HIV+ cases may be as a result of the clinically stable state of the HIV+ individuals. HIV-induced disruption to immune system function is minimal during the earlier stages of infection causing the cytokine profile of these individuals to overlap. The HIV+ individuals thus present similar immunological profiles to HIV- individuals making it impossible for LDA and logistic regression to accurately distinguish and classify these cases.

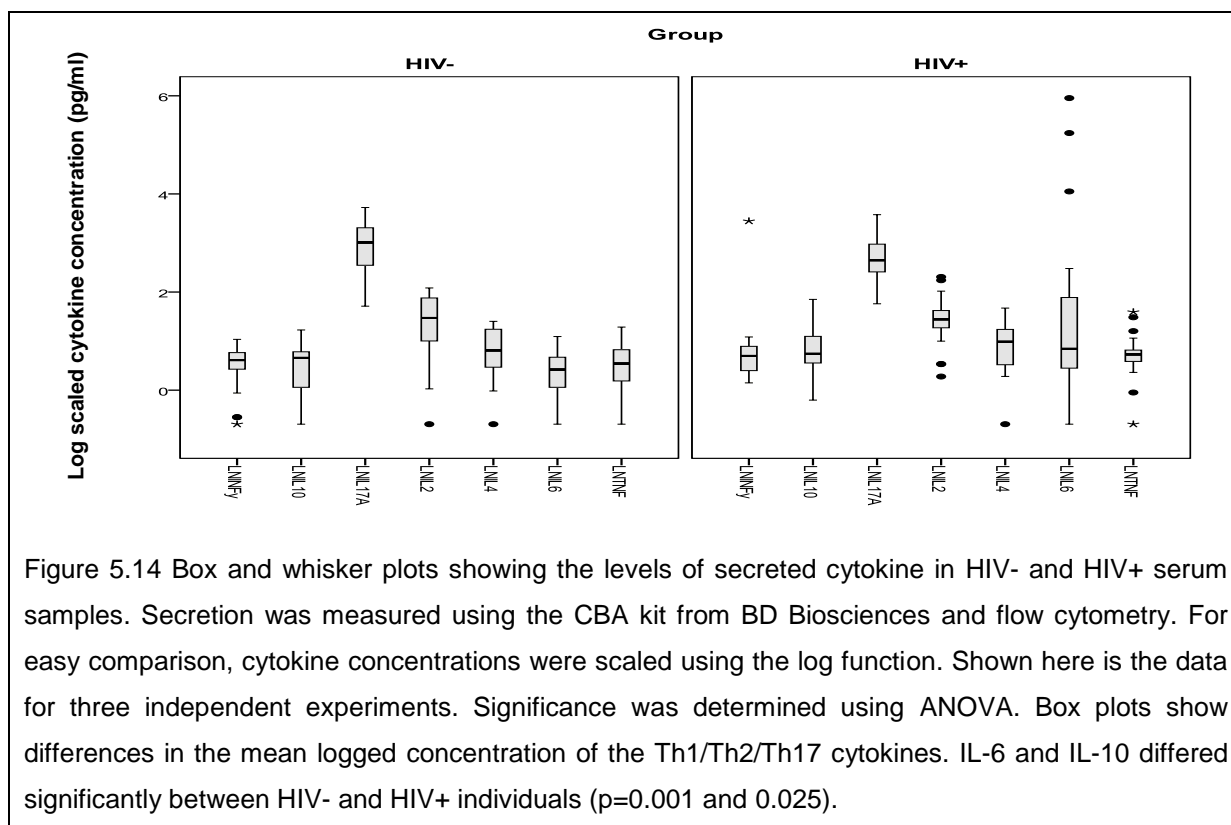
In addition to multivariate approaches (such as LDA and logistic regression) ANOVA was performed to allow for a comparison of the data when different statistical methods are used. Of the seven cytokines measured, ANOVA identified IL-6 and IL-10 to be dissimilar and differentiating the two groups from each other (visual representation in Figure 5.14, $p=0.001$ and 0.025), whilst the remaining cytokines resulted in a profile that was generally indistinguishable for the two groups. The two cytokines capable of discriminating between the two groups namely IL-6 and IL-10 were plotted on a scatter diagram (Figure 5.15). Poor separation of the two groups was evident and is to be expected because of the clinically stable condition of the HIV+ individuals causing them to display profiles equivalent to that of uninfected individuals. The majority of samples therefore showed an overlap in terms of the cytokine profile and this is in agreement with the high number of misclassified cases obtained with LDA and logistic regression for the patient group.

Table 5.1 Classification of experimental cases as HIV- or HIV+ using stepwise linear discriminant analysis

| Classification Results ^a | | | | | | | | | |
|---|---------|---------------|----------------------------|------|--------|-----------|-----|--------|------|
| | | | Predicted Group Membership | | Total | | | | |
| | | | HIV- | HIV+ | | | | | |
| Count | HIV- | | 31 | 3 | 34 | | | | |
| | HIV+ | | 12 | 13 | 25 | | | | |
| % | HIV- | | 91.2 | 8.8 | 100.0 | | | | |
| | HIV+ | | 48.0 | 52.0 | 100.0 | | | | |
| a. 74.6 % of original grouped cases correctly classified. | | | | | | | | | |
| Variables Entered/Removed ^{a,b,c,d} | | | | | | | | | |
| Step | Entered | Wilks' Lambda | | | | Exact F | | | |
| | | Statistic | df1 | df2 | df3 | Statistic | df1 | df2 | Sig. |
| | | | | | | | | | |
| 1 | LNIL6 | .819 | 1 | 1 | 57.000 | 12.615 | 1 | 57.000 | .001 |

Table 5.2 Classification of experimental cases as HIV- or HIV+ using stepwise logistic regression

| Classification Table ^a | | | | | | | |
|--|----------|-----------|------|--------------------|------|--------|-------|
| | | Predicted | | | | | |
| | | Group | | Percentage Correct | | | |
| Observed | | HIV- | HIV+ | | | | |
| Group | HIV- | 31 | 3 | 91.2 | | | |
| | HIV+ | 14 | 11 | 44.0 | | | |
| Overall Percentage | | | | 71.2 | | | |
| a. The cut value is .500 | | | | | | | |
| Variables in the Equation | | | | | | | |
| | B | S.E. | Wald | df | Sig. | Exp(B) | |
| Step 1 ^a | LNIL6 | 1.476 | .553 | 7.127 | 1 | .008 | 4.377 |
| | Constant | -1.261 | .432 | 8.519 | 1 | .004 | .283 |
| a. Variable(s) entered on step 1: LNIL6. | | | | | | | |



This overlap in the cytokine profile is explained by the fact that during the asymptomatic phase of infection, the infected individual presents with minimal phenotypic changes representative of HIV-induced immunological alterations. The result therefore suggests the immunological profile of treatment naive, clinically stable individuals to not differ significantly from that of uninfected controls. The bivariate plot also showed three cases to be clustered further away implying a drastic alteration in the cytokine profile of these cases. Upon inspection, it was found that these were samples having low CD4 counts. The viral load for the top most of the three samples was in excess of 8 million copies/ml plasma. This suggests that as HIV infection advances to AIDS (high viremia and low CD4 counts) the discriminatory power of cytokines improve. Findings here are thus supportive of the view that laboratory markers are more abnormal with advanced disease (Fahey, 1998). Hewer et al (2006) investigated whether HIV-, HIV+ and HIV+/AIDS on ART could be distinguished based on their NMR metabolic profiles. In the investigations of this author, “healthy” as well as sick HIV+ patients were included. The viral load measurements for these individuals were widespread i.e. low and high viral loads were recorded. Separation of the groups based on the measured metabolic profiles was observed. Unlike Hewer et al’s (2006) metabolic data, the cytokine profile measured here was influenced by viral load, yielding clearer separation profiles where samples representative of the advanced stage of the disease were included. Few cases having low CD4 counts were retained in the study

because all selection criteria were met (HIV+ and naive of ART). These samples therefore provided insight into how AIDS affected cytokine changes. Where those cases having low CD4 counts were removed from the analysis, the mean concentration of IL-6 and IL-10 was still discriminatory for HIV infection although with a lower degree of significance. Similar to [Jansen et al \(2006\)](#), it seems that viral load is the driving force in determining the extent of the immune response. [Keating et al \(2011\)](#) also showed this influence of viral load on the extent of cytokine perturbation (cytokines were less altered when viremia was lowered through treatment).

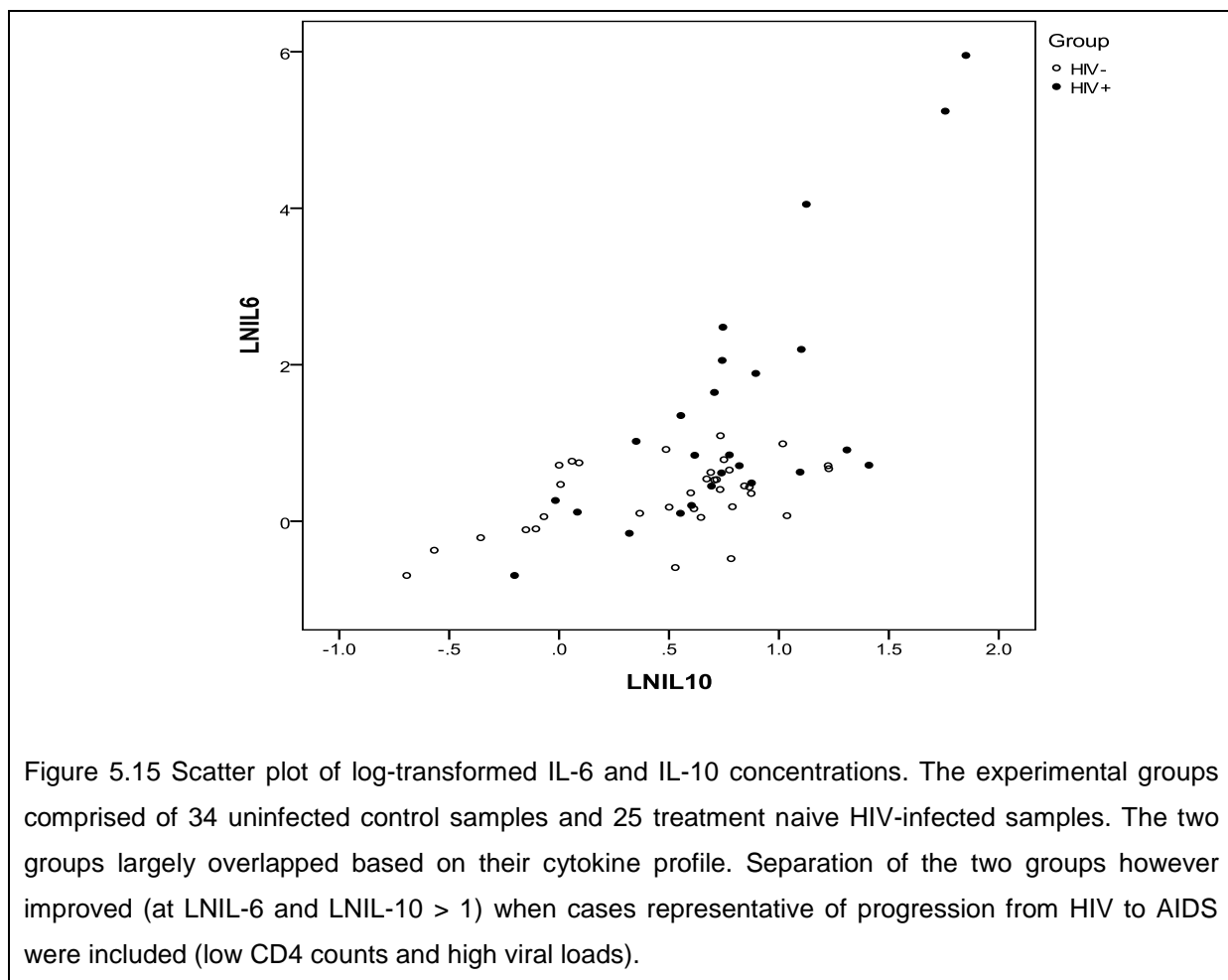


Figure 5.15 Scatter plot of log-transformed IL-6 and IL-10 concentrations. The experimental groups comprised of 34 uninfected control samples and 25 treatment naive HIV-infected samples. The two groups largely overlapped based on their cytokine profile. Separation of the two groups however improved (at LNIL-6 and LNIL-10 > 1) when cases representative of progression from HIV to AIDS were included (low CD4 counts and high viral loads).

According to the data presented here IL-6 and IL-10 appear to function as potential markers during HIV infection. The fact that more than one marker was identified as being altered as a result of HIV infection is confirmatory of the fact that cytokines do not function in isolation. IL-6 and IL-10 is representative of Th2 type cytokines and are thus associated with disease progression. Elevated levels of IL-6 are associated with HIV infection as well as aging ([Nixon and Landay 2010](#)). Although the mean age of the HIV+ individuals were

notably higher than that of the uninfected control group, age could not have served as a confounding factor in this case since one of the samples which clustered further away was from a young individual (22 years of age). IL-6 levels are further elevated during advanced disease (Fahey, 1998) and support the results presented here. The use of IL-6 and IL-10 as markers of sepsis and predictors of mortality (Tárnok et al., 2003) has been investigated. These cytokines have also been identified as markers for bacterial infection, with IL-10 ranked as highly prognostic (Tang et al., 2011; Tang et al., 2008). In an acute model of HIV infection, Roberts et al (2010) showed IL-6 and IL-10, amongst other cytokines, to be elevated. These authors also showed IL-10 to be correlated to viral load. IL-2 and IL-6 were key cytokines elevated in SIV-infected monkeys with encephalitis (Keating et al., 2011). The trend of increased IL-6 and IL-10 levels definitely shows perturbation of immune function by the HI virus and suggests a role for these cytokines in the immune response to HIV infection.

The concept of Th1 and Th2 cells was introduced in Section 2.7. Type 1 cytokines generally include IL-2 and IFN- γ whilst type 2 cytokines include IL-4, IL-5/6 and IL-10 (Clerici and Shearer 1993). Type 1 cytokine responses are associated with resistance to disease whilst type 2 responses are associated with disease progression. This led Clerici to propose the Th1 to Th2 switch for HIV disease progression where IL-2 is decreased and IL-4 as well as IL-10 is elevated (Graziosi et al., 1994). Such a shift also serves as a marker for the failure of CMI (Klein et al., 1997). Cell mediated immune responses are especially important to remove/decrease HIV+ cells because infection primarily occurs from cell-to-cell and there is usually limited free virus in the system. IL-6 and IL-10 is representative of Th2 type cytokines and are thus associated with disease progression. Cytokine profiles analyzed through multivariate statistics have therefore enabled us to distinguish between HIV- and HIV+ groups and led to the identification of immune markers associated with HIV disease progression in situations where ART has not yet been administered.

