Profiling of Host Response Genes, miRNAs and Metabolites in HIV/AIDS patients

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Doctor of Philosophy

By

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Dedication

This thesis is dedicated to all the HIV positive people who took part in this study.

I salute their fighting attitude and hope for their victory against HIV/AIDS.

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Declaration

I hereby declare that the research work described in this thesis entitled "**Profiling of Host Response Genes, miRNAs and Metabolites in HIV/AIDS patients**" has been carried out by me under the supervision of Dr. Shahid Jameel in the Virology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India.

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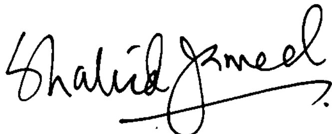
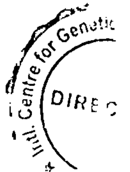
Certificate

This is to certify that the research work described in this thesis entitled **"Profiling of Host Response Genes, miRNAs and Metabolites in HIV/AIDS patients"** has been carried out by Mr. Saif Ullah Munshi in the Virology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India. This work is original and no part of this thesis has been submitted for the award of any other degree or diploma to any other university.





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Last but not the least, many have not been mentioned but none forgotten.

Abbreviations and Symbols

µg	Microgram
µl	Microliter
MIP	Macrophage inflammatory protein
%	Percent
°C	Degree Celsius
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency Syndrome (AIDS)
APCs	Antigen-presenting cells
APOBEC3G	Apolipoprotein B mrna-editing, enzyme-catalytic, polypeptide-like 3G
ART	Antiretroviral therapy
ARV	Anti-retroviral
AUC	Area under curve
AZT	Azidothymidine (AZT)
CASP3	Caspase 3, apoptosis-related cysteine peptidase
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CCL2	Chemokine (C-C motif) ligand 2
CCL8	Chemokine (C-C motif) ligand 8
CD	Cluster of differentiation
cDNA	Complementary DNA
Ct	Threshold cycle
CTLs	Cytotoxic T Lymphocytes
CXCR4	Chemokine (C-X-C motif) receptor 4
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
FCS	Fetal Calf Serum
FIDs	Free Induction Decays
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GMBs	Genes involved in miRNA biosynthesis
HAART	Highly active antiretroviral therapy
HDFs	HIV-dependency factors
HIV	Human immunodeficiency virus
HMGA1	High mobility group AT-hook 1
hsa-miR	homo sapience miRNAs
HSQC	Heteronuclear Single Quantum Coherence
HTLV-III	Human T-lymphotropic virus type III
IDU	Injecting drug users
IL10	Interleukin 10
IL1ß	Interleukin 1, beta
INIs	Integrase inhibitors
IRGs	Immune response genes
LAV	Lymphoadenopathy-associated virus
LDLR	Low density lipoprotein receptor
liquid N_2	Liquid Nitrogen
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MCP-1	Macrophage chemoattractant protein-1
MCP-2	Macrophage chemoattractant protein-2
MHC	Major histocompatibility complex
min	Minute/ Minutes

miRNA	Micro-Ribonucleic acid
miTGs	miRNA target genes
MMA	Methyl Malonic acid
mRNA	Messenger RNA
MS	Mass spectrometry
MSM	Men sex with men
ng	Nanogram
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
Oligo dT	Deoxy-thymine nucleotides
PBMCs	Peripheral blood mononuclear cells
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCs	Principal components
PIC	Preintegration complex
PLS	Partial least squares
PLS-DA	Partial least squares discrimination analysis
PLWHA	People Living with HIV/AIDS
PMN	Polymorphonuclear neutrophils
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
RNU-44	Nucleolar RNA-44
ROC	Receiver operating characteristic
RT	Reverse transcriptase
RT-PCR	Reverse Transcriptase-Polymerase chain reaction
RTC	Reverse transcription complex
RTIs	Reverse transcriptase inhibitors
SERPINC1	Serpin peptidase inhibitor, clade C (antithrombin), member 1
STAT1	Signal transducer and activator of transcription 1, 91kda
STAT3	Signal transducer and activator of transcription 3
s	Second/Seconds
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
VIP	Variable Importance in Projection
WB	Whole blood
WHO	World health organization

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Chapter 1: Introduction and Objectives of the study

Introduction

The human immunodeficiency virus (HIV) selectively infects and impairs the functions of CD4 T-lymphocytes, macrophages (MCs), microglia and dendritic cells (DCs), and causes immunodeficiency in infected persons (Dalgleish et al.1984). The initial infection with HIV is followed by an asymptomatic period of variable duration. The progressive, slow, and irreversible destruction of the immune system by HIV is not clinically apparent for many years. Although the median interval between HIV-1 infection and the development of Acquired Immuno Deficiency Syndrome (AIDS) in adults is 10 to 11 years (Muñoz et al., 1989), some infected persons rapidly progress to AIDS in less than 5 years (Phair et al., 1992), but some patients may remain asymptomatic for more than 6 years without evidence of immunologic decline (Sheppard et al., 1993). The biological basis of this variability is not known, but differences in viral strains, host immune responses (Kaslow et al., 1990) and exposure to microbial (Webster et al., 1989) or environmental cofactors, probably contribute to the phenotype.

For an individual, the likelihood and timing of the development of clinical AIDS following seroconversion is not readily predictable. Despite improvements in the life span of HIV/AIDS patients following the introduction of anti-retroviral therapy (ART), problems of non-adherence, side effects and development of resistance results in therapy failure within a few years. The variability of the outcome, related changes in the natural infection and the situation after ART introduction all contribute to the complexity of HIV disease. Physicians who deal with HIV-infected persons and AIDS patients draw on a number of indicators of immunologic function that are associated with an increased probability of disease progression. Identification of laboratory tests that help to predict progression to AIDS in people infected with HIV is desirable because of the implications for both clinical management and counseling of the patient (Lifson et al., 1992). These laboratory tests are very valuable during the period of clinical latency and subsequently supplement various clinical parameters, and response to ART can be monitored using these prognostic tests (Dar et al., 1999). The commonly used surrogate markers for disease progression and staging are CD4 T cell counts and viral loads, both of which

are costly and labor intensive, and have procedural and inter-laboratory variations. Moreover, it is believed that CD4 T cell counts and viral loads were important predictors before the introduction of ART, but these cannot correctly predict the outcome in patients on ART (Kawado et al., 2006). Therefore, clinical decisions made on the basis of these markers should be interpreted with caution. Since these tests are costly and require trained personnel to perform, and treatment decisions are critically dependent on their availability, it is causing significant burden on health services and economies worldwide, especially in resource-poor regions of Africa and Asia. This situation demands more studies on different biological parameters like host gene expression, protein modulation, the levels of regulatory RNAs and metabolites from patients at different stages of the disease.

Genes and metabolites are the two ends of complex biological systems. During any disease process, infectious or otherwise, there are several changes that occur in gene expression, miRNA expression and levels of metabolites, which are reflected in blood cells and body fluids of the human host. Blood, which includes plasma and cells, is an important and easily accessible source of information on the pathophysiological conditions of an individual. Plasma is filtered through the kidneys to produce urine, which contains waste products including metabolites. A complex network of proteins, metabolites and normal flora maintains homeostasis in the oral cavity. Because the changes in genome and metabolome of the human biological system are best reflected in blood cells, plasma and other body fluids, genomic and metabonomic studies on these compartments for gene expression, miRNAs and metabolites in normal and diseased conditions using high throughput techniques like microarrays, real-time polymerase chain reaction and nuclear magnetic resonance (NMR), are relevant. These methods have been used previously to identify biomarkers of autoimmune diseases, coronary heart disease and cancer (Ishii et al., 2005; van de Vijver et al., 2002; Vasilescu et al., 2009; Brindle et al., 2002; Coen et al., 2005).

Although the molecular details of HIV-1 infection are understood in much detail, comparatively little is known about the changes in host gene

expression, miRNAs and metabolites in blood and various body fluids such as plasma, urine and saliva during different stages of HIV disease, including in patients on ART and those who failed therapy. Due to the variable clinical expression of HIV infection, including the different stages after ART initiation, the identification of markers of clinical disease has become critically important for patient management. Till date there is little or no published report of profiling samples from patients with different stages of HIV disease for identifying biomarkers of disease progression using genomic, microRNAomic or metabonomic platforms. A comprehensive profiling of HIV-mediated effects on patient blood and other body fluids would provide information on the pathological changes and may lead to the identification of possible biomarkers for monitoring different stages of HIV infection and early ART failure.

In the present study we have selected four groups of HIV/AIDS patients – (1) Asymptomatic HIV seropositive persons (without ART, CD4 > 350/μl), (2) AIDS patients (CD4 < 200/μl, prior to initiating ART), (3) Patients on ART (>1 year, CD4 > 300/μl), and (4) patients failing ART (fulfilling WHO treatment failure criteria). This study is focused around the following issues.

1. Are there any differences in gene expression patterns of whole blood cells and the PBMC compartment? Is it possible to identify any biomarker(s) - either gene or protein expression patterns from whole blood cells or PBMCs?

2. Are there any changes in the expression of some T-cell specific miRNAs in PBMCs? Is it possible to use these miRNAs as biomarker of HIV/AIDS progression and therapy?

3. Are there any global metabolic changes in the biofluids (plasma, urine, saliva) of HIV patients? What are the metabolites responsible for these changes?

Objectives:

In view of the focused questions posed above, the objectives of this study are outlined below.

1. To characterize gene expression profiles of whole blood and PBMCs of HIV/AIDS patients in different stages of disease and therapy.

2. To characterize miRNA expression profiles in PBMCs of HIV/AIDS patients in different stages of disease and therapy, and identify the relationships with their probable target genes (miTGs).

3. To characterize metabolite signatures in the plasma, urine and saliva of HIV/AIDS patients in different stages of disease and therapy.

Chapter 2: Review of Literature

Review of Literature

2.1. HIV infection

HIV is the causative agent of the progressive disease AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1983), one of the leading causes of death worldwide (UNAIDS, 2008). A French research group led by Luc Montagnier first identified HIV in 1983 (Barre-Sinoussi et al., 1983). Immediately after that an American research group headed by Robert Gallo also published their findings of a novel virus isolated from AIDS patients (Popovic et al., 1984). Both the French and the American groups similarly noted findings that the virus infected T-lymphocytes and named it as lymphoadenopathy-associated virus (LAV) and human T-lymphotropic virus type III (HTLV-III, due to the resemblance with HTLV-I), respectively. It was later shown that the two groups had in fact isolated the same virus, and the virus was renamed as "HIV" in 1986 (Coffin et al., 1986).

2.2.1. Epidemiology of HIV infection

AIDS is one of the leading causes of death in young adults globally. According to the World Health Organization (WHO), 33.3 million (31.4-35.3 million) people were estimated to be living with HIV in 2009 (UNAIDS, 2010). This was an increase of more than 20% with respect to the numbers in 2000 and was three-fold higher than the prevalence in 1990. The rate of new infections was also very high with approximately 2.7 million in 2008. At the same time, an estimated 2 million people died on AIDS-related illnesses. It is estimated that AIDS has already caused the death of more than 25 million people worldwide. Today, no region of the world can be considered as unaffected (Inciardi and Williams, 2005) (Figure 1) and Sub-Saharan Africa remains as the epicenter of the HIV pandemic. High rates of infection are still seen in this poor region of the world and the rapid spread of HIV-1 is alarming. Even though the intravenous route (due to drug abuse) is one of the most important modes of transmission in Europe and in Central and Southeast Asia, heterosexual transmission still accounts for 85% of all HIV-1 infections worldwide (Hayes and Weiss, 2006). A quarter of all new infections are seen in adults aged younger than 25 years (UNAIDS, 2007). Women now make up

about 42% of those infected worldwide and 70% in Sub-Saharan Africa; this has added implications for mother-to-child transmission of HIV. The prevalence of HIV varies greatly between different regions of the world. Sub-Saharan Africa, accounting for two thirds of all people living with HIV and three fourths of AIDS deaths in 2007, is by far the most severely affected region. In countries such as Botswana, South Africa and Swaziland, almost every 5th adult is infected with HIV. In contrast, most countries in Western and Central Europe have prevalence rates of about 0.1% (UNAIDS, 2008).

2010: A global view of HIV infection

33.3 million people [31.4–35.3 million] living with HIV, 2009

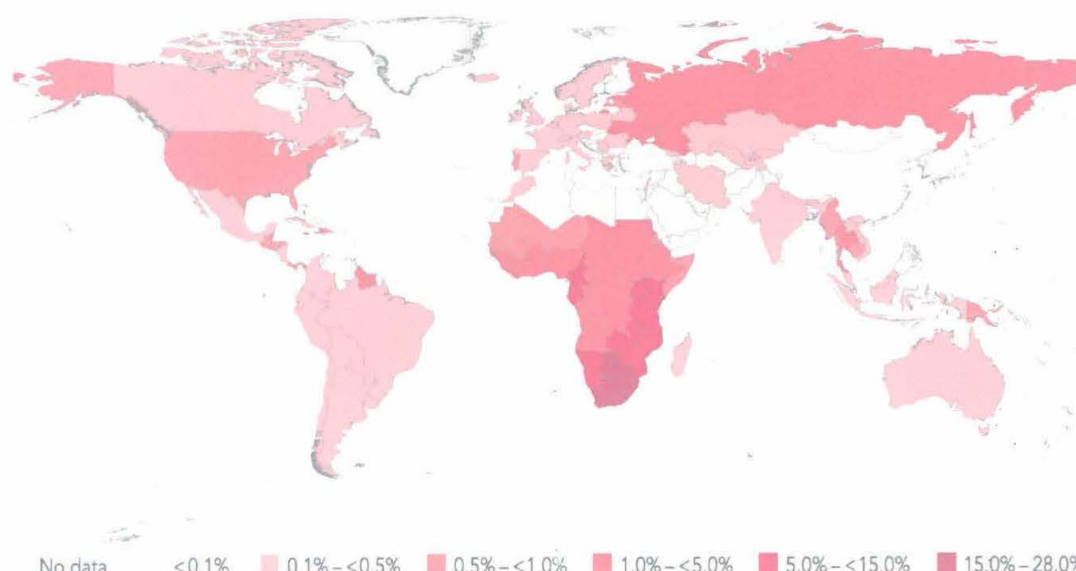

Figure 1: Global prevalence of HIV, 2009. UNAIDS estimates that there were 33.3 million [31.4 million–35.3 million] people living with HIV at the end of 2009 compared with 26.2 million [24.6 million–27.8 million] in 1999—a 27% increase (UNAIDS, 2010)

Another main difference between the regions lies in the primary modes of transmission. While the epidemic in Sub-Saharan Africa is mainly due to heterosexual exposure and often due to mother-to-child transmission, men who have sex with men (MSM) are the most affected group in Latin America. Similarly, most countries in North America and Western and Central Europe show a clear tendency towards increased transmission among the MSM while the rates of new infections in another high-risk group of injecting drug users (IDU) decreased in the last years. However, injecting drug use appears to

remain the primary route of transmission in Eastern Europe, Asia, the Middle East and North Africa. Such disproportionate high risk groups also indicates the fact that in Sub-Saharan Africa women account for about 60% of infections whereas in North America and in Western and Central Europe men outnumber women in both HIV prevalence and incidence by more than 2:1 (UNAIDS, 2009). In addition to the health of the infected people, AIDS has also made a great impact on people as a whole in countries most heavily affected, both economically and demographically. In Swaziland, a high-prevalence country, the average life expectancy decreased by almost half from 65 years in 1991 to 37.4 years in 2005 (Whiteside et al., 2006). Such increases in mortality lead to reduced numbers of skilled population and labor force, which affects economic growth (Bell et al., 2003).

2.2.2. The Indian scenario

India is one of the largest and most populous countries in the world, with over one billion inhabitants. Among them, it is estimated that around 2.4 million people are currently living with HIV (UNAIDS, 2010). At the beginning of 1986, despite over 20,000 reported AIDS cases worldwide (Bureau of Hygiene & Tropical Diseases, 1986), India had no reported cases of HIV or AIDS (Ghosh, 1986). In 2006, UNAIDS predicted that there were 5.6 million people living with HIV/AIDS (PLWHA) in India, which meant that there were more people with HIV in India than in any other country in the world (UNAIDS, 2006). This information was corrected in the next survey in 2007 in which the estimated number of PLWHA was between 2 million and 3.1 million (UNAIDS 'Press release', 2007). In 2008, the figure was estimated to be 2.31 million (UNAIDS, 2008). In 2009, the estimated number of PLWHA was 2.4 million, which equates to a prevalence of 0.3% (UNAIDS, 2010). Though it may seem low, because of India's large population size, it is the third highest in the world in terms of the number of PLWHA. With a population of around a billion, a mere 0.1% increase in HIV prevalence would increase the estimated number of PLWHA by over half a million.

2.3. Brief History of HIV/AIDS

In 1981 a group of gay men in New York and Los Angeles were recognized with symptoms not usually seen in people with healthy immune status (Hymes et al., 1981; CDC 1981; Gottlieb, 2006). As the disease was first observed in gay men, it was initially named gay compromise syndrome or gay-related immune deficiency (GRID) (Brennan and Durack, 1981). The affected population demonstrated a more aggressive form of Kaposi's sarcoma or a rare lung infection called *Pneumocystis carinii* pneumonia. Soon after these reports, other cases with similar symptoms were identified leading to the conclusion that some unknown agent was responsible for this new disease associated with immune system disorder. In 1983, a group led by Luc Montagnier and Françoise Barré-Sinoussi at the Institute Pasteur in Paris, France first isolated HIV (Barre-Sinoussi et al., 1983), a discovery for which they were awarded with the Nobel Price for Physiology or Medicine 25 years later in 2008. The Montagnier group named the virus Lymphadenopathy-Associated virus (LAV) and suggested that LAV might be the cause of this immune deficiency. About a year later, in 1984, the United States Health and Human Services Secretary Margaret Heckler announced that Robert C. Gallo of the National Cancer Institute had isolated the virus causing AIDS and called it HTLV-III. A very controversial debate including allegation about the first discovery of HIV started, but it soon became obvious that both groups had isolated the same virus. The difficulty with the different names was solved in 1986, when the International Committee on the Taxonomy of Viruses (ICTV) decided to drop both names and replaced them by a new term, Human Immunodeficiency Virus (HIV) (Coffin et al., 1986). Although Dr. Gallo's group was not the first to isolate the virus, they were recognized to be the first to exhibit that HIV causes AIDS (Popovic et al., 1984). Within 20 years of its first isolation, in 2003, the WHO declared a pandemic of HIV/AIDS.

2.4. Taxonomy of HIV

HIV belongs to the genus *Lentivirus* of the *Retroviridae* family. Retroviruses are characterized by their single strand positive RNA diploid genome and by the enzyme reverse transcriptase. This enzyme is capable of RNA-directed

DNA synthesis, which results in a viral genome DNA intermediate that is integrated in the host genome (Bishop, 1978; Coffin, 1997). Lentiviruses vary from other retroviruses mainly due to their long incubation period before the clinical manifestations of infection. Moreover, lentiviruses share a common morphogenesis and morphology, a tropism for MCs, extensive genetic and antigenic variability, and accessory regulatory genes not found in the other groups of retroviruses (Narayan and Clements, 1989; Coffin, 1997).

There are two types of HIV, namely HIV-1 (Barre-Sinoussi et al., 1983; Gallo et al., 1983), and HIV-2 (Clavel et al., 1986). Both types of viruses cause AIDS, but HIV-2 represents a considerable minority of all HIV infections, which are distinguished by slow disease progression and poor transmission, a lower plasma viral load and a low rate of CD4+ T cell decline (Marlink et al., 1994; Whittle et al., 1994; Jaffar et al., 1997). Phylogenetic studies have revealed three groups of HIV-1 isolates: group M (Major), group O (Outlier) and group N (non-M, non-O). The M group includes more than 90% of viral isolates associated with HIV/AIDS cases; these are further subdivided into eleven clades (from A to K) as well as circulating recombinant forms (CRFs) (Geretti, 2006). Among these viruses, HIV-1 subtype C is associated with about 50% of cases and rapid progression to the disease state. The subtype C viruses are commonly present in Sub-Saharan Africa and the Indian subcontinent. A new HIV sequence was reported in 2009 and proposed as Group P (Plantier et al., 2009). The HIV-2 strains are rare and have only been isolated from West Africa (Horsburgh and Holmberg, 1988) and India (Kannangai et al., 1999)

2.5. HIV-1 structures

2.5.1. Morphology

The mature HIV-1 virions are spherical in shape with a diameter of approximately 100-120 nm. The virion consists of an envelope and a core structure (Figure 2). The viral envelope is derived from the host cell membrane and consists of host lipids and proteins besides the viral surface (gp120 or SU) and the viral envelope is derived from the host cell membrane

and consists of host lipids and proteins besides the viral surface (gp120 or SU) and transmembrane (gp41 or TM) glycoproteins. The host components

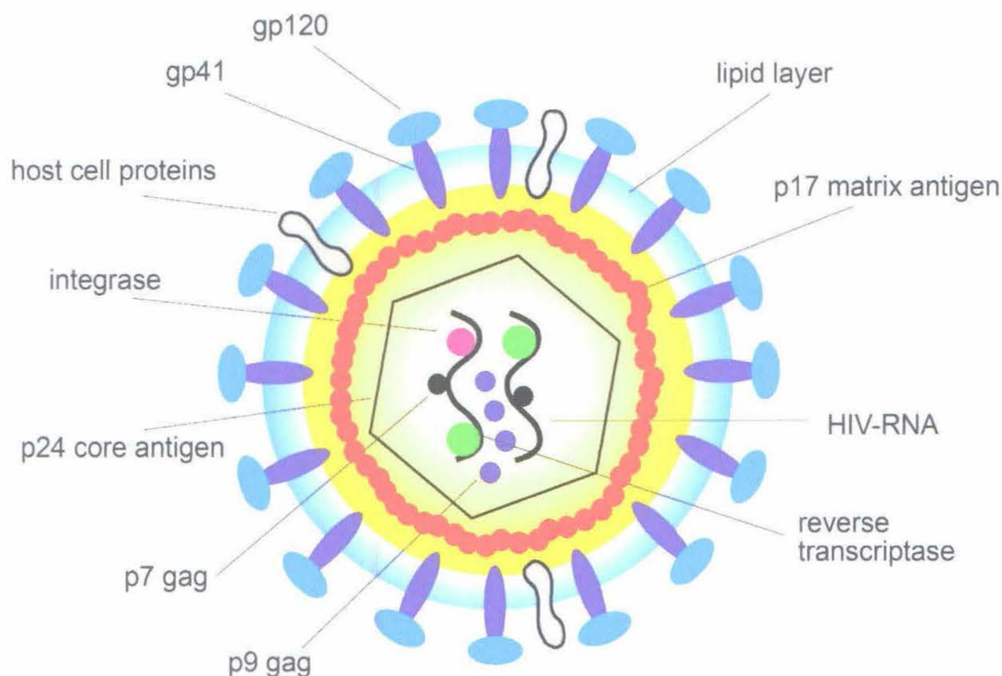

Figure 2: Structure of an HIV virion particle (http://hivbook.com/2011/10/28/3-pathogenesis-of-hiv-1-infection/)

vary depending upon the cell type in which the virus has replicated and from which it was released. The gp120 protein has high affinity for the CD4 molecule, which is present on the surface of the T-helper lymphocytes, monocytes, macrophages and dendritic cells, and acts as the HIV entry receptor.

The viral core (internal structure) is composed of a diploid RNA genome, structural proteins and other proteins, and is separated from the external structure by protein-coated material, which is composed of *gag* gene product. The *pol* gene encodes internal proteins and the associated enzymes, which participate in integration and replication. These enzymes are reverse transcriptase (RT), protease (PR) and integrase (IN).

2.5.2. Genes and Structure

The HIV-1 virion contains two copies of single-stranded RNA (ssRNA) as well as the viral PR, RT and IN enzymes packaged in its core. The length of each ssRNA is approximately 9.7kb, which is strongly bound to the nucleocapsid p7

protein. The core is protected by a cone-shaped capsid consisting of around 250 hexamers and precisely 12 pentamers of the viral p24 capsid (CA) protein (Pornillos et al., 2009). The viral cone itself is enclosed by a sphere-shaped matrix comprised of p17 (matrix, MA) proteins, which is enclosed by the viral envelope. A total of 72 spikes, each composed of a trimer of gp120 and gp41 viral proteins are part of the viral envelope (Gelderblom, 1991). A schematic illustration of the viral genome is shown in Figure 3.

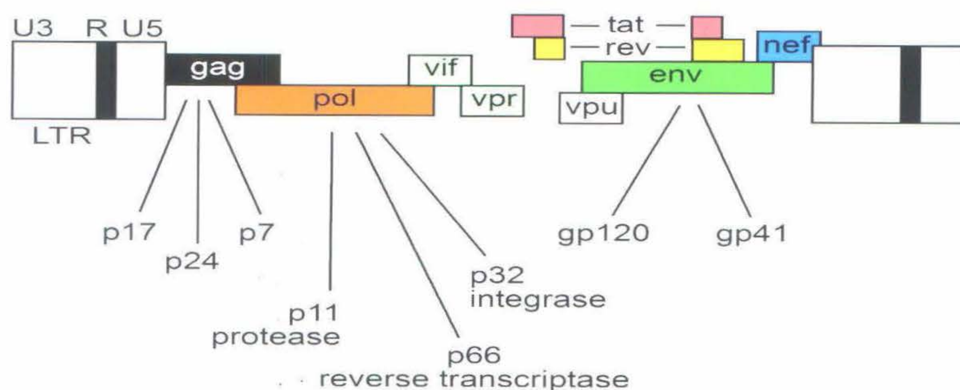

Figure 3: The HIV genome (http://hivbook.com/2011/10/28/3-pathogenesis-of-hiv-1-infection/)

The ssRNA encodes 15 viral proteins from spliced RNAs, partly in overlapping reading frames (Frankel and Young, 1998). These proteins are synthesized from nine genes - three that encode polyprotein precursors (*gag, pol, env*) and six that code for regulatory (*Rev, Tat*) and accessory *(Nef, Vif, Vpr, Vpu)* proteins with auxiliary functions. The Gag (group-specific antigen) precursor is cleaved by the viral protease into the mature Gag proteins that encapsidate the viral genome - matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), and p6 (Freed, 2001). The viral protease cleaves the Gag-Pol precursor into the viral enzymes - protease (PR), reverse transcriptase (RT) and integrase (IN). The RT enzyme reverse transcribes the viral ssRNA genome back into a dsDNA copy, which is integrated into the host genome by the IN enzyme. The *env* gene encodes the glycoprotein precursor gp160, which is cleaved by a cellular protease into the two subunits of the viral spike, the mature gp120 and the gp41. The regulatory and accessory proteins serve other important

functions such as transactivation (*Tat*), RNA export from the nucleus (*Rev*), and modulation of the host response (*Nef, Vif, Vpr and Vpu*). The accessory proteins are not required for viral replication *in vitro* but are essential for efficient virus production and disease in the infected host.

2.6. HIV-1 Replication Cycle

The replication of HIV-1 is a complex process, with an anticipated turnaround time of 1.5 days. It depends on several factors, including target cell type and activation status, and is divided into two overlapping phases: early and late. The early phase includes the steps until the viral genome is integrated into the host genome while the late phase is characterized by the production of new virions and includes gene expression, assembly and release. In the following sections, the individual steps of the viral replication cycle are described.

2.6.1. Viral Entry

For infection and subsequent replication, HIV-1 requires a host cell, which expresses the CD4 receptor. As a result, the virus largely targets CD4+ T lymphocytes, monocytes/macrophages, microglia, and dendritic cells. It attaches through its gp120 envelope protein to the CD4 receptor on the host cell (Dalgleish et al., 1984). This interaction causes some conformational changes in gp120, which facilitate the exposure of a second binding site for a chemokine receptor (known as coreceptor). In vivo, chemokine CC receptor 5 (CCR5) and chemokine CXC receptor 4 (CXCR4) act as coreceptors for macrophage and T cells, respectively (Berger et al., 1999; Wyatt and Sodroski, 1998). Binding to such a coreceptor activates gp41, which reorients parallel to viral and cellular membranes, allowing its N-terminal fusion peptide to insert into the cellular membrane. Both the membranes are then brought into near proximity, which facilitates their fusion, following which the viral core is released into the cytoplasm of the target cell.

2.6.2. Reverse Transcription, Nuclear Import and Integration

Following release of the viral core into the cell, uncoating occurs and a large reverse transcription complex (RTC) is formed. The RTC consists of genomic RNA coated with nucleocapsid protein molecules and other components such as the matrix, the capsid and Vpr molecules together with the viral enzymes RT and IN (Mougel et al., 2009). The viral RNA is transcribed into double stranded DNA (dsDNA) by the RT enzyme. Since the reverse transcriptase has no proofreading activity and can "jump" from one template to another, this process is generally error-prone and the resulting dsDNAs are not exact genetic copies of the original RNAs. Therefore, there is a high occurrence of genetic recombination, together with a high mutation rate resulting in a highly varied population of newly synthesized virions, termed as quasispecies (Freed, 2001; Jung et al., 2002).