5.4 Conclusion

HIV-1 subtype C impacts on the immune system of even clinically stable patients. The various immune parameters measured i.e. elevated ROS, apoptosis of PBMCs and T cells are thus in agreement with dysfunctional mitochondrial activity as measured through GC-MS profiling of organic acids. For example, the increased detection of hydroperoxyl molecules indicated oxidative damage to membranes and is in agreement with the increase in fatty acids detected through GC-MS, the activated immune state (represented by increased IL-6) and apoptosis. Molecules such as IL-6, IFN- γ and TNF- α are associated with elevated levels of fatty acids, triglyceride content and apoptosis as well as wasting. Profiling of T cell apoptosis gives an indication of disease status or patient well-being as the increase in CD8

cell apoptosis (Figure 5.6 D) was attributed to CD4 cells being present and supplying the soluble factor necessary to trigger apoptosis.

Stimulation with low concentrations of mitogen can surprisingly be toxic depending on the activation and proliferation status of cells (low PHA-P concentration was shown to be toxic to HIV- cells, Section 5.3.4.1). Similarly; PMA commonly known to reduce the expression of cell surface markers, can have an opposite effect depending on co-use of the protein transport inhibitor, GolgiPlug (5.3.4.1). Although the immunological assays used are not new, the data obtained with these were relevant for confirming the GC-MS data and in some cases yielded novel information. IFN- γ and TNF- α production or CMI responses for that matter have not been shown for host derived cellular antigens like R7V before this. [Negredo et al \(2010\)](#) reported on HIV+ patients using HAART who progressed to AIDS quicker due to intrinsic apoptosis of CD4 cells. That less CD4 versus CD8 cells were undergoing early apoptosis is thus confirmatory of the clinically stable nature of these patients. The immune parameters measured thus further describe our experimental groups.

The production of intracellular IFN- γ and TNF- α indicates that the cells of the HIV+ individuals were still functional and able to elicit protective cellular responses when challenged with antigen whilst analysis of secreted cytokines through CBA analysis showed HIV to cause a shift from the Th1 to Th2 cytokine profile and is therefore associated with disease progression. Inducing favourable intracellular cytokine responses *in vitro* is best achieved through the use of peptide pools. Based on data presented for DS 50 (Figure A7 E and F) and others (Figures 5.10 and 5.11); it can be concluded that cellular antigens (R7V) have a role to play in stimulating beneficial immune responses and perhaps to the same extent as viral antigens (Gag peptide).

Cytokinomics approaches can disclose information on cytokines as markers defining the advanced stage of HIV infection and may be developed into a methodology for monitoring disease progression prior to development of severe clinical symptoms. The detection of the immunological markers was influenced by viral load (as was metabolite detection which is presented in Chapter 4).

CHAPTER 6

CONCLUDING CHAPTER

6. OVERVIEW

HIV/AIDS once dubbed the most devastating pandemic of our time is now manageable following the introduction of HAART. The use of these therapies has increased the life span of infected individuals and together with persistent chronic HIV infection these drugs contribute to the development of metabolic complications. Despite the success associated with treatment, the use of HAART still has numerous drawbacks linked to its use. Of primary concern is the fact that the drugs are toxic and that it induces various side effects ([Montaner et al., 2003](#)). Patients therefore do not adhere to prescribed regimens and as a result contribute to the development of drug resistant strains. Vaccine strategies and improvement of existing therapies or developing new ones gets priority whilst HIV-induced complications/changes (metabolic in particular) are neglected. If addressed; conventional, laborious, insensitive technologies are applied. Current diagnostic and prognostic markers are also not infallible and inform the need for investigations into HIV-induced changes which could translate into HIV-specific biomarkers which are currently lacking.

HIV is primarily known for causing immunodeficiency and the disrupted immune processes along with the virus, perturb mitochondrial function causing metabolic change in the host. The host metabolism is thus an important system to study during HIV infection as it influences disease pathogenesis largely because HIV relies entirely on the host machinery for its survival. Host metabolism also influences the rate of the immune response ([Cable et al., 2007](#)). Because of the association between the metabolic and immune systems, the concurrent effect of HIV on these systems, the limited application of metabolomics to the study of HIV-infected biofluids and the lack of an integrated analysis of the two systems; characterization of the metabolic and immune profiles of clinically stable HIV-infected individuals was done using sensitive, multi-parametric, analytical approaches. The focus of this study was on metabolic changes linked to HIV's effect on mitochondria and immune parameters having a role in metabolism. Organic acids were chosen as the metabolites of interest as they are established biomarkers of mitochondrial dysfunction and play a role in numerous metabolic pathways. Analysis of the metabolic profile of clinically stable individuals was done using an MS-based metabolomics approach while the analysis of

immune parameters was performed using spectroscopy and flow cytometry. The different stages of HIV infection are associated with different biochemical changes leaving room for a large array of biomarkers to be discovered.

A brief outline of the methodologies used and a summary of the main findings are provided next in order to revisit the hypothesis that was developed and to provide answers for the research questions proposed in Chapter 2 (Section 2.9). The significance and limitations of this work is then highlighted followed by recommendations and future considerations.

6.1 Metabonomics Profile of HIV infected Individuals

Organic acids were extracted from the sera, cell lysates and urine of HIV- and HIV+ individuals and analyzed through GC-MS. MS-based analysis was performed in batches because this produced reasonable sample sizes and accommodated the time frames associated with sample availability. GC-MS analysis yielded chromatograms which showed differences in the organic acid profile of HIV- and HIV+ individuals (Figure 4.3). Differences in the metabolic complexity of the three biofluid types were also observed (Figure 4.3-4.5). Urine displayed more peaks and was interpreted as having a more complex metabolic profile (Figure 4.5) than sera (Figure 4.3) and cells (Figure 4.4). **Different metabolic profiles** are expected since some biochemical pathways have their end products ending up in different biofluids, some in blood and others in urine for example. The difference in sensitivity of the respective biofluids to a stimulus such as HIV could also account for the different metabolic profiles detected. It can also be that the different biofluids have the **same metabolic profiles** but fail to show peaks for some of the metabolites as a result of concentration differences ([Jellum, 1981](#)).

To investigate the less obvious differences that may exist between samples that were analyzed through GC-MS, peak deconvolution and processing was done using three software programmes for which only the data of one was reported. Missing values are a common but unfavourable feature of metabonomics datasets as it influences the distribution of the variables and subsequent multivariate statistical approaches ([Behrends et al., 2011](#)). For the data shown in this thesis, MET-IDEA produced statistically sound data matrices comprising of limited missing values (Table 4.6). Data pre-processing (curation, variable selection etc) facilitated with the reduction of the data matrices. Upon investigating the quality of the GC-MS experiments RSDs within the acceptable 30 % range was obtained for sera and urine but not cells. RSDs were much higher where cells were used (± 50 %). Metabolite extractions were performed using a defined concentration of cells. Based on

experiments conducted by [Munger et al \(2006\)](#), it is expected that improved RSD values may be obtained if extractions from cells are based on protein content instead.

Scatter plots of the variables' logged intensities (Figure 4.8 a-c) showed the organic acid content of HIV+ biofluid to be mainly elevated. Only a few of these metabolites were present at low concentrations. Metabolites measured by [Wikoff et al \(2008\)](#) showed significant increases during SIV-induced encephalitis which in a way supports the findings recorded here. The GC-MS data was finally analyzed using uni- and multivariate statistics. PCA score plots showed overlapping and separated organic acid profiles for HIV- and HIV+ sera, cells and urine (Figure 4.9-4.11). Where HIV- and HIV+ groups separated well, this was attributed to an increase in viral load and thus greater metabolic burden. Overlapping profiles were as a result of the "healthy" status of the patient group utilized here. Instrument sensitivity ([Philippeos et al., 2009](#)) as well as "masking" of HIV-induced metabolic complications by the presence of large numbers of uninfected cells in the vicinity of infected ones ([Weber, 2001](#); [Rosenberg and Fauci 1991](#)) may have contributed to large metabolic differences not being observed (see Section 4.3.5.1).

Mention was made for why 5×10^6 cells/ml was used for the extraction of metabolites from cells (Section 4.3.5.1) and that more metabolites and potentially more information can be expected when higher cell concentrations are used. Although more metabolites may be extracted from a higher cell number, the amount and type of biological information retrieved may not necessarily increase/change. This is deduced from urine data where increased metabolite detection did not translate into more biological information being retrieved i.e. similar numbers of metabolites were finally detected for the biofluids (see Section 4.3.5.2). In fact, by using more cells for the extraction of metabolites an increase in the concentration of existing metabolites may potentially occur. Overall; the number of metabolites detected for the respective biofluids was similar. The types of molecules detected was different but overlapped in terms of the biological information which could be obtained. The urinary organic acid profile seemed to be less perturbed compared to that of sera and cells (PCA plots overlap in Figure 4.11a and PCA variation declared is lower, Table A1).

It is possible to be exposed to HIV without becoming infected ([Clerici and Shearer 1993](#)). This occurs when individuals are exposed to non-infectious HIV antigens and when there is limited viral infection ([Clerici and Shearer 1993](#)). HIV interacts with CD4 and CCR5 or CXCR4 (surface receptors) to gain entry into host cells. Some individuals have a mutation in their CCR5 gene which prevents HIV from binding to the receptor and as such confers resistance against HIV infection. For other individuals, HIV-specific CTL activity confers this protection against HIV infection ([Rowland-Jones et al., 1995](#)). In cases such as the ones

outlined above, individuals remain resistant to infection (this can be confirmed through PCR and viral culture) even after repeated/regular exposure to HIV through unprotected sexual contact, sharing of needles with infected individuals, etc. Based on the PCA score plot in Figure 4.9a where an uninfected but HIV-exposed individual clustered with HIV+ samples (blue arrow); metabolic change due to HIV exposure is implied and warrants further investigation. Immunological changes as a result of exposure to the HI virus have been previously reported (Rowland-Jones et al., 1995; Clerici and Shearer 1993). In the investigations of these authors, cells from HIV- individuals who were at high risk of contracting HIV (e.g. prostitutes and gay men) were isolated and treated with HIV peptides *in vitro*. Strong CTL and IL-2 responses were measured for **exposed HIV-** individuals signalling immunological change as a consequence to HIV exposure. The responses were found to be associated with protective immunity against HIV infection. In the case of **unexposed HIV-** individuals, the response to *in vitro* peptide stimulation was minimal or absent. Since the metabolic and immune systems are linked; metabolic change as a consequence to HIV exposure is therefore not unexpected.

From the PCA and PLS-DA plots (Figure 4.9-4.11) it can further be deduced that there is still a large degree of heterogeneity between and within the HIV- and HIV+ groups bearing in mind the selection criteria applied (Section 3.6). Molecules significantly altered as a result of HIV-induced mitochondrial dysfunction were identified and interpreted in context to infection. The main metabolites detected (see Section 4.3.6 and Table 4.7-4.9) were associated with altered mitochondrial function (e.g. succinic, fumaric, adipic and suberic acid also identified in this same context by, Reinecke et al., 2011 and Barshop, 2004) and defective respiratory chain complexes which are crucial for ATP production. Due to mitochondria not functioning properly, β -oxidation of fatty acids becomes limited. Consequently, ATP is reduced and glycolytic processes induced. The activity of neurotransmitter molecules such as tyrosine/tyramine is activated (Reinecke et al., 2011; Korzeniewski, 2001) and believed to (as in our case) assist with ATP production. This explains the high oxygen consumption by infected individuals as measured by others (Lane and Provost-Craig, 2000; Hommes et al., 1990). The detection of adipic acid signalled a potential sugar disorder in the HIV patients whilst various lipid molecules were indicative of the risk for developing lipodystrophy (glyceryl palmitate), oxidative stress (3-heptenedioic acid, 4-trimethylsilyloxy-, bis(trimethylsilyl) ester), oxidative membrane damage (a feature synonymous with intrinsic apoptosis) and thus mitochondrial damage. Several markers were identified as being associated with wasting (oleamide); cardiovascular disease (lauric/elaidic acids) and neurodegeneration (arachidonic acid, quinolinic acid, pyroglutamic acid) to name but a few. HIV-associated dementia is a consequence of HIV infection which presents later during

infection. The detection of neurometabolic markers thus indicates the potential risk for the development of this condition and suggests metabonomics to detect disorders for which symptoms are not yet visible. The patient group in this study was clinically stable and therefore asymptomatic (not showing any symptoms for the metabolic complications suggested above). Additional markers indicating malnourishment (4-hydroxyphenylacetic acid, 3-hydroxysebacic acid) and impaired pyrimidine synthesis (orotic acid) were also detected. Many of these metabolites share in the biochemical processes which they form a part of. For example, in addition to pyroglutamic acid signalling neurodegeneration, this metabolite also gives an indication of the degree of oxidative stress. The overall effect of HIV infection on host metabolism thus seems to be; impaired organelle functioning, reduced ATP and an increase in neurological/stress markers which further damage the cell and its membranes (lipids altered) to eventually cause cell death which is detected as apoptosis. To compensate for the reduction in ATP, processes such as glycolysis are activated (sugar and energy metabolism). In a recent investigation where the metabolic profile of CD4 cells and a macrophage cell line was measured following *in vitro* HIV infection, an increase and decrease in glycolysis was measured respectively (Hollenbaugh et al., 2011). The difference in the metabolic profile of the CD4 and macrophage cells was based on the fact that the cell line is long-lived. The PBMCs used in our study were also representative of short-lived cells and yielded metabolic data which is in agreement with that obtained for the CD4 cells.

Metabolite detection was found to be influenced by viral load. Very few metabolites were found to be common between sera, cells and urine, showing the different sensitivities of these biofluids to the HIV stimulus. It must be noted that although the detected metabolites are characterized as potential markers, they can also participate in the pathophysiology of the syndrome (Dunn et al., 2007). Overall; the detected metabolites were found to be representative of known metabolic complications induced by HIV (Section 2.8.1). The metabonomics approach used thus showed metabolic disturbances during the asymptomatic stage of HIV infection in a setting prior to the use of ART.

6.2 Immune Profile of HIV-infected individuals

Immunological assessments were done in parallel to metabolic analysis. The type of metabolites detected through GC-MS (fatty acids, neurological and oxidative stress markers etc) guided the choice of immune parameters to measure. In addition, parameters having a role in metabolic regulation were considered. The oxidative, apoptotic and cytokine profile of HIV- and HIV+ biofluids was determined using spectroscopy and flow cytometry respectively.

Using a colorimetric assay an increase in hydroperoxyl and therefore ROS molecules was measured in the sera of HIV-infected patients compared to controls (Figure 5.2). This is in agreement with the literature cited in Section 2.7.2. Hydroperoxides signals damage to cellular membranes and therefore cell death. When infected with HIV the immune system is activated. Increased amounts of ROS (as detected here) add to this activated state causing the immune system to become prone to apoptosis. In addition; fatty acids such as *cis-paranic* acid serve as markers of oxidative damage to membranes (Miro et al., 2004) and apoptosis. Based on the detection of ROS, the associated degree of immune activation and fatty acid detection, the apoptosis profile of HIV- and HIV+ individuals was measured.

Apoptosis was significantly higher in HIV-infected samples ($p < 0.0001$) compared to uninfected controls (Figure 5.4 B) due to their activated immune state (Herbein et al., 1998; Meygaard et al., 1992). Controversy exists as to the occurrence of apoptosis in CD4 and CD8 cells. In this investigation, apoptosis was highest in the cells of HIV+ individuals presenting surface CD8 ($p = 0.0269$, Figure 5.6 D) possibly due to the availability of CD4 cells (providing a necessary soluble factor for apoptosis of CD8 cells, as explained by Holm and Gabuzda 2005). During advanced HIV infection, CD4 cells are depleted but sufficient numbers of these cells are still present during asymptomatic, chronic infection providing a source for the soluble factor needed for apoptosis. A small degree of spontaneous apoptosis was measured and attributed to the fragile nature of the cells following isolation.

After detecting an increase in oxidative stress and apoptosis in the biofluid of HIV+ individuals (indicative of eventual disease progression), the ability of the participating donors' cells to produce intracellular cytokine in response to *in vitro* HIV peptide stimulation was investigated using flow cytometry. Whereas the levels of ROS and apoptosis were indicative of **HIV's effect** on the immune system (no exogenous stimulant added); treating the cells with HIV peptide *in vitro* informed on the possible **effects of the cells** on HIV, primarily the cells' ability to produce cytokines having antiviral activity. HIV-specific responses following treatment of the cells with HIV peptide would also provide prognostic information. For example, the inability of cells to produce IL-2 and IFN- γ after *in vitro* stimulation with HIV peptide was shown by Jansen et al (2006) to be associated with disease progression. Unstimulated cells produce minimal or no cytokine and therefore requires stimulation with mitogen or antigen (O'Neil-Andersen and Lawrence 2002). A low concentration of PHA-P (2 $\mu\text{g/ml}$) was used to stimulate cells but was found to be toxic to especially HIV- cells. PHA-P is a proliferating agent and might have increased cells numbers so much that the growth surface area and nutrient supply of the cells were depleted causing cells to become stressed and die. PMA-ionomycin is known to affect and reduce the expression of cell surface markers (Biselli et al., 1992) but had an opposite effect, possibly due to the immediate

addition of GolgiPlug after this stimulant. The treatment of cells with PMA-ionomycin causes an activation of PKC, an increased association of CD4/8 with coat proteins, endocytosis and eventually a reduction in surface CD4/8 (Anderson and Coleclough 1993). GolgiPlug containing brefeldin A (according to Nebenführ et al., 2002) prevents this process by impairing the formation of coat proteins thus reducing internalization and the subsequent endocytosis of surface molecules (also explained under Section 5.3.4.1). When exposed to foreign antigen the ability of cells to produce soluble immune system proteins *in vitro* was detected. Cells challenged with host-derived R7V and virus-derived Gag generally produced more IFN- γ than TNF- α (Figure 5.11 A and Figure 5.12 A versus Figure 5.11 B and Figure 5.12 B). This result was indicative of protective Th1 type responses. Cytokine production in response to these peptides also suggests virus incorporated cellular antigens (like R7V) to have a role in stimulating beneficial immune responses and perhaps to the same extent as virus-derived antigens (e.g. Gag pool). More of the T cells produced cytokine in response to the pooled Gag peptide than to R7V which represented a single epitope (Figure 5.11 and Figure 5.12). More (and different) epitopes were displayed and recognized following exposure to the Gag peptide pool.

To account for IFN- γ possibly secreted from cells prior to activation with mitogen/antigen and prior to GolgiPlug having an effect, the secretion of this cytokine was measured in the supernatant of untreated cells and cells activated for intracellular IFN- γ detection. A unique HIV+ case with 100 % of its T cells producing intracellular IFN- γ was identified (Figure A7 E and F) and the supernatant of these cells included as part of the ELISA analysis. Significant amounts of secreted IFN- γ was detected in the supernatant of HIV+ R7V-treated cells compared to untreated cells and is explained by the inclusion of the HIV+ case that responded almost completely to R7V (see Section 2f of the Appendix, Figure A8). In the absence of this sample; no significant levels of the cytokine was secreted.

Having confirmed the ability of the cells to recognize HIV antigen *in vitro* and to produce Th1 type cytokines with antiviral activity (i.e. **the effect these cytokines would have on virus**), the secretion of seven endogenous serum cytokines was also measured to investigate **HIV's influence on these molecules**. Whereas the IFN- γ ELISA was used to measure one cytokine and the data thereof analyzed using univariate statistics, CBA technology and flow cytometry was used to measure the secretion of multiple cytokines and the data analyzed using multivariate statistics. HIV+ individuals presented with high levels of IL-6 and IL-10 (Figure 5.14 and Figure 5.15) which correctly classified > 70 % of cases as HIV- and HIV+ respectively (Table 5.1 and 5.2). The increased IL-6 confirms the activated immune status of HIV+ individuals and therefore supports the elevated ROS measured for these individuals. This cytokine together with TNF- α and IFN- γ plays a role in tissue wasting

and generally stimulates leptin production but decreases lipogenic enzymes (Cossarizza et al., 2002) causing an increase in lipid synthesis and triglyceride content (supporting the metabolic changes detected in this study which are well-associated with HIV infection, Table 4.7-4.9). IL-6 and IL-10 are Th2 cytokines and associated with increased apoptosis as well as with the development of HIV to AIDS.

6.3 Linking metabolic and Immune changes

Organic acids are known indicators of mitochondrial dysfunction and were detected here as indicators of HIV-induced mitochondrial damage. Because of the pivotal role of mitochondria in apoptosis, the levels of this form of cell death in patients for whom metabolic malfunction had been demonstrated was also investigated. HIV+ patients experienced higher levels of apoptosis in both the PBMCs and CD8 T cells. The higher levels of apoptosis were in agreement with the increased ROS, IL-6, IL-10 and organic acid profiles measured. Cytokines detected here (IL-6, IL-10, TNF- α) promote catabolism and induce weight loss in HIV-infected patients. IL-6 particularly leads to an increase in triglycerides and glucose. In Table 4.8; glyceryl palmitate and stearate were identified as highly elevated in HIV+ cells. These molecules consist of glycerol linked to a fatty acid and serve as intermediates in triglyceride metabolism. A number of other metabolites involved in lipid metabolism were also altered (Table 4.7-4.9). ROS attacks lipid membranes. This rise in oxidative damage especially of the membranes is measurable through the fatty acid content of PBMCs (Miro et al., 2004) and explains the detected increase in fatty acids. The increase in oxidative stress, apoptosis and endogenous IL-6 and IL-10 indicates a progressive state from HIV to AIDS for the patient group investigated here. However; cells exposed to HIV antigen *in vitro* can also produce Th1 cytokines having antiviral activity. Del Llano and colleagues used colorimetric assays and flow cytometry to investigate metabolic and immunological parameters of SIV-infected cells. In this project more sensitive analytical techniques (MS and multi-laser flow cytometry) were used to analyze the metabolic and immunological parameters of clinically stable HIV-infected individuals.

6.4 Answers to Questions raised

The stated **hypothesis** for this PhD project was that HIV disrupts the function of the metabolic and immune systems, primarily through its effect on mitochondria. Since these systems are linked to each other through these organelles, mitochondrial dysfunction should then be visible by modifications in the processes of both systems and be detectable through MS-based metabonomics and flow cytometry respectively. To address this hypothesis several questions were raised in Section 2.9 and will now be answered after having performed experiments to prove or disprove the statements made.

a. Metabolic Profile

In response to the questions raised in Section 2.9:

[1] Is the extraction of organic acids possible from serum and cells infected with HIV?

Traditionally organic acids have primarily been extracted from urine. Reports of the profile of these metabolites in serum and plasma exist but are limited and have not been profiled in context to HIV infection. Here these molecules were successfully extracted from HIV-infected biofluids and demonstrated metabolic aberrations in patients in the early stages of infection (stage 2).

[2] Does the organic acid profile of HIV- and HIV+ individuals differ?

This study was designed to show that early infection with HIV induces mitochondrial damage and therefore metabolic changes which are detectable by monitoring organic acid changes. PCA score plots (Figures 4.9-4.11) showed non-overlapping and overlapping profiles for the various batches of samples. In Figure 4.9a there is separation of HIV- and HIV+ groups which improved if the patient's viral load was higher suggesting a role for organic acids as indicators of metabolic changes later in infection as well (Table 4.1 batch 1). With lower viral load measurements (Figure 4.9a, middle plot) patients probably experience less metabolic stress and therefore display an overlap in organic acid content. The organic acid profile of HIV- and HIV+ individuals therefore differs but the larger the metabolic stimulus (viral load) the larger the difference.

[3] Do the measured profiles provide information on the use of organic acids as reliable indicators of HIV-induced mitochondrial dysfunction?

This study was targeted towards the detection of organic acids in HIV-infected biofluid. The fact that we were able to link organic acid changes in these patients to immune parameters known to be associated with mitochondrial damage and dysfunction (increase ROS, apoptosis etc) shows that these molecules definitely have merit as indicators of HIV-induced mitochondrial dysfunction. The data also indicates that an improved separation between HIV- and HIV+ groups is possible for cases having high viral loads (for example see Figure 4.9 and Table 4.1) suggesting progressive mitochondrial dysfunction with increased viral load.

[4] How does the organic acid profile in the different biofluids compare?

Urine presented with the most complex chromatogram (Figure 4.5) and therefore the most complex metabolic profile followed by serum then cells. Data analysis suggests that the higher metabolite number in urine did not translate to more biological data being deduced. Blood-based biofluids provided useful information about the experimental groups which was no less important than that obtained from urine. Only three common metabolites were extracted from serum and cells and only one from serum and urine (Figure 4.14). Metabolites between the biofluids therefore differed in identity but were similar in context to the biological information extracted.

[5] Does the data generated from the different software differ substantially?

Data collected through AMDIS and an in house library failed to identify most of the peaks (Table 4.4). Concentrations of the detected metabolites were very low and upon trying to merge various samples for simultaneous analysis using “R”; matrices contained a lot of missing values which were subsequently filled with zeros (Table 4.5), a characteristic known to affect further analysis of the data. Where MET-IDEA was used, there were less zeros in the data matrices leading to a better distribution of the variables (Table 4.6). There is thus a substantial degree of variation in data from different software.

[6] Is one software better suited than another?

The software to use is dependent on the research question being asked and the type of data collection technique as well as statistical analysis that is to be applied. One software programme is therefore not superior over the other but may be better suited depending on the application.

b. Immune Profile

[1] Can hydroperoxides be detected and show significant differences when profiled in HIV- and HIV+ serum?

Hydroperoxides are produced when there is peroxidation of membranes. This damage to the membranes ultimately results in cell death which primarily occurs through apoptosis. Mitochondria play a central role in apoptosis. In addition, HIV infected individuals are documented to be under constant oxidative stress (Pace and Leaf 1995) and to be prone to apoptosis. HIV acts directly on the regulation of pathways associated with apoptosis (Pinti et al., 2010). A colorimetric assay was performed and detected higher levels of hydroperoxyl and thus ROS in the HIV+ sera (Figure 5.2 A and B as well as Figure A3, 1 and 2) showing

that these molecules are indeed detected and differ significantly ($p = 0.004$) in concentration between HIV- and HIV+ individuals.

[2] Which subset of (immune system) cells was undergoing apoptosis?

In the patient group analyzed in this study the CD8 cells of HIV-infected individuals showed higher levels of apoptosis than did the CD4 cells (Figure 5.6 D). The patient group was relatively healthy having moderate to high CD4 counts. Infected CD4 cells release a soluble factor required for apoptosis of CD8 cells to occur, this was explained by [Holm and Gabudza 2005](#) (for primary cells infected *in vitro*) and is what we believe happened in this case as well.

[3] Are the cells of clinically stable HIV-infected patients still functional when treated with mitogen and antigen *in vitro*?

Loss of cellular function has been reported to occur even during asymptomatic infection ([Sarih et al., 1996](#)). The production of IFN- γ and TNF- α by the cells challenged with mitogen and antigen suggests the cells to be functional (Figure 5.11 and Figure 5.12). For these cells to have produced cytokine suggests that they were activated/stimulated by the antigens and recognized the respective epitopes presented.

[4] Are the HIV-specific immune responses (as detected by single and pooled peptides) more prominent than a non-specific response (memory versus no memory)?

HIV-specific responses were less prominent than the non-specific responses (induced by PHA-P and PMA-ionomycin) but were still relevant for informing on disease progression. R7V is a seven amino acid peptide whereas Gag consists of a pool of peptides (fifteen amino acids in length overlapping by eleven amino acids). According to [Betts et al \(2001\)](#), cells respond better to peptide pools since more potential epitopes are displayed and more information on the HIV-specific responses can be extracted based on the response to several epitopes. As expected, cells challenged with Gag contained more cytokine producing cells than those exposed to/stimulated with R7V (Figure 5. 11 and Figure 5.12). Gag has a high epitope density and T cells often target multiple regions of this protein ([Kaushik et al., 2005](#); [Venturini et al., 2002](#)).

[5] Is the detectable *in vitro* response (cytokine production) anti- or proinflammatory i.e. IFN- γ or TNF- α and how does the result contribute to understanding HIV/AIDS pathogenesis?

IFN- γ is representative of an anti-inflammatory, antiviral cytokine whilst TNF- α is a pro-inflammatory cytokine. The latter cytokine can however be bi-functional in that it can increase or inhibit the survival of HIV through the activation of the transcription factor, NF- κ B or by inducing CMI responses respectively (Imperiali et al., 2001; Kaushal Sharma et al., 2003; Reeves and Todd 1996). IFN- γ and TNF- α were produced in response to both peptides (Figure 5.11 and Figure 5.12). IFN- γ production was however higher each time. This is a Th1 cytokine having antiviral activity. TNF- α despite it being a pro-inflammatory cytokine has been shown, when at low levels, to inhibit viral replication suggesting CTL activity which is generally orchestrated by CD8 cells. Intracellular cytokines such as IFN- γ are generally associated with non-progression to AIDS. The treatment-naive HIV+ cells analyzed here therefore undergo increase apoptosis and are at risk of progressing to AIDS whereas the same cells activated *in vitro* can produce antiviral cytokines.

[6] Which cytokine profile is observed; Th1, Th2 or Th17, and what does this mean in terms of disease pathogenesis?

After showing that cells respond to *in vitro* activation by producing intracellular IFN- γ and TNF- α , the endogenous Th1/Th2/Th17 cytokine profile of serum was measured and analyzed using a multivariate approach. Elevated IL-6 was detected. IL-6 is a Th2 cytokine associated with increased immune activation and therefore chronic infection, apoptosis and progression to AIDS (Clerici et al., 1997). The detection of this cytokine is in line with detected metabolites (Table 4.7-4.9) which were also characteristic of disease progression (i.e. metabolites representative of oxidative stress, neurodegeneration, wasting and malnutrition). This means that although the patients looked clinically well their immune and metabolic conditions were indicative of disease progression which would soon necessitate initiating treatment.