The dsDNA forms the preintegration complex (PIC) with other proteins of cellular and viral origin and is transported to the nucleus (Freed, 2001). The viral IN, which remains in the PIC, catalyzes the insertion of the dsDNA into the host chromosome (Freed, 2001). The integrated dsDNA, also known as the provirus is transcribed using the host cell machinery analogous to other cellular genes. Integration is an important irreversible part of HIV life cycle (Simon et al., 2006).

2.6.3. Gene Expression

In the beginning, the integrated viral genes are transcribed by the cellular machinery at a comparatively lower basal rate. However, this soon changes because the Tat (trans-activator of transcription) protein, produced early in replication, begins a positive feedback loop that augments viral RNA transcription, gene expression and its own production by orders of magnitude. This switching leads to production of very high quantities of HIV-1-specific RNA and protein synthesis (Cullen, 1991; Kim and Sharp, 2001). Whilst Tat increases its own expression, the viral protein Rev (regulator of virion) down regulates its own synthesis and thus early class mRNAs. The early class viral mRNAs are multiply spliced; these spliced mRNAs encode the viral regulatory proteins Tat, Nef, and Rev. On the contrary, in the late replication phase,

unspliced and partially spliced transcripts are produced encoding the structural proteins. The difficulty faced by HIV-1 is that nearly all cellular mRNAs are fully spliced before they transport out from the nucleus. The Rev

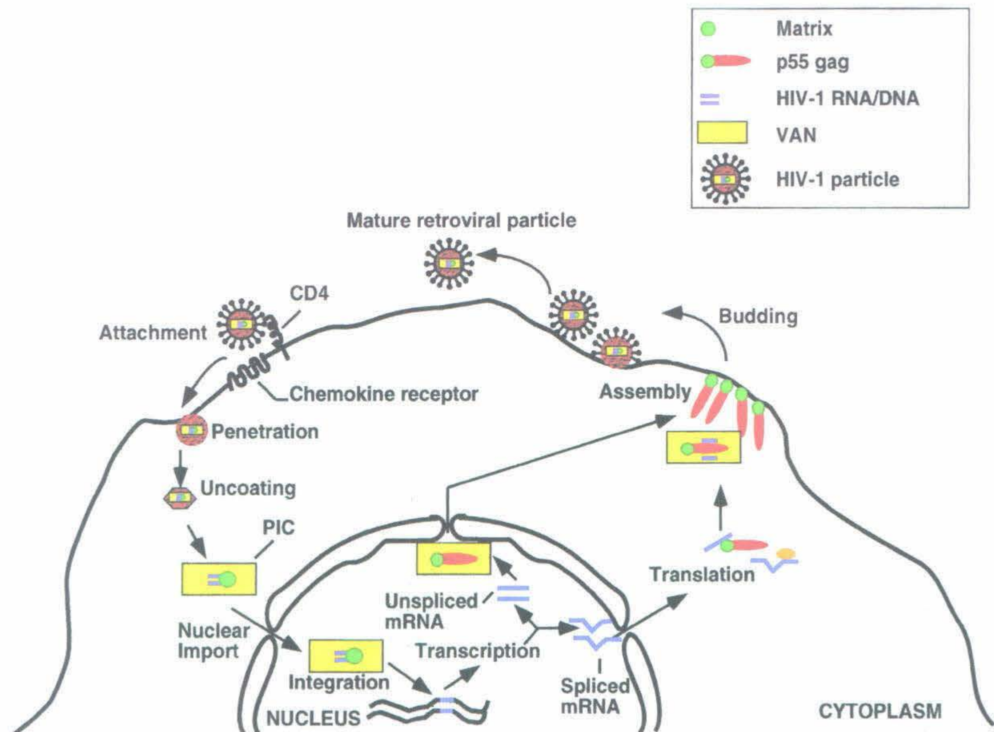

Figure 4: Life cycle of HIV (Gupta K et al., 2000)

protein resolves this problem together with the Rev responsive element (RRE), a highly structured RNA element that is located in the *env* gene and which is present in all unspliced and partially spliced HIV RNAs. Binding of around eight *Rev* protein molecules with one RRE gives a configuration that enables it to interact with the cellular nuclear export machinery and thus transport the unspliced or partially spliced viral RNAs from the nucleus to the cytoplasm. However this process requires a critical threshold level of Rev so that it extends the entry into the late phase in cells as long as they are incapable of supporting efficient HIV-1 gene expression (Cullen, 1991; Freed, 2001).

2.6.4. Budding and Maturation

The envelope polyprotein gp160 is transported to the cellular membrane via the endoplasmic reticulum and the Golgi complex. It then undergoes trimerization and a host protease cleaves it into gp120 and gp41 subunits

(Freed, 2001; Moulard and Decroly, 2000). Both subunits are then transported to the cell membrane where gp41 anchors the *env* complex into the membrane and links non-covalently with gp120. The precursor proteins Gag and Gag-Pol also associate at the cell membrane where the generation of new virion starts. The immature virus particles bud from the host cell and acquire a part of the plasma membrane as their envelope. The maturation of virus starts during or shortly after budding from the cell membrane. The viral PR being part of the Gag-Pol precursor protein gets activated when the respective domains of two Gag-Pol precursors dimerize. The PR then cleaves Gag and Gag-Pol polyproteins into their subunits. The structural proteins of the Gag polyprotein form the matrix, the capsid, and the nucleocapsid, which rearrange to form the mature particle. Finally, only fully mature viruses are able to infect other target cells.

2.7. Pathophysiology of HIV Infection

2.7.1. Acute Infection

Transmission of HIV occurs through body fluids, mostly via sexual contact. In addition, it can also be transmitted through blood transfusions from HIV carriers, contaminated needles shared among IDUs, and from mothers to their children during pregnancy, childbirth and breast-feeding.

After initial infection, Langerhans cells (LCs) or dendritic cells (DCs) pass the virus to local lymph nodes (LNs) within the first week of infection (Figure 5). From there it can spread to other sites, particularly to gut-associated lymphoid tissue (GALT) (McMichael, 2006), which is known to be the major replication site and reservoir for HIV (Arthos et al., 2008). Lymphoid tissue supports its persistence even during long-term suppression of plasma viremia while on ART (Chun et al., 2008). After about 3 weeks of exposure, the virus appears in blood with high viral loads of often more than 10 million copies/ml (McMichael, 2006). This is generally known as the acute phase of HIV infection and is associated with rapid depletion of about half of the CD4+ T cells (Mattapallil et al., 2005) and results in flu-like symptoms (Figure 6). This massive viral replication and widespread destruction of CD4+ memory T cells

leads to an irreversible damage to the immune system setting the stage for its eventual failure to respond against pathogens (Picker, 2006). At the end of

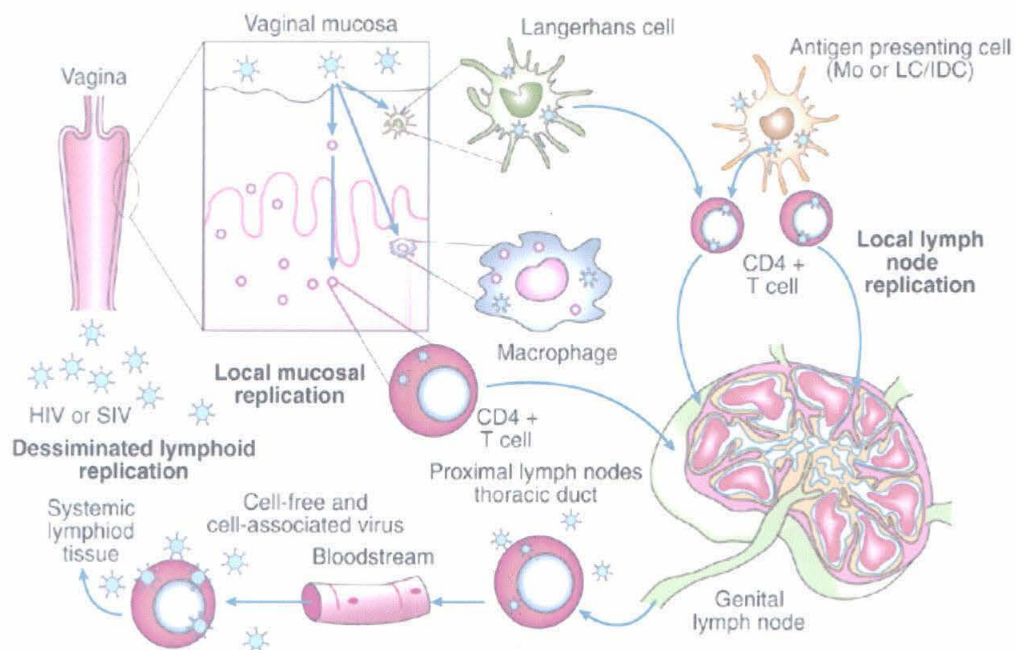

Figure 5: Target cells and dissemination pathways in vaginal HIV transmission (Miller, 2007).

the acute phase, plasma viral load drops 10- to 100-fold to a relatively stable set point between 2 and 6 months after infection while the number of CD4+ T cells again increase. This rapid and spontaneous decline in viral load is supposed to be due to limited target cell population (Phillips, 1996) after the initial exponential expansion and because of the development of specific immune responses (Borrow et al., 1994; Safrit et al., 1994). In general, the viral set point is a good predictor of how fast an individual will progress to develop AIDS - the higher it is the faster a person will progress to disease (Mellors et al., 1996; Fauci, 1996; Lyles, 2000). After infection with HIV, some people may remain asymptomatic, while others experience a flu-like illness with symptoms that include fever, general malaise and sore throat within 2 - 4 weeks of infection. These symptoms generally last for about 1-2 weeks.

In females, after exposure of the genital mucosa, HIV infects intra-epithelial CD4+ T cells and intra-epithelial LCs of the intact epithelium. In addition, trauma or ulceration of the genital epithelium allows HIV to interact directly

with CD4+ T cells, MCs and immature DC in the lamina propria. Inflammatory cytokines and chemokines induce HIV-infected LC, lamina propria DC and MCs to travel through afferent lymphatic vessels to the genital LN (Miller, 2007).

In males, HIV-1 is most likely to enter the penis through the inner aspect of the foreskin and frenulum, which have little protective keratin and many superficial LC expressing both CD4 and CCR5 receptors. HIV binds to these receptors stimulating the LC to migrate to the regional LNs, transporting the HIV to resident T cells.

In LNs, normally the antigen-presenting cells (APCs) interact with CD4+ T cells to present foreign antigens, but in HIV infection, the APCs pass the virus to highly susceptible CD4+ T cells. Once in the LNs, the infection quickly spreads out and disseminates through the lymphatic system to the blood and then to all lymphoid tissues. Based on studies in rhesus monkeys infected with SIV, this whole process can take around 48-72 hours following exposure. After the initial infection, with a decrease in CD4+ T cells and a rise in CD8+ T cells, the virus titers increase sharply, resulting in viremia, which rapidly returns to normal. Mucosal CD4+ T cells are lost during the early acute infection stage, never to recover fully, while others are lost later during the chronic phase of infection. The B cells produce HIV specific antibodies, which may take up to 6 months, and this period is called the "window period" (Benjamini et al., 2000).

2.7.2. Chronic Latent Stage

The characteristic of second stage of HIV infection is immune response from B and T lymphocytes. The cell-mediated immune response increases to counteract the rapidly replicating virus. At this phase, the virus has a half-life of 5-6 hours. The HIV-infected cells are destroyed either by the immune system reacting against them or by the virus itself. Some infected CD4 cells can survive longer and may go into a resting memory stage, wherein these cells will not express any viral protein, and only specific antigen from pathogens can reactivate the HIV provirus genome inside. These are known as memory T cells and can live for many years (Benjamini et al., 2000). The

viral load is then reduced by the strong immune response, resulting in clinical latency. However, replication of the virus continues in the LNs.

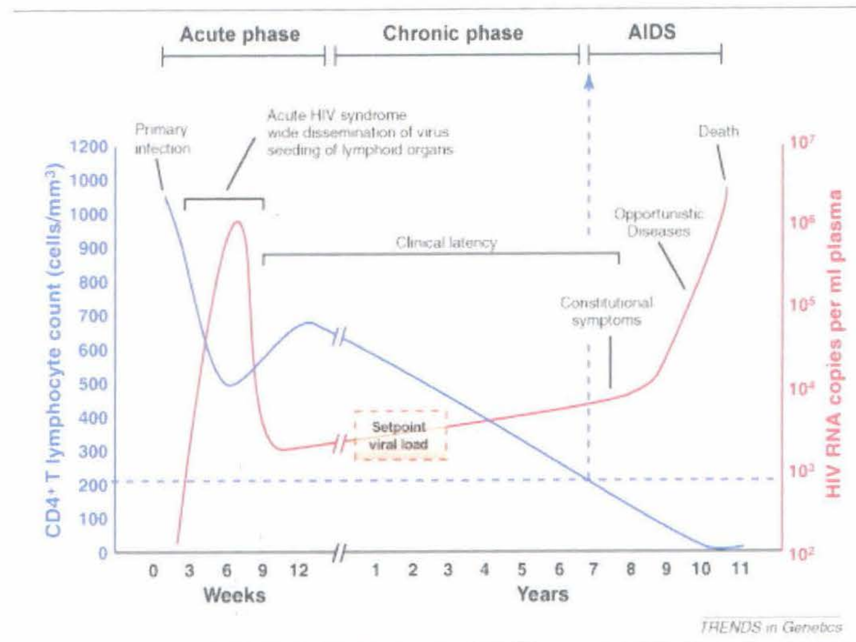

Figure 6: A general graph of the association between HIV copies (viral load) and CD4 counts in different phases of untreated HIV infection and AIDS; any particular individual's disease course may vary significantly. (An and Winkler, 2010)

2.7.3. AIDS

After a prolonged latent period of HIV infection, which may last 1-15 years, the immune system begins to fail to control the infection. The numbers of CD4+ T cells are reduced and reach below the levels of 200 cells/µl. The numbers of CD4+ T cells are used as markers of the stage of the infection. Loss of control over the virus results in immunosuppression, which makes the host susceptible to infections by opportunistic bacteria, fungi, protozoa and viruses. At this stage, the virus replicates uncontrollably. This stage of the combination of HIV infection and AIDS is usually abbreviated as HIV/AIDS, which was classified into different stages by WHO (Table 1).

Table 1: Revised WHO clinical staging of HIV/AIDS for adults and adolescents (WHO, 2005)

Primary HIV infection

Asymptomatic
Acute retroviral syndrome

Clinical stage 1

Asymptomatic
Persistent generalized lymphadenopathy (PGL)

Clinical stage 2

Moderate unexplained weight loss (<10% of presumed or measured body weight)
Recurrent respiratory tract infections (RTIs, sinusitis, bronchitis, otitis media, pharyngitis)
Herpes zoster
Angular cheilitis
Recurrent oral ulcerations
Papular pruritic eruptions
Seborrhoeic dermatitis
Fungal nail infections of fingers

Clinical stage 3

Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations

Severe weight loss (>10% of presumed or measured body weight)
Unexplained chronic diarrhoea for longer than one month
Unexplained persistent fever (intermittent or constant for longer than one month)
Oral candidiasis
Oral hairy leukoplakia
Pulmonary tuberculosis (TB) diagnosed in last two years
Severe presumed bacterial infections (e.g. pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia)
Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis

Conditions where confirmatory diagnostic testing is necessary

Unexplained anaemia (<8 g/dl), and or neutropenia (<500/mm^3) and or thrombocytopenia (<50 000/ mm^3) for more than one month

Clinical stage 4

Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations

HIV wasting syndrome
Pneumocystis pneumonia
Recurrent severe or radiological bacterial pneumonia
Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration)
Oesophageal candidiasis
Extrapulmonary TB
Kaposi's sarcoma
Central nervous system (CNS) toxoplasmosis
HIV encephalopathy

Conditions where confirmatory diagnostic testing is necessary:

Extrapulmonary cryptococcosis including meningitis
Disseminated non-tuberculous mycobacteria infection
Progressive multifocal leukoencephalopathy (PML)
Candida of trachea, bronchi or lungs
Cryptosporidiosis
Isosporiasis
Visceral herpes simplex infection
Cytomegalovirus (CMV) infection (retinitis or of an organ other than liver, spleen or lymph nodes)
Any disseminated mycosis (e.g. histoplasmosis, coccidiomycosis, penicilliosis)
Recurrent non-typhoidal salmonella septicaemia
Lymphoma (cerebral or B cell non-Hodgkin)
Invasive cervical carcinoma
Visceral leishmaniasis

2.8. Immunobiology of HIV infection

2.8.1. Innate immunity

Innate immunity is the first line of defense against invading pathogens, which includes external barriers such as skin and mucus membranes with various cells, especially leucocytes and proteins. In HIV infection, different components of the innate immune system affect the outcome of the infection. For example, Natural killer (NK) cells, DCs and MCs produce chemokines such as Regulated on Activation Normal T cell Expressed and Secreted (RANTES) and Macrophage Inflammatory Protein (MIP) type 1 α and β, which are the natural ligands for CCR5 that is used as a co-receptor by HIV. These molecules thus prevent CCR5-tropic HIV from infecting cells and interestingly, these chemokines are upregulated in exposed but uninfected individuals (Iqbal et al., 2005; Koning et al., 2004). In addition, intracellular APOBEC3G and tripartite motif protein 5a (TRIM5a) molecules are the two important anti-HIV proteins produced by the innate immune system. The APOBEC3G protein impedes the pro-viral DNA by causing mutations through deamination (Lecossier et al., 2003; Marin et al., 2003; Zhang et al., 2003) and TRIM5a prevents viral uncoating by binding with viral capsid (Yap et al., 2004; Stremlau et al., 2004). In addition, antigen presentation and signaling by the innate immune system is essential for the development of functional HIV-specific adaptive immunity (Hoebe et al., 2004).

2.8.2. Adaptive immunity

Adaptive immunity acts in a more specific manner than the innate immune system and is dependent upon memory to a specific antigen, to which it was exposed earlier. It is however slower in action than the innate system and requires days or weeks to develop. The system is divided into a cellular and a humoral arm, coordinated by the CD4+ T-helper type 1 or 2 (Th1 or Th2) cells, respectively. As CD4+ T cells are the major target cells in HIV infection, it hampers the functions of both arms of the adaptive immune system. Moreover, HIV down-regulates MHC molecules from the host cell membrane, which helps it to escape from the immune system (Collins et al., 1998) and constantly changes the amino acid sequences of the immunogenic epitopes,

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which together result in reduced recognition of virus-infected cells by the immune cells (Goodenow et al., 1989; Whitcomband and Hughes, 1992).

2.8.2.1. Humoral immune responses

The B cells are the main elements of the humoral immune system and express membrane-bound antibodies. These antibodies bind and engulf the antigens, which are then processed, associated with MHC class II molecules and presented on the B cell membrane for recognition by the T cell receptor (TCR) on Th cells. In the presence of proper co-stimulatory signals on the B cell, the Th cells secrete cytokines that allow the B cells to differentiate into antibody-secreting plasma cells and to undergo isotype switch from IgM or IgD to IgG, IgA and IgE. Vaccine-induced neutralizing antibodies are correlated with for most microbial vaccines developed to date. In order to prevent HIV infection with a vaccine, it should be able to induce broadly neutralizing antibodies. Despite the difficulties in inducing such neutralizing antibodies by active vaccination, there are some broadly neutralizing antibodies that have been isolated from HIV-infected individuals. These monoclonal neutralizing antibodies can prevent SHIV infection in macaques when administered post challenge (Ferrantelli et al., 2004). More recently passive immunization with neutralizing antibodies has been shown to delay the rebound after treatment interruption of ART in HIV infected individuals (Trkola et al., 2005). These antibodies do, however, most often have unusual characteristics, due to which it is difficult to induce similar antibodies by immunization (Burton et al., 2005). Several viral factors have complicated the generation of anti-HIV neutralizing antibodies. HIV has vast genetic diversity, which due to pressure from the immune system results in constantly changing gp120/gp41 envelope proteins (Goodenow et al. 1989). Moreover, these proteins are heavily glycosylated, which sterically hinders antibodies from binding the proteins (Li et al. 2008; Wyatt et al. 1998). The existence of non-functional gp120/gp41, which misleads antibody responses, has also been suggested to contribute as an impediment for the production of neutralizing antibodies (Crooks et al., 2007; Moore et al., 2006). In addition to the steric hindrance of the virus-host cell contact arbitrated by neutralizing antibodies, these can also trigger the complement system and support cell

killing by antibody-dependent cellular cytotoxicity (ADCC). This occurs as immune cells interact with the Fc-part of a bound antibody. The importance of Fc-receptor mediated activity was demonstrated in an experiment by removing the Fc part of a broadly HIV neutralizing antibody, which resulted in reduced protection from challenge in the SHIV/macaque model (Hessell et al., 2007).

2.8.2.2. Cellular immune responses

The CD8+ Cytotoxic T Lymphocytes (CTLs) are the major cells of the adaptive cellular immune system, which identifies antigens present on MHC class I molecules at the surface of infected cells. After an encounter with the MHC/antigen complex, the CD8+ cells develop into antigen-specific memory CTLs, effector CTLs or both (McMichael and Rowland-Jones, 2001). The effector CTLs can produce strong immune responses, together with HIV-specific antibodies that can contribute to controlling viral replication during different phases of the infection. In the case of HIV, it is assumed that the CTLs are incapable of preventing infection. However, several observations indicate the importance of cellular immune responses in controlling HIV infection. Rhesus monkeys were shown to be unable to control primary SIV infection when monoclonal antibody against CD8+ cells was administered prior to challenge (Schmitz et al., 1999). Some individuals who are repeatedly exposed but are not infected with HIV also demonstrate HIV-specific CTLs but lack antibody responses (Rowland-Jones, et al., 1998). The elite controllers who are infected with HIV but are capable of suppressing the virus to undetectable levels also show an over-representation of individuals expressing the HLA-B57 and B27 alleles, which are both associated with a better outcome of the HIV infection (Altfeld et al., 2003; Jansen et al., 2005). All these examples imply the importance of CTLs in controlling HIV infection.

2.9. Treatment of AIDS

2.9.1. Highly Active Antiretroviral Therapy (HAART)

Antiretroviral (ARV) drugs against HIV were introduced in the early 1990s, and these were found to reduce viral replication to undetectable levels. At the beginning of the ART era, monotherapy with azidothymidine (AZT) was tried, but it failed due to the high mutation rate of HIV resulting in the development of resistance and virus replication even in the presence of the drug. Later, HAART was introduced, which consists of combinations of drugs that act by different mechanisms (Baum et al., 1999; Williams, 1997). The HAART therapy has been used successfully to suppress viral replication, to delay the development of AIDS, and has considerably increased the quality of life for HIV-infected individuals. There are currently more than 30 approved ARVs that can be divided into four separate classes based on their modes of action (Table 2). The major class of ARVs includes reverse transcriptase inhibitors (RTIs) that are either nucleoside/nucleotide (NRTI) or non-nucleoside (NNRTI) in structure. The NRTIs inhibit HIV reverse transcription as they are incorporated into newly synthesized viral DNA and prevent further elongation. The NNRTIs directly bind the RT enzyme and inhibit its activity (Hang et al., 2007). Integrase inhibitors (INIs) prevent integration of the provirus into host chromatin by interfering with viral integrase binding or the strand transfer events during integration (Sato et al., 2006). The fourth class of ARVs currently available is the protease inhibitors (PIs) that prevent cleavage of viral polyproteins in immature budding viruses (Flexner, 1998), thus preventing the next cycle of infection.

In addition to these conventional targets for HIV inhibition, novel ARV drugs affecting different stages of the viral life cycle have been licensed over the last years. These includes drugs targeting integration by IN (Evering and Markowitz, 2008), viral/host cell fusion (Bianchi et al., 2005; Marr and Walmsley, 2008), and co-receptor binding (Kuhmann and Hartley, 2008). However, so far no ARV drugs are able to cure the infection and the access to HAART is limited in low-income countries.

Table 2: FDA approved ARVs (Piliero, 2004)

Medscape® www.medscape.com

Table. Antiretroviral Agents Approved by the FDA

Drug	Dosing frequency	Daily pill burden	Common side effects
Entry inhibitors			
Enfuvirtide (T20)	Twice daily	2 SC injections	Injection site reactions
Protease inhibitors			
Saquinavir mesylate	Twice daily (with ritonavir)	12	GI
Indinavir	3 times daily	6	Nephrolithiasis, GI
	Twice daily (with ritonavir)	6 - 8	
Ritonavir	Twice daily	12	GI, dyslipidemia
Nelfinavir	3 times daily	9	GI
	Twice daily	4 - 10	
Saquinavir soft-gel	3 times daily	18	GI
	Twice daily (with ritonavir)	12	
Amprenavir	Twice daily	16	Rash
	Twice daily (with ritonavir)	10	
	Once daily (with ritonavir)	10	
Lopinavir/ritonavir	Twice daily	6	GI
Atazanavir	Once daily	2	Benign hyperbilirubinemia
	Once daily (with ritonavir)	3	
Fosamprenavir	Twice daily	4	Rash
	Twice daily (with ritonavir)	4	
	Once daily (with ritonavir)	4	
			de effects
Nucleoside reverse transcriptase inhibitors			
Zidovudine	Twice daily	2	GI
Didanosine	Twice daily	2 - 4	Peripheral neuropathy
	Once daily	1	
Zalcitabine	3 times daily	3	Peripheral neuropathy
Stavudine	Twice daily	2	Peripheral neuropathy
Lamivudine	Twice daily	2	GI
	Once daily	1	
Lamivudine/zidovudine	Twice daily	2	GI
Abacavir	Twice daily	2	GI, allergic reaction
Abacavir/lamivudine	Once daily	1	GI, allergic reaction
Abacavir/lamivudine/zidovudine	Twice daily	2	GI
Emtricitabine	Once daily	1	Headache, GI
Nucleotide reverse transcriptase inhibitors			
Tenofovir	Once daily	1	GI
Tenofovir/emtricitabine	Once daily	1	Headache, GI
Nonnucleoside reverse transcriptase inhibitors			
Nevirapine	Twice daily	2	Rash, elevated liver enzyme levels
Delavirdine	3 times daily	6	Rash
Efavirenz	Once daily	1 or 3	Rash, nervous system symptoms

Source: AIDS Read © 2004 Cliggott Publishing, Division of SCP Communications

As the RT enzyme lacks proof-reading activity, it often induces mutations during the viral genome replication. It is estimated that roughly 3 nucleotide substitutions are introduced per 10^5 incorporated nucleotides during each round of replication (Mansky and Temin, 1995). Moreover, RT promotes further genetic diversity by recombination of two strands of viral RNA (Jetzt et al., 2000). Together with the high mutation and recombination rate with a rapid viral turnover and an extremely efficient production of new progeny virus (Ho et al., 1995; Wei et al., 1995), HIV easily escapes recognition by both ARVs and the adaptive immune response. The induction of drug resistance mutations is, however, usually prevented when treated with the HAART regimen.

2.9.2. ARV resistance and side effects

Sooner or later HIV develops resistance to all of these drugs, especially when administered alone or in combination with drugs with impaired efficacy. Drug resistance generally begins from genome variation due to misincorporation of nucleotides during reverse transcription. While mutations are always occurring in a viral population, resistance mutations are only evident when the respective viruses are still viable and when they have a selective advantage in comparison to the other viruses in the quasispecies population. In case of ARV drugs this benefit is obvious since non-resistant viruses are typically not able to replicate anymore. The time for a virus to become resistant and adapt to ARV drugs can be different and depends on several factors. If the drug has been given earlier, then drug resistant viruses usually emerge immediately. This happens because HIV integrates into the human genome and therefore "older versions" can be easily recovered from proviral depots in diverse host tissues. If there is no archived resistant variant, the time to become resistant depends primarily on the genetic barrier, the size of the viral population, and on (bad) luck. The term “genetic barrier” means “difficulty of the virion to develop resistance”. In case of a high genetic barrier typically several mutations are required to develop resistance while in the case of a low genetic barrier only a few (sometimes just one) mutations are needed. As every mutation occurs more or less independently by chance, the probability

of developing drug resistance is of course lower with a high genetic barrier and as a result acquiring resistance on average takes longer.

The larger the viral population and higher the viral turnaround, the higher are the chances of finding a resistant variant. It is possible that the viral RT generates all necessary mutations within the first round of replication but it might also happen that all viruses acquire a mutation at the same time that is lethal for the virus or that they never find the resistance mutations. The extraordinary high turnover, with a total daily production of more than 10^{10} virions in the initial stages of the infection, coupled with a replication time of only 1-3 days and a high mutation rate is the main reason why resistant variants appear within the first weeks of monotherapy (Simon and Ho, 2003). It was therefore recommended to combine several drugs into a combination therapy. The idea was to use several drugs to raise the genetic barrier, which in turn should make it more difficult to accumulate resistance mutations (Hirsch, 1990). In 1995, this hypothesis was first proven effective in two clinical trials where it was demonstrated that the combination of two drugs, namely AZT with ddI or ddC, is more effective than treatment with AZT alone leading to the fact that dual therapy comprising two NRTIs then became the standard of care (Choo, 1995; Torres, 1995).