[7] How does uni- and multivariate analysis of the cytokine data compare? Are new conclusions reached with the latter?

In addition to the multivariate analysis, ANOVA was applied to the Th1/Th2/Th17 cytokine data to confirm immune molecules significantly altered during HIV infection. IL-6 and IL-10 were found to be significantly elevated ($p=0.001$ and $p=0.025$). IL-10 similar to IL-6 is a Th2 cytokine and is confirmatory of the immunological processes at hand. Uni- and multivariate approaches thus complement each other in this case.

[8] Does the Th1/Th2/Th17 cytokines allow for discrimination between HIV- and HIV+ groups?

Log IL-6 and IL-10 were displayed on a scatter plot (Figure 5.15) and classified over 70 % of HIV- and HIV+ cases correctly (Table 5.1 and Table 5.2). These two molecules could thus serve as potential biomarkers of infection.

In conclusion, mitochondrial dysfunction which is commonly measured as apoptosis in the cells of HIV+ individuals can be shown through an additional approach i.e. investigating organic acid changes using GC-MS metabonomics and correlating this to other immune data. All the objectives of this project were therefore reached i.e. metabolic and immune profiles were measured, significantly altered metabolic (Table 4.6-4.9) and immune markers (IL-6 and IL-10, Figure 5.14 and 5.15) were detected and linked to each other. MS and flow cytometry were shown to be suitable for measuring HIV-induced changes early on during infection. Indications are that these same methodologies will be suited to the study of advanced disease. The findings obtained are in favour of the proposed hypothesis in Section 2.9 and showed that HIV does indeed cause a malfunction of the metabolic and immune systems (seen by the change in organic acid levels and the levels of various immune markers) and that these changes can be detected through mass spectrometry and flow cytometry.

6.5 Significance of the Project

Metabonomics characterizes metabolic changes in response to a stimulus. Development of this approach for infectious diseases such as HIV/AIDS allows for the identification of biomarkers which may provide biological information about the status of infection and/or progression of disease. HIV-specific metabolites may eventually be used to discriminate between uninfected and infected groups, predict the outcome of infection, could potentially be used to monitor intervention strategies ([Dettmer et al., 2007](#)) and be utilized to manage disease outcome. By evaluating the properties of the metabolites and the different metabolic pathways they affect, further information regarding the mechanisms of viral infection as well as information regarding viral-host interactions could be obtained. Rapid detection of pathways that are malfunctioning as a result of infection will allow for earlier interventions and recommendations for lifestyle changes, if needed. "Snapshots" of the person's (patho) physiological status can thus be achieved within a relatively short period. In addition, new methods based on metabonomic assessments could be developed to rapidly and quantitatively assess HIV-induced complications.

Organic acid profiling of HIV-infected biofluids adds to the information currently known about HIV infection and mitochondria. Based on the molecules detected, new information about the mechanisms through which mitochondrial dysfunction and related metabolic complications occur, can be obtained. Having shown that organic acid profiles change during asymptomatic infection (al be it moderately) and that distinct differences between the groups increase with viral load, researchers are made aware of the possible prognostic application of these molecules in pathological states (i.e HIV infection) other than inborn errors metabolism where these molecules are primarily investigated.

Because of its sensitivity, MS requires very little sample for analysis. The analysis is done in a shorter space of time (minutes to hours per sample) in comparison to other clinically relevant techniques (days) allowing for rapid collection of usable data/information. The majority of individuals infected with HIV most often do not know their status and only seek medical attention with the onset of symptoms. Mass spectrometry metabonomics provides phenotypic information before the onset of symptoms thus the need for running a range of other confirmatory, costly medical tests is decreased or prevented.

Because flow cytometry also provides multi-parametric information of single cells, both major techniques employed are cost effective in that increased information is obtained from limited sample volumes and numbers. Because health problems can be addressed before the onset of symptoms, treatment or intervention can be recommended early on and even individualized to the specific patient. Interventions can also be monitored using the same techniques.

By characterizing the immune profile of HIV-infected individuals an indication of the current health status of the individuals was obtained. An indication of the degree of disease pathogenesis was obtained through cytokine profiling (e.g. CBA analysis showed disease progression through IL-6 and IL-10 whilst intracellular cytokine staining showed that protective immune responses can be stimulated following exposure to antigens). IL-6 and IL-10 were able to discriminate between HIV- and HIV+ groups by more than 70 %. Since samples were collected locally, the information obtained will be relevant to patients in Africa (particularly South Africa) where infections are dominated by the virulent subtype C strain.

This study was a novel attempt at utilizing metabonomics to detect indicators of HIV-induced mitochondrial dysfunction and linking this data to the immune status of the patients to see whether there was a correlation between the two. This was successfully done but an improvement of the data is possible if patient criteria and patient numbers are optimized. The data obtained does however suggest other strategies for monitoring HIV disease progression.

6.6 Limitations of this study

There were several limitations to the study:

- It was difficult to obtain asymptomatic HIV+ individuals to donate blood. Most individuals are only tested for HIV when they seek medical assistance for symptoms and are already sick from opportunistic infections. The bulk of patients who know their status are on treatment. This project was designed for people not on treatment and these patient groups were not easy to find. This aspect limited sample number. Based on the metabolites identified this study is still important and shows that HIV-induced mitochondrial disruption can be demonstrated through organic acid profiling.
- In cases where donors were available, collapsing of veins sometimes limited the volume of sample obtained. This in turned prevented replicate analysis for the same blood sample from being performed.
- There were time frames associated with sample availability which meant that the data analysis strategy had to incorporate batch analysis. Although batch analysis produced reasonable sample sizes to work with for extraction purposes and the potential for errors was less, data analysis was more exhaustive using this route.
- Nurses at the respective clinics sometimes recorded incomplete demographic and other patient information.
- Metabolic values were not uniformly obtained (diet, time of day, etc) and were thus representative of a real life situation where nothing about the patients' habits was known. Still the approach was able to supply useful biological information.
- This study is not representative of all ethnic and racial groups and men were generally reluctant to supply blood samples for various reasons including a fear of needles.

6.7 Novel Aspects

This study differs from other similar studies in that a profile of metabolic intermediates and related immune parameters was screened. These revealed HIV-induced changes in the host prior to disease development and the initiation of ART. It also showed that the immune response to HIV is not only measurable through immune parameters (cytokines, apoptosis) but is reflected in the host metabolism. Similarly, metabolic changes are also reflected in immune processes.

The proposed methodology proved effective for the detection and characterization of metabolic and immune markers affected by HIV and allows for a distinction between HIV- and HIV+ groups based on this. This has important implications for disease prognosis, disease management and for understanding disease mechanisms as well as viral-host interactions.

To date, there are no reports which have concurrently investigated HIV-induced metabolic and immune changes using such sensitive analytical technologies. This type of work is one of few worldwide addressing HIV/AIDS metabolic influences using metabonomics and is novel as it represents the first of its kind attempting to link HIV-induced metabolic (through organic acids) and immune changes of the host using such sensitive technology.

6.8 Recommendations and Future Considerations

The experiments performed were largely dependent on patient willingness and therefore sample availability. Where sample collection occurs over time, it is recommended that QC samples be prepared to combine data from batches of samples (batch correction) and enable one to analyze the variability between batches. QC samples comprise of a pool of all serum/plasma/cell samples used in a study ([Bijlsma et al., 2006](#)) or a pool of representative samples from each condition being investigated (e.g. HIV- and HIV+; [Dunn et al., 2011](#)). These samples also correct for drifts in signal and retention time. The use of MET-IDEA and the need for QC samples was not part of the initial study design. Limited starting sample volumes also meant that these pooled samples could not be prepared afterward. Serum samples which can be aliquoted and extracted alongside test samples of an analytical batch are now being made available commercially through Sigma-Aldrich. In the HUSERMET PROJECT the detected metabolite distribution and concentration in the serum of the test samples differed from that of the commercial QC sample. It is thus best to take aliquots of the first available set of samples to prepare the QC especially in those cases where all samples are not obtained at once ([Dunn et al., 2011](#)).

Organic acid extractions from cells were done at a defined cell concentration (5×10^6 cells/ml). The quality of the GC-MS approach was evaluated through the RSD of the internal standard's intensity signal. RSDs for cells were much higher than that of sera and urine. In the work of [Munger et al \(2006\)](#) metabolite extractions from cells was done following normalization to protein content. Standardizing extractions according to protein content and not cell concentration is thus a future possibility. Extracting metabolites from higher cell numbers may also still be worth considering for increasing metabolite concentrations and detection.

Even though the study design entailed using treatment naive, clinically stable HIV+ individuals, the CD4 count range selected was still too broad as it included anybody with CD4 counts above 200 cells/ μ l of blood. Although the mean CD4 count was in the range of 300 cells/ μ l of blood, viral load for these patients varied significantly. Metabonomics and cytokinomics revealed that the samples of HIV- and clinically stable HIV+ individuals overlap in terms of their organic acid and cytokine profile when there is moderate metabolic and immune stress but that the separation profile improved with viral load and thus metabolic and immunological stress (Figure 4.9-4.11, Figure 5.14). A comparative study of patients defined as having AIDS versus clinically stable patients is advised to further confirm and validate the prognostic application of the metabonomics and cytokinomics approach, this time with a larger more “controlled” cohort. In context to the South African setting these samples may be difficult to obtain (limited centres with stored samples, if patients are at the AIDS stage HAART is usually implemented etc). Stricter criteria in terms of CD4 counts and viral loads will more accurately show the overlap or separation of groups based on metabolic and cytokine profile.

Several metabolites were altered in response to HIV infection (Table 4.7-4.9). Although the significance of these molecules were assessed through statistics, further confirmation of the increase or decrease of metabolites can be done through gene expression studies (Wikoff et al., 2008) and through enzymatic studies. With future investigations where larger cohorts are to be used, correlation analysis between current markers of HIV infection (CD4 and viral load) and the identified metabolites is proposed.

An HIV- individual repeatedly exposed to HIV (i.e. having unprotected sex with her HIV+ partner for over three years, as per clinic records) was found to cluster with the HIV+ samples (blue arrow in Figure 4.9a, left plot). This led to the speculation that exposure to virus (no confirmed seroconversion and infection) induces metabolic change. A study into this aspect is warranted especially since exposure-induced immunological changes have been previously reported (Rowland-Jones et al., 1995; Clerici and Shearer 1993). The potential use of systems biology approaches, metabonomics included, for investigating HIV-induced changes in this group of individuals was also reviewed (Burgener et al., 2010).

This study targeted the organic acid metabolome specifically. Targeted approaches yield better quality data because the method is exclusive for the group of metabolites (Álvarez-Sánchez et al., 2010). However; the section of the metabolome investigated is smaller versus that investigated during an untargeted approach (i.e. one where all metabolites are analyzed), limiting the differences that may be observed. Regions where other significant changes occur may therefore be excluded from the analysis. This may also be why

moderate metabolic changes were detected. An untargeted analysis of HIV-infected biofluid is recommended since analysis of the entire metabolome leaves room for detecting larger metabolic differences and room for obtaining a better distinction between the experimental groups. Preliminary data collected through ultra performance liquid chromatography (UPLC)-MS is shown in the appendix (Figure A11-A12). In this case, all the metabolites that can be extracted from sera (in acetonitrile containing 1 % formic acid) were analyzed. The untargeted approach detected multiple metabolites which through simple visualization (Figure A11) already showed differences which were amplified through statistical evaluation (Figure A12).

In Chapter 2 (Section 2.7.1.1) mention was made of the fact that HIV infection of a host does not imply infection of all immune cells. As a result, [Herbein et al \(1998\)](#) used a HIV reporter virus expressing GFP to label and differentiate uninfected from HIV-infected cells. The authors did this to obtain clarity about the subset of cells primarily experiencing apoptosis. Since the objective of this thesis was to characterize the metabolic and immune profile of HIV-infected biofluid; specifically labelling and selecting only the infected cells and assessing metabolic and immune perturbation in these may yield more virus-specific information. In a recent investigation based on acute HIV infection (ours was based on chronic infection), [Hollenbaugh et al \(2011\)](#) first selected the HIV+ CD4 and macrophage cells prior to performing metabolite extractions.

It is clear that the indirect and direct influence of HIV causes a multifunctional disruption of mitochondrial function which is augmented by other disruptive effects from products of the immune system (like the effect of pro-inflammatory cytokines). A lot more needs to be done with regards to the simultaneous or stepwise analysis of HIV-induced metabolic and immune disruptions, and the work presented forms a small but relevant drop in the ocean.

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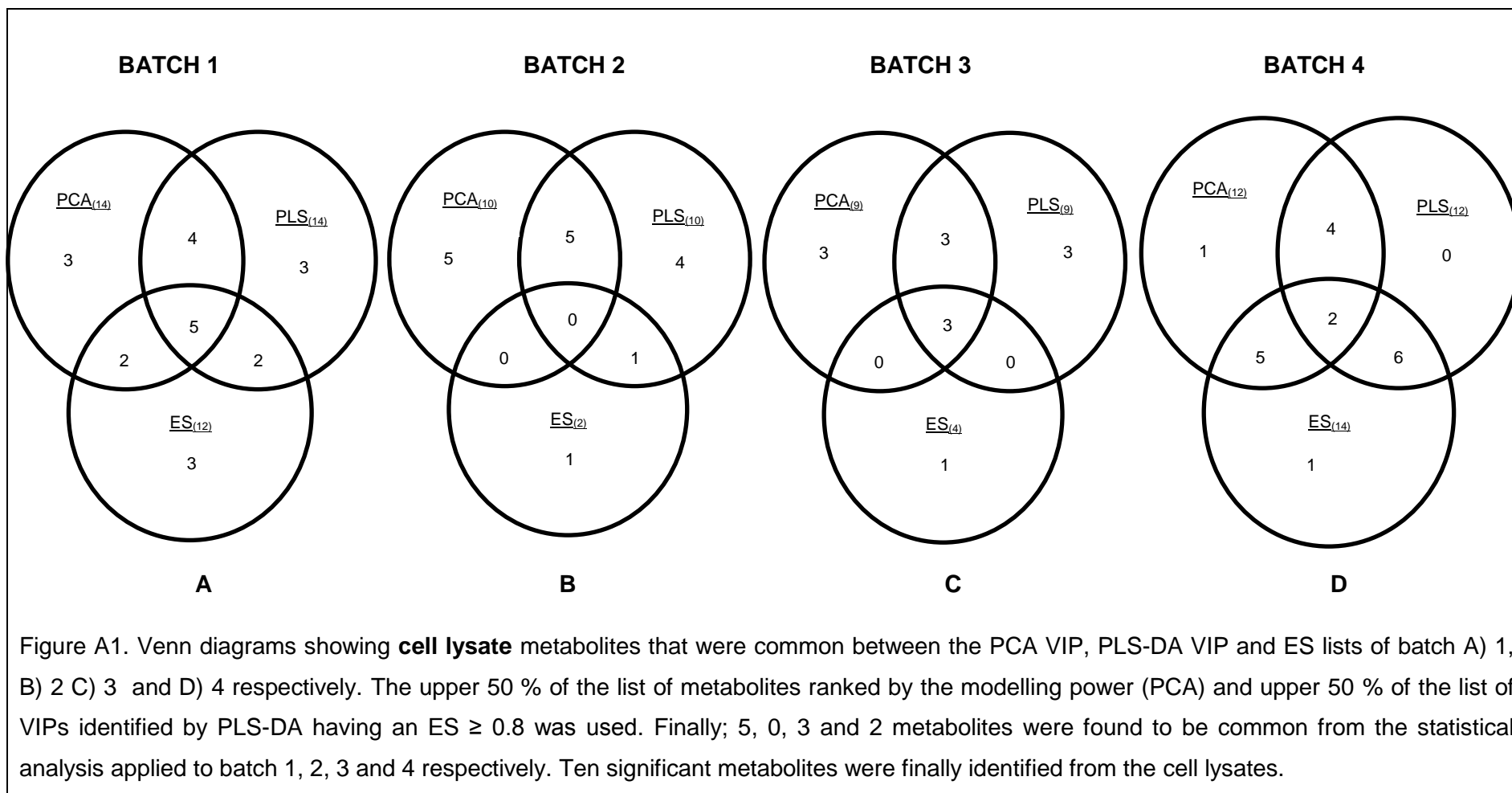
APPENDIX