An additional key breakthrough was achieved when the first drugs from the new group of drugs of PIs and NNRTIs were approved in 1995 and 1996, respectively. This permitted simultaneous targeting of the viral replication cycle and viral genome at different points, further increasing the genetic barrier. This HAART containing at least three drugs of at least two classes has been shown to outperform purely NRTI-based combinations in a number of clinical trials and has considerably reduced HIV-related morbidity and mortality. As a consequence, HAART has now become the standard of care. However, despite the success of HAART, the emergence of resistant viruses is still a major concern. The constant residual viral replication from the infected cells that do not get into contact with the ARV drugs in high enough concentrations is one of the principal reasons for the appearance of resistant viruses. Over time, resistance mutations also accumulate from low-level replication and in due course lead to therapy failure. Moreover, adherence of

the patients becomes a much more difficult issue in the era of HAART because more drugs in the regimen also imply that patients have to take more pills and have to deal with more side effects, such as headache, diarrhea, and lipodystrophy etc. This non-compliance reduces the pressure on the virus and permits it to escape to resistance more easily. HAART has improved the quality of life of PLWHA significantly, but it is no panacea, and existing treatments must be maintained for life (Richman et al., 2009). The long-term side effects of life-long therapy are poorly understood. HIV-associated neurocognitive disorders and HIV-associated dementia are known, but it is not clear to what extent such effects can be attributed to the virus or the drugs. However, when considering the large numbers of pills an HIV-infected patient has to take, it is obvious that this cannot be good over time (Hawkins, 2010). Therefore, low-toxicity compounds are becoming increasingly important, with pharmaceutical companies also shifting their focus towards these.

2.10. Search for Biomarkers

2.10.1. Biomarkers

A biomarker is a quantifiable indicator of a specific biological condition, mainly one relevant to the risk of contraction, the presence or the stage of disease. The definition of a biomarker as proposed by the Biomarkers Definitions Working Group is that "a biomarker may be applicable as a diagnostic and/or prognostic tool, staging or classification of the extent of a disease, or to be used for prediction and monitoring of clinical response to an intervention". Some biomarkers may also be developed and used as surrogate endpoints instead of, or in combination with, clinical endpoints. The molecular and cellular bases of a disease could also be assayed by biomarkers (Atkinson et al., 2001). It could be an alternative term of molecular signatures, which includes qualitative and quantitative pattern of groups of biomolecules (mRNA, miRNA, proteins, peptide or metabolites) in a cell, tissue, biological fluid, or in an organism. Biomarkers can be used clinically for diagnosis or for monitoring the status of diseases and to guide molecularly targeted therapy or evaluate therapeutic response (Etzioni et al., 2003). For physicians, biomarkers can provide the following useful information (Cohen, 2007).

1. Biologic state of an individual;
2. Disease risk;
3. Disease diagnosis;
4. Disease progression;
5. Treatments of choice;
6. Monitoring responses to treatment; and
7. Endpoints for assessing treatment efficacy.

2.10.2. Biomarkers of HIV/AIDS

Several clinical and laboratory biomarkers have been used to evaluate the stages of HIV-1 infection. Markers of development of AIDS include HIV related symptoms (Redfield et al., 1986), reduction of CD4+ T cells (Fahey et al., 1990), cutaneous anergy (Redfield et al., 1986; Blatt, 1993), elevated serum β2-microglobulin (β2-m), neopterin levels (Grieco et al., 1984), HIV-1 p24 (core) antigenemia (Allain et al., 1987), anti-leucocyte antibodies (Kiprov et al., 1985), sTLR2 (soluble toll-like receptors) (Heggelund et al., 2004), C-reactive protein (Lau et al., 2006) and HLA (human leukocyte antigen) markers (Fernandes et al., 2003). Currently more research studies are looking at neuropathogenic biomarkers predicting HIV infection of the central nervous system (Gendelman et al., 2007).

The markers used for monitoring HIV infected individuals have depended predominantly on detecting the effects of HIV on the immune system rather than estimating viral load (Dwyer et al., 1997). There is prognostic importance of both immune deficits (declining number of CD4+ T cells) and immune activation (increased levels of neopterin, β2-m, soluble IL-2 receptors (sIL-2R) and IgA). The levels of sIL-2R reflect the activation of T cells and the numbers of CD8+ T cells suggest the stimulation of that population. Generally the levels of IgA and β2-m reflect B cell activation and lymphoid activity respectively (Fahey et al., 1990). The role of C-reactive protein in HIV pathogenesis is still uncertain (Carbone et al., 2004), and elevated levels of sphingomyelin and ceramides are due to oxidative stress, which indicates loss of cellular homeostasis during HIV infection (Sacktor et al., 2004). Data on the high viral turnover in HIV infection and the value of viral load measurements in

ARV trials have led to the use of markers that directly quantify viral load or other viral features in clinical practice (Saag et al., 1996, Volberding, 1996). The CD4 cell count, plasma viral load and other markers should not be used alone in making decisions on patient care, nor is it advisable to base decisions on a single phenotype (Koot et al., 1993). Unfortunately all of these markers have limitations in sensitivity, specificity, or predictive power. The single best predictor of AIDS onset identified thus far is the percentage or absolute numbers of circulating CD4+ T cells (Fahey et al., 1990) but less variable and earlier markers of risk for AIDS are needed (Mellors et al., 1995).

2.10.3. Limitations of HIV/AIDS Biomarkers

Inspite of a large number of surrogate markers for HIV/AIDS, their medical use still remains disputed, as they fail to fulfill some important goals such as

1. Role in the natural history of HIV-induced disease,
2. Measurable in the majority of infected persons,
3. Variation with clinical status in both progression and remission of disease, and
4. Quantifiable changes after a therapeutic intervention or no change following failure of therapy.

Moreover, very few studies have shown the result of treatment on surrogate markers and long-term survival. There is a necessity for validation of these studies in larger trials before surrogate marker measurements would be established commonly as clinical end-points (Tsoukas et al., 1994). Existing research in the area of biomarkers related to HIV/AIDS continues to be experimental and lacking in validated biomarkers. There is a shift in the cellular markers of disease progression from lymphocyte predominance to other cellular markers such as monocyte-macrophage system (Gendelman, 2007). These preliminary experimental studies need to be followed by more longitudinal studies, which discover newer biomarkers that are associative as well as predictive of disease progression.

2.10.4. Biomarker Quest: Profiling through "Omics"

The discovery, qualification and application of diagnostic and prognostic biomarkers remain the holy grail of the current "omics" paradigm. To

understand the complex biological systems such as cells, tissues, or the entire system (i.e. the human body), it is not sufficient to discover and characterize the individual molecules in the system. It is also obligatory to have a thorough understanding of the interactions between molecules and pathways. It has now become easier to examine complex cellular processes at the molecular level with recent technological advances, which helps in changing focus towards understanding the data generated by these "omics" technologies including genomics (DNA), transcriptomics (mRNA), proteomics (protein), miRnomics (miRNA), metabolomics (metabolites), glycomics (glycoproteins and carbohydrates), lipidomics (lipids), etc. These advances are now used regularly to identify biomarkers associated with different diseases.

The genomics methods include DNA copy-number assessment with comparative genome hybridization to DNA microarrays, DNA sequencing, genotyping, and mutation-specific PCR. The assessment methods for transcriptomics include gene-expression profiling with DNA microarrays, multiplex PCR and PCR arrays. For miRnomics, microRNA expression profiling is done either with DNA microarrays or multiplex PCR. The evaluation methods for proteomics include two-dimensional electrophoresis followed by mass spectrometry (MS) or proteomic profiling with mass spectrometry; phosphoproteomic profiling is also done with mass spectrometry but after immunoprecipitation with phosphotyrosine-specific antibodies. The methods used for metabolite profiling in metabolomics includes mass spectrometry and Nuclear Magnetic Resonance (NMR) (Sawyers, 2008) (Figure 7).

2.10.5. Features of "Omics" technologies

These "Omics" technologies have three distinct features. First, they allow analyses on different molecular levels, such as the DNA, RNA, protein, or metabolite. These different molecular levels sometimes act asynchronously - that is, although some proteins are highly abundant in a cell, the levels of the corresponding mRNAs from which they are produced may be very low or vice versa. Because asynchronous actions can indicate the effects of complex

regulatory interactions, it is important to investigate the role and level of synchronization of the transcriptome, proteome, metabolome, etc. The omics technologies permit researchers to discover interactions of different biomolecules systematically and integrate them into models intended to capture the required regulatory features of a pathway.

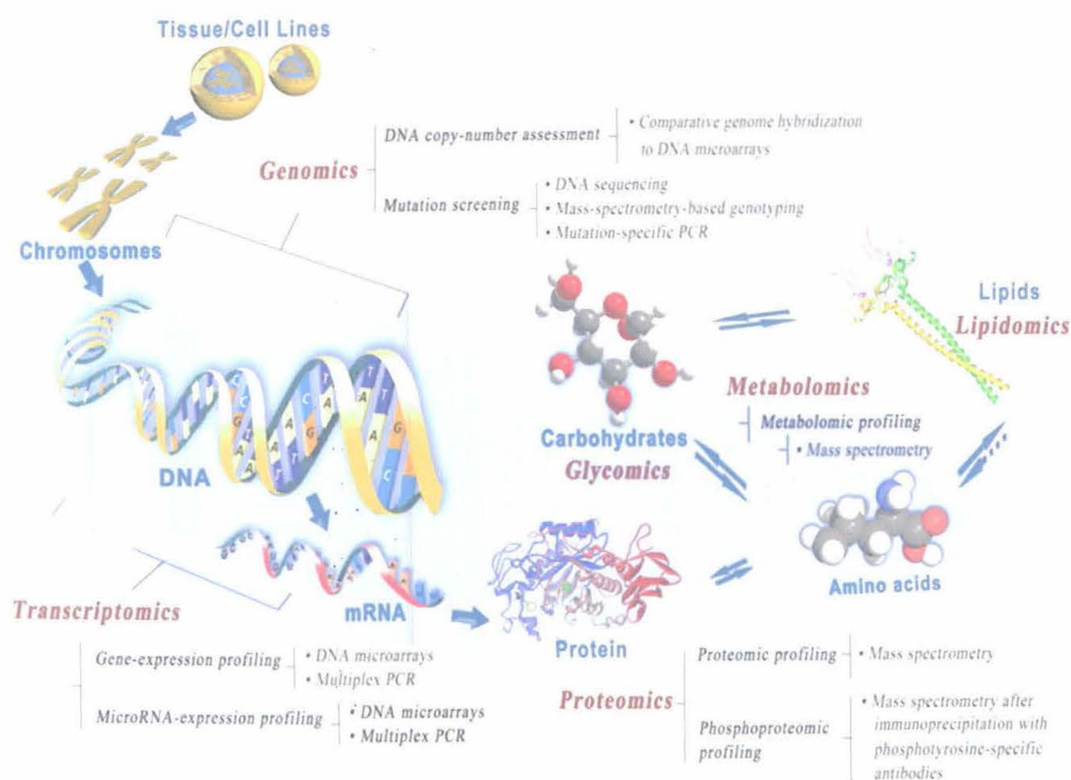


Figure 7: Diagram of omics technologies, their corresponding analysis targets, and estimation methods. DNA (genomics) is first transcribed to mRNA (transcriptomics) and translated into protein (proteomics), which can catalyze reactions that act on and give rise to metabolites (metabolomics), glycoproteins and carbohydrates (glycomics), and various lipids (lipidomics). (Sawyers, 2008).

Second, the omics technologies are parallel. This means that for a particular biological sample many distinctive biological "readouts" can be measured concurrently. For example, through microarrays the expression of all genes of an organism can be measured simultaneously rather than performing several individual experiments focusing on different genes. Such parallelization aids scientists to identify not only the possible but also unanticipated responses of an organism. For instance, microarrays in toxicology research allow for a broad screen for unexpected biological side effects caused by new drugs

(Ulrich and Friend 2002; Waters and Fostel 2004). The high degree of parallelization also allows researchers to uncover functional interactions between different genes and proteins and acquire complete descriptions of evolving properties of a cell or organism. This is particularly important for complex cellular processes such as proliferation, apoptosis or the response to infection, all of which can involve several hundred different types of molecules.

Third, the omics technologies are highly automated, permitting investigators to handle and process large numbers of biological samples. These techniques simplify the sample processing and experimentation. The ease of use of omics technologies is appropriate for clinical studies, in which large patient populations must be tested to obtain a sound statistical basis for confirming drug efficacy and identifying potential side effects. The huge amount of data generated from metabonomics require tools to process automatically, compare, and interpret in a way that identifies the most relevant pieces of information, which can be used to create models or identify biomarkers of a disease.

2.10.6. "Omics" Techniques

2.10.6. 1.1. Transcriptomics

Following the sequencing of the human genome, it was predicted that the human genome encodes more than 100,000 transcripts, collectively known as the transcriptome. However, this approximation went through considerable modification until bioinformatics approaches predicted the number of transcripts encoded by the human genome to be 20,000–25,000. It is important to mention that all of these genes are not expressed in every type of cell. The subsequent wave of exploration was then focused on which genes are expressed in a specific cell/tissue type or on a comparative analysis of transcripts between two conditions or cell/tissue types. These analyses took benefit of the human genome sequence and the information of expressed sequences either from open reading frame (ORF) and intron-exon analysis, or from sequencing cDNAs cloned from different tissues. Oligonucleotides corresponding to every ORF were arrayed onto a high-density slide or chip,

and these microarrays were then hybridized with target cDNA (or cRNA) generated from RNA isolated from a cell- or tissue-type. A thoughtful insight into changes of gene expression in different diseases and developmental stages was obtained from these studies.

2.10.6.1.2. Transcriptomic Methods

A number of technologies have been developed to profile gene expression in cells or tissues at the level of the mRNA transcripts. Northern blot is the oldest and simplest process used to determine whether a gene is expressed in a sample or not (Alwine et al., 1977). It requires transferring of electrophoretically separated RNA molecules from an agarose gel to nitrocellulose membranes. Specific RNA molecules can be detected by hybridization with ^{32}P-labeled DNA or RNA probes followed by autoradiography (Melton et al., 1984). Non-radioactive probes and detection methods have also been used. The RNase protection assay is more sensitive method than Northern blots. It is the solution hybridization of a single-stranded, discrete-sized ^{32}P-labeled anti-sense probe(s) to a RNA sample. Non-hybridizing (single-stranded) RNA species are digested with RNase enzyme followed by the extraction and electrophoretic isolation of hybridized fragments. For quantifying mRNA levels, the intensities of hybridized probe fragments are compared with the intensities generated from either an endogenous internal control (relative quantification) or known amounts of sense-strand RNA (Prediger, 2001). Like Northern blots, this method has the disadvantage of being low throughput and only few genes can be analyzed in parallel.

Quantitative PCR is increasingly becoming the ideal choice to prove differential expression of known genes (O'Garra and Vieira 1992; Freeman, 1998). It is more sensitive than Northern blotting and the RNase protection assay, and one can determine differences in the expression levels of even those mRNAs that are present in small amounts per cell. Comparing with the internal control, relative amounts of PCR products are determined. Currently, fluorescence-based kinetic i.e. real-time PCR permits monitoring amplification

during a PCR, and more correctly reflects the relative expression levels of target genes (Bustin, 2000).

The Serial Analysis of Gene Expression (SAGE) allows the serial analysis of short cDNA sequences or 'tags' resulting from a defined position within a cDNA, and permits both qualitative and quantitative analysis (Velculescu et al. 1995). SAGE is based on the serial sequencing of 15-bp tags that are unique to each and every gene. These gene-specific tags are produced through a series of molecular biological manipulations and then concatenated for automated sequencing.

The use of cDNA and oligonucleotide arrays, on either filters or glass slides ('chips'), has become the standard in large-scale differential gene expression analysis. Microarrays do not necessitate extensive sequencing, as opposite to SAGE and therefore present a truly high throughput method. In this technique, PCR-amplified cDNA fragments (ESTs) are spotted at high density (10-50 spots per mm^2) onto a microscope slide and probed against fluorescently or radioactively labeled cDNA (Schena et al., 1995). The signal intensity observed in cDNA microarray is assumed to be proportional to the amount of transcript present in the RNA population being studied. Differences in intensity reflect differences in transcript levels between treatments. Statistical and bioinformatics analyses are then performed, usually with the goal of creating hypotheses that may be further tested with established molecular biological procedures (Brown and Botstein, 1999).

The second common method for parallel analysis of gene expression is the use of oligonucleotide microarrays, also branded by the trademark Affymetrix GeneChip® (Lockhart et al., 1996; Lipshutz et al., 1999). The manufacture of GeneChip arrays uses photolithography and solid-phase chemistry to create arrays containing hundreds of thousands of 25-mer oligonucleotide probes packed at extremely high densities. The probes are designed to maximize sensitivity, specificity and reproducibility, permitting reliable discrimination between specific and background signals, and between closely related target sequences. In a standard eukaryotic gene expression assay, labeled cDNA or cRNA targets resulting from the mRNA of an experimental sample are

hybridized to nucleic acid probes attached to the solid support. By screening the amount of label associated with each DNA location, it is possible to understand the abundance of each mRNA species. The combination of miniaturization technology and increasing amounts of sequence information has expanded the scale at which gene expression can now be studied.

2.10.6.2.1. miRNomics

It appears that roughly one-third of the transcripts in an animal cell may possibly be controlled by microRNAs (miRNAs). The new paradigm of RNA interference (RNAi) discovered in 1998 supplemented an extra layer to the regulation of gene expression in an animal cell (Fire et al., 1998). The RNAi phenomenon elucidated the regulatory role of non-coding RNAs or miRNAs, and these small RNAs occupy an important position in the hierarchy of gene regulation (Ambros, 2004; He and Hannon, 2004). Though array-based platforms exist for global profiling of miRNAs (miRNomics) in a cell or tissue, the significance of a specific profile is complex to explain, because a single miRNA can target hundreds of target sequences in the human genome and modulate several cellular pathways. Newer experimental or bioinformatic methodologies are evolving to allow high-throughput discovery of the most likely target of a specific miRNA in a particular cell or tissue under a given condition.

2.10.6.2.2. MicroRNAs (miRNAs)

The miRNAs are about 19-22-nucleotide, short, non-coding RNAs that are considered to regulate gene expression by sequence-specific base pairing with target mRNAs. Through molecular cloning and bioinformatics prediction approaches hundreds of miRNAs have been identified in worms, flies, fish, frogs, mammals and flowering plants (Lagos- Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lim et al., 2003a; Llave et al., 2002; Reinhart et al., 2002; Watanabe et al., 2005; Zamore, 2005). MicroRNAs are transcribed as long RNA precursors, the primary miRNAs (pri-miRNAs) that include a stem-loop structure of around 80 bases. These pri-miRNAs are processed in the nucleus by the RNase III enzymes Drosha and DGCR8/Pasha, which remove the stem-loop to form the pre-miRNA (Figure 8) (Denli et al., 2004;

Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003). The pre-miRNAs are exported from the nucleus by Exportin-5 (Bohnsack et al., 2004). In the cytoplasm, another RNase III enzyme, Dicer,

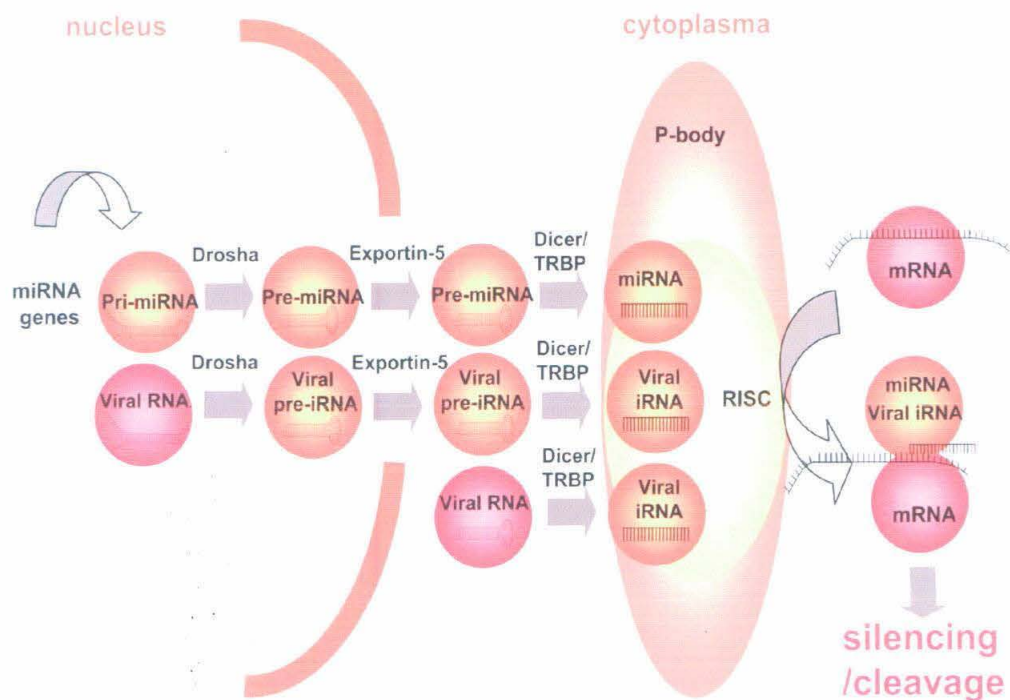


Figure 8: Biosynthesis and activity of miRNAs (Corbeau, 2008).

cleaves the pre-miRNA to produce the mature microRNA as part of a short RNA duplex. The RNA is then unwound by a helicase activity and incorporated into a RNA-induced silencing complex (RISC). Most miRNAs in animals are thought to function through the inhibition of effective mRNA translation of target genes by imperfect base pairing with the 3' untranslated region (3'UTR) of target mRNAs (Bartel, 2004). The underlying mechanism is still poorly understood, but it seems to work through the inhibition of translational initiation (Pillai et al., 2005). MicroRNA targets are largely unidentified, but it is estimated based on target predictions using a variety of bioinformatics approaches that each miRNA may target upto 100 genes (John et al., 2004).

2.10.6.3. 1. Human Metabonomics

The human metabolome may be defined as the complete set of small molecules such as amino acids, fatty acids, mono- and di-carbohydrates, etc. that are derived from metabolism (Oliver et al., 1998). It is a complex system where the chemical substances may be derived from human metabolism, gut microflora, etc. of various origins and environments (Nicholson et al., 2005). Metabonomics was introduced as a scientific field only in the past decade (Nicholson, 1999) and shortly thereafter, the term "Metabolomics" was coined (Fiehn et al., 1999). However, metabonomics is often referred to as being NMR based and used to express multiple (but not necessarily comprehensive) metabolic changes caused by a biological perturbation. Metabolomics, on the contrary, has been established as being MS-based and places a higher importance on comprehensive metabolic profiling, regardless of the species being investigated.

In addition of being a potential tool for the discovery of new biomarkers for diagnosis, prognosis and treatment of disease, metabolomics could also provide information on probable metabolic pathways in a disease condition. However, identification of the altered metabolites is of high importance for success. Identification is still one of the major obstacles for metabolomics since few resources are available (Gibney et al., 2005). The Human Metabolomics Database (HMDB) is the most complete and comprehensive database that collectes metabolite and human metabolism data and was developed only recently (Wishart et al., 2007).

2.10.6.2. Metabonomics Methods

There are a number of different platforms used for metabolite profiling, of which NMR spectroscopy and mass spectrometery (MS) are the most widely used and differ in specificity and sensitivity. While NMR can simultaneously measure all kinds of metabolites, it has limited sensitivity and should therefore only be applied to samples with high concentrations of compounds. For enabling detection of compounds with low concentration, more sensitive methods such as MS should be considered. Usually, MS is used to identify and quantify metabolites after separation by different techniques. The most

established combinations of separation and quantification techniques are: gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), Fourier transform ion cyclotron resonance/mass spectrometry (FTICR-MS) and capillary electrophoresis/mass spectrometry (CE-MS) (Soga et al., 2006; Lee et al., 2007; Han et al., 2008).

2.10.6.3. Nuclear magnetic resonance (NMR)

In the 1980s and 1990s, high-field proton NMR became widespread for metabolite studies (Nicholson et al., 2002) and has since been used extensively for metabolomics research in areas as diverse as plant metabolism (Bligny and Douce, 2001), Duchenne muscular dystrophy (Griffin et al., 2001), neurological disorders (Holmes et al., 2006), and hepatotoxicity and nephrotoxicity in rodents (Bollard et al., 2005; Lenz et al., 2005). One key benefit of using NMR for metabolomics is the requirement of very small amounts of sample, often involving no more than pH buffering plus the addition of a deuterated solvent such as chloroform, methanol, or acetonitrile (Defernez and Colquhoun, 2003) for the study of biofluids, cell lysates, and tissues. Other advantages of NMR include both rapid and quantitative analysis (Dunn et al., 2005) and preservation of the sample, which is particularly important when the amounts of available material are small.

2.11. HIV and Host Factors

The immune response against HIV is modulated by multiple host genetic determinants, many of which are directly or indirectly implicated in recognition of the virus. These include chemokine receptors, human leukocyte antigen (HLA), T-cell receptor (TCR), antibodies, killer immunoglobulin-like receptors (KIRs), toll-like receptors (TLRs), immune cell trafficking (including chemokines and receptors, adhesion molecules), and immune response amplifiers (includes molecules involved in signaling pathways, cytokine genes) (Kaur et al., 2009).

HIV-1 utilizes multiple host proteins during infection. These proteins participate in a broad array of cellular functions and are associated with pathways in the viral life cycle. These proteins are known as HIV-dependency

Gene	SNP reference number and name	Mechanism	Effect	Refs
HIV entry				
Chemokine receptors				
CCR5	rs333, Δ32	Truncated HIV-1 coreceptor	Δ32/Δ32: prevents HIV acquisition	[2]
			Δ32/+: delayed AIDS	[2]
	rs1799987, promoter SNP, 59029A[b]	Upregulate CCR5 expression	Accelerate AIDS	[21]
CCR2	rs1799864, V64I	LD with *CCR5*?	Delayed AIDS	[97]
CXCR6	rs2234355, E3K		Increased survival time after PCP diagnosis	[98]
Chemokines				
SDF1	rs1801157, 3′A (3′UTR)	Increased levels of CXCL12 mRNA and enhanced mRNA stability compared to 3′G [99]	Delayed AIDS	[100]
CCL5	rs2280789, In1.1C (intronic)	Down-regulate *CCL5*	Accelerated AIDS, risk for HIV acquisition in AA[b] and Thai [32], infection in Chinese [33] and German [31]	[28,30,34,65]
	Haplotype R3+R5		Accelerated AIDS in EA[b] [28] and under HAART	[28]
	rs2107538, -403		Increased risk of infection	[28]
	rs1800825, -28	Up-regulation	Delayed progression in Japanese [29] and Thai [32]	[29]
	3′222C		Increased risk of infection	[28]
CCL3L1	Copy number polymorphism at 17q11.2	Correlate with CCL3L1 level	Increasing copy number may be associated with favorable outcomes	[37,39,40, 63,101]
CCL2-CCL17-CCL11	Hap 7 (31 kb) at 17q11.2-q12	Immune modifiers?	Prevents HIV acquisition	[1]
CCL18-CCL3-CCL4	rs1719153, rs1719134, 47 kb haplotype at 17q12		Accelerated AIDS	[1]
DC-SIGN	rs4804803, −336G	Lower DC-SIGN expression on dendritic cells	Increased risk of HIV acquisition in EA	[102]
Post-entry cellular viral cofactor				
CUL5	rs7117111, SNP5	Unknown	Accelerated AIDS in AA	[53]
	rs11212495, SNP6	Transcription factor binding	Accelerated AIDS in AA	[53]
	rs7103534, SNP4	Unknown	Delayed progression in EA	[53]
PPIA/CypA	rs8177826, 1604G	Binding of transcription factors	Accelerated AIDS in EA and AA	[64]
	rs6850, 1650G	Increased HIV infectivity	Increased risk of HIV acquisition	[64,65]
Tsg101	Haplotype C		Delayed progression	[86]
APOBEC3G	rs8477832, H186R		Accelerated AIDS in AA, high viral load in Africans	[44,46,93]
	rs3736685, 197193C	Intronic, in LD with H186R	Accelerated AIDS in AA	[44]
	rs2294367, 199376C	Intronic	Accelerated AIDS in EA	[44]
	C40693T	Intronic	Increased risk of HIV acquisition	[43]
APOBEC3B	Δ3B/Δ3B	Deletion of *APOBEC3B* gene	Increased risk of HIV acquisition	[52]
TRIM5	rs16934386, promoter SNP2, rs7127617, promoter SNP 3	Regulate TRIM5 expression in T-cell?	Increased risk of HIV acquisition	[56]
	rs10838525, R136Q	Better anti-HIV-1 activity	Prevents HIV acquisition	[56]
	rs3740996, H43Y		Prevents HIV acquisition	[56]
Cytokines				
IL10 (Th2 cytokine)		Down-regulates IL10	Accelerated AIDS	[103]
IFNG (Th1 cytokine)	rs2069709, -179T	Aberrant IFNG regulation	Accelerated AIDS	[104]
IRF-1	rs17848395, 619A, rs17848424, 6516G	Down-regulation and lowered response to IFN-r	Prevents HIV acquisition	[16]
CXCR1 (IL8 receptor)	Haplotype Ha	Modulation of CD4 and CXCR4 expression	Delayed progression	[17]
HLA system				
HLA	Class I A,B,C homozygotes	Reduced epitope recognition repertoire	Accelerated AIDS	[9,95]
	HLA class I Concordance	Narrow epitope recognition repertoire	Increased risk of sexual and mother-to-infant transmission	[12]
	B*35-Px	Weak epitope binding helps HIV immune escape	Accelerated AIDS	[10]
	B*27	Hinders HIV immune escape	Delayed AIDS	[7]
	B*57	Hinders HIV immune escape	Delayed AIDS	[7]
	rs9264942, 5′ upstream of *HLA-C*	Regulates *HLA-C* expression	Deceased viral set point	[71]
HCP5	rs2395029-G	LD with B*57	Deceased viral set point	[71]
KIR	*KIR*3DS1 + *HLA* Bw4-80I	Signaling the NK cells to kill the HIV infected cells	Delayed AIDS	[7]
	*KIR*3DS1 in absence of ligand	Poor regulation of NK cell activity	Accelerated AIDS	[7]

Table 3: Published host genetic factors of HIV/AIDS (An and Winkler, 2010).

factors (HDFs). Recently through genome-wide screening technologies by performing *in vitro* infection studies, a total of 1,254 genes were found to be important during HIV infection; these represent about 5% of all human protein-coding genes. The nine genome-wide surveys carried out this far yielded an overlap of only 257 genes (Bushman et al., 2009), some of which are listed in Table 3.

2.12. RNA Interference and HIV

Viral interfering RNAs (viRNAs) and the miRNA machinery may perhaps interfere in a variety of ways (Figure 9). First, the viRNAs produced by the cell from viral RNAs may target back viral RNAs (pathway 1 or autosilencing). In case of HIV infection, a nef- and LTR-specific HIV miRNA that inhibits LTR-driven transcription was proven (Omoto et al., 2004, Omoto and Fujii, 2005). In some cases the host cell transforms a viral RNA into a viRNA with some degree of homology with a cellular mRNA (pathway 2). *In silico* analyses have shown that regions of base complementarity between HIV-1 sequences and human genes involved in HIV infection, e.g., CD4, CD28, CD40L, IL-2, IL-3, IL-12, and TNF beta (Couturier et al., 2005) but also cellular mRNAs that happen to share some sequence homology with them. The cellular miRNAs may also target viral RNAs and silence their expression (pathway 3). Huang et al. have shown that miR-28, -125b, -150, -223, and -382, cellular miRNAs that are over expressed in quiescent T4 lymphocytes, target sequences in the 3' end of HIV-1 RNA, thereby silencing almost all viral mRNAs (Huang et al., 2007). Finally, the cell may produce miRNAs that control the expression of a cellular protein necessary for the virus life cycle (pathway 4). Triboulet et al. have shown that the cellular miRNAs miR-17-5p and miR-20 silence the mRNA encoding the histone acetylase PCAF. The PCAF protein has been previously presented as a host cofactor for Tat-mediated transactivation of the HIV LTR, and as being recruited by Tat for remodeling the histone architecture in the vicinity of the LTR, thus promoting HIV gene expression (Triboulet et al., 2007). The course of HIV-1 infection in cells is influenced by the action of hundreds of host proteins (Lama et al., 2007; Brass et al., 2008; König et al., 2008, Zhou et al., 2008, Goff 2008). Viral replication appears to be modulated also by the expression of human miRNAs (Huang et al., 2007, Hariharan et al., 2005, Wang et al., 2008, Yeung et al., 2007). In turn, the expression of HIV-1 proteins in cells (Yeung et al., 2005) or the *in vivo* infection by virus (Triboulet et al., 2007) can change human miRNA profiles. Among the 372 miRNAs studied, 62 were differentially regulated and a majority was down regulated. The T-cell specific miRNAs such as miR-223, mR-150, miR-146b, miR-16, and miR-191 are significantly down regulated in

PBMCs following HIV infection. The observed miRNA changes are likely indirect bystander effects resulting from systemic changes in the activation status or cytokine levels in the infected individuals (Houzet et al., 2008).

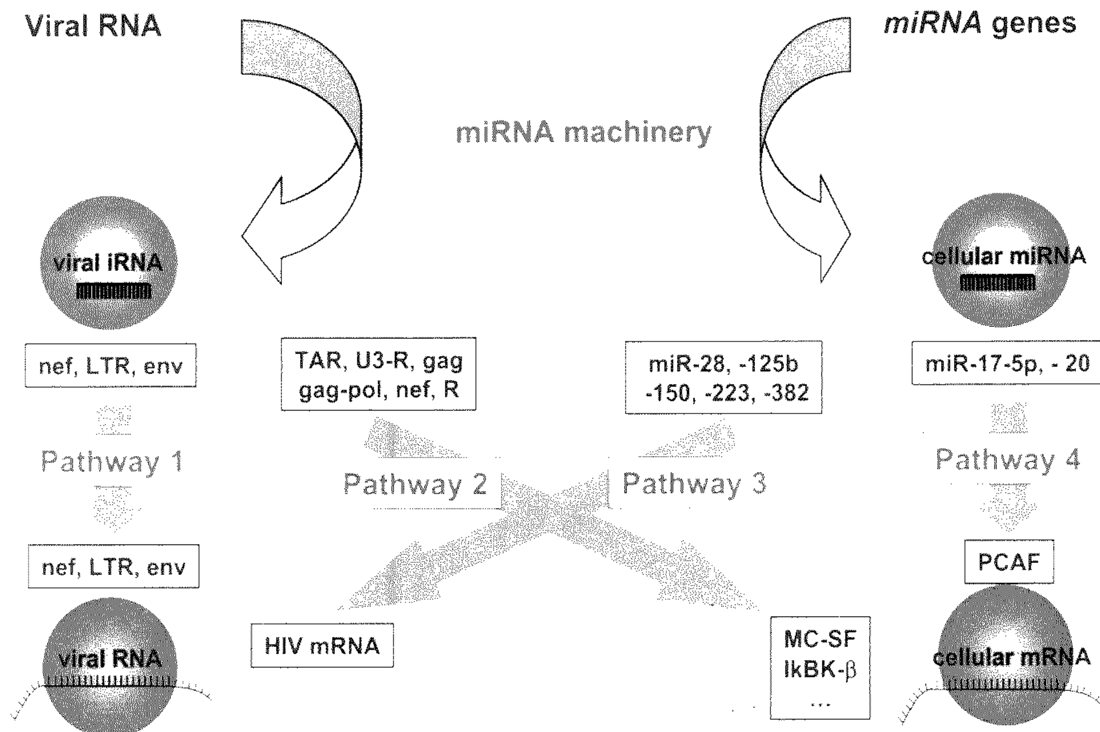


Figure 9: Interactions between miRNAs, cellular mRNAs, and HIV RNAs. (Corbeau, 2008).

2.13. Metabolites and HIV infection

HIV infected patients encounter an increasing number of complications and in particular age-related comorbidities occurring earlier than in the general population. These include cardiovascular disease, dyslipidemia, diabetes, osteoporosis, liver and kidney failure, neurocognitive impairment and non-AIDS defining cancers. All these diseases are related with host metabolism.

Through NMR techniques so far three studies were done on biological fluids collected from HIV patients without and with ART. By studying serum metabolites it was possible to distinguish between HIV-positive individuals on ART or otherwise (Philippeos et al., 2009), and between the HIV/AIDS patients on ART and HIV-1 negative individuals (Hewer et al., 2006). In another study on oral wash samples, a total of 12 metabolites (e.g., allantoin, phenylalanine, tryptophan etc.) were found increased and 15 (e.g. fucose,fumarate, and N-acetylglucosamine etc.) were decreased in HIV-infected patients.

In different studies, it was found that metabolite levels vary in concentration during the course of HIV infection. In HIV infection, plasma levels of metabolites such as glucose, neopterin and choline were increased (Reeds et al., 2006). There were also changes in amino acid levels in HIV infection. Significantly increased levels of plasma citrulline, oral sarcosine (Wanchu et al., 2002; Ghannoum et al., 2011), and lower levels of plasma threonine and methionine were previously seen in HIV infection (Hortin et al., 1994; Laurichesse et al., 1998).

As discussed above, it is clear that host genes and miRNAs found in different cells and metabolites in different biofluids are differentially regulated during HIV infection, and some of these are altered after the introduction of ART. We assumed that to develop a unique biomarker or a group of biomarkers to follow the different stages of HIV infection including patients on therapy and those going through therapy failure, and to understand the role of this differential regulation in disease pathogenesis, it would be worthy to explore the expression of selective host genes and miRNAs in cells and metabolites in the biofluids of HIV/AIDS patients at different stages of disease. The following chapters give and in-depth account of the findings from HIV/AIDS patients after profiling of 84 host genes through PCR array, five T-cell specific miRNAs through real time-PCR and metabolites in plasma, urine and saliva through NMR.

Chapter 3: Materials and Methods

3. Materials and Methods

3.1. Materials

All the materials and their sources used in this study are presented in a tabulated form in Table 3.1 (Appendix 1).

3.2. Methods

3.2.1. Participants

The participants in this study were recruited from the ART Clinic of Dr. Ram Manohar Lohia (RML) Hospital and Maulana Azad Medical Collage Hospital (MAMC), New Delhi, India.

The study subjects were divided into five groups.

Group 1: HIV infected asymptomatic (without ART); CD4 > 350/μl

Group 2: AIDS patients; CD4 < 200/μl, prior to initiating ART

Group 3: Patients on ART (at least 1 month); CD4 > 300/μl

Group 4: Patients failing ART (fulfilling WHO treatment failure criteria)

Group 5: HIV negative; samples were collected from laboratory personnel at ICGEB, New Delhi and other healthy volunteers. Their HIV-negative status was confirmed by HIV-1/2 rapid tests.

Patients were coded and their anonymity was maintained throughout. The clinical, immunological (CD4 counts), ART and other related data were collected in a Data Sheet (Appendix 2) by interviewing the study subjects and from the hospitals.

After proper written consent was obtained on a Consent Form (Appendix 3), blood, urine and saliva were collected from the participants with no known current metabolic or other medical conditions like HBV or HCV infection and other immunological diseases. All participants were also free of active tuberculosis and were not receiving any anti-tubercular drugs.

3.2.2. Samples

Blood, urine and saliva were collected from the study subjects following standard operating procedures.

3.2.2.1. Blood collection and separation

The blood collection process and the separation of different components were as follows.

1. Approximately 5 mL of blood was collected following laboratory's standard procedures from each study subject.
2. The study subjects were positioned and site for venipuncture was selected.
3. The venipuncture site was prepared with alcohol swabs and allowed to dry.
4. The tourniquet was applied 3-4 inches above the selected venipuncture site.
5. The needle shield was removed from 10 ml of syringe and venipuncture was performed with patient's arm in a downward position.
6. The tourniquet was removed as soon as blood was drawn.
7. After withdrawing the needle fully, pressure was applied with the cotton ball over the puncture site and hold pressure. Patient was asked to apply pressure for 3 to 5 minutes until the bleeding stops.
8. The needle was destroyed with needle cutter and syringe was discarded into the biohazard container.
9. The collected blood in the syringe was divided in to two parts. First half was poured directly into the Tempus ™ Blood RNA Tube, containing liquid reagents for whole blood RNA isolation and another half was collected in to vacutainer containing EDTA to collect plasma and to isolate PBMCs.
10. To ensure uniform contact with the sample, immediately after collection of blood the Tempus™ tube was stabilized by shaking vigorously for 10 seconds.
11. The collected samples were taken to the lab within 3 hours of collection. On arrival the Tempus ™ Blood RNA tubes were preserved at -70°C and PBMCs were isolated from blood collected in the vacutainer with EDTA.

3.2.2.2. Urine collection and preparation

Random urine samples (150-200 ml) were collected in 250 ml bottles and kept immediately on ice. To remove cellular and particulate matter, the urine was centrifuged at 1000xg for 10 min after reaching the laboratory and was frozen in aliquots at -70°C.

3.2.2.3. Saliva collection and preparation

A standard protocol was used for the collection of saliva samples. Whole, unstimulated saliva (1-3 ml) was collected over a 5 min period by the draining method. Subjects were advised to rinse their mouth thoroughly with deionized water and to take rest for 5 min and then saliva was allowed to drip off the lower lip into a sterile tube. The specimen was centrifuged to remove cells, and supernatant was stored in aliquots at -70°C.

3.2.3. PBMC isolation

The PBMC was isolated using Ficoll-Paque density gradient technique and the isolated PBMCs were preserved in liquid N_2 as described below.

1. The Ficoll-Paque PLUS bottle was inverted and using aseptic techniques, the required volume of Ficoll-Paque was withdrawn and 1.5 ml of Ficoll-Paque PLUS solution was added in to each of the centrifuge tube.
2. Two ml of blood sample was carefully layered on Ficoll-Paque PLUS solution. The tubes were centrifuged at 400 x g for 30 minutes at 4°C and the upper layer was drawn off using clean blue tips, leaving the lymphocyte layer undisturbed at the interface.
3. Using a clean Pasteur pipette the lymphocyte layer was transferred to a clean centrifuge tube and 3 volumes of balanced salt solution were added to the lymphocytes in the test tube.
4. The cells were suspended by gently drawing them in and out of blue tips by repeated pipetting and then the tubes containing isolated cells were centrifuged at 60-100 × g for 10 minutes at 18-20°C.
5. The supernatant was removed and the lymphocytes were suspended in 4 ml of balanced salt solution by gently drawing them in and out of the blue tips by repeated pipetting.
6. Again the tubes were centrifuged at 60-100 × g for 10 minutes at 18-20°C

and the supernatant was removed and the lymphocytes were suspended to preserved in 5% DMSO with Fetal Calf Serum (FCS).

7. To produce FCS with 5% DMSO, 500 µL of DMSO was added to 9.5 ml of FCS in a 15 ml of falcon tube.
8. 400 µL of the mixture was added to the tube with isolated PBMC and pipetted to the cryovial for preservation.
9. The cryovials were kept at -20°C for 3 hours and then shifted to -70°C for 2 days and then finally to liquid Nitrogen jar for longer preservation.

3.2.4. Isolation of RNA

3.2.4.1. Isolation of RNA from Whole Blood

Total RNA was extracted from whole blood that was collected in Tempus™ Blood RNA Tubes by using Tempus™ Spin RNA Isolation Kit according to the manufacturer's instructions. The process is described below.

1. The frozen whole blood sample collected in Tempus ™ Blood RNA Tubes was thawed at room temperature (18 to 25°C).
2. The cap was removed from the Tempus ™ tube and the content was poured into a clean 50-mL falcon tube.
3. 3 mL of 1-phosphate-buffered saline (PBS; Ca2+/Mg2+-free) was pipetted into the tube to bring the total volume to 12 mL.
4. The tube was closed by the cap and vortexed vigorously for 30 seconds to ensure proper mixing of the contents.
5. The tube was centrifuged at 4°C at 3,000 x *g* (rcf) for 30 minutes and after that the supernatant was carefully pour off and the tube was left inverted on absorbent paper for 1 to 2 minutes.
6. The remaining drops of the liquid were blotted off the rim of the tube with clean absorbent paper and 400 µL of RNA Purification Resuspension Solution was pipetted into the tube and vortexed briefly to resuspend the RNA pellet.
7. The RNA purification filter was labeled and then inserted into a waste collection tube.
8. The filtration membrane was pre-wetted with 100 µL of RNA Purification Wash Solution and then the total RNA that was around 400 µL, which was resuspended in 50 mL falcon tube, was pipetted into the purification filter

and then centrifuged.

9. The purification filter was removed and liquid waste collected in the waste tube was discarded and then the purification filter was re-inserted into the waste tube.
10. 500 µL of RNA Purification Wash Solution 2 was pipetted into the purification filter and then centrifuged.
11. The purification filter was removed and the liquid waste collected in the waste tube was discarded, then the purification filter was re-inserted into the waste tube.
12. Again 500 µL of RNA Purification Wash Solution 2 was pipetted into the purification filter and then centrifuged.
13. The purification filter was removed and the liquid waste collected in the waste tube was discarded. The purification filter was re-inserted into the waste tube and centrifuged to dry the membrane.
14. The purification filter was transferred to a new-labeled collection tube to collect the eluate.
15. 100 µL of Nucleic Acid Purification Elution Solution was pipetted into the purification filter. The cap was closed and the entire tube was incubated at 70°C for 2 min and then centrifuged.
16. The collected RNA eluate was pipetted back into the purification filter, then centrifuged. The purification filter was discarded and then approximately 90 µL of the RNA eluate was transferred to a new, labeled collection tube.
17. Isolated RNA was stored at −70°C in the new collection tube.

3.2.4.2. Isolation of RNA from PBMC

Total RNA was extracted from PBMC that was isolated and stored at -70°C in cryovial by using the miRNeasy Mini Kit according to the manufacturer's instructions. The process is described below.

1. To disrupt the cells of the stored PBMC, 700 µL of QIAzol Lysis Reagent was added and properly vortexed.
2. The tube containing the homogenate was placed on the benchtop at room temperature (15–25°C) for 5 min.

3. 140 µl chloroform was added to the tube containing the homogenate and capped securely. The tube was shaken vigorously for 15 s.
4. The tube containing the homogenate was placed on the benchtop at room temperature for 2–3 min.
5. The tube was centrifuged for 15 min at 12,000 x g at 4°C.
6. The upper aqueous phase of the tube was transferred to a new collection tube and then 1.5 volumes of 100% ethanol were mixed thoroughly by pipetting up and down several times.
7. Without any delay up to 700 µl of the sample was pipetted, including any precipitate that may have formed, into a miRNeasy Mini spin column in a 2 ml collection tube. The lid was closed gently and centrifuge at ≥8000 x g (10,000 rpm) for 15 s at room temperature (15–25°C). The flow-through was discarded.
8. Step 7 was repeated using the remainder of the sample and the flow-through was discarded.
9. 700 µl of buffer RWT was added to the miRNeasy Mini spin column. The lid was closed gently and centrifuged for 15 s at 8000 x g (10,000 rpm) to wash the column and the flow-through was discarded.
10. 500 µl Buffer RPE was pipetted onto the miRNeasy Mini spin column and the lid was closed gently and centrifuged for 15 s at 8000 x g (10,000 rpm) to wash the column. Then the flow-through was discarded.
11. Again another 500 µl buffer RPE was added to the miRNeasy Mini spin column. The lid was closed gently and centrifuged for 2 min at 8000 x g (10,000 rpm) to dry the RNeasy Mini spin column membrane.
12. The miRNeasy Mini spin column was transferred to a new 1.5 ml collection tube. 30–50 µl miRNase-free water was pipetted directly onto the miRNeasy Mini spin column membrane. The lid was closed gently and centrifuge for 1 min at 8000 x g (10,000rpm) to elute the RNA. The RNA was stored in -70°C.

3.2.5. PCR Array

For PCR array analysis, the Human HIV Infection and Host Response RT Profiler™ PCR Array (SuperArray Biosciences Corporation, Frederick, MD, USA) was used. It profiled 84 genes related to host response to HIV Infection with five reference genes, RNA quality control, reverse transcription and PCR

efficiency controls, utilizing the StepOne Plus real time PCR machine (Applied Biosystems). Each well of the 96 well array plate contained lyophilized primers for a gene involved in HIV Infection and Host Response as indicated in Figure 3.1 below and Table 3.2 in Appendix 1.

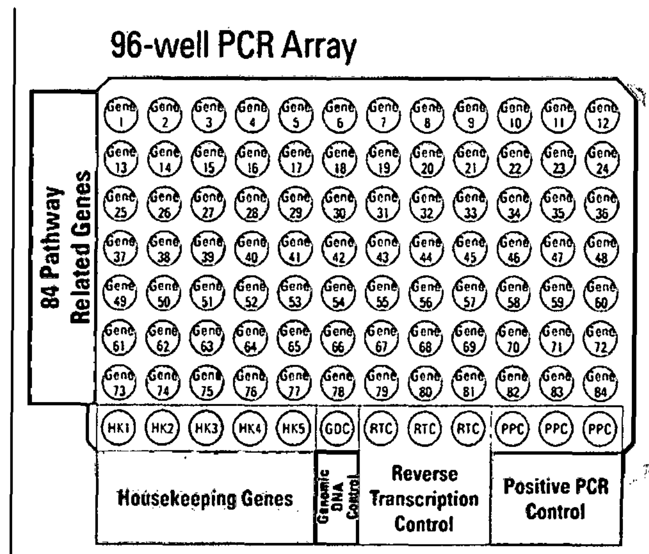


Figure: 3.1. Layout of the 96-well PCR array. Here columns designated as A, B, C, D, E, F, G, H and row indicates number of the wells from 1-12. Wells A1 through G12 contained a real-time PCR assay for genes that are related with HIV/AIDS. Wells H1 through H5 contained a housekeeping gene panel to normalize PCR Array data. Well H6 contained the Genomic DNA Control (GDC). Wells H7 through H9 contained replicate Reverse Transcription Controls (RTC). Wells H10 through H12 contained replicate Positive PCR Controls (PPC).

A total of 500 ng of RNA was reverse transcribed using the RT^2 First Stand Kit and RT-PCR carried out as described below. The resulting cDNA was diluted in 2X RT qPCR Master Mix (SA Biosciences) and water as described below, which was subsequently aliquoted to each well of the PCR array plate. The real time PCR was performed using the StepOne Plus (Applied Biosystems) real time PCR machine and data was analyzed with the Excel-based PCR Array Data Analysis Template uploaded in the SuperArray website (Appendix 4) and normalized to the expression level of reference control genes provided on the arrays.

RT^2 Profiler™ PCR Array Human HIV Infection and Host Response

RT reaction

1. All reagents were briefly (10-15 seconds) spun down.

2 The Genomic DNA Elimination Mixture (GE) was prepared as described below –

a. For each RNA sample, the followings were combined in a sterile PCR tube:

Total RNA	25.0 ng to 5.0	μg
GE** (5X gDNA Elimination Buffer)	2.0	μl
H_2O to a final volume of	10.0	μl

b. The contents were mixed gently with a pipette followed by brief centrifugation and incubated at 42°C for 5 min.

c. The tube was chilled on ice immediately for at least one minute.

2. The RT Cocktail was prepared as described below –

RT Cocktail	1 reaction	2 reactions	4 reactions
BC3 (5X RT Buffer 3)	4 μl	8 μl	16 μl
P2 (Primer & External Control Mix)	1 μl	2 μl	4 μl
RE3 (RT Enzyme Mix 3)	2 μl	4 μl	8 μl
H_2O	3 μl	6 μl	12 μl
Final Volume	10 μl	20 μl	40 μl

3. First Strand cDNA Synthesis Reaction:

a. 10 μl of RT Cocktail was added to each 10-μl Genomic DNA Elimination Mixture and mixed well gently with a pipette.

b. The tubes were incubated at 42°C for exactly 15 min and then immediately stopped the reaction by heating at 95°C for 5 minutes.

d. A total 91 μl of H_2O was to each 20-μl of cDNA synthesis reaction and mixed well.

e. The finished First Strand cDNA Synthesis Reaction was held on ice until, the real time PCR was carried out.

Real-Time PCR:

1. All reagents were spun down briefly (10-15 seconds).
2. Experimental Cocktail was prepared as described below:

Mix the following components in a 5-ml tube or a multi-channel reservoir:			
Plate Format: **Plate Format Designation:**	**96-well** **A, C, D, & F**	**384-well (4x96)** **E & G**	**384HT** **E & G**
2X SABiosciences RT^2 qPCR Master Mix	1350 μl	550 μl	2000 μl
Diluted First Strand cDNA Synthesis Reaction	102 μl	102 μl	102 μl
H_2O	1248 μl	448 μl	1898 μl
Total Volume	2700 μl	1100 μl	4000 μl

3. Loading the PCR Arrays

a. The PCR Array plate was removed carefully from its sealed bag.
b. 25 μl of the Experimental Cocktail was added to each well of the PCR Array plate, using an eight-channel pipette.
4. Performing Real-Time PCR Detection:
a. The PCR Array plate was tightly sealed with the optical adhesive film.
b. The plate was centrifuged for 1 full minute at room temperature at 1000 g to remove bubbles. The plate was visually inspected from underneath of the plate to ensure no bubbles are present in each well.
c. Plate was placed in real-time thermal cycler and under appropriate program (written below) the real time PCR was performed.

ABI:	**Cycles**	**Duration**	**Temperature**
StepOnePlus	1	10 minutes	95 ^{O}C
	40	15 seconds	95 ^{O}C
		1 minute	60 ^{O}C

d. Threshold Value was set manually by using the Log View of the amplification plots and place it as .1 for all the PCR plates.
e. All the results from different plates with different types of samples were copied on an Excel-based PCR Array Data Analysis Templates and uploaded on Web-based PCR Array Data Analysis Software (Appendix 4).

3.2.6. Selection of miRNAs and miTGs

We chose to assay the expression of miR-16, miR-146b-5p, miR-150, miR-191 and miR-223 in the PBMCs of healthy controls and HIV-infected persons in the four groups outlined above. These miRNAs were differentially expressed in PBMCs of HIV/AIDS patients (Houzet et al., 2008). The DIANA-microT 3.0 algorithm (Appendix 4) was used to identify the probable gene targets of the 5 miRNAs. These were compared to host genes identified through genome-wide siRNA screens to be involved in HIV infection (Brass et al. 2008, König et al. 2008, Zhou et al. 2008, Yeung et al. 2009). We also searched for validated targets of the select miRNAs from miRecords (Appendix 4), which is another web resource containing high-quality data for

experimentally proven miRNA targets. The miTGs identified through a combination of these methods were divided into host dependency factors (HDFs), immune response genes (IRGs) and genes involved in miRNA biosynthesis (GMBs). As down regulation of miRNA is likely to result in upregulation of the miTGs, we sorted out genes or proteins that showed increased expression following HIV infection either *in vivo* or *in vitro* in different types of cells using literature sources in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and Google Scholar (http://scholar.google.co.in/). The HDFs selected for evaluation were: DDX3, MAP4, STMN1, UGP2, MDN1, FOXO3, CD43 and LDLR. The IRGs were selected from the same groups of patients and controls using a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) array (SA Bioscience, Frederick, MD, USA) in the previous part of the study. The upregulated genes in the arrays were analyzed using miRWalk (Appendix 4), which is a database of predicted and validated miRNA targets. The IRGs targeted by the 5 miRNAs - SERPINC1, HMGA1, CASP3, STAT3, STAT1, CXCR4, TNFSF-10 and CASP8, were included in the study. Using the same database we also found the miRNAs to target the following GMBs – DICER, DROSHA, AGO1 and AGO2. The expression levels of the selected miTGs were estimated by quantitative Real-time PCR as below.

3.2.7. Real-time PCR for Expression of genes/miTGs

Relative quantitation with real-time RT-PCR was performed using specific primers and conditions outlined in Table 3.3. (Appendix 1). The protocol for real time PCR for the gene/miTG is described below. Each sample was assayed in duplicate and the median threshold cycle (Ct) values were used to calculate the expression ratios.

Real-time PCR for Expression of genes/ miTGs

1. cDNA synthesis

For 50 µl of Reverse Transcription (RT) reaction

a. Reaction mixture A:

Total RNA (From PBMC or Whole Blood): 1.0 µg

Oligo dT (500 ng): 2.0 µl

Nuclease free H_2O to a final volume of	33.5 µl

This reaction mixture was kept at 65°C for 5-10 minutes, and then snap chilled in ice-water for 5 minutes.

b. Reaction mixture B: Master Mix

5 X buffer	10.0 µl
dNTPs	2.5 µl
RNAse inhibitor	2.0 µl
RT enzyme	2.0 µl

c. Reaction mixture A and B were added and kept at 42°C for 1 hour.

d. To dilute the RT product, 150 µl of nuclease-free water was added to 50 µl of RT product and used for real time PCR immediately or stored at -20°C for future use.

2. Real time PCR

For 20 µl of reaction

EvaGreen	4.0 µl
Diluted Forward primer of a gene/ miTG	0.5 µl
Diluted Reverse primer of a gene/ miTG	0.5 µl
Nuclease free H2O	11.0 µl
Diluted RT product	4.0 µl
Total	20.0 µl

The PCR reaction for different genes / miTGs were set with the PCR condition mentioned in Table 2.3

3.2.8. miRNA Assay

The miRNAs were assayed individually in each sample using Taqman MicroRNA® Assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol shown below.