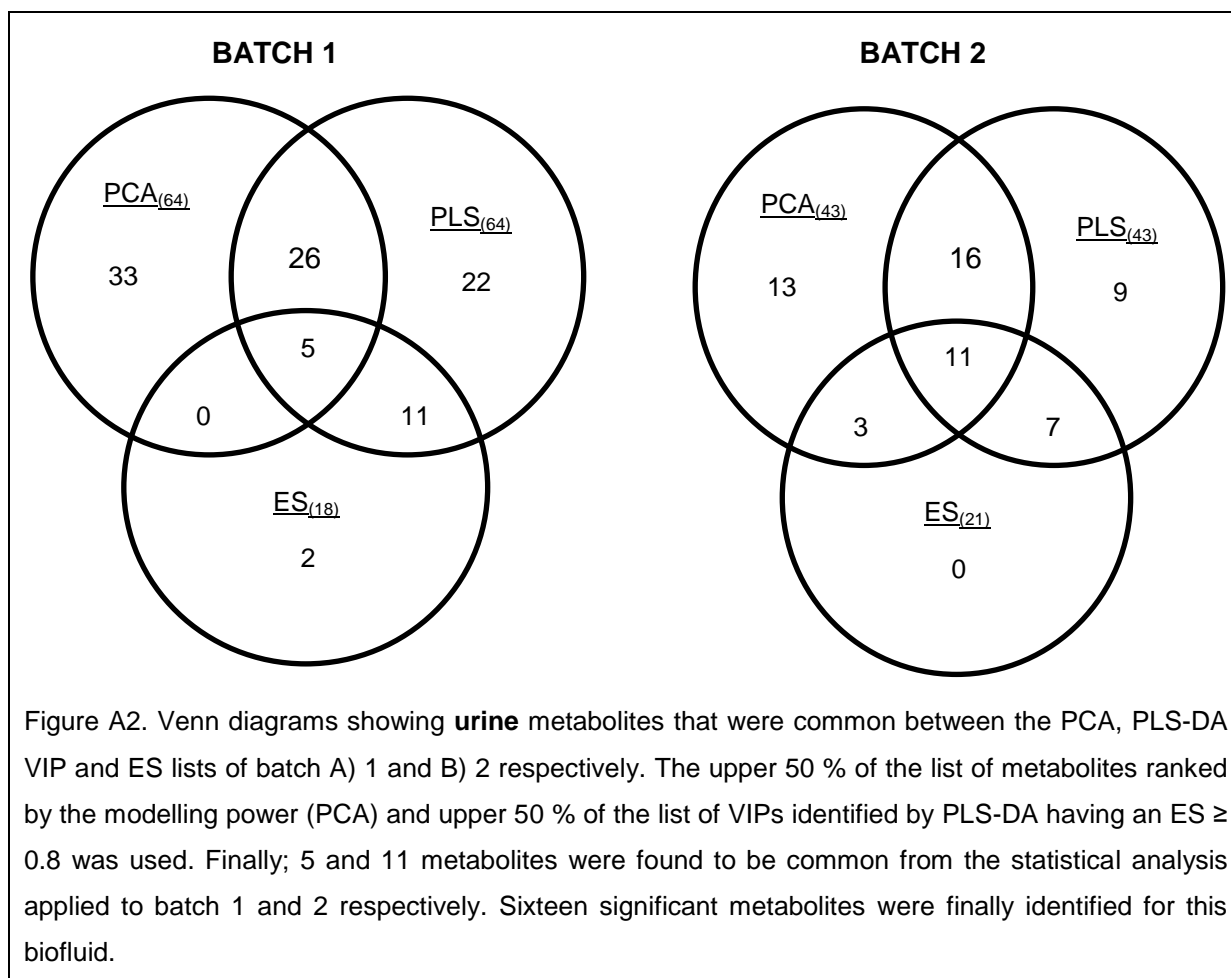
This section contains additional information and data which was not important for achieving the main objectives of this work but which supports some of the main findings obtained.

1. Metabonomic Analysis

a. Identification of organic acid molecules affected by HIV Infection

Organic acids were extracted from sera, cells and urine. Extracts were subjected to GC-MS analysis. GC-MS data was then deconvoluted, pre-treated (curation etc) and analyzed using multi- (PCA, PLS-DA) as well as univariate statistics (ES). These statistical methods helped with identifying metabolites that were significantly altered by HIV infection. Since the data analysis strategy included batch analysis, a resource for assessing the relationship and comparing the information of the three statistical approaches (PCA, PLS-DS and ES) and the different biofluids had to be incorporated. Venn diagrams were therefore constructed and because of the important information extracted from these, a representative Venn diagram of the sera (Figure 4.12) was regarded important to show in the main text (Section 4.3.5.2). Similar information was obtained for the cell lysates and urine and is therefore shown as supplementary data here. Figure A1 shows that for the batches of cells, the number of metabolites that were common between the statistical lists was different (i.e. 5, 0, 3 and 2 respectively). The three statistical methods are completely different and can be expected to differ in terms of the number of metabolites they identify. Using the Venn diagram, 10 metabolites were finally shown to be significantly altered in the cells of the patients utilized here. Figure A2 shows that 16 metabolites were significantly altered in urine (5 for batch 1 and 11 for batch 2). The numbers of metabolites altered in the respective biofluids were thus similar.





b. PCA and PLS-DA Percentage Variations Declared

PCA and PLS-DA analysis was done to assist with the classification of HIV- and HIV+ groups based on organic acid content. Based on the grouping trends of the two groups potential organic acids linked to HIV-induced mitochondrial dysfunction were identified. Overlapping and non-overlapping profiles were observed. To quantitatively account for these observed profiles, the percentage variation declared for the respective biofluids was reviewed. In Table A1 below, a summary of the variations declared by the first three PCA PCs and the first two PLS-DA components (X and Y space) are shown. Whereas the PCA components of sera and cells explain over 70 % of the variation each time, the variation displayed for urine samples was much lower. This supports the idea that the organic acid/metabolic profile of urine collected from asymptomatic HIV+ individuals is less altered versus that of sera and cells causing it to overlap with that of HIV- individuals (see also Section 4.3.5.1).

The validity of PLS-DA models is estimated through Q^2 values which should preferably equate to one. For predictive purposes only one component should be extracted based on the estimated Q^2 value. In this study we extracted two components to identify

possible biomarkers linked to HIV-induced mitochondrial dysfunction. The low Q^2 values obtained for the datasets analyzed in this study suggested that PLS-DA would have poor predictive power at classifying HIV- and HIV+ groups based on their organic acid profile and supports why the model was used in an explorative context only. In Figures 4.9-4.11 the second PLS-DA component does contribute to group separation and the unfavourable/low Q^2 value extracted using two components might be caused by small sample sizes. Two components were extracted nevertheless. Batch 1 and 2 of the serum were the only datasets having somewhat higher Q^2 values (0.72 and 0.55 respectively, Table A1) meaning the models developed with these samples would possibly function well for prediction purposes.

Table A1. The table shows variations declared by the first three PCA principal components and the first two PLS-DA components in the X and Y space, respectively. The PLS-DA approach was used in an explorative context. Q^2 values show poor predictive application of the model and supports the use of this model for explorative purposes in this study.

| BIOFLUID | | | | | | |
|--------------|-----|------------|------------|----------------------|----------------------|--|
| | PCA | PLS-DA (X) | PLS-DA (Y) | Q^2 component 1 | Q^2 component 2 | |
| SERUM | | | | | | |
| BATCH 1 | 84 | 72 | 87 | 0.72 | -0.55 | |
| BATCH 2 | 75 | 50 | 80 | 0.55 | -1.01 | |
| BATCH 3 | 72 | 49 | 51 | 0.15 | -0.4 | |
| CELLS | | | | | | |
| BATCH 1 | 90 | 69 | 64 | 0.13 | -2.17 | |
| BATCH 2 | 84 | 45 | 63 | 0.21 | -0.34 | |
| BATCH 3 | 87 | 41 | 62 | 0.37 | -0.44 | |
| BATCH 4 | 85 | 61 | 57 | 0.38 | -0.49 | |
| URINE | | | | | | |
| BATCH 1 | 55 | 36 | 70 | 0.34 | -0.5 | |
| BATCH 2 | 62 | 42 | 75 | 0.28 | -1.07 | |

2. Immune Analysis

a. Batch Effect (ROS)

The availability of samples can contribute to the development of batch effects (i.e. samples collected on one day will have the same degree of variance compared to samples collected on another day). Such batch effects are sometimes unavoidable (Luo et al., 2010)

and can lead to incorrect interpretation of biological data. Shown below in Figure A3 is a representation of data from sera that was assessed for oxidative stress on three different occasions (for detailed protocols please refer to Section 5.2.3). This effect was observed even after including a control with which to normalize the data. To avoid such batch effects it is best to freeze aliquots of the test sample and to perform experiments when enough samples have been accumulated.

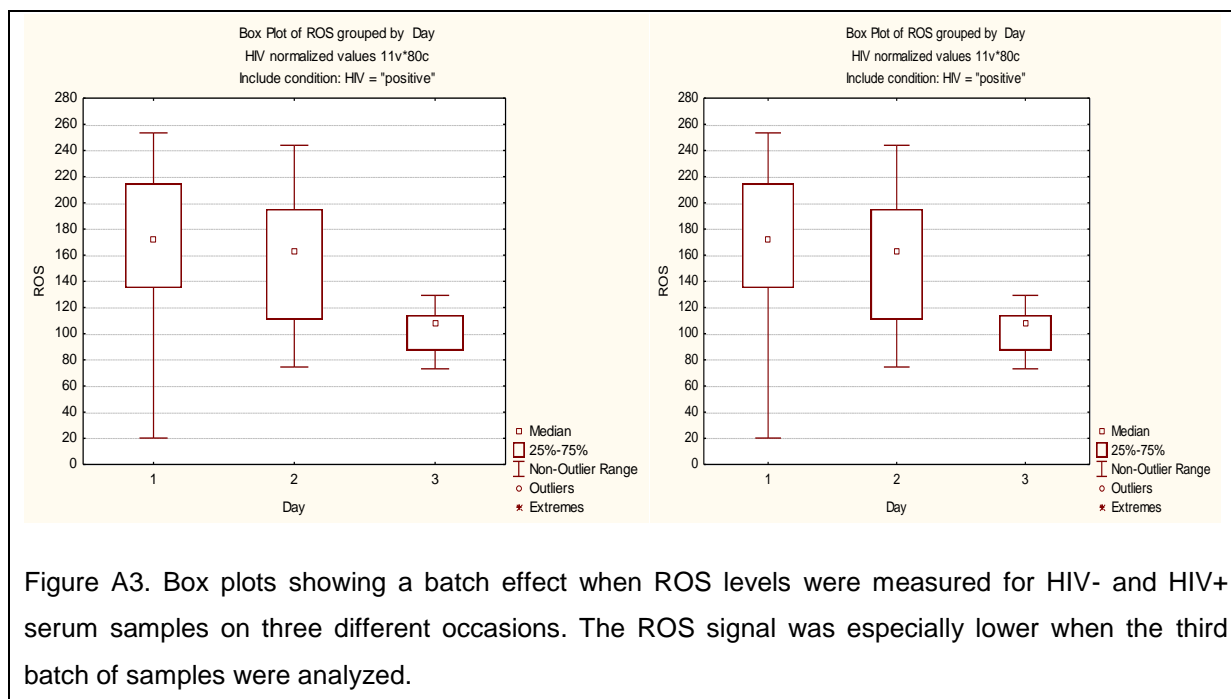
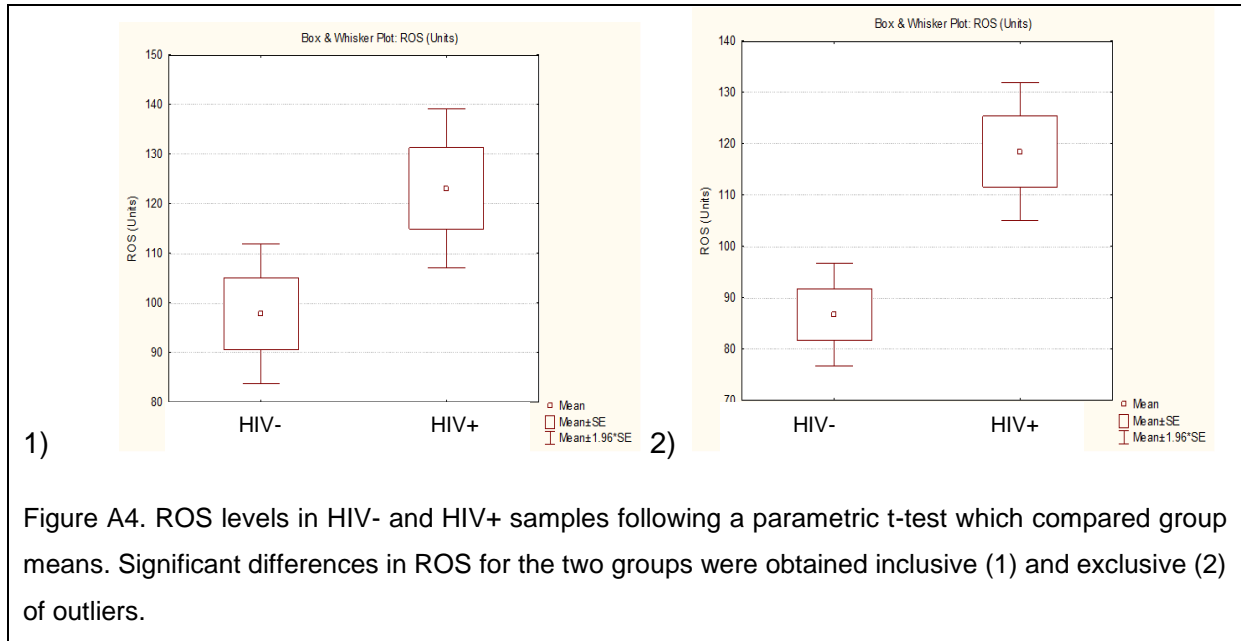


Figure A3. Box plots showing a batch effect when ROS levels were measured for HIV- and HIV+ serum samples on three different occasions. The ROS signal was especially lower when the third batch of samples were analyzed.