Preparation of RT master mix using the TaqMan MicroRNA Reverse Transcription Kit

1. The kit components were allowed to thaw on ice.
2. In a polypropylene tube, the RT master mix was prepared by scaling the volumes listed below to the desired number of RT reactions. To account for pipetting losses, 10% overage was added.

Component	Master Mix Volume/ 15-µL Reaction[a]
100mM dNTPs (with dTTP)	0.15
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00
10 Reverse Transcription Buffer	1.50
RNase Inhibitor, 20U/µL	0.19
Nuclease-free water	4.16
Total	7.00

Each 15 µL of RT reaction consists of 7 µL of master mix, 3 µL primer, and 5 µL RNA sample.

3. The mixture was mixed gently and centrifuged to bring solution to the bottom of the tube.
4. The RT master mix was placed on ice until the microRNA reaction was prepared.

Preparation of the RT Reaction

1. For each 15 µL of RT reaction, 7 µL RT master mix was combined with 5 µL total RNA
2. The solution was mixed gently and centrifuged to bring the solution to the bottom of the tube.
3. The RT Primer tubes were thawed before opening the tubes on ice and mixed by vortexing and then centrifuged.
4. For each 15 µL RT reaction, 12.0 µL of RT master mix containing total RNA was dispensed into a 0.1 mL polypropylene reaction tube.
5. 3 µL of RT primer (tube labeled RT Primer) was transferred from each assay set into the corresponding RT reaction tube or plate well.
6. The tube was sealed and solution was mixed gently. To bring solution to the bottom of the tube was centrifuged.
7. The tube was incubated on ice for 5 min and keep on ice until the thermal cycler was ready to load.

Performing Reverse Transcription in PCR machine

1. The following parameter values were set to program the thermal cycle

Step Type	Time (min)	Temperature (°C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	∞	4

2. The reaction volume was made up to 15.0 μL.
3. The reaction tubes or plate were loaded into the thermal cycler.
4. The reverse transcription was started.

Preparation of the Real time PCR Reaction

1. The volumes listed below were scaled to the appropriate number of RT reactions. The solution was prepared on ice.

Reagent	Master Mix Volume for One 20-μL Reaction
TaqMan 2 Universal PCR Master Mix, No AmpErase UNG	10.00
Nuclease-free water	7.67
Total Volume	**17.67**

2. The solution was mixed gently and centrifuged to bring solution to the bottom of the tube.
3. 17.67 μL of the PCR master mix/water was added per 20 μL PCR reaction into a polypropylene tube (the PCR reaction tube), as shown in the following example.

Volume for One 20-μL Reaction	Example: Volume for 4 replicates[a]
17.67	17.67 μL x 4 replicates = 70.68 μL + 8.8 μL excess = 79.48 μL

4. 1.0 μL of 20 X TaqMan MicroRNA Assay mix (labeled Real Time) was transferred into the PCR Reaction tube, as shown in the following example.

Volume for One 20-μL Reaction	Example: Volume for 4 replicates[a]
1.0	1.0 μL x 4 replicates = 4 μL + 0.5 μL excess = 4.5 μL

5. 1.33 μL of the RT product was transferred from the RT reaction tube into the PCR reaction tube.

6. The mixture was mixed gently and centrifuged to bring solution to the bottom of the plate.
7. The PCR reaction plate was prepared by dispensing 20 µL of the complete PCR master mix (including primer and RT product) into each of four wells.
8. The plate was sealed with an optical adhesive cover, and then the plate was centrifuged briefly to spin down the contents and eliminate any air bubbles.

Set Up the Real time PCR machine

The following parameter values were set to program the real time PCR machine.

Step	AmpliTaq Gold® Enzyme Activation	PCR	
	HOLD	CYCLE (40 cycles)	
		Denature	Anneal/ Extend
Time	10 min	15 sec	60 sec
Temp (°C)	95	95	60

3.2.9. Viral Load Measurement

HIV-1 plasma viral loads were quantitated using an in-house reverse-transcriptase TaqMan real-time PCR assay using specific primers and probes selected in the *gag* region of the Indian HIV-1 subtype C strain (Table 2.3). A plasma sample that contained 150,000 copies/ml (NIH AIDS Reagent Bank) was similarly treated and used to obtain the standard curve. The viral load assay procedure is outlined below.

Plasma HIV RNA isolation

QIAamp Viral RNA Mini Kit was used for isolation of HIV RNA. The procedure was as follows-

1. 5.6 µl of RNA carrier was added to 560 µl of AVL solution and mixed
2. 560 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube.
3. 100 µl plasma was added to the Buffer AVL–carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 s. The tube was

incubated at room temperature (15–25°C) for 10 min.

4. 560 µl of ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 15 s. After mixing, the tube was centrifuged briefly to remove drops from inside the lid.
5. 630 µl of the solution from step 4 was carefully applied to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. The cap was closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate was discarded. If required, this step was repeated.
6. The QIAamp Mini column was opened carefully, and 500 µl of Buffer AW1 was added. The cap was closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed in a clean 2ml collection tube, and the tube containing the filtrate was discarded.
7. The QIAamp Mini column was opened carefully, and 500 µl of Buffer AW2 was added. The cap was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.
8. The QIAamp Mini column was placed in a new 2 ml collection tube, and centrifuged at full speed for 1 min.
9. The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube. The old collection tube containing the filtrate was discarded. The QIAamp Mini column was opened carefully and 60 µl of Buffer AVE equilibrated to room temperature was added. The cap was closed and the tube was incubated at room temperature for 1 min and then centrifuged at 6000 x g (8000 rpm) for 1 min.
10. Viral RNA collected in the tube was stored at –20°C.

Plasma HIV RNA estimation by real time PCR

1. cDNA synthesis

a. Reaction mixture A:

Random primers	0.5 µl
dNTPs (100mM)	0.1 µl
Oligo dT	0.5 µl
H_2O	0.9 µl
Viral RNA	10 µl

Total 12.0 µl

This reaction mixture was kept at 65°C for 5-10 minutes, and then snap chilled in ice-water for 5 minutes.

b. Reaction mixture B:

5 X First Strand buffer 4 µl

0.1 M DTT 2 µl

RNAse Inhibitor 1 µl

Total 7 µl

The mixture was kept in room temperature for 25°C for 2 minutes, and then 1 µl of SuperScript® II Reverse Transcriptase was added to the tube.

c. Reaction mixture A and B were added (Total 20 µl) in PCR tube and PCR was done after setting the following parameter values to program the machine.

Duration	**Temperature**
10 minutes	25°C
50 minutes	42°C
15 minutes	70°C

2. Real time PCR

TaqMan Master MIx 10.0 µl

Diluted HIV primer 1 and 2 0.5 µl each

Probe 2 µl

Nuclease free H2O 2 µl

Diluted RT product 5 µl

Total 20 µl

Following parameter values were set to program real time PCR machine

ABI:	**Step**	**Duration**	**Temperature**
StepOnePlus	Hold	2 minutes	50°C
		10 minutes	95°C
	Cycles (40)	15 seconds	95°C
		1 minute	60°C

3.2.10. miRNA-Targeted Pathway Analysis

To categorize the molecular pathways potentially altered by miRNA

expression, we first used a web-based computational tool called DIANA-mirPath (Appendix 4). Moreover, as miRNA expression is tissue-specific, we also analyzed the miRNA-mediated regulation of signaling pathways that were likely to be affected in the blood compartment, using the miTALOS online tool (Appendix 4).

3.2.11. Multiplex Cytokine Assay

3.2.11.1. Preparation of reagents for immunoassay

3.2.11.1.1. Preparation of Antibody-Immobilized Beads

The premixed bead bottle was sonicated for 30 seconds and then vortexed for 1 minute before use.

3.2.11.1.2. Preparation of Quality Controls

Before use, vial named as "Quality Control 1" and "Quality Control 2" were reconstituted with 250 µL-deionized water. The vials were inverted several times to mix and vortex. The vial was allowed to sit for 5-10 minutes and then the controls were transferred to appropriately labeled polypropylene microfuge tubes. Unused portion was stored at -20°C.

3.2.11.1.3. Preparation of Wash Buffer

The 10X wash buffer was brought to room temperature and mixed to bring all salts into solution. 30 mL of 10X wash buffer was diluted with 270 mL deionized water.

3.2.12.1.4. Preparation of Serum Matrix

1.0 mL of deionized water was added to the bottle containing lyophilized Serum matrix and mixed well. At least 10 minutes were allowed for complete reconstitution.

3.2.11.1.5. Preparation of Human Cytokine Standard

Prior to use, the Human Cytokine Standard was reconstituted with 250 µL-deionized water to give a 10,000 pg/mL concentration of standard for all analytes. The vial was inverted for several times to mix. The vial was vortexed

for 10 seconds and allowed to sit for 5-10 minutes and then transferred to an appropriately labeled polypropylene microfuge tube. That was used as the 10,000 pg/mL standard; the unused portion was stored at -20°C.

Preparation of Working Standards: Five polypropylene microfuge tubes were labeled as 2000, 400, 80, 16, and 3.2 pg/mL. 200 µL of assay buffer was added to each of the five tubes. Serial dilutions were prepared by adding 50 µL of the 10,000 pg/mL reconstituted standard to the 2000 pg/mL tube, mixed well and 50 µL of the 2000 pg/mL standard was transferred to the 400 pg/mL tube, mixed well and 50 µL of the 400 pg/mL standard was transferred to the 80 pg/mL tube, mixed well and 50 µL of the 80 pg/mL standard was transferred to 16 pg/mL tube, mixed well and 50 µL of the 16 pg/mL standard was transferred to the 3.2 pg/mL tube and mixed well. The assay buffer was used as the 0 pg/mL standard (Background).

3.2.11.2. Immunoassay procedure

All reagents were allowed to warm to room temperature (20-25°C) before use in the assay.

The placement of Standards [0 (Background), 3.2, 16, 80, 400, 2000, and 10,000 pg/mL], Controls 1 and 2, and Samples was drawn on Well Map.

All the assays were performed in duplicate.

1. The filter plate was preweted by pipetting 200 µL of assay buffer into each well of the Microtiter Filter Plate and sealed and mixed on a plate shaker for 10 minutes at room temperature (20-25°C).

2. Assay buffer was removed by vacuum. Excess assay buffer was blotted from the bottom of the plate with an absorbent pad or paper towels.

3. 25 µL of each Standard or Control was added into the appropriate wells. The assay buffer was used for the 0 pg/mL standard (Background).

4. 25 µL of assay buffer was added to the sample wells.

5. 25 µL of serum matrix was added to the background, standards, and control wells.

6. 25 µL of Sample was added into the appropriate wells.

7. 25 µL of Premixed Beads was added to each well

8. The plate was sealed with a plate sealer and covered with the lid. A rubber band was wrapped around the plate holder, plate and lid and incubated with agitation on a plate shaker for 1 hour at room temperature (20-25°C).

9. The fluid was gently removed by vacuum.

10. The plate was washed 2 times with 200 µL/well of wash buffer; wash buffer was removed by vacuum filtration between each wash. Excess wash buffer was blotted from the bottom the plate by an absorbent pad or paper towels.

11. 25 µL of Detection Antibodies was added into each well.

12. The plate was sealed and covered with lid, and incubated with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).

13. 25 µL Streptavidin-Phycoerythrin was added to each well containing the 25 µL of Detection Antibodies.

14. The plate was sealed and covered with lid, and incubated with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).

15. All contents were gently removed by vacuum.

16. The plate was washed 2 times with 200 µL/well wash buffer; wash buffer was removed by vacuum filtration between each wash. Excess buffer on the bottom of the plate was wiped with a tissue paper.

17. 150 µL of sheath fluid was added to all wells. The beads were resuspended on a plate shaker for 5 minutes.

18. Plate was ran on Luminex 100™ IS.

19. Data was saved and analyzed in the Median Fluorescent Intensity (MFI) data using a weighted 5- parameter logistic.

3.2.12. ^{1}H-NMR and Heteronuclear Single Quantum Coherence (HSQC) spectroscopy

For ^{1}H NMR spectroscopy, 100 µl of plasma, 400 µl of urine or 100 µl of saliva were used; the volume was made up to 500 µl with D_2O (Deuterium oxide, 99.8%) and 0.0005% DSS. These were placed in 5 mm NMR tubes (Wilmad, Sigma Aldrich, USA). All NMR experiments were carried out at 298 K on a Bruker Avance III spectrometer equipped with cryogenic triple-resonance probes, operating at the field strength of 500 MHz. One-dimensional ^{1}H NMR spectra were measured with 32 scans with a relaxation delay of 4 sec. For each sample free induction decays (FIDs) were collected with a spectral width of 6009.62 Hz and acquisition time of 2.73 sec (t_{1max}). The plasma, urine and saliva spectra were referenced to internal reference DSS at 0 ppm. All NMR spectra were processed using Topspin 2.1 (Bruker AG). The spectral region between 6.0 and 4.5 ppm was set to zero integral to remove the effects of difference in the suppression of the water resonance and the effects of variation in the urea signal caused by partial cross-solvent saturation by means of solvent-exchanging protons.

Heteronuclear Single Quantum Coherence (HSQC) experiments (^{1}H-^{13}C) were carried out using liquid state high resolution NMR spectroscopy and a Bruker Avance 500 MHz NMR spectrometer equipped with a 5 mm TCI cryoprobe, variable temperature supply and pulsed field z gradients. The ^{1}H-^{13}C gradient enhanced NMR spectra were acquired using the standard library gradient enhanced pulse sequence at the central frequencies of 500.1323541 MHz (^{1}H, F2 dimension) and 125.7691082 MHz (^{13}C, F1 dimension).

Statistical analysis and annotation of the NMR spectra were carried out using 'MetaboAnalyst' (Appendix 4). Through different statistical analyses a list of NMR peaks was developed and corresponding metabolites for those peaks were identified from the metabolites library in the Human Metabolome Data Bank (HMDB) and MetaboMiner, which are linked with MetaboAnalyst. In addition, we confirmed the list of metabolites by comparing their ^{1}H and ^{13}C chemical shifts and coupling patterns with corresponding values of metabolites that were previously published (Lindon et al., 1999; Nicholson et

al., 1996) or available from HMDB (Appendix 4). We identified the pathways most involved in HIV/AIDS infection by using the MetPA (Metabolomic Pathway Analysis), which is web based pathway analysis software (Appendix 4).

3.2.13. Statistical Analysis

DataAssist™ v2.0 was used to estimate the expression differences of miRNAs between different groups of patients and controls. This software uses the comparative Ct (ΔΔCt) method for relative quantitation of gene expression. To test the relationship between paired miRNA-miTG profiles, the Pearson correlation coefficients and p-values were computed using SPSS (SPSS, version 17; SPSS Inc., Chicago, Ill). In order to evaluate the predictive value of each miRNA in defining the status of HIV/AIDS patients, Receiver Operating Characteristic (ROC) curve analysis was applied and area under the curve (AUC) was calculated. All the comparisons between the fold changes and box plots were prepared using Excel software and SSP (Mac OS X) respectively. In all the statistical tests, $p < 0.05$ was considered to be significant.

Chapter 4: Results and Discussion

Result and Discussion 1

Objective:

To characterize gene expression profiles of whole blood and PBMCs of HIV/AIDS patients in different stages of disease and therapy.

Summary:

HIV infects CD4+ cells and disease progression is associated with (1) a decline in the numbers of CD4+ T cells that far outnumber infected cells, and (2) a steady loss in the ability of HIV-specific CD8+ T cell responses to limit viral replication. Death due to AIDS occurs not directly due to HIV infection, but due to opportunistic infections, rare cancers or metabolic disorders. The control of infections caused by different bacteria depends on the concerted action of immune cells including neutrophils, eosinophils, macrophages, T and B cells. Moreover, cytokines and other chemical mediators produced from these cells also have an important role in disease pathogenesis. While numerous studies have used arrays to observe the differential expression of genes in individual cell types involved in HIV/AIDS pathogenesis, cellular gene expression in whole blood has not been studied earlier. In this study we have analyzed the gene expression differences between the whole blood cells and PBMC compartments by isolating RNA directly from these samples and applied to PCR arrays to identify differentially expressed genes in these two sets of cells among the different groups of patients mentioned above. We selected 12 genes - CASP3, CASP8, CCL2, CCL5, CCL8, CXCR4, HMGA1, IL10, IL1 ß, STAT1, STAT3 and TNFSF-10 from the PCR array results. These were differentially regulated in whole blood and PBMCs among the patients for validation on a larger set of samples. Through a multiplex cytokine assay, we have also checked the plasma levels of the cytokines CCL2/MCP-1, CCL8/MCP-2, IL10, IL-1ß and TNFSF-10 to correlate gene expression changes that were determined in whole blood and PBMCs by real-time PCR. Our results indicate that gene expression of whole blood cells differ markedly from that of PBMCs. A more comprehensive understanding of HIV/AIDS pathogenesis will require moving beyond just looking at the PBMC fraction or whole blood during infection and disease.

Result:

PCR Array analysis:

To elucidate differential gene expression in whole blood and the PBMC fraction, real time-PCR-based gene array was carried out on RNA extracted directly from these samples belonging to 5 groups of study subjects – healthy controls, HIV+ asymptomatic, HIV+ symptomatic, HIV+ on ART and HIV+ showing ART resistance. The PCR array plate contained primer pairs for 84-host response genes known to be differentially expressed following HIV infection and development of AIDS. Three individual subjects were randomly chosen from each of the groups listed above for the PCR array analysis. For selection of differentially regulated genes, the genes that showed >1.5-fold up regulation or <0.5-fold down regulation and were statistically significant ($p<0.05$) in any group of patients, either in whole blood or PBMCs was set as a criteria. According to these criteria, 12 genes showed differential regulation in asymptomatic and symptomatic patients, after ART initiation and following ART resistance (Figure 4.1.1). These were CASP3, CASP8, CCL2/MCP-1, CCL5, CCL8/MCP-2, CXCR4, HMGA1, IL-10, IL-1β, STAT1, STAT3, and TNFSF-10.

Validation of PCR array:

To validate the PCR array results, independent real-time PCR analyses were carried out on the genes of interest. The correlation between PCR array data and real-time PCR was confirmed by plotting the fold changes of PCR array and real-time PCR results (Figure 4.1.2 & Figure 4.1.3). There was a good correlation between the results obtained from PCR array and real-time PCR of whole blood and PBMC (Pearson correlation coefficient 0.63 and 0.59 with $p < 0.0001$, respectively).

The overall expression of CASP3 in whole blood showed no change and in PBMC it showed up regulation. Compared to healthy controls, the whole blood CASP3 profile showed no significant change, except in patients on ART ($p<0.05$), while in PBMCs it was progressively upregulated, peaking in patients showing ART resistance ($p = 0.01$) (Figure 4.1.4). Alternatively,

CASP8 showed an overall down regulation trend in the whole blood sample but an upregulation trend in the PBMCs of all groups of patients.

On looking at the data on chemokine-related genes, while in PBMCs, CCL2/MCP1 showed an upward trend in symptomatic and ART groups of patients, the changes were not significant. On the other hand in the whole blood sample from the same patients, we observed significant up regulation in symptomatic and ART resistance groups when compared to healthy controls ($p<0.05$). In other words, CCL2/MCP1 expression in whole blood is significantly down regulated on commencing ART and is upregulated as patients show ART resistance. The expression of CCL5 also showed similar downward trend in asymptomatic and symptomatic groups in whole blood and PBMC, and after initiation of ART significant up regulation was observed ($p<0.05$). The expression of CCL8/MCP2 showed no significant change in PBMCs, but was found to be significantly upregulated in the whole blood samples from symptomatic and ART resistance patients. In other words, the expression of CCL8/MCP2 in whole blood went down on commencing ART ($p<0.05$) and went up again as patients developed ART resistance ($p<0.05$).

Coming to the expression of cytokine genes and their related factors, STAT1 expression showed an upward trend in both categories of cells from symptomatic patients. The expression of STAT3 did not show any significant change in either whole blood or PBMCs from any group of patients. The expression of IL-1β was not significantly regulated in whole blood or PBMCs of any of the groups, except in patients failing ART, in whom it showed reduced expression. Though IL-10 expression in PBMC showed an upward trend in various groups of patients compared to healthy controls, the levels were not statistically different. However, in the whole blood , IL-10 expression was significantly increased in various groups of patients. There was significant up regulation in the expression of TNFSF-10 in both types of samples; this marker remained upregulated during the whole disease process. However, on ART introduction the expression levels of TNFSF-10 reduced significantly when compared with symptomatic patients.

The expression levels of the HIV-1 coreceptor CXCR4 showed down regulation in the whole blood and PBMC fraction of asymptomatic persons as well as in the PBMCs of patients on ART; all other groups of patients showed no significant change. The expression of HMGA1 also did not change significantly in any group of patients.

Overall there was good agreement between the data obtained from the 84-gene array (Figure 4.1.1) and the real-time PCR validation carried out on whole blood (Figure 4.1.2) and PBMC (Figure 4.1.3) fractions from individual patients. But, there was little agreement between the gene expression data derived from whole blood and PBMCs. This is apparent from the heat map (Figure 4.1.1) and the box plots for whole blood (Figure 4.1.2) and PBMCs (Figure 4.1.3). These results call for caution in comparing studies that use one or the other sample.

Cytokine levels in patient plasma:

Among the 12 differentially regulated genes, 5 cytokines i.e. CCL2/MCP1, CCL8/MCP2, IL10, IL1β and TNFSF-10 were selected for further validation at the protein level in the plasma of subjects belonging to the five study groups (Figure. 4.1.5). The Multiplex Cytokine Assay was used for that purpous. These cytokines were chosen to correlate whether their gene expression levels in whole blood and/or PBMCs were also reflected in their protein expression levels. Compared to healthy controls the IL-1β levels were significantly higher in the asymptomatic ($p<0.001$) and symptomatic ($p<0.01$) groups. In patients on ART these reduced significantly ($p<0.01$) and remained low in patients who developed ART resistance ($p<0.05$). A similar upward trend was also observed in the case of IL-10 whose levels in the asymptomatic and symptomatic groups were significantly higher than healthy controls ($p<0.001$). Following the introduction of ART, the levels of IL-10 were significantly reduced ($p<0.01$). Interestingly IL-10 also remained low in the ART resistance group. Compared to healthy controls, the plasma levels of CCL2/MCP-1 were significantly raised in the symptomatic and ART resistance groups. Though the levels of CCL2/MCP-1 were lower in the ART group, it showed a significant increase following the development of ART resistance.

Significantly increased levels of MCP-2 were observed only in symptomatic AIDS patients in comparison to healthy controls. Compared to healthy controls, there was a significant increase in TNFSF-10 levels in symptomatic ($p<0.001$) and ART resistant ($p<0.01$) patients, while ART introduction significantly reduced the amounts of plasma TNFSF-10 ($p<0.01$). Following the development of ART resistance, the TNFSF-10 levels again went up ($p<0.05$).

Protein - Protein interaction:

We were also interested in understanding the inter-relationships between the differentially regulated genes (proteins) identified in this study. To investigate this, we searched the STRING 8.0 database for protein interactions, and found these proteins to be highly interlinked (Figure 4.1.6). Further, all the identified genes were functionally grouped into different gene ontology (GO) groups (Table 4.1.1). The clusters developed from these 12 genes (proteins), indicated that in HIV/AIDS, several molecular functions are modulated, which include cytokine receptor binding, protein binding, signal transducer activity, etc. The STAT3 protein appears to a node that links various parts of this network.

Discussion:

Blood being a complex tissue, it contains a variety of cell types that include granulocytes, T-cells, B-cells, monocytes, NK cells, etc. Neutrophils are the most abundant cell type in blood, the abundance of which normally varies in the range of 30-70% of white blood cells in healthy adults. The numbers of monocytes are 2-10% of PBMCs (Kaspekasr et al., 2005; Kuby, 1997) and within the lymphocyte subsets; the comparative proportion of T- and B-lymphocytes can vary from 61-85% and 7-23%, respectively (Reichert et al., 1991). In addition, the ratio of CD4+ T-cells to CD8+ T-cells can fluctuate from <1.0 to 2.0 (Shahabuddin, 1995). The relative proportion of all these cell types can vary between individuals and with states of health and disease, and in response to stimuli.

The pathophysiology of a disease is under systemic control in the infected

host. Therefore, studying whole blood cells along with individual cell types would provide a more representative scenario of the diseased state. Unfortunately whole blood cells and/or granulocytes/neutrophils are frequently excluded from analyses of gene expression in human blood. There is almost no work done on gene expression of whole blood cells or granulocytes or neutrophils in HIV infection. On the contrary, many studies are reported based on PBMCs. In HIV infection, in comparison to PBMC, there are fewer publications on the role of neutrophils or their gene expression profiles but following HIV infection, several bacterial conditions are reported that are related with abnormalities of neutropenia or neutrophil dysfunction, including abnormal chemotaxis, phagocytosis, oxidative metabolism and bacterial killing (Ellis et al., 1988; Pitrak et al., 1993; Pitrak et al., 1997). Though opportunistic infections due to poor cell-mediated immunity are the characteristics of AIDS, infections with common bacteria also occur with increased rates of recurrence and severity in HIV-infected patients with high rates of morbidity and mortality (Witt et al., 1987; Nichols et al., 1989, Kielhofneer et al., 1992, Pitrak et al., 1993; Barat et al., 1996; Stein et al., 1992). In addition, cytokines are thought to play an important role in the HIV-specific immune responses (Cocchi et al., 1995; Nicastri et al., 2001) and these are produced from different blood cells and other tissues. Therefore, an overall view of HIV/AIDS pathogenesis obtained through studying cellular gene expression changes in whole blood cells along with individual cell types would provide valuable information in its own right.

In the present study, we performed a PCR array analysis on whole blood cells and PBMCs and selected 12 genes with differential regulation for further validation. These genes are CASP3, CASP8, CCL2/MCP-1, CCL5, CCL8/MCP-2, CXCR4, HMGA1, IL-10, IL-1β, STAT1, STAT3 and TNFSF-10. We discovered that the patterns of regulation in whole blood and in PBMCs are not always in the same direction with regards to disease progression, therapy and failure of therapy. Further, the gene expression pattern is not always reflected in the plasma levels of the secreted proteins. We discuss below the roles significantly modulated genes (proteins) in cellular pathways and relate these to HIV/AIDS pathogenesis.

Apoptosis: Caspase 3 and 8

An elevated level of apoptosis is observed in HIV-1 infected individuals (Ameisen et al., 1991), which can be tracked to various types of cells (Salmen et al., 2004; van Grevenynghe et al., 2011; Cummins et al., 2010). Since Caspase 3 is the central executor molecule in apoptosis and Caspase 8 is important for transducing external death signals, it was important to test their expression at various stages of HIV disease progression and therapy. Though not always statistically significant in the sample size tested, we found both genes to be upregulated in the PBMCs of symptomatic patients. However, when tested in whole blood the expression levels for these genes either showed no overall change or even a downward trend. The main constituents of PBMC are T-cells, B-cells and monocytes. In earlier studies, CASP3 was found to be upregulated in activated CD4+T cells but was down regulated in a chronically infected HIV-1 cell line H61 (Sedaghat et al., 2008; Olivares et al., 2009). Moreover, in a group of HIV patients, CASP3 expression was found to be elevated in blood myeloid dendritic cells (mDC), but was depressed in lymph node mDCs (Dillon et al., 2011). Though in a T-cell line and in primary CD4+ T cells, the HIV Tat protein (Bartz et al., 1999) showed increased expression and activity of CASP8, we did not find any changes in the PBMC of asymptomatic HIV infected person, but observed increased CASP8 expression in symptomatic patients.

The expression of CASP3 was increased in the PBMCs of patients showing ART resistance compared to those on therapy. This may be due to the augmented apoptotic state in PBMCs or more specifically apoptosis in B cells by nucleoside or non-nucleoside reverse transcriptase inhibitors (NRTI/ NNRTI). Our study population was on AZT, which is known to cause apoptosis in B cells (Feola & Garvy, 2005; Chong et al., 2004). It has been shown earlier that Lamivudine, another antiretroviral drug, which was used in treating our study population, is associated with persistent T-cell apoptosis (de Oliveira et al., 2002). Increased CASP3 activity in the PBMCs of HAART recipients was reported earlier (Weinberg et al., 2004). Further, increased expression of CASP8 in the PBMCs of patients on ART in our study is supported by previous findings of higher mean value of CASP8 activity in the

PBMCs of HAART recipients compared to healthy controls (Weinberg et al., 2004).

In whole blood, while CASP3 showed no significant change, CASP8 was down regulated in symptomatic patients and its expression levels increased following ART, which is opposite of the gene expression patterns observed in PBMCs. This may be due to reduced apoptosis of neutrophils or due to neutropenia caused by the NNRTI and NRTI drugs (Moyle et al., 2004; Moore et al., 2001). These results indicate that in HIV infection the regulation of apoptotic genes occur in a divergent manner in different cellular compartments during the natural course of infection and even after commencement of ART.

Cytokine expression:

CCL2/MCP1

The macrophage chemoattractant protein 1(MCP-1), also known as CCL2, binds to the CCR2 receptor and acts as a chemoattractant for monocytes, T lymphocytes and NK cells *in vitro* (Rollins, 1996; Carr et al., 1994; Matsushima et al., 1989). In the present study, significantly higher plasma levels of MCP-1 were observed in symptomatic AIDS patients, the levels subsequently dropping down on commencement of ART. Similar findings were reported earlier (Haissman et al., 2009; Yao et al., 2007). The levels of MCP1 were again increased in patients showing ART resistance. Ansari et al (2006) found higher MCP1 mRNA and protein levels in PBMC and plasma respectively and a higher percentage of MCP1-expressing CD14+ monocytes in viremic patients who are on HAART. We observed significant upregulation of MCP1 mRNA in whole blood as well as PBMCs of symptomatic patients; these levels went down after ART and increased again in whole blood (but not PBMCs) following ART resistance. Such regulation of the MCP1 gene is likely to be due to the differential regulation of MCP1 in polymorphonuclear neutrophils (PMN), which is reflected in whole blood but not in PBMCs. Earlier it has been shown that PMN exposed *in vitro* to CCR5-utilizing HIV can produce significant amounts of MCP1 (Yoshida et al., 2007). Additionally, HIV can also infect vascular smooth muscle cells *in vivo* and *in vitro* and can

increase the secretion of CCL2/MCP1 (Eugenin et al., 2008).

CCL8/MCP2

We found plasma macrophage chemoattractant protein 2 (MCP-2) levels to be significantly higher in symptomatic patients. The MCP2 protein binds with high affinity to the CCR5 chemokine receptor, which is the major co-receptor for macrophage-tropic strains of HIV-1. *In vitro*, CCL8/MCP2 was found to block CD4/CCR5-mediated HIV-1 entry (Gong et al., 1998) and works as a chemoattractant for monocytes and T cells (Balestrieri et al., 2008). The MCP2 protein is produced mainly from peripheral blood leukocytes (Van Damme et al., 1994) but in our study, though the MCP2 mRNAs were up regulated in PBMCs, it was not significant. Rather we found MCP2 gene expression to be significantly upregulated in whole blood of all classes of HIV-infected persons, being highest in symptomatic patients and those showing ART resistance. This suggests that granulocytes may also have some role in the production of MCP2. Interestingly, granulocytes are regarded as inefficient MCP2 producer *in vitro* (Van Damme et al., 1994). It thus remains to be studied in case of HIV infection from which type of cells MCP2 is released *in vivo*. Alternatively, interleukin-1 and interferon-γ induced fibroblasts and epithelial cells could also be a good source of MCP2 (Van Damme et al., 1994; Struyf et al., 1998). Though ART could only bring a small change in the MCP2 mRNA levels in PBMCs, observed earlier by others (Wasmuth et al., 2004), we found significant down regulation MCP2 mRNA in whole blood following ART and its increase again following ART resistance.

IL-10

As shown previously (Haissman et al., 2009) we found significant increase in the plasma levels of IL-10 in asymptomatic and symptomatic patients, which was decreased following ART administration. Several cell types within PBMCs including monocytes, macrophages, T-cells and B-cells secrete IL-10 (Howard and O'Garra, 1992). Though it was previously shown that IL-10 mRNA levels in the total PBMC fraction from HIV-infected viremic individuals were significantly elevated relative to healthy controls (Brockman et al., 2009), we did not find any significant change in the expression of IL-10 in PBMCs

among the different groups of patients. This is also supported by another study (Brazille et al., 2003). On the other hand, in whole blood cells, IL-10 mRNA showed significant upregulation in HIV patients, which indicates that granulocytes may be a major source of IL-10 in HIV infection; this was previously shown *in vitro* as well (Yoshida et al., 2007).

IL-1 beta.

IL-1β is a pro-inflammatory cytokine expressed by many types of cells including monocytes, macrophages, NK cells and neutrophils. We found decreased levels of IL-1β mRNA in the whole blood fraction of HIV patients who were symptomatic and showed ART resistance, the latter also matched by the PBMC fraction. However, plasma IL-1β levels were significantly elevated in infected persons, coming down with ART. There are several conflicting reports on the levels of IL-1β in HIV patients. While Reddy et al (1989) found no significant change, Belec et al (1994) found increased levels in AIDS patients and Ullum et al (1997) found decreased levels. The primary sources of plasma IL-1β are monocytes and tissue macrophages. In animal studies, after stimulation with LPS, splenic macrophages show the highest local production of IL-1β (Ge et al., 1997). In HIV infection, higher levels of IL-1β may be due to its increased production from the spleen or other organs. This could explain the discrepancy between gene expression in peripheral blood and plasma IL-1β levels.

TNFSF-10/TRAIL

We found the mRNA levels of TNFSF-10 in whole blood and PBMCs to broadly agree with the plasma levels of this cytokine in the various patient groups. Moreover this is the only cytokine, which changed significantly in each group and corresponded with the clinical stages of infection and therapy. TNFSF-10/TRAIL is secreted by leukocytes, including T lymphocytes (Kayagaki et al., 1999), NK cells (Smyth et al., 2001), dendritic cells (Vidalain et al.2000; Vidalain et al., 2001), monocytes and macrophages (Herbeuval et al.,2003), and is thought to have a role in killing T cells in HIV infection (Esser et al.,2001). High serum interferon-alpha (IFN-α) levels found in HIV/AIDS patients (Grunfeld et al., 2004) may stimulate the expression of TNFSF-

10/TRAIL mRNA and the release of high amounts of its soluble form from neutrophils and monocytes (Tecchio et al., 2004). In addition, HIV-1 infection of human lymphoid tissue *ex-vivo* resulted in increased TRAIL production (Herbeuval et al., 2005). This implies that in HIV infection almost all the blood cells significantly modulate TRAIL secretion in all the stages of infection, suggesting that it could be a good biomarker of HIV/AIDS disease progression and prognosis after ART.

CCL5/RANTES

CCL5, more commonly known as RANTES, is the natural ligand for the HIV-1 coreceptor CCR5 (Samson et al., 1996). It suppresses HIV-1 infection by directly competing with the CCR5-tropic envelope gp120 of HIV for it's binding to the coreceptor and by down regulating the cell surface levels of CCR5 (Cocchi et al., 1995). We found decreased expression of the CCL5 gene in the whole blood of symptomatic patients but increased expression in the PBMCs of symptomatic patients with or without ART. *In vitro* CD4+ clones from non-progressors and CD8+ clones from HIV/AIDS patients were found to secrete high levels of RANTES (Saha et al., 1998). The reported levels of RANTES during HIV infection have varied in different studies. Though some subsets of PBMCs showed positive correlation of RANTES with non-progression, high levels of RANTES had no correlation and even correlated with rapid progression to AIDS (Zanussi et al., 1996; Polo et al., 1999).

Co-receptor expression:

CXCR4

The CXCR4 protein is a chemokine receptor, which regulates leukocyte migration as well as other immunological and developmental processes. Additionally, CXCR4 was also recognized as a cofactor or coreceptor for HIV entry into T cells (Feng et al., 1996). The CXCR4 mRNA is constitutively expressed in almost all types of leukocytes (Nagasawa, et al., 1999; Forster et al., 1998). Though down regulation of CXCR4 expression on lymphocytes in HIV-infected subjects compared with seronegative controls was found earlier (Ostrowski et al., 1998; Giovannetti et al., 1999; Nicholson et al.,

2001), we did not find any significant changes in the level of CXCR4 mRNA in either whole blood or PBMCs from HIV patients. This indicates that the down regulation of CCR4 on the surface of leucocytes may be due to intracellular sequestration of the CXCR4 protein or due to the direct effects of HIV proteins on its synthesis and transport to the cell surface (Choi et al., 2008).

Transcription factors expression:

STAT1 and STAT3

STAT1 and STAT3 are latent cytoplasmic transcription factors, which are phosphorylated by the Janus kinases (JAKs) in response to pro-inflammatory and regulatory factors (Darnell, 1997) and are related to the pathogenesis of HIV infection (Shrikant et al., 1996; Magnani et al., 2003). We found significant upregulation of STAT1 in the whole blood and PBMCs of symptomatic patients. The expression of STAT3 was down regulated in whole blood from patients in all stages of disease, but was upregulated in the PBMCs of those showing ART resistance. Though in HIV infection the activation of STAT1 takes place in a majority of individuals with progressive HIV disease (Bovolenta et al., 1999), increased STAT1 expression was observed in monocytes *in vitro* (Herbeuval et al., 2004) and in patients who were off ART (Alhetheel et al., 2008). In cell culture, CD4+ T cells showed significant changes in STAT3 protein levels but not in the levels of STAT1 protein (Herbeuval et al., 2005b). These findings indicate that differential regulation of STAT1 and STAT3 may occur in some subsets of blood cells, but that is not reflected in the whole blood or PBMC fractions.

Differences between plasma cytokine levels and mRNA expression in whole blood cells and PBMCs

In the present study, the mRNA levels of the cytokines studied in PBMCs and whole blood were not always reflected in the levels of plasma cytokines. In several circumstances this type of discrepancy was observed which might be due to additional cellular sources of circulating cytokines. Besides white blood cells, many other cells in various types of tissues and organs secrete cytokines (5). During prolonged exercise IL-6 mRNA expression levels were found not to increase in monocytes, but in skeletal muscle, and that increase

was reflected in increased plasma cytokine levels (Starkie et al., 2001; Ostrowski et al., 1998). Similar observations were made in polymyalgia rheumatica (PMR) in which the increased circulating IL-6 levels were not reflected in increased IL-6 transcript levels, leading the authors to suggest that the inflamed tissue might be the source of circulating IL-6 (Alvarez-Rodríguez et al., 2010). It was demonstrated earlier that IL-8 transcription occurs in leucocytes before and during cardiopulmonary bypass but the circulating IL-8 was not secreted from these leucocytes, rather from other cell types (John et al., 1998). Activation of endothelial cells may enhance the production of IL-8, MCP-1 and RANTES (Nilsen et al., 1998). Along with circulating lymphocytes, RANTES is produced from the platelets and tissue cell monocytes (Cavusoglu et al., 2007). In advanced non-small-cell lung cancer (NSCLC), which is also observed in HIV/AIDS, the tumor may itself be the major source of circulating cytokines (Ru¨be et al., 2008). In addition, the plasma levels of produced cytokines may be influenced by plasma half-life and presence of inhibitors or antagonists in the plasma against cytokines (http://www.arthritis-treatment-and-relief.com/plasma-cytokines-half-life.html) or may be affected by receptor binding, metabolism of cytokines, processing technique and the assay type used for measurement of cytokines (Jason, 2001).

These data indicate that alterations in plasma cytokine concentrations in HIV/AIDS infection are not always indicative of changes in the production of cytokines by the circulating leukocytes and thus it may not always correlate with mRNA expression in a subset of PBMCs, whole PBMCs or even the whole blood cells. Thus studies related with overall plasma / serum cytokine production in relation with any specific cell type(s) should be interpreted cautiously.

Protein-Protein Interaction

In several *in vitro* studies we observed that the genes (proteins) differentially regulated in HIV patients in this study to be functionally linked. For example, TNFSF-10, which we found to be increased in different stages of HIV infection and reduced after introduction of ART, induces the expression of MCP-1

(Trauzold et al., 2006) and mediated the activation of Pro-caspase 8 (Fu et al., 2011). Interestingly we also observed higher levels of MCP-1 mRNA in whole blood and PBMC and increased protein levels in plasma. In nitric oxide (NO) modulated expression of caspase-8, phosphorylated STAT-1 binds to the caspase-8 promoter and increases the expression of its mRNA (Li et al., 2007). In our study though we did not find any remarkable changes in expression of STAT-1 and caspase-8, their upward expression trend was noticed. The CCL5 protein mediates glial activation caused by HIV-Tat protein and morphine in mice and thus aggravates HIV-1 neuropathogenesis in opiate users. Here, CCL5 preferentially increases production CCL2/MCP-1 by astrocytes, resulting in recruitment and activation of macrophage/microglial cells (El-Hage et al., 2008). In different studies and in different cell types IL-1 was observed to stimulate the expression of MCP-1 mRNA (Villiger et al., 1992; Rovin et al., 1994). In other studies, STAT3 was found to be involved in the production of RANTES and MCP-1 (Kovacic et al., 2010; Ray et al., 2008). IL-10 regulates MCP-1 gene expression (Ikeda et al., 2002) and induces its own expression in monocytes via STAT3 (Staples et al., 2007). IL-1β induced the accumulation of CXCR4 mRNA in normal bronchial epithelial cells in a time-dependent manner (Eddleston et al., 2002). Though in the current study, we observed the possible functional interactions of a few genes, we believe it would be an important research question to explore in future in HIV patients of different stages of disease.

Conclusion:

In the present study, we observed differential expression of several genes of whole blood cells and PBMCs in HIV/AIDS infection and alternations in them were found after the commencement and development of resistance to ART. We revealed that the gene expression pattern in whole blood cells and PBMC compartment was not always in the similar direction. Moreover, expression of genes related with cytokines in whole blood cells and PBMCs are not always correlated with circulating plasma cytokines concentrations. Therefore, we believe that production of mRNA in any specific cell type(s) and correlating this with the plasma /serum protein levels will be an oversimplification of the fact at least in case of HIV/AIDS infection.

Figures and Tables

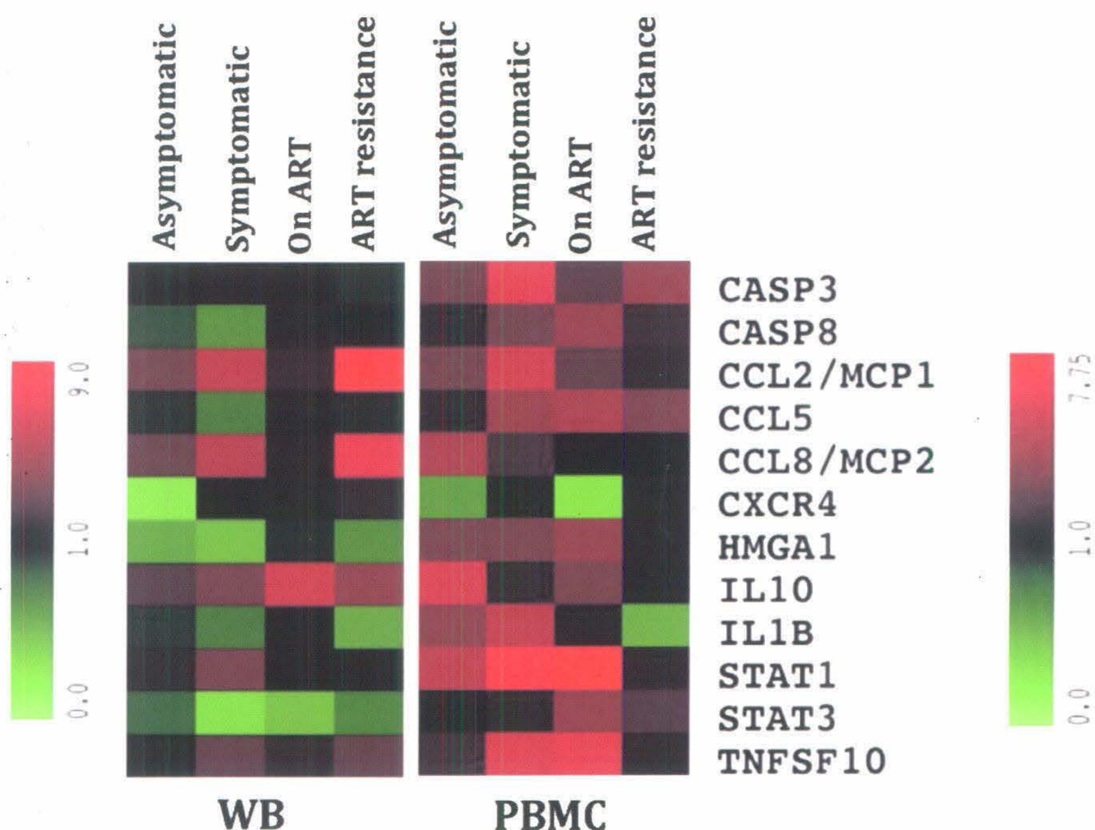


Figure 4.1.1: A heat map showing fold up or down regulation of gene expression determined through PCR array analysis of whole blood (WB) and PBMC from HIV-infected asymptomatic, symptomatic, on ART and ART resistance patients. Of the 84 genes whose expression was analyzed, twelve genes written on the right hand side showed high discriminate expression among the groups. Fold-change values greater than one indicate positive- or up-regulation and fold-change values less than one indicate negative or down-regulation of the gene.

Whole Blood

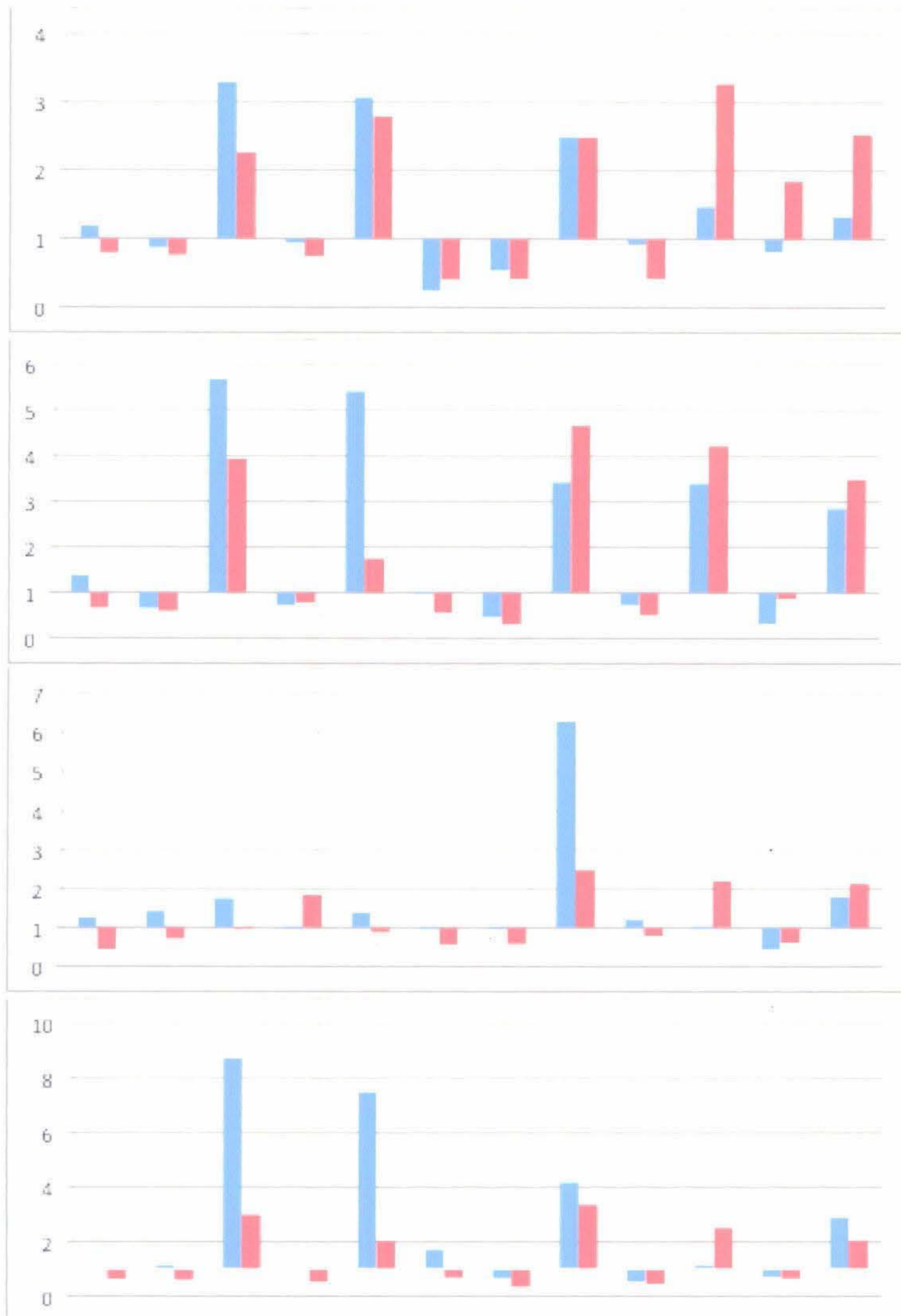


Figure 4.1.2: Validation of the PCR array data by quantitative real-time RT-PCR analysis of 12 selected genes in whole blood samples. The results were assessed by comparing the fold up or down regulation from PCR array (blue bar) with real-time PCR (red bar) analysis. The x-axis represents the genes arranged from left to right - CASP3, CASP8, CCL2/MCP-1, CCL5, CCL8/MCP-2, CXCR4, HMGA1, IL-10, IL-1β, STAT1, STAT3, TNFSF-10, and the y-axis shows the fold changes. The patient groups shown from top to bottom are asymptomatic, symptomatic, on ART and ART resistance. All comparisons are to healthy controls.

PBMC

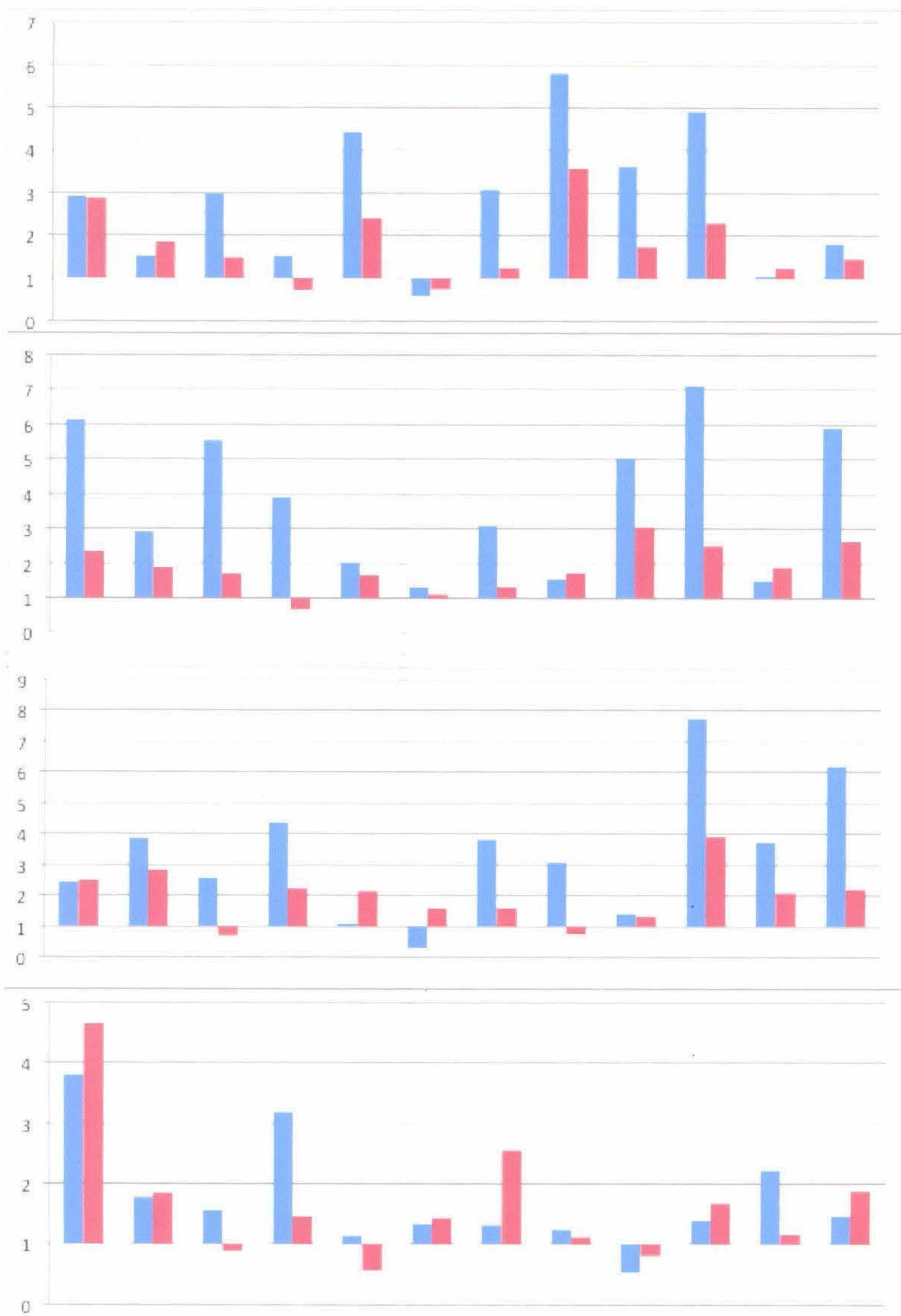
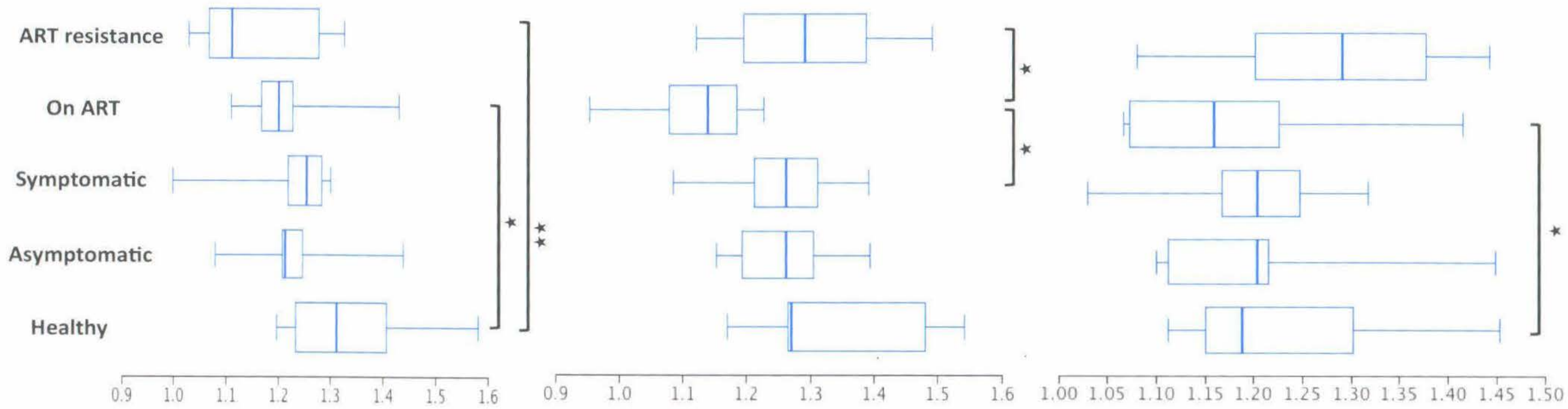
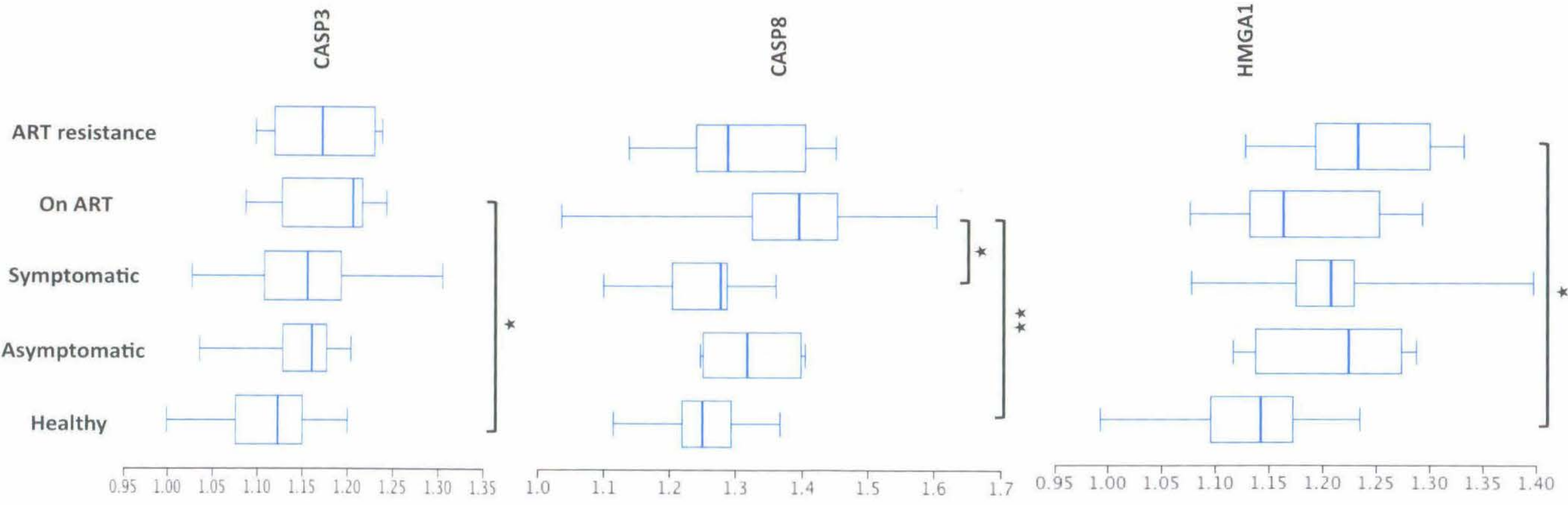
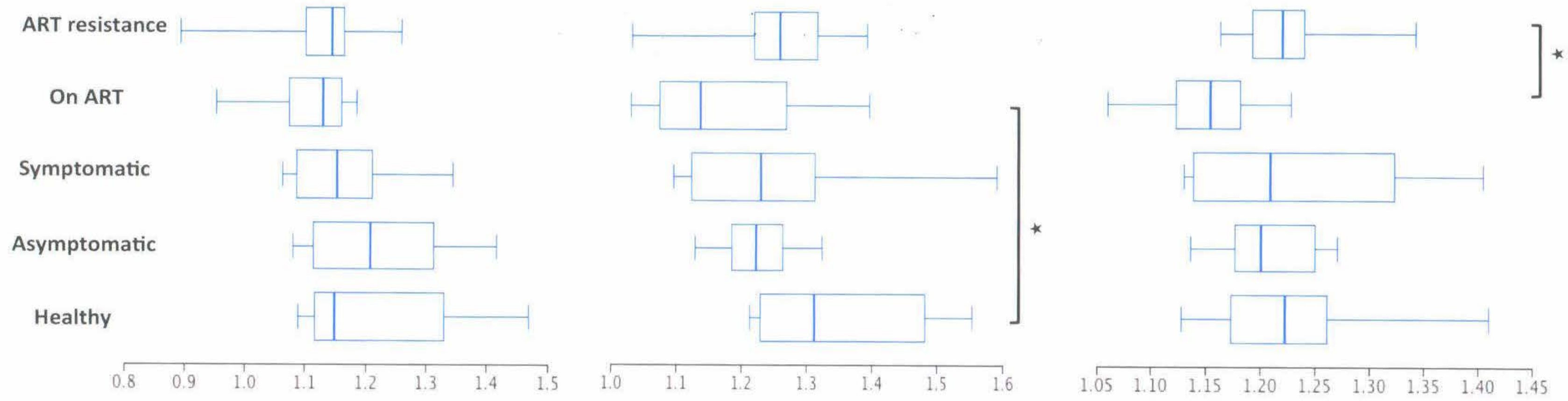
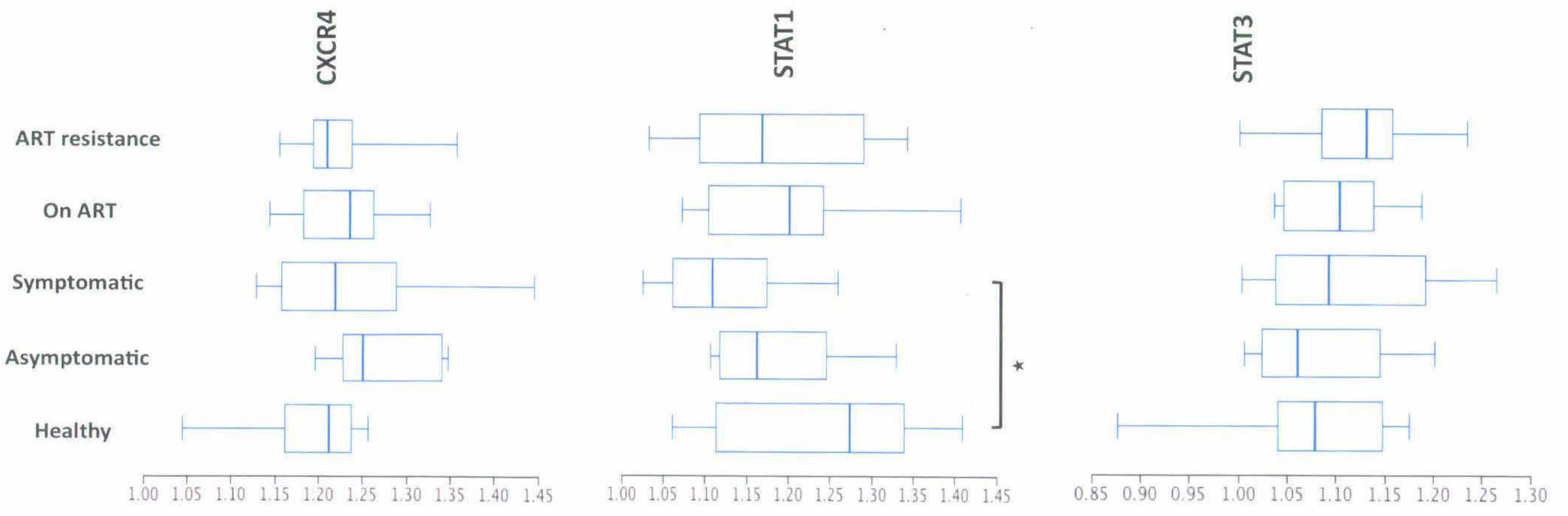