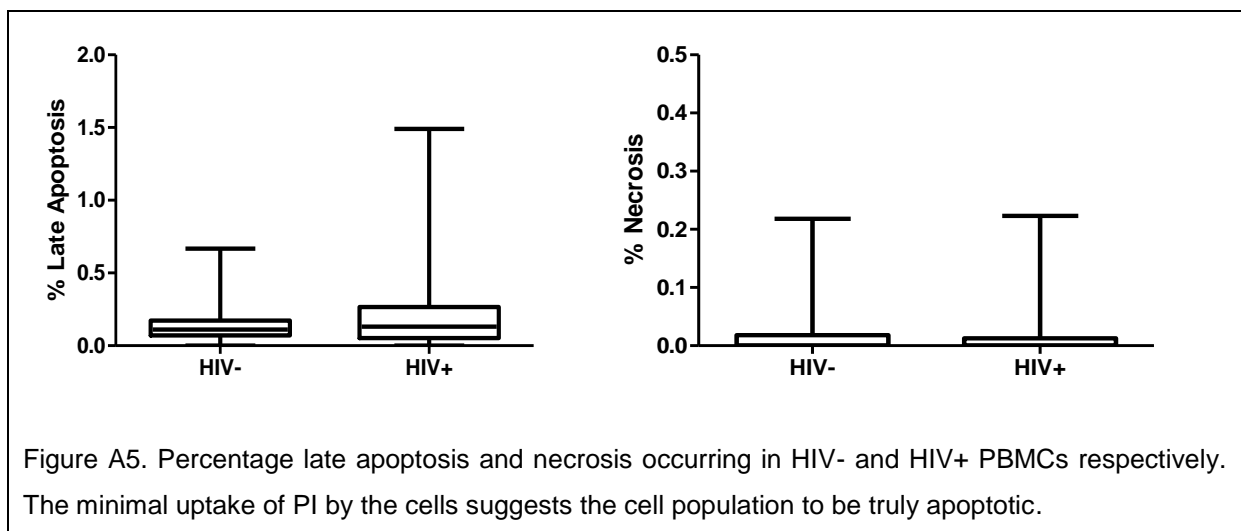
b. Parametric Analysis (ROS)

Significant differences in the redox status of HIV- and HIV+ serum was shown using the non-parametric t test (Figure 5.2). Here the same data is shown (Figure A4) but using the parametric t-test. This was done to illustrate that the spread in the data observed in Figure 5.2 is not as obvious when displaying the results of such a test. This is largely due to the fact that parametric tests compares the means of two groups while nonparametric tests (as used in Figures 5.2 A and B) compares the medians of groups. Parametric tests are however suitable to use when there is an increase in sample numbers irrespective of whether the data is distributed normally or not (this was the case for the samples analyzed here). The nonparametric data was however chosen for presentation as it was most representative of the “health status” of the samples and depicted the variability in ROS that is associated with samples from different individuals.



c. PBMC Apoptosis (Late apoptosis measurements and necrosis)

In addition to detecting apoptosis, cells were also evaluated for other forms of cell death; namely necrosis through the uptake of PI. This dye is usually excluded by viable cells and taken up by dead ones. The minimal uptake of PI by the cells below (Figure A5) suggests the cells to be a truly apoptotic population. Morphological changes were confirmatory of this apoptotic state i.e. HIV+ cells showed a decrease in FSC properties and therefore cell size. Minimal necrosis during HIV infection was observed by other research groups (Potter et al., 1999).



d. Surface markers in samples analyzed for T cell Apoptosis

To determine the subset of immune cells undergoing apoptosis (data shown in Figure 5.4 D); cells were labelled by staining for characteristic molecules which they present on their surface. Fluorescently labelled monoclonal antibodies were thus used to stain for CD3, CD4 and CD8 representing T cells, T helper and T cytotoxic cells respectively. The percentage CD3 on the surface of HIV- and HIV+ cells was similar (Figure A6, 1). However; the percentage CD4 on the surface of HIV+ cells was significantly lowered (Figure A6, 2) while that of CD8 was significantly elevated (Figure A6, 3). HIV infection is characterized by a decline in CD4 (Cummins and Badley 2010; Gougeon and Montagnier 1999). The data obtained is thus confirmatory of the health and clinical status of the participating donors.

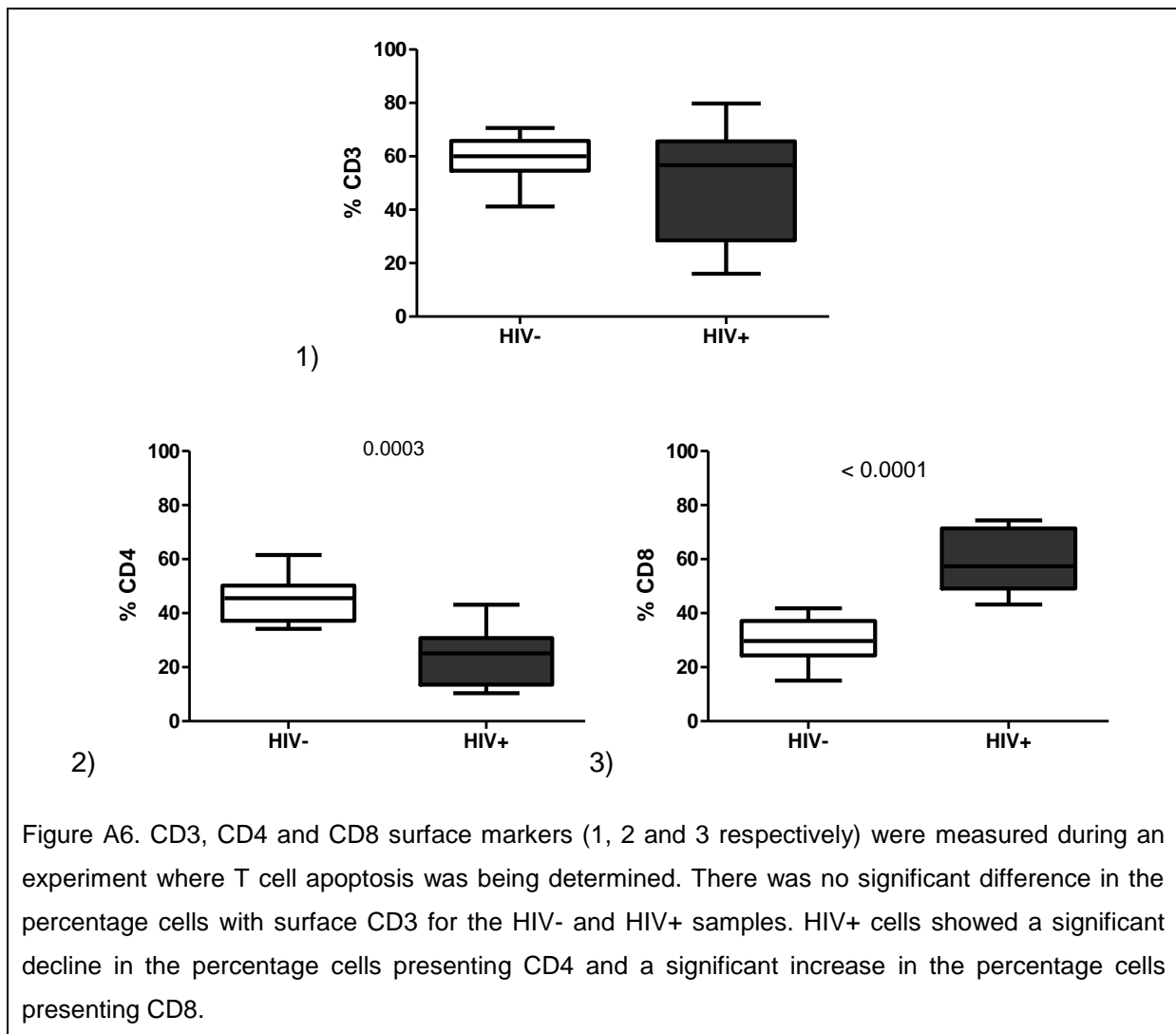


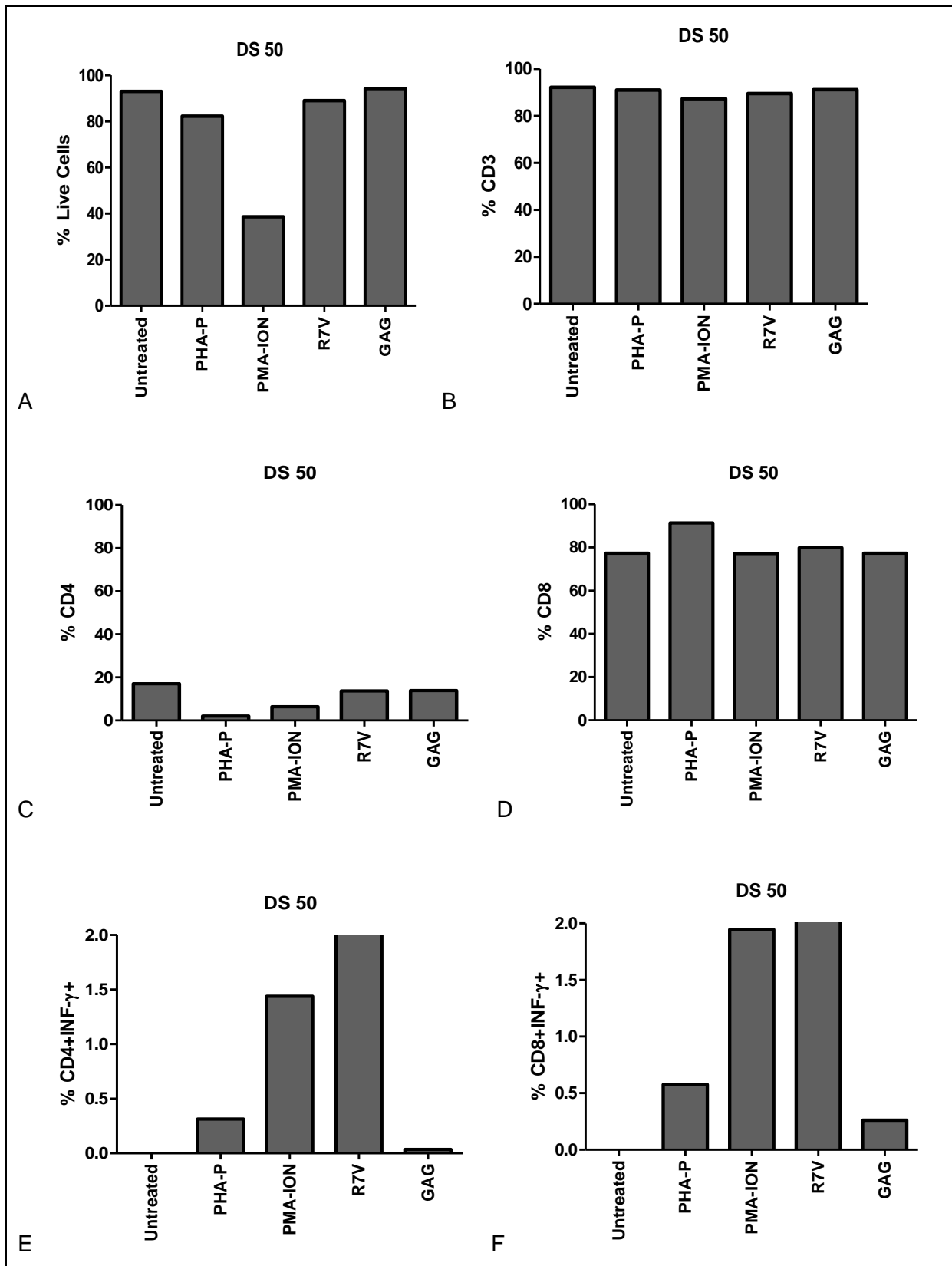
Figure A6. CD3, CD4 and CD8 surface markers (1, 2 and 3 respectively) were measured during an experiment where T cell apoptosis was being determined. There was no significant difference in the percentage cells with surface CD3 for the HIV- and HIV+ samples. HIV+ cells showed a significant decline in the percentage cells presenting CD4 and a significant increase in the percentage cells presenting CD8.

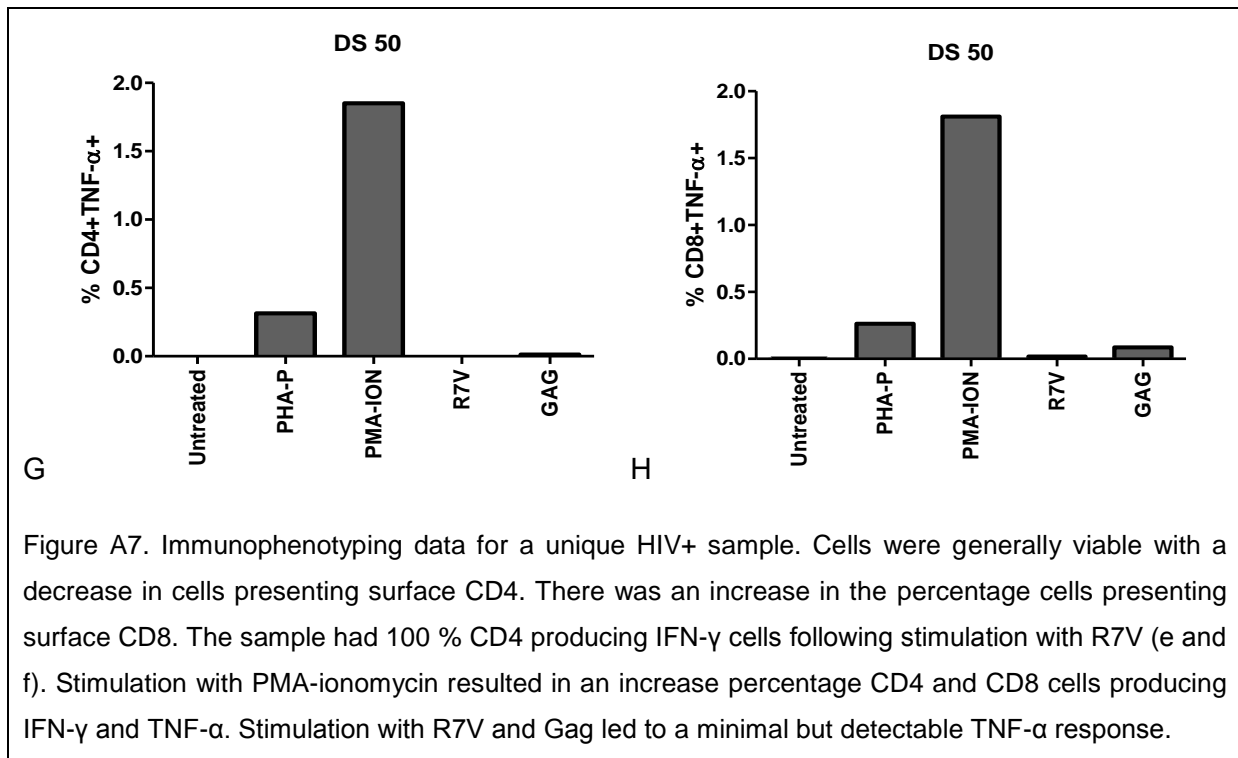
e. Intracellular cytokine staining

A unique HIV+ case having almost all of its T cells responding to *in vitro* R7V stimulation was identified when intracellular cytokine staining was performed (for detailed protocol see Section 5.2.6). The data of this particular individual is summarized below.