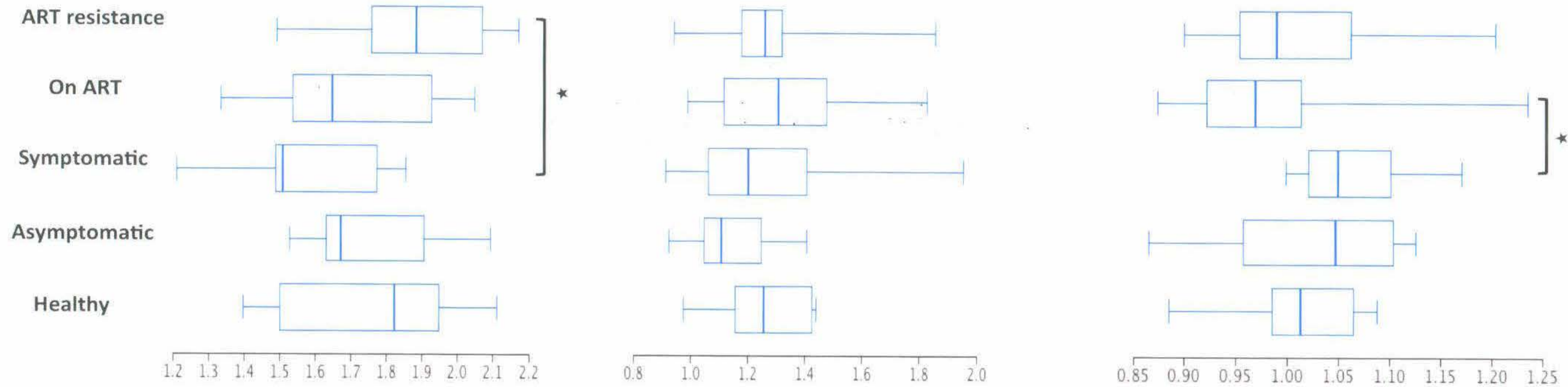
Figure 4.1.3: Validation of the PCR array data by quantitative real-time RT-PCR analysis of 12 selected genes in PBMC samples. The results were assessed by comparing the fold up or down regulation from PCR array (blue bar) with real-time PCR (red bar) analysis. The x-axis represents the genes arranged from left to right - CASP3, CASP8, CCL2/MCP-1, CCL5, CCL8/MCP-2, CXCR4, HMGA1, IL-10, IL-1β, STAT1, STAT3, TNFSF-10, and the y-axis shows the fold changes. The patient groups shown from top to bottom are asymptomatic, symptomatic, on ART and ART resistance. All comparisons are to healthy controls.

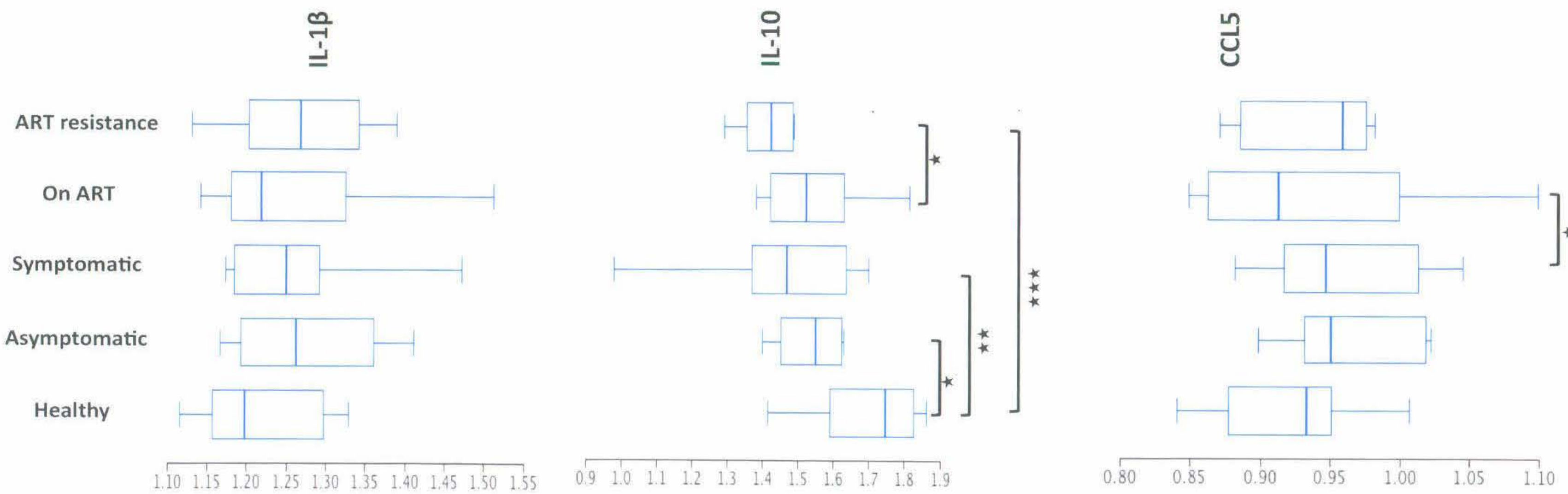

PBMC
ART resistance
On ART
Symptomatic
Asymptomatic
Healthy
0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6
0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6
1.00 1.05 1.10 1.15 1.20 1.25 1.30 1.35 1.40 1.45 1.50
CASP3
CASP8
HMGA1

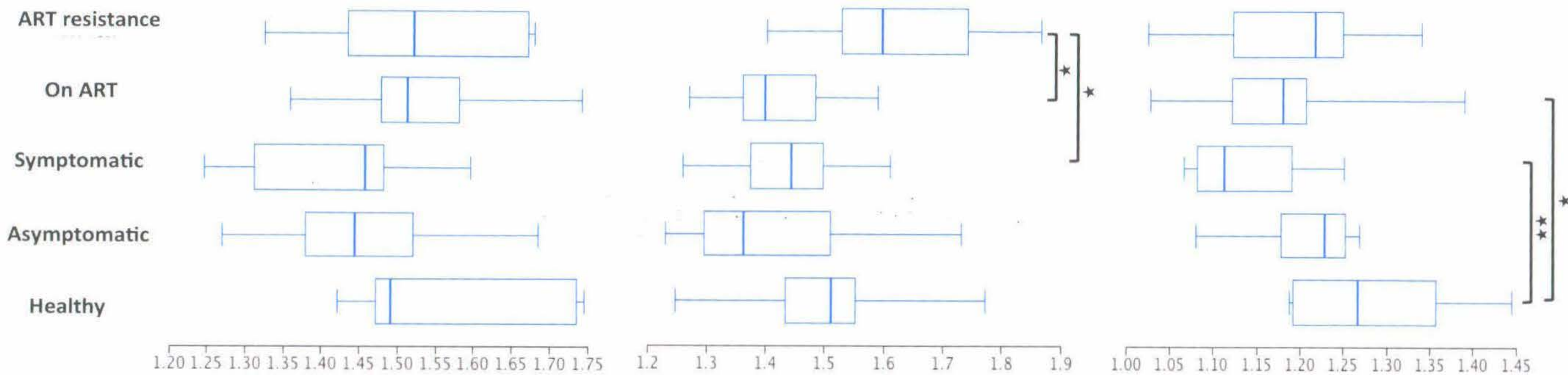

Whole Blood Cells
ART resistance
On ART
Symptomatic
Asymptomatic
Healthy
0.95 1.00 1.05 1.10 1.15 1.20 1.25 1.30 1.35
1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7
0.95 1.00 1.05 1.10 1.15 1.20 1.25 1.30 1.35 1.40


PBMC
ART resistance
On ART
Symptomatic
Asymptomatic
Healthy
*
*
0.8 0.9 1.0 1.1 1.2 1.3 1.4 1.5
1.0 1.1 1.2 1.3 1.4 1.5 1.6
1.05 1.10 1.15 1.20 1.25 1.30 1.35 1.40 1.45


CXCR4
STAT1
STAT3
Whole Blood Cells
ART resistance
On ART
Symptomatic
Asymptomatic
Healthy
*
1.00 1.05 1.10 1.15 1.20 1.25 1.30 1.35 1.40 1.45
1.00 1.05 1.10 1.15 1.20 1.25 1.30 1.35 1.40 1.45
0.85 0.90 0.95 1.00 1.05 1.10 1.15 1.20 1.25 1.30


PBMC
ART resistance
On ART
Symptomatic
Asymptomatic
Healthy
1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 2.0 2.1 2.2
0.8 1.0 1.2 1.4 1.6 1.8 2.0
0.85 0.90 0.95 1.00 1.05 1.10 1.15 1.20 1.25


IL-1β
IL-10
CCL5
Whole Blood Cells
ART resistance
On ART
Symptomatic
Asymptomatic
Healthy
1.10 1.15 1.20 1.25 1.30 1.35 1.40 1.45 1.50 1.55
0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9
0.80 0.85 0.90 0.95 1.00 1.05 1.10


PBMC
ART resistance
On ART
Symptomatic
Asymptomatic
Healthy
1.20 1.25 1.30 1.35 1.40 1.45 1.50 1.55 1.60 1.65 1.70 1.75
1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9
1.00 1.05 1.10 1.15 1.20 1.25 1.30 1.35 1.40 1.45
MCP-1
MCP-2
TNFSF-10

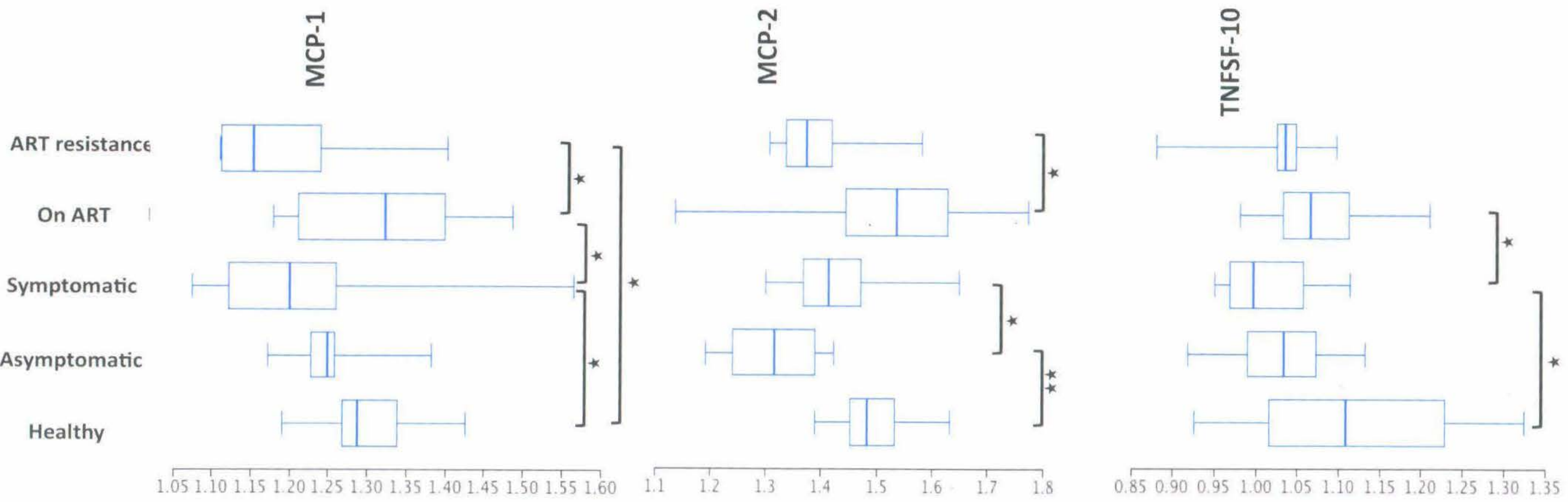

Whole Blood Cells
ART resistance
On ART
Symptomatic
Asymptomatic
Healthy
1.05 1.10 1.15 1.20 1.25 1.30 1.35 1.40 1.45 1.50 1.55 1.60
1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8
0.85 0.90 0.95 1.00 1.05 1.10 1.15 1.20 1.25 1.30 1.35

Figure 4.1.4: Boxplot of mRNA levels of 12 selected genes observed in whole blood cells and PBMC. Each Ct value of particular gene was converted into a ratio by dividing with the Ct value of the housekeeping gene GAPDH, and subjected to Kruskal-Wallis Test. Here lower the expresion ratio higher the gene expression. ★ p <0.05, ★★ p < 0.01, ★★★ p < 0.001.

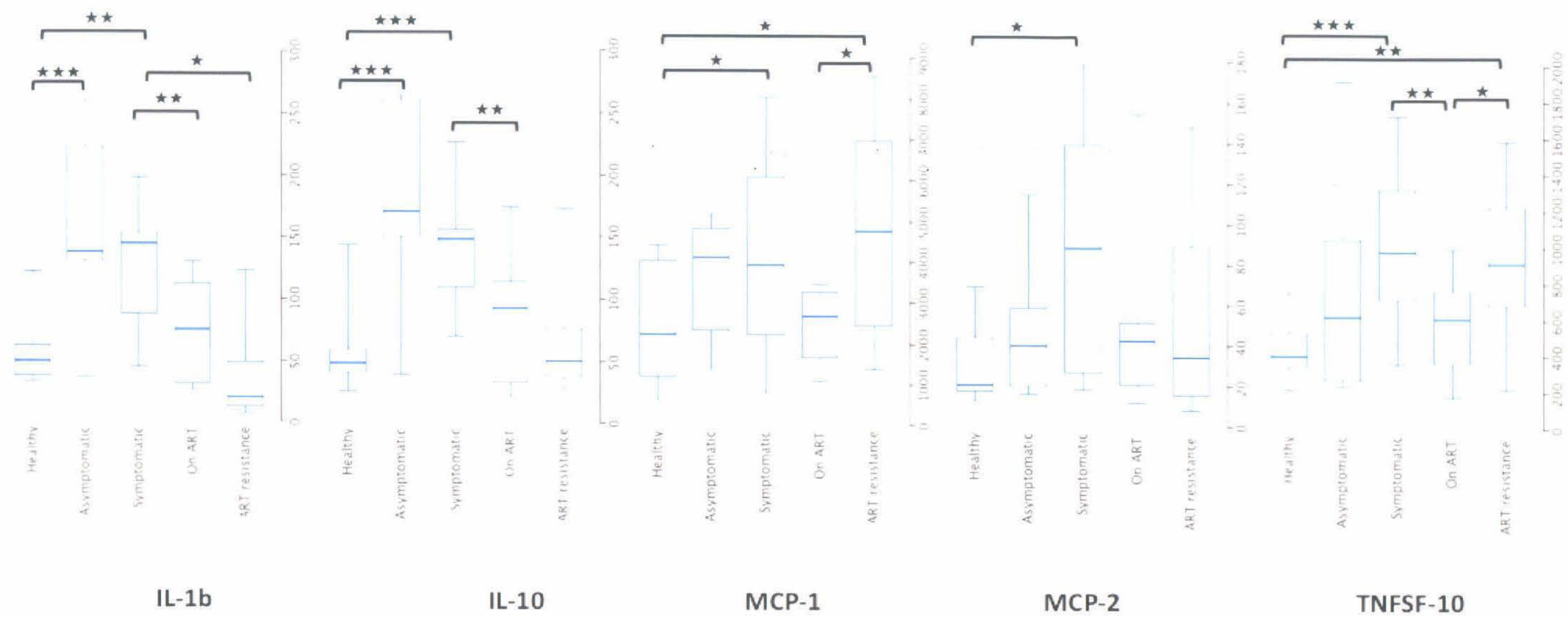


Figure 4.1.5: Boxplot of 5 selected cytokine levels. Units of the cytokine levels were expressed as MFU (Mean flurecence unit) observed in plasma of study subjects in various groups. Kruskal-Wallis Test was done. Here ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$.

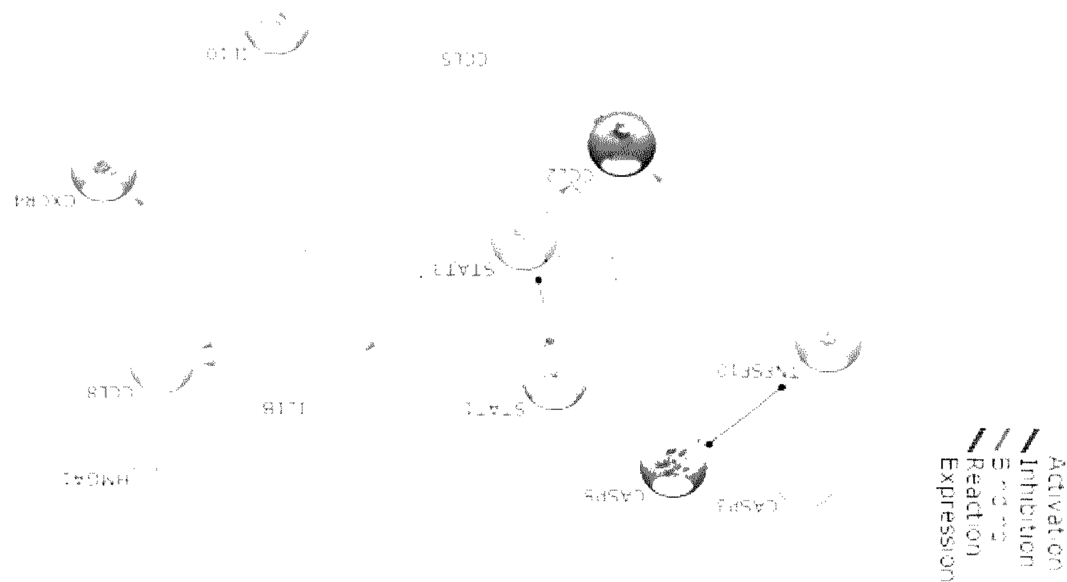


Figure 4.1.6: Human protein interaction relationships among the 12 differentially regulated genes in HIV infection found in this study. The relationships were mapped and visualized in a network structure using the STRING software. The interactions are represented as the lines that run in between the bubbles, and the color refers to the type of interaction.

Table 4.1.1: Genes found to be differentially regulated in this study modulate several host molecular functons. The modulated genes were analyzed with the STRING software and were separated into several clusters on the basis of Gene Ontology (GO) terms. Here only those GO clusters with p value < 0.05 are considered. The extreme right column indicates the genes involved in a particular GO term.

GO id	Term	Genes
GO:0005126	Cytokine receptor binding	IL1 ß, TNFSF-10, CCL8, CCL2, IL10, CCL5
GO:0005125	Cytokine activity	IL1 ß, TNFSF-10, CCL8, CCL2, IL10, CCL5
GO:0005102	Receptor binding	HMGA1,IL1ß, TNFSF-10, CCL8, CCL2, IL10, CCL5
GO:0005515	Protein binding	CASP3, CASP8, CCL2, CCL5, CCL8, CXCR4, HMGA1, IL-10, IL-1 ß,STAT1, STAT3,TNFSF-10
GO:0008009	Chemokine activity	CCL8, CCL2, CCL5
GO:0004197	Cysteine-type endopeptidase activity	CASP3, CASP8
GO:0070851	Growth factor receptor binding	IL10, IL1 ß
GO:0008201	Heparin binding	CCL2, CCL8
GO:0004871	Signal transducer activity	CCL8, CCL2, CCL5, STAT1, STAT3, CXCR4
GO:0005539	Glycosaminoglycan binding	CCL2, CCL8
GO:0001664	G-protein-coupled receptor binding	CCL8, CCL2, CCL5
GO:0008083	Growth factor activity	IL10, IL1 ß
GO:0043565	Sequence-specific DNA binding	STAT1, STAT3, HMGA1
GO:0019899	Enzyme binding	STAT3, CXCR4, HMGA1
GO:0008134	Transcription factor binding	STAT3, HMGA1
GO:0003700	Sequence-specific DNA binding transcription factor activity	STAT1, STAT3, HMGA1
GO:0001071	Nucleic acid binding transcription factor activity	STAT1, STAT3, HMGA1
GO:0004175	Endopeptidase activity	CASP3, CASP8
GO:0030246	Carbohydrate binding	CCL2, CCL8
GO:0008233	Peptidase activity	CASP3, CASP8
GO:0005509	Calcium ion binding	STAT1, STAT3

Result and Discussion 2

Objective:

To quantify select microRNAs in the PBMCs of HIV/AIDS patients at different stages of disease and therapy, and to identify their relationships with probable target genes (miTGs).

Summary:

The surrogate markers that are used to assess the progression of HIV/AIDS include CD4 T cell counts and plasma viral loads. However, the reliability of these markers has been questioned in patients on anti-retroviral therapy (ART). MicroRNAs have been shown to be good markers of progression and severity for some diseases. An earlier report found five miRNAs – miR-16, miR-146b-5p, miR-150, miR-191 and miR-223 – that could be used to assign HIV/AIDS patients into groups with varying CD4 T cell counts and viral loads. We quantified the levels of these miRNAs in the PBMCs of HIV/AIDS patients with progressing disease and ART. Our results show miR-150 levels to correlate well with CD4 T cell numbers, and to reliably distinguish between HIV-infected asymptomatic persons and those on ART from symptomatic patients and those failing first-line ART. We also carried out target and pathway analyses to identify host genes and cellular pathways that are potential targets of the five miRNAs. Selected targets were verified by quantitative reverse transcription polymerase chain reaction. Good inverse correlation was observed between the miRNAs and some of their host gene targets involved in immunoregulation, HIV replication and miRNA biogenesis. Using pathway analysis tools, we also predict cellular pathways that are likely to be affected due to the deregulation of these miRNAs. Our results show these pathways to be involved in cell survival, metabolism and cellular transformation. Besides finding miR-150 as a potential biomarker for disease progression, these studies also provide insights into HIV/AIDS pathogenesis.

Results:

Differences in miRNA expression:

All the five miRNAs were down regulated in HIV-infected persons belonging to all disease groups, compared to healthy controls (Figure 4.2.1), except miR-146b-5p and miR-150 in patients who were on ART. Compared to healthy controls, the down regulation of miR-146b-5p and miR-150 was statistically significant ($p<0.05$) for HIV-infected symptomatic but not asymptomatic subjects. Following at least 6 months of ART, the expression levels of both of these miRNAs increased to levels similar to healthy controls (Figure 4.2.1). However, in patients who became resistant to first-line ART, the expression levels of miR-146b-5p and miR-150 again fell with respect to controls to average values of 0.72 ($p=0.31$) and 0.42 ($p<0.05$), respectively.

Relationship of miRNA expression to CD4 cell counts and viral loads:

To determine whether any of the miRNA measurements could be utilized for monitoring HIV/AIDS patients with or without ART, we determined the correlation between miRNA levels and existing surrogate markers - CD4 T cell counts or viral loads. We calculated the miRNA expression ratio for each sample by dividing the expression value of each miRNA (Ct value) with that of the RNU44 endogenous control for that sample. In this representation, higher the ratio for a given miRNA, lower will be its expression. The normalized expression ratio for miR-150 negatively correlated with CD4 T cell counts across 33 samples belonging to different disease stages, with a Pearson correlation of -0.661 and a p-value of <0.001 (Figure 4.2.2). Thus the expression levels of miR-150 decrease with reducing CD4 T cell counts. The other four miRNAs did not show any significant relationship to CD4 T cell counts. In the same manner, we compared miRNA expression ratios for each sample with its viral load and found the expression ratios of miR-16 to positively correlate with viral loads with a Pearson's correlation of 0.383 and a p-value of 0.03. Thus, the expression levels of miR-16 decrease with increase in viral loads.