i. Unique case responding to R7V Stimulation

The viability of the patient's cells was generally high (Figure A7, A). Following treatment with PHA-P and PMA-ionomycin, the viability of these cells decreased. The treatments applied had no effect on the percentage cells presenting surface CD3 (Figure A7, B). The percentage cells with CD4 on the surface were severely decreased while those with CD8 were elevated (Figure A7 C-D). These results are characteristic of HIV infection. IFN- γ production by CD4 and especially CD8 cells is representative of a strong antiviral or CTL response. HIV+ individuals with strong HIV-specific cellular responses are associated with slow progression of HIV to AIDS. That an HIV+ sample would fully respond to R7V as in the case of DS 50 makes this an important individual to consider in the design and testing of anti-HIV therapeutics or vaccines. Since many of the treatments under development are aimed at inhibiting the virus, but fail to restore immune function, an immune response such as that of DS 50 suggests that the immune cells of this individual can mount protective responses through cytokine production and could work synergistically with potential therapies to improve immune responses whilst concurrently inhibiting viral replication. The immune response of such an individual could also shed light on viral-host interactions, correlates of protection and thus disease pathogenesis. Stimulation of the cells with PMA-ionomycin resulted in an increase in the percentage of CD4 and CD8 cells producing IFN- γ and TNF- α whereas stimulation with R7V and Gag led to minimal TNF- α responses. The beneficial role of low TNF- α levels have been highlighted before (Section 6.4, b).





f. Secreted Cytokine

Secreted cytokine in the culture supernatant of cells previously stimulated with mitogen and antigen was measured (see detail below in [i]). This was to detect endogenous cytokine secreted before *in vitro* activation and before GolgiPlug has had a chance to work. Secreted IFN- γ measured here differs from serum cytokines (including IFN- γ) measured during CBA analysis with flow cytometry in that it was activated to be produced and secreted versus endogenous serum IFN- γ . The ELISA used to measure secreted IFN- γ can detect only one cytokine at a time whereas the CBA analysis facilitated the detection of multiple cytokines. IFN- γ data acquired through ELISA analysis was analyzed using univariate statistics whereas multiple cytokines detected with CBA and flow cytometry was analyzed using multivariate statistics. This ELISA assay differs from intracellular cytokine assays performed with flow cytometry in that the cells responsible for cytokine production cannot be identified unless they are first purified. The ELISA was only performed for IFN- γ since this cytokine was produced at slightly higher levels than TNF- α .

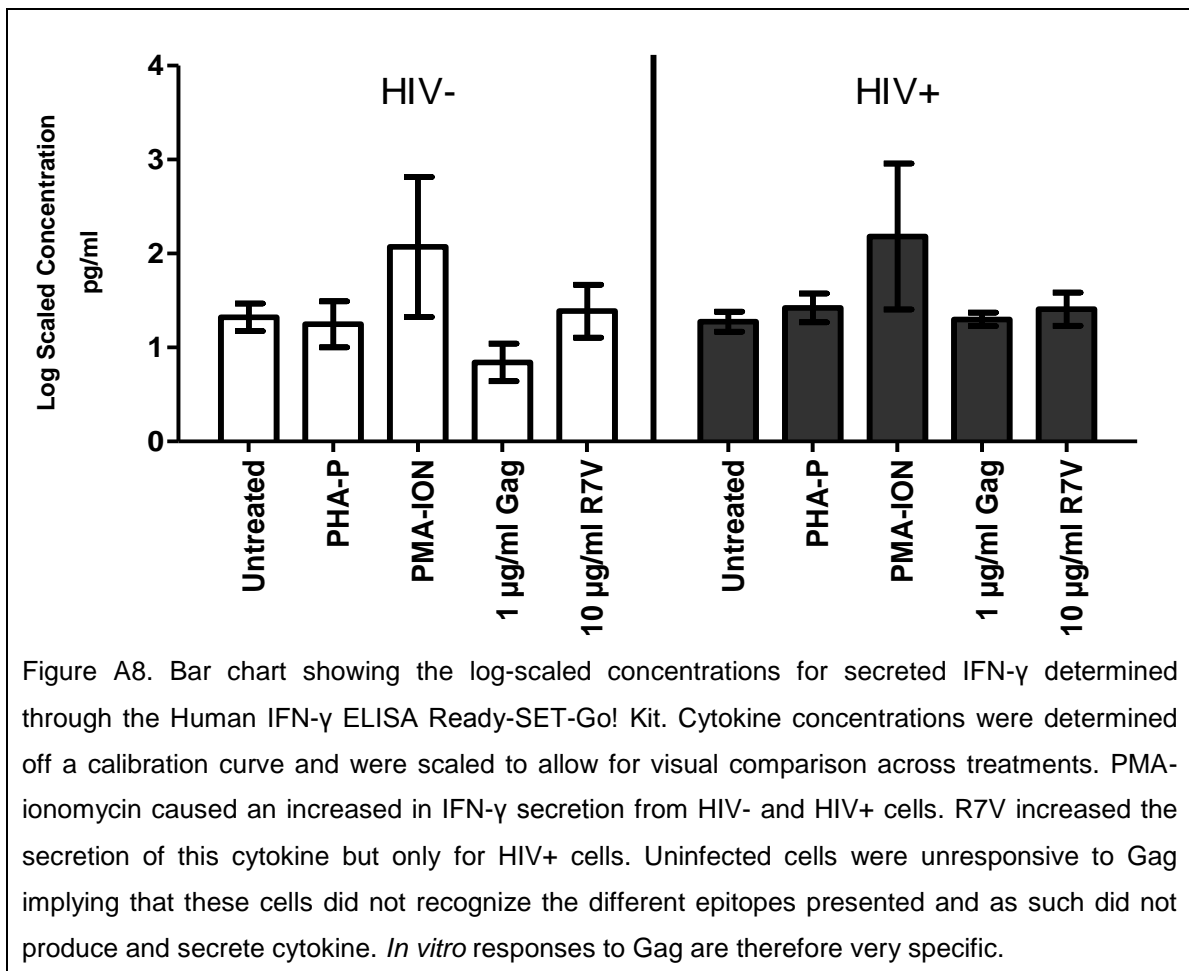
i. (IFN- γ)

Method: Secreted IFN- γ produced by PBMCs after stimulation with media, PHA-P, PMA-ionomycin, R7V and Gag (Section 5.2.6.1) was measured for 15 selected culture supernatant samples (HIV- = 7 and HIV+ = 8) using the Human IFN- γ enzyme linked immunosorbent assay (ELISA) Ready-SET-Go! kit from eBioscience, San Diego, USA.

Secreted cytokine produced after 6 hours was measured according to the manufacturer's instructions. Briefly, coating buffer (0.01M PBS, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) was prepared. One hundred microlitres of capture antibody dissolved in coating buffer was used to coat a Corning Costar 9018 ELISA plate which was incubated at 4 °C overnight. The coating buffer was aspirated the following day and the plate washed 5 x with 280 µl Wash Buffer (0.05 % Tween in PBS 20, v/v). The wells were then blocked with 200 µl of 1 x assay diluent for 1 hour at room temperature. Assay diluent was aspirated and the plate washed as described previously. Recombinant standards of the cytokine were prepared by 2-fold serial dilutions in assay diluent. Standards (100 µl) were plated in the respective wells in duplicate. Culture supernatant was diluted 3 fold and 100 µl plated in the appropriate wells in duplicate. Plates were sealed and incubated overnight at 4 °C. The supernatant was then aspirated and the plate washed as described before. Biotin-conjugate anti-human IFN-γ detection antibody diluted in assay diluent (100 µl) was added to each well and incubated for 1 hour at room temperature. The detection antibody was aspirated and the plate washed 5 x. Avidin-horseradish peroxidase (HRP) diluted in assay diluent (100 µl) was then plated and incubated for 30 minutes at room temperature. The wells of the plate were then washed 7 times followed by the addition of 100 µl tetramethylbenzidine substrate solution. The plate was incubated for 15 minutes at room temperature after which 50 µl stop solution (2N H₂SO₄) was added. Absorbancies were recorded at 450 nm using the MultiScan Ascent Plate reader from Thermo Labsystems, Helsinki, Finland.

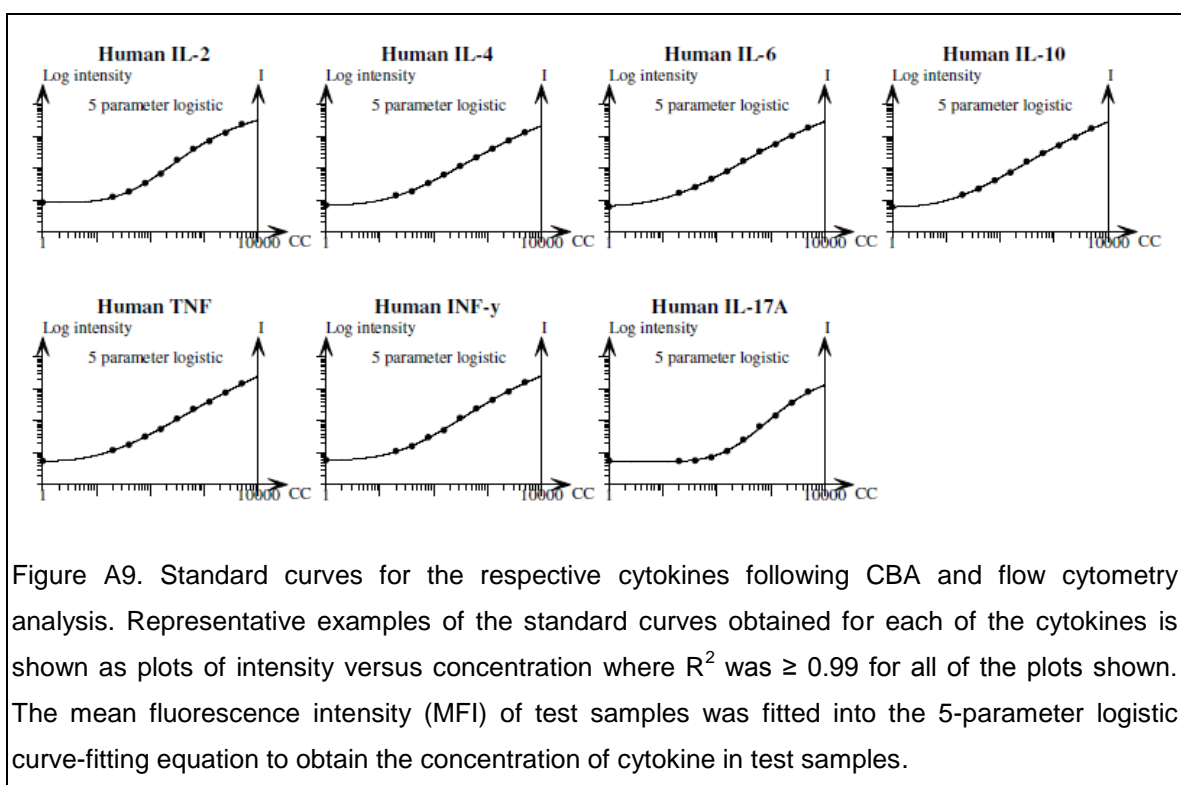
Results and Discussion: HIV- and HIV+ PBMCs were stimulated with media, 2 µg/ml PHA-P, 10 ng/ml PMA-ionomycin, 10 µg/ml R7V and 1 µg/ml Gag respectively. Following stimulation, the supernatant was collected and analyzed for IFN-γ secretion. HIV- and HIV+ PBMCs secreted minimal IFN-γ in the absence of stimulation (Figure A 8). Background IFN-γ is however expected since HIV activates the immune system causing IFN-γ production and release (Pala et al., 2000). Resting cells generally produce and secrete minimal or undetectable levels of cytokine thus the need for stimulation of cells with mitogen or antigen (O'Neil-Andersen and Lawrence 2002). Treatment of HIV- and HIV+ cells with PHA-P did not cause the cells to secrete any more of the cytokine. PMA-ionomycin being the positive control (Pala et al., 2000) and also a test for cell functionality, showed HIV- and HIV+ to secrete a significant amount of IFN-γ in comparison to untreated cells (p = 0.0156 for both groups). IFN-γ secretion by HIV- cells in response to the Gag peptide was below the levels secreted by unstimulated cells. This implies that the epitope was not recognized and that the uninfected cells were therefore unresponsive to this peptide causing the response between HIV- and HIV+ to differ significantly. Exposing HIV+ cells to Gag did not induce IFN-γ secretion. IFN-γ was produced following treatment with Gag (Figure 5.11 A and Figure 5.12

A) but not secreted (Figure A8). HIV+ but not HIV- cells secreted slightly elevated amounts of IFN- γ when stimulated with R7V. DS 50 was the HIV+ case that was presented on its own in Section e above (Figure A7 E-F) since all the T cells of this individual produced IFN- γ in response to R7V treatment. The cell supernatant of DS 50 was one of the selected few chosen for the ELISA analysis of IFN- γ . IFN- γ secretion in response to R7V treatment is then mainly due to the inclusion of this sample. This implies that cytokine could no longer be retained in the cells of this individual despite the addition of GolgiPlug-when maximum cytokine has been produced, the excess is possibly secreted. There is also the possibility of minimal cytokine secretion due to the activated state of the immune cells. Cytokine may have “leaked” from the cells prior to stimulants and GolgiPlug being added/taking effect, but this possibility can be excluded based on non-significant IFN- γ secretion by HIV+ untreated cells. When this sample is removed from the analysis, non-significant background levels of IFN- γ are measured.



g. Cytometric Bead Array (CBA) standard curves

Seven endogenous cytokines in the serum of HIV- and HIV+ individuals were simultaneously detected using CBA technology and flow cytometry (for the principles of CBA technology refer to Section 5.2.6.2.1). To enable the quantification of these cytokines, standard curves were plotted. These plots were constructed after capture beads (specific for IL-2, 4, 6, 10, 17, IFN- γ and TNF- α) were reacted with a known concentration of each cytokine.



3. Confirmation of Subtype C infection

Subtype C infection was confirmed through nested PCR even though enough evidence exists for the predominant HIV-1 subtype in this region of Africa to be subtype C.

Method: A nested PCR was performed as specified by [Yagyu et al \(2005\)](#) with slight modifications and confirmed subtype C infection. Blood was collected in EDTA vacutainers. Samples were heat-inactivated at 56 °C for 1 hour and stored at -20 °C. On the day of the experiment, samples were thawed, centrifuged (12 000 \times g, 10 minutes) and the supernatant discarded. Genomic DNA was then extracted from 200 μ l of blood using 700 μ l phenol:chloroform:isoamyl (25:24:1). This extraction solution was mixed with the sample followed by 1 hour incubation at 56 °C and shaking for 10 minutes thereafter. Samples were centrifuged at 10 000 \times g for 10 minutes. The supernatant was transferred to a clean tube

and the DNA ethanol-precipitated (2.5 volumes of 100 % ethanol) at -70 °C for 1 hour followed by centrifugation at 12,000 × g for 20 minutes. Supernatant was removed and the pellet washed (12,000 × g for 20 minutes) with 1 ml 70 % ethanol (room temperature). This was repeated 2 times and the DNA left to air dry for approximately 30 minutes. The DNA pellet was dissolved in 20 µl Tris-EDTA (TE) buffer. Two µl DNA diluted in 48 µl TE buffer was used for spectrophotometric quantification of the DNA (Gene Quant Pro, Amersham Biosciences). The remaining DNA was stored at -20 °C until used. On the day of analysis, the DNA was thawed on ice and a PCR performed using BECO5 (5'-GGCATCAAACAGCTCCAGGCAAG-3') and BECO3 (5'-AGCAAAGCCCTTTCTAAGCCCTGTCT-3') as forward and reverse primers respectively. A reaction mixture comprising of 2.5 µl 10 × PCR buffer, 2.5 µl deoxynucleotide triphosphates (dNTPs, 25mM each), 0.25 µl Ex Taq, 1 µl of each primer (10 µM each), 1 µl of template DNA solution and distilled water (dH₂O), up to 25 µl was made and analyzed on a GeneAmp® PCR System 9700 (Applied Biosystems). The cycle conditions were as follows: 95 °C for 1 min, 50 °C for 1.5 min, and 72 °C for 2 min, for 30 cycles. Amplification products were subjected to 2 % agarose gel electrophoresis at 100 V for 30 min. For the second round of PCR; BE-ANCH (5'-TCCTGGCTGTGGAAAGATACCTA-3') and C-SPEC (5'-AGACCCCAATACTGCACAAGACTT-3') was used as primers. A reaction mixture as outlined above was made up except that amplified DNA from the first round of PCR now served as the DNA template. Cycle conditions were also as outlined above. Amplification products of the second PCR were subjected to 2 % agarose gel electrophoresis. Molecular weight markers which were run alongside the amplicons were used to estimate the size of the products. Gels were visualized following staining with the Gel Red Nucleic Acid (Anatech) and an image recorded using the Gel Doc Imager (Bio-Rad Laboratories, Milan, Italy).

Results and Discussion: The world and the African continent are burdened with HIV-1 subtype C infections. Sub-Saharan Africa accounts for 67 % of global HIV infections. Travel and migration has caused a shift in the geographical distribution of the virus. In addition there has been an increase in the development of CRFs. Infection with a particular subtype can therefore no longer be defined as occurring only in a particular region but should be confirmed. DNA extracted from HIV-infected blood collected within South Africa was analyzed through a nested PCR using subtype C specific primers. Following gel electrophoresis of the amplicons, the samples were confirmed as being infected with HIV-1 subtype C as it showed amplification of the 697 bp gp41 region (Figure A10). There is literature which documents Sub-Saharan Africa to be laden with almost all subtypes (McCutchan, 2006). As shown here, the samples collected in the intended region were

indeed representative of subtype C infection. The assay still requires further optimization as smears which prevented the visualization of the amplified product occurred frequently. There was also the occurrence of primer dimers signalling non-specific amplification. Since the primers used were specific for subtype C (Yagyu et al., 2005) no further confirmation of subtype C infection was needed through sequencing for those samples where the amplicon was visible.

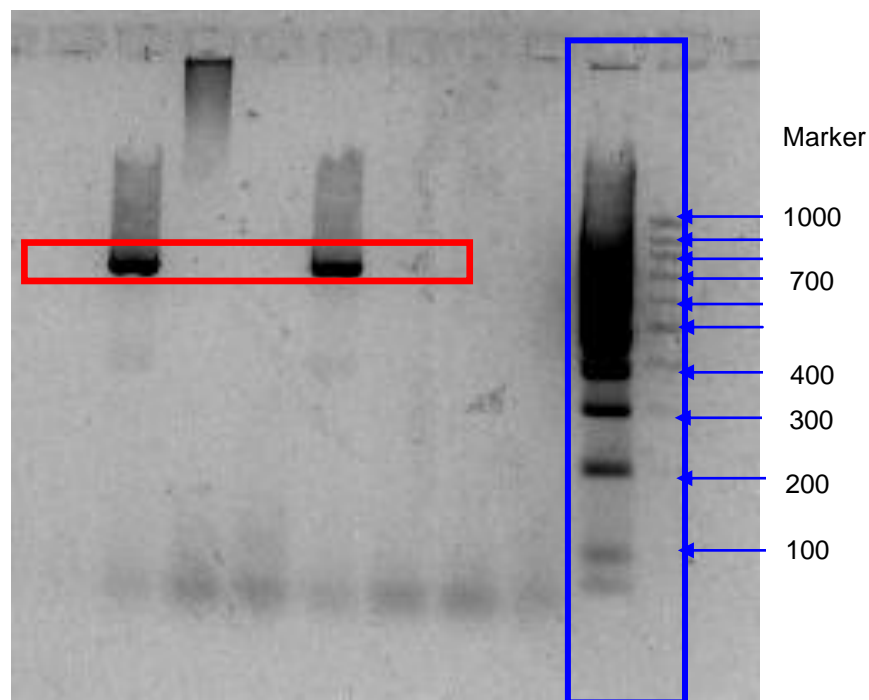


Figure A10. A 2 percent agarose gel showing the separation of DNA amplicons following nested PCR. Lanes shown are representative of HIV-infected samples analyzed at different DNA concentrations. The molecular weight marker (O'GeneRuler™, 100 bp DNA ladder) which was loaded in the last two lanes is circled in blue. Although the assay is flawed and requires further optimization, amplicons (circled in red) with an approximate size of 697 bp were detected and confirmatory of subtype C infection as per the use of subtype C specific primers. Primer dimers were also visible signalling non-specific amplification.

4. Ultra performance liquid chromatography mass spectrometry analysis of HIV-infected biofluid

Preliminary UPLC-MS data not targeted to a specific group of molecules is shown below in support of a recommendation that was made in Section 6.8. Differences between the metabolic profiles of HIV-, HIV+ and HIV+HAART+ individuals were visible prior to the use of statistics (Figure A11) and amplified after multivariate analysis (Figure A12).

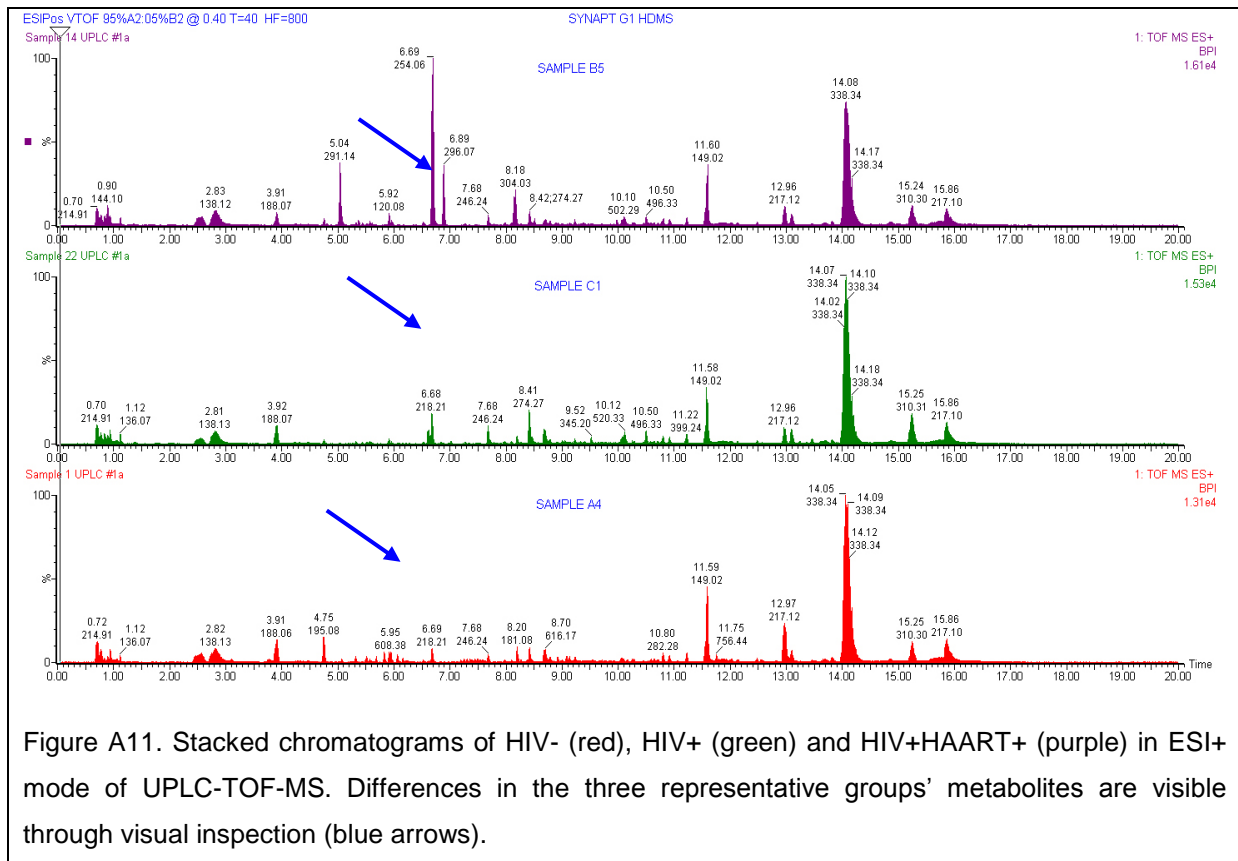


Figure A11. Stacked chromatograms of HIV- (red), HIV+ (green) and HIV+HAART+ (purple) in ESI+ mode of UPLC-TOF-MS. Differences in the three representative groups' metabolites are visible through visual inspection (blue arrows).

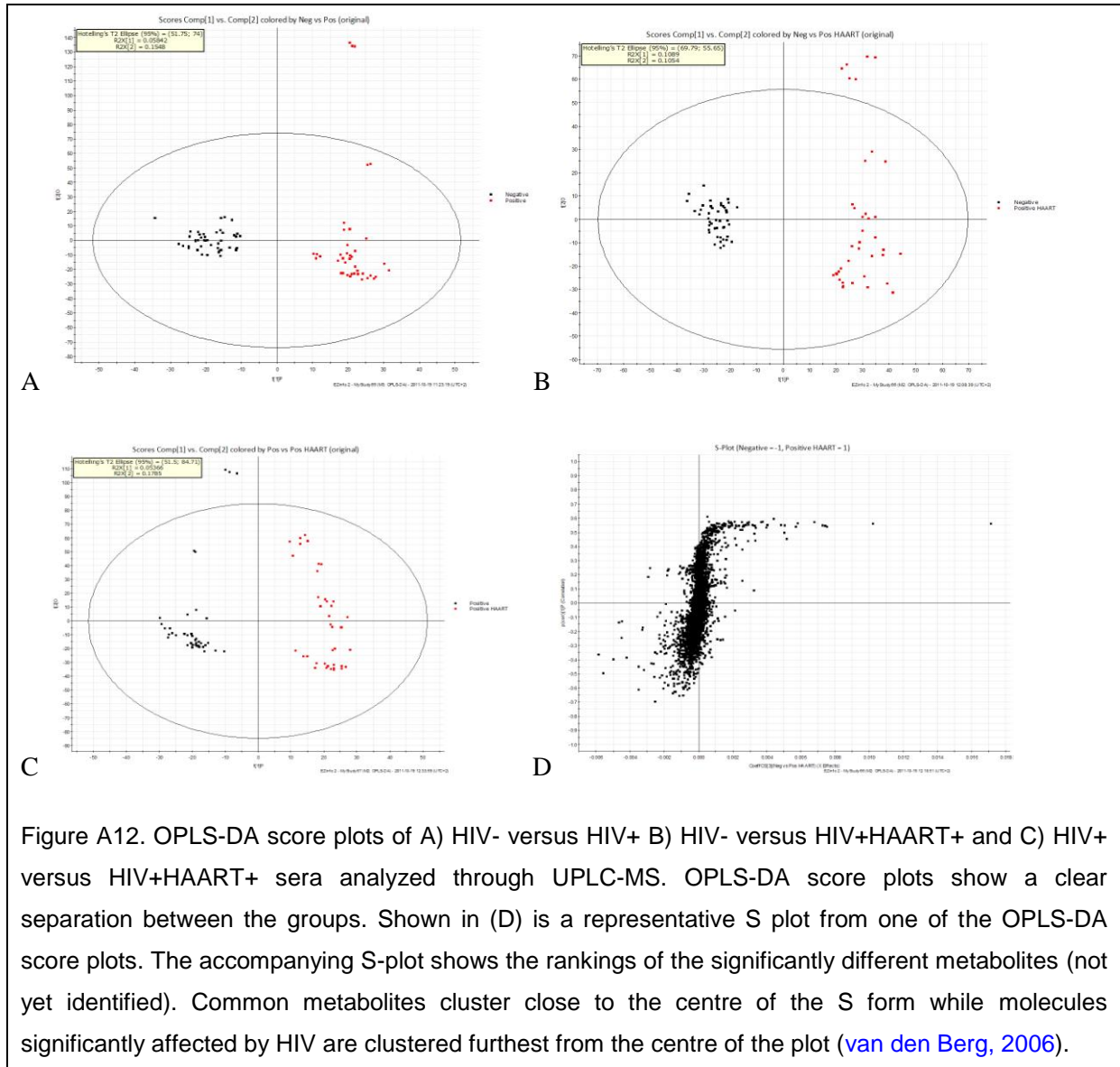


Figure A12. OPLS-DA score plots of A) HIV- versus HIV+ B) HIV- versus HIV+HAART+ and C) HIV+ versus HIV+HAART+ sera analyzed through UPLC-MS. OPLS-DA score plots show a clear separation between the groups. Shown in (D) is a representative S plot from one of the OPLS-DA score plots. The accompanying S-plot shows the rankings of the significantly different metabolites (not yet identified). Common metabolites cluster close to the centre of the S form while molecules significantly affected by HIV are clustered furthest from the centre of the plot (van den Berg, 2006).