Predictive value of miRNAs as biomarkers:

We were interested in knowing whether the measured miRNA levels, especially those for miR-150 and miR-146b-5p are able to differentiate between different stages of HIV disease. This diagnostic accuracy was evaluated by Receiver Operating Characteristic (ROC) curve analysis. The results showed the area under curve (AUC) value for miR-150 to be 0.8238 (SE 0.0673, $p<0.05$) in predicting CD4 T cell counts (Figure 4.2.3A). In a similar analysis, miR-146b-5p showed an AUC value of 0.5061 (SE 0.1054, $p<0.05$). Since miR-16 levels also showed significant correlation with viral loads (Figure 4.2.2), we also carried out ROC analysis for its ability to predict viral loads in patients with different stages of disease. This gave an AUC value of 0.6076 (SE 0.0886, <0.05) (Figure 4.2.3B). The diagnostic accuracy of other individual miRNAs as well as the combination of all five miRNAs was found to be poor.

Expression relationships between miRNA and miTGs:

To evaluate the correlation between the miRNAs and their target genes, we analyzed the expression of randomly selected immune response genes (IRGs), genes involved in miRNA biogenesis (GMBs) and HIV host dependency factors (HDFs) in the PBMCs of patients belonging to the different disease groups by quantitative RT-PCR. The Ct values of mRNAs were converted into an mRNA expression ratio by dividing with the Ct value of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH); this was then correlated with the miRNA expression ratio. As shown in Figure 4.2.4, among the IRGs we found significant inverse correlation of SERPINC1 and HMGA1 with miR-16, STAT1 with miR-146b-5p and TNFSF-10 with miR-150. Among the GMBs, AGO2 showed significant inverse correlation with miR-16, miR-191 and miR- 223, while DROSHA levels correlated with miR-16. Among the HDFs, we found only LDLR to have a significant inverse correlation with miR-150.

Pathways targeted by the selected miRNAs:

Through the DIANA-miRpath application, we found 122 pathways that were targeted either by an individual miRNA or all the five miRNAs (union). We filtered

these putative pathways by setting the -ln[p-value] > 3 and found 36 host pathways that were likely to be affected in HIV infection (Figure 4.2.5 and Table 4.2.1). These pathways, identified through the miRNAs, are mainly involved in signaling, metabolism or cellular transformation (cancers). We then used miTALOS, which utilizes information on tissue-specific expression of miRNA targets. Using default settings of an enrichment score, E=1 and its significance, p(E)=0.05, we found 11 pathway associations with the five miRNAs (Table 4.2.2 Part A). These were again involved in signaling, metabolism and cancers, and were understandably, a subset of the pathways discovered through the DIANA-miRpath algorithm. Since the E score focuses only on target enrichment without any concern for network topology, we also queried miTALOS for the five miRNAs using the settings P=0.4 and p (P)=0.05. This showed three networks – the MAPK signaling pathway, natural killer cell cytotoxicity and B cell receptor signaling pathway (Table 4.2.2 Part B). These networks did not change even when the proximity score was relaxed to its limit of P=1. Pathway visualization using KEGG pathway maps showed the MAPK signaling pathway to be central to the other two pathways. Understandably then, when target enrichment as well as network proximity were considered using the settings E=0.4, p(E)=0.05, P=0.4, p(P)=0.05, only the MAPK kinase signaling pathway showed up. The association of these pathways with HIV infection was verified in published literature using information available in PubMed and Google Scholar. As shown in Table 4.2.2, many targets within each of these pathways are modulated during HIV infection. These upregulated genes, which are targets for one or more of the five miRNAs that we and others have found to be down regulated during HIV/AIDS, mainly encode proteins that are part of cell survival pathways such as the MAPK (ELK1, CRKL, MAPK13, SGK1) and PI3K/Akt (PDPK1, PIK3R1) signaling pathways, or are involved in regulation of the cell cycle (E2F3, TP53, CCND1, CDC27) or protein ubiquitination (CBL-B, CDC27, SMURF2, BTRC).

The top pathways showing up in the DIANA-miRpath and miTALOS analyses were visualized using the KEGG maps. These include the insulin signaling pathway (Figure 4.2.6) and the MAPK signaling pathway (Figure 4.2.7). Multiple genes in these pathways are predicted to be targets of the miRNAs studied in HIV/AIDS patients.

Discussion:

In this study, we assayed five abundantly expressed T cell specific miRNAs in the PBMCs of HIV/AIDS patients at different stages of disease. These miRNAs were previously shown to broadly correlate with CD4 T cell counts and viral loads in HIV-infected persons (Houzet et al., 2008). This analysis of only select miRNAs was a proof-of-principle pilot study to assess their utility in developing possible biomarkers of disease and response to therapy in infected persons, and to develop possible correlations between miRNA levels, the expression of select miTGs and host cell pathways. Compared to healthy controls, there was a generalized down regulation of all the five miRNAs in patients belonging to all the study groups, except in the case of patients receiving ART, who had miRNA levels that were similar to healthy controls. In ART-naïve symptomatic patients the levels of these miRNAs were down regulated compared to healthy controls. Following ART, their levels became similar to that in healthy controls, but on developing resistance to ART, there was again a fall in the levels of these miRNAs, to pre-ART levels. Since the differences were significant for miR-150 and miR-146b-5p, we suggest that these could be good surrogate markers for disease progression and therapy in HIV/AIDS. In addition to their effects on host pathways, miR-150 and miR-223 are also regarded as anti-HIV-1 miRNAs because they can also target the 3' untranslated regions of HIV-1 transcripts (Huang et al., 2007). The infection-related down regulation of miR-150 in CD4+ T cells during chronic HIV-1 infection was also reported earlier (Swaminathan et al., 2009). Though we studied the miRNAs in PBMCs, of which only 30-40% are T cells and only less than 15% of PBMCs are infected with HIV (Bagasra et al., 1992), the down regulation of miR-150 is clearly observed in this compartment. In another study, miR-150 levels were significantly reduced in the PBMCs and plasma of sepsis patients, correlated well with disease severity, and is now considered a prognostic marker for sepsis (Vasilescu et al., 2009). Interestingly, of the patients who have severe sepsis, about 10% also have HIV infection (Mrus et al., 2005). Reduced miR-146b-5p levels are reported in patients with melanoma and glioma (Jukic et al., 2010; Katakowski et al., 2010), two conditions that are also often reported in AIDS patients (Gildenberg et al., 2000; Rodrigues et al., 2002).

As CD4 T cell counts and viral loads are the most commonly used surrogate markers, the miRNA expression levels were compared to these. We found the levels of miR-150 to significantly correlate with CD4 T cell counts but not with viral loads. Alternatively, the levels of miR-146b-5p and miR-16 correlated with viral loads; only the latter was statistically significant. For their predictive values as potential biomarkers, ROC analysis was carried out for the miRNAs with HIV-positive asymptomatic persons and those on ART compared to symptomatic patients and those showing ART resistance. This analysis revealed miR-150 to have a high AUC value (0.823), suggesting that it can be used reliably to assess disease progression in HIV/AIDS patients.

Several studies have shown that changes in miRNA levels lead to altered expression of their target genes, or miTGs (Lim et al., 2005; Wang and Wang 2006; Grimson et al., 2007). We therefore performed correlative mRNA studies on all the study groups using the same RNA extracted from PBMCs. During selection of miTGs we focused on host dependency factors (HDFs) and immune response genes (IRGs), and on genes involved in miRNA biosynthesis (GMBs). The HDFs were selected because HIV-1 depends upon cellular proteins to support its replication (Brass et al., 2008; König et al., 2008; Zhou et al., 2008; Yeung et al., 2009); generalized down regulation of miRNAs in T cells or monocytes, may result in increased expression of HDFs to facilitate HIV-1 entry and replication in these target cells. We selected 7 HDFs (DDX3X, MAP4, STNM1, UGP2, FOXO3, CD43 and LDLR) that were previously reported to be upregulated *in vitro* or *in vivo* (Yedavalli et al., 2004; Vahey et al., 2008; Salgado et al., 2011; Ringrose et al., 2008; Dabrowska et al., 2008; Gallego et al., 2001; van 't Wout et al., 2003; van 't Wout et al., 2005). Of these only LDLR was found to have a significant inverse correlation with miR-150 expression. Chronic immune activation has a major role in AIDS pathogenesis and the IRGs control the ability of the immune system to respond to foreign antigens, including viruses. Of the 8 IRGs studied here, four - SERPINC1, HMGA1, STAT1 and TNFSF-10 showed significant inverse correlation with their corresponding miRNAs. Finally, since there appears to be generalized down regulation of miRNAs during HIV infection, we checked whether GMBs were also differentially regulated. The expression levels of DICER, DROSHA, AGO1 and AGO2 showed significant inverse

correlation with their corresponding miRNAs. Thus, despite an increase in the expression of these GMBs, the miRNAs levels were largely down regulated during HIV infection. This deserves more attention in future studies.

Altered miRNA levels result in deregulated levels of target transcripts, leading to altered host pathways and the development of a disease phenotype (Thum et al., 2008). Searching through DIANA-miRpath, we found a large number of host pathways to be potential targets for the five miRNAs studied here (Figure 4.2.5). Of the 34 such pathways showing high probability for change, at least 20 are altered in HIV infection (Table 4.2.1). The functional analysis of miRNA-mediated pathway regulation was improved using miTALOS, an interactive tool that incorporates tissue and pathway filters. Using a combination of target enrichment and network proximity filters, the MAPK, NK cell cytotoxicity and B cell receptor signaling pathways were revealed. Within the pathways that are likely to be modulated by the studied miRNAs, several genes are already known to be upregulated during HIV infection (Table 4.2.2 and references therein). Genes that are part of the cell survival network, such as those involved in the MAPK and PI3K/Akt signaling pathways are broadly upregulated. The same is the case with genes that encode proteins involved in the initiation (E2F, Cyclin D1), G2/M block (p53) and mitotic (Cdc27) phases of the cell cycle. Cell survival as well as activation of the cell cycle and mitosis would positively impact a replicating virus. Interestingly, p53, which is apoptotic, but also blocks the cell cycle at the G2/M boundary, is increased as well. This would again positively impact HIV whose replication rates are highest in the G2 phase of the cell cycle. A number of other direct correlations to HIV infection are also apparent from these analyses. For example, the PI3K regulatory subunit (PI3KR1) interacts with the HIV Nef protein to activate the Nef-associated p21-activated kinase (PAK), which increases HIV production (Linneman et al., 2006). The F-box/WD repeat-containing protein 1A (BTRC), which interacts with the HIV-1 Vpu protein, is the substrate recognition component of a SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complex and mediates the ubiquitination and subsequent proteasomal degradation of target proteins, including CD4 in infected T cells (Margottin et al., 1998). The histone acetyltransferase 300 (EP300) is recruited by the HIV Tat protein and is likely to be involved in chromatin remodeling of proviral genes. All of

these examples indicate an impact of select miRNA down modulation of the studied miRNAs on HIV biology and pathogenesis and demand greater attention in future studies.

The DIANA-miRpath (Table 4.2.1) and miTALOS (Table 4.2.2) analyses showed the Wnt signaling and insulin signaling pathways as the top hits, respectively. In an earlier study (Kumar et al., 2008) the Wnt/β-catenin signaling pathway was shown to inhibit HIV replication in PBMCs *in vitro*. Further, ART-naïve patients who were being treated with lithium, an inducer of β-catenin signaling, also showed a reduction in viral loads (Kumar et al., 2008). There are reports of increased insulin resistance and type 2 diabetes in ART-naïve HIV patients (Brown et al., 2005) and this has been correlated with increased release of pro-inflammatory cytokines from peripheral leucocytes (Limone et al., 2003). One of the genes targeted by miR-16 is fatty acid synthase, which is involved in the insulin-signaling pathway and whose serum levels are higher in HIV-infected patients (Aragonès et al., 2010). There is also a large body of literature, which implicates a role for MAPK signaling in HIV pathogenesis, especially in its ability to manipulate the host immune response (Furler and Ulttenbogaart, 2010). Recent evidence also suggests a role for MAPK signaling in miRNA biogenesis (Paroo et al., 2009). Thus, the MAPK signaling pathway, which is revealed in our studies through the analysis of miRNAs in different disease states, is likely to have a complex interaction with HIV. Further, many clinical syndromes observed in HIV-infected patients may also be related to miRNA deregulation during the progression of HIV infection to AIDS.

Conclusion:

In this study we have built a new strategy to evaluate disease progression, response to therapy and ART failure in HIV/AIDS patients by comparing the levels of T-cell specific miRNAs in PBMCs. Studies have shown that CD4 T cell counts and viral loads are not reliable markers of disease progression in HIV patients who are receiving ART. Our pilot study proposes miR-150 and possibly also miR-146b-5p as potential biomarkers of disease progression, response to and failure of therapy. These should be evaluated in larger and longitudinal patient cohorts. With a view on pathogenesis, we have also correlated deregulated miRNA levels

to those of their target genes, and have predicted cellular pathways that are likely to be affected during different clinical phases of HIV disease. Our proposals are validated through independent reports in literature on the deregulation of many of these cellular pathways following HIV infection. This constitutes a novel approach that demands further exploration to fully understand the pathogenesis of HIV/AIDS.

Figures and Tables

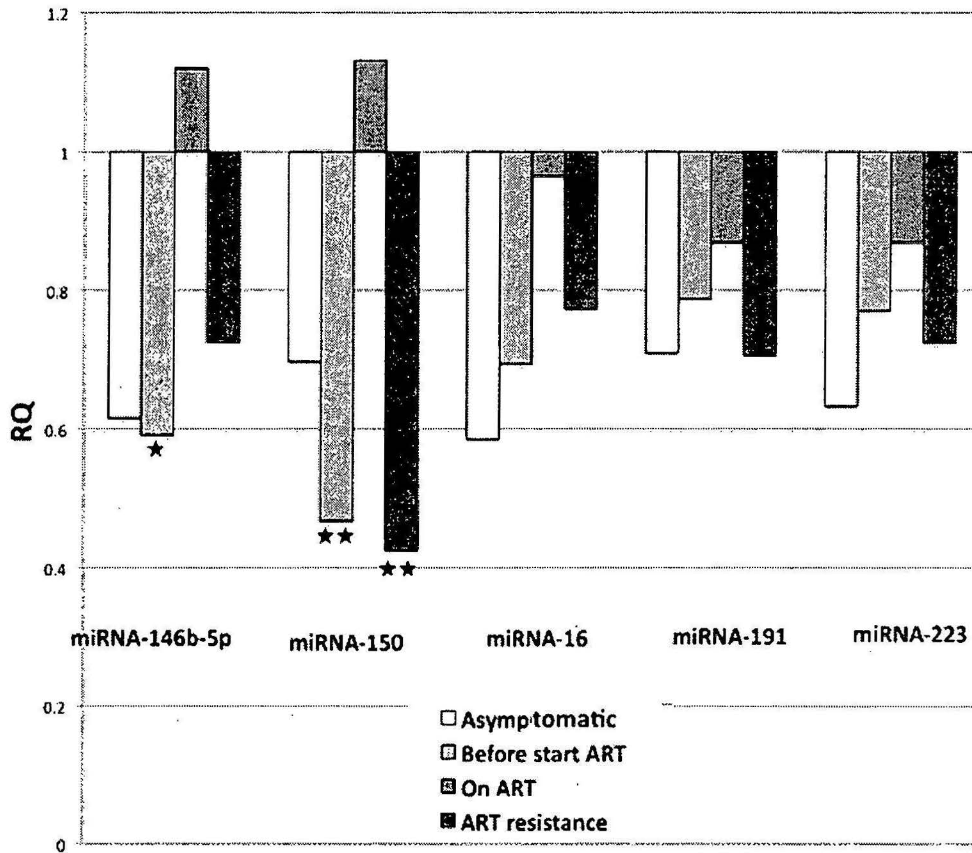
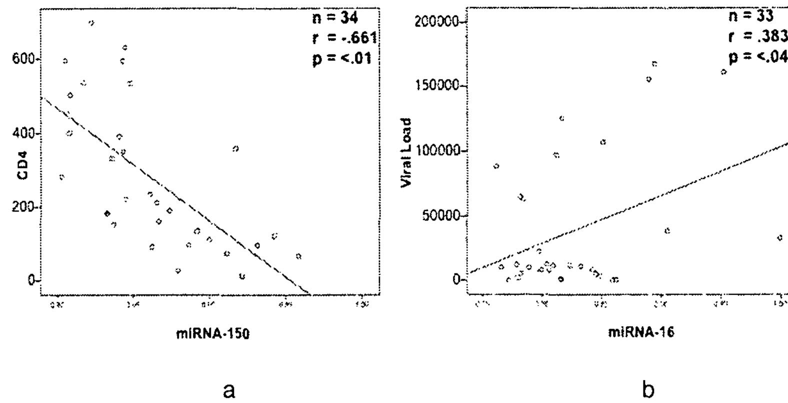


*Figure 4.2.1: Expression levels of 5 select miRNAs at different stages of HIV/AIDS, before and after ART. The Ct value for each miRNA was normalized by dividing it with the Ct value for RNU44. RQ, relative quantification of gene expression is expressed in arbitrary units, where controls are regarded as 1 unit and compared with the test samples. * Indicates significance ($p<0.05$); ** indicates high significance ($p<0.001$).*



miRNA	CD4 count		Viral load	
	Pearson Correlation	Sig. (2-tailed)	Pearson Correlation	Sig. (2-tailed)
miRNA-16	- 0.009	0.959	0.383	0.030*
miRNA-146b	- 0.244	0.178	0.318	0.077
miRNA-150	- 0.661	0.000**	0.102	0.580
miRNA-191	- 0.045	0.798	0.221	0.224
miRNA-223	0.044	0.802	0.320	0.074

*Figure 4.2.2: Scatterplots of (a) CD4 counts vs miR-150 expression ratio; (b) Viral loads vs miR-16 expression ratio. The table shows Pearson Correlation and Significance (2-tailed) p values for each miRNA expression ratio compared to CD4 cell counts and viral load. * indicates significance (p<0.05); ** indicates high significance (p<0.001).*

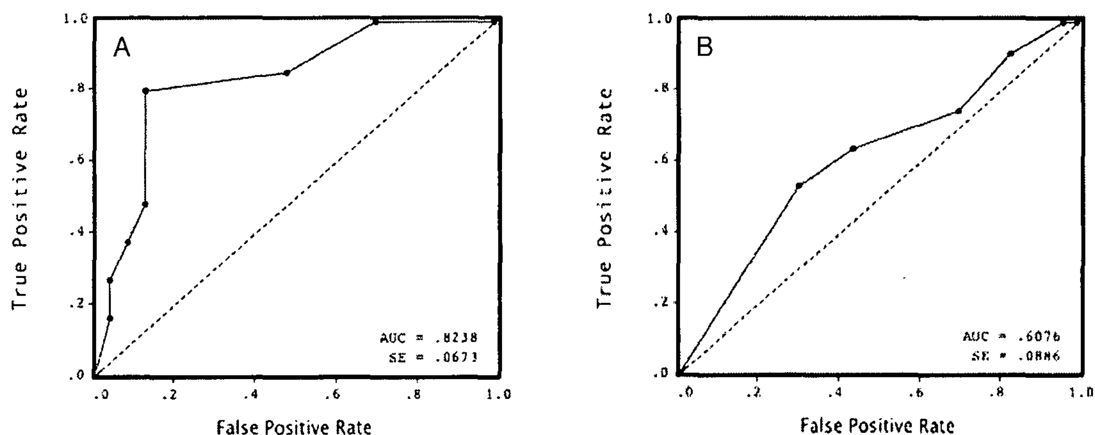


Figure 4.2.3: Area under the receiver-operating curve (ROC) for (A) miR-150 expression ratio and CD4 cell counts, and (B) miR-16 expression ratio and viral load.

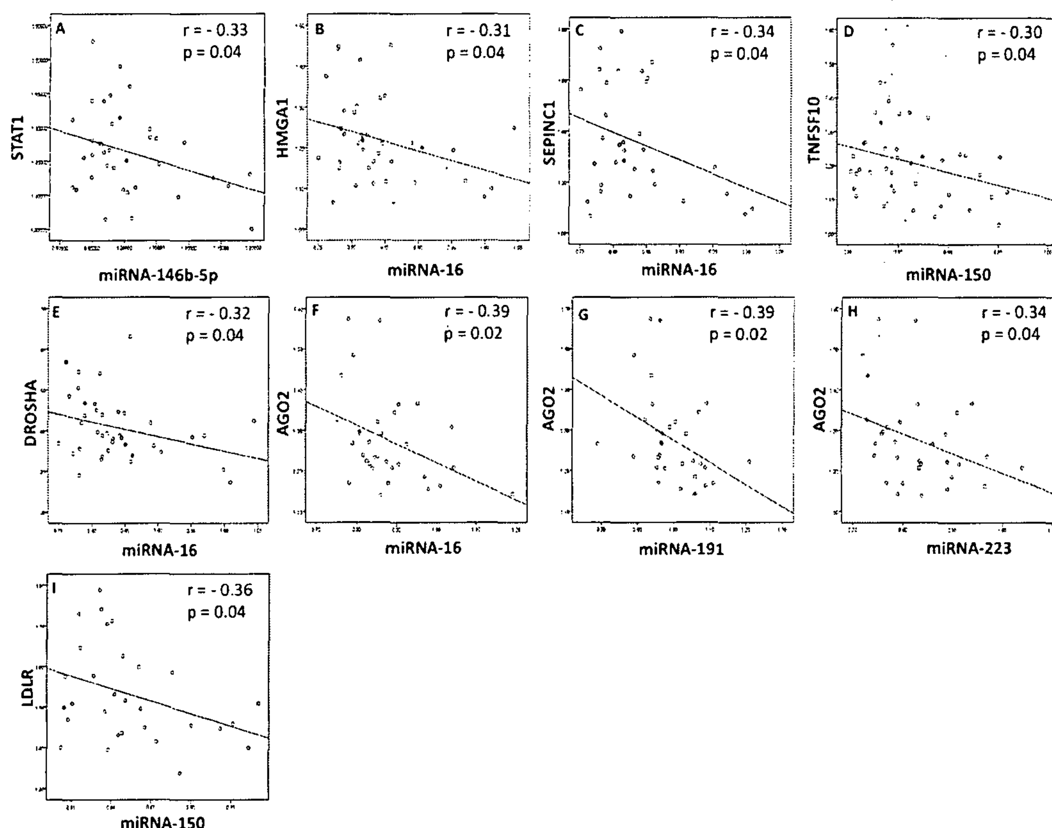


Figure 4.2.4: Scatterplots of miTG expression ratios (Y axis) compared to miRNA expression ratios (X axis) as described in Materials and Methods. The miTGs are arranged as IRGs (A to D), GMBs (E to H) and HDF (I). Pearson Correlation (r) and significance (p) of the inverse correlations are shown inside each box.

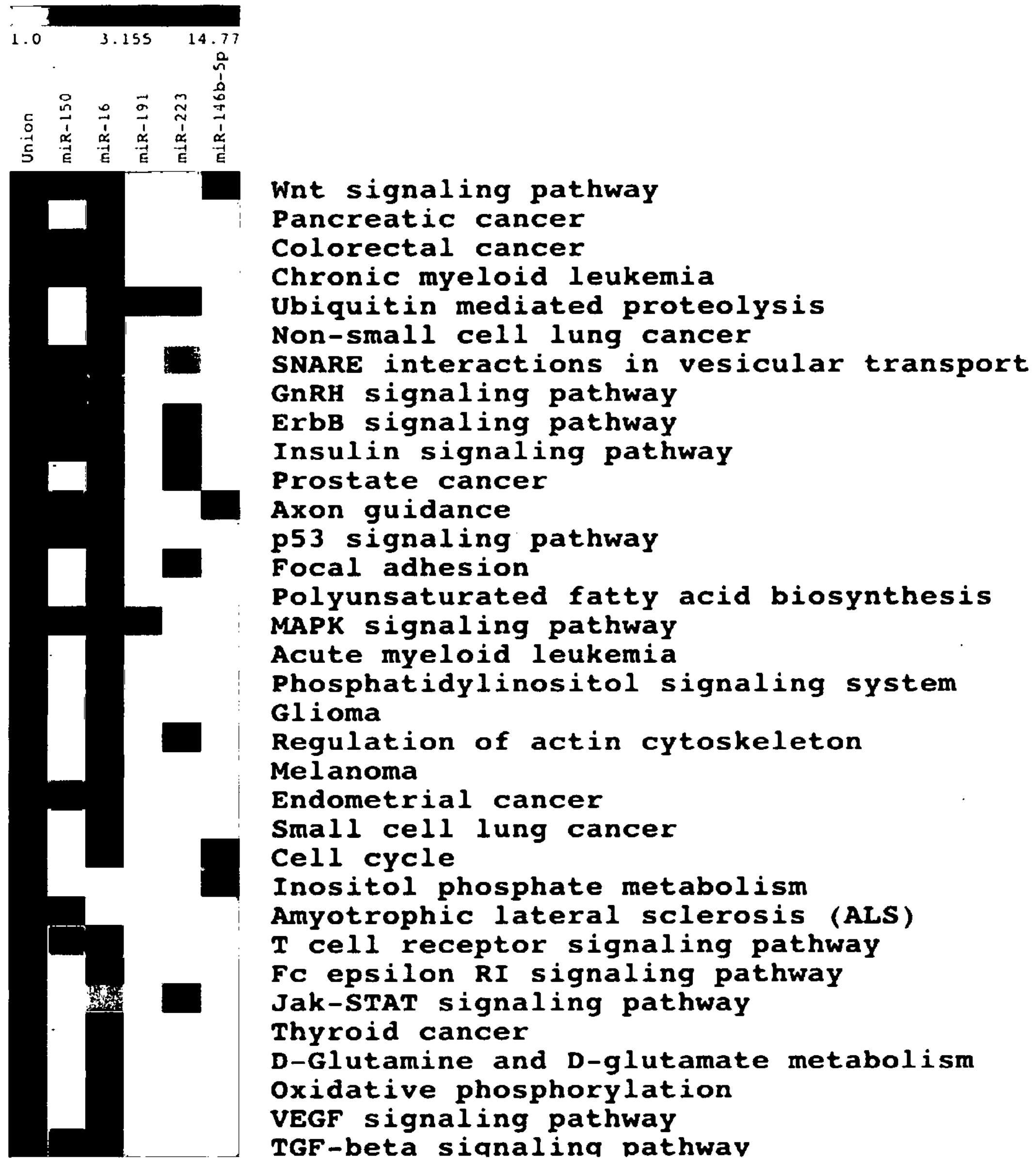


Figure 4.2.5 : Pathways affected by the miTGs of all the five miRNAs altered in HIV infection assayed in this study. The KEGG Pathways were enriched and the results of the analysis were converted into a heat map using the -ln (p-value). See Table 4.2.1 for details.

Table 4.2.1: Host pathways targeted by the five miRNAs during HIV infection, analyzed using the DIANA-miRpath algorithm.

KEGG pathway	KEGG ID	Genes	-ln (P value)	Ref *
Wnt signaling pathway	hsa04310	32	16.84	I
Colorectal cancer	hsa05210	20	11.71	II
Pancreatic cancer	hsa05212	18	11.64	
Insulin signaling pathway	hsa04910	26	9.54	III
Chronic myeloid leukemia	hsa05220	17	9.01	IV
D-Glutamine and D-glutamate metabolism	hsa00471	3	8.34	V
SNARE interactions in vesicular transport	hsa04130	10	7.9	
Focal adhesion	hsa04510	31	7.57	
Axon guidance	hsa04360	22	7.02	
Regulation of actin cytoskeleton	hsa04810	32	6.87	VI
GnRH signaling pathway	hsa04912	18	6.65	
Endometrial cancer	hsa05213	12	6.65	VII
Prostate cancer	hsa05215	17	6.44	VIII
Non-small cell lung cancer	hsa05223	12	6.15	IX, X
Ubiquitin mediated proteolysis	hsa04120	22	6.15	XI
Acute myeloid leukemia	hsa05221	12	5.92	XII
ErbB signaling pathway	hsa04012	16	5.38	XIII
MAPK signaling pathway	hsa04010	35	5.26	XIV
TGF-beta signaling pathway	hsa04350	16	5.07	
Polyunsaturated fatty acid biosynthesis	hsa01040	6	4.92	
p53 signaling pathway	hsa04115	13	4.9	XV, XVI
Thyroid cancer	hsa05216	7	4.55	
Small cell lung cancer	hsa05222	15	4.55	XVII
Phosphatidylinositol signaling system	hsa04070	13	4.4	
Glioma	hsa05214	12	4.34	XVIII, XIX
Melanoma	hsa05218	13	4.24	XX
Adherens junction	hsa04520	13	4.24	
Cell cycle	hsa04110	18	4.22	XXI
Oxidative phosphorylation	hsa00190	3	3.87	XXII
Amyotrophic lateral sclerosis (ALS)	hsa05030	5	3.51	XXIII
Type II diabetes mellitus	hsa04930	8	3.47	XXIV
VEGF signaling pathway	hsa04370	12	3.43	XXV
Inositol phosphate metabolism	hsa00562	9	3.33	XXVI
Antigen processing and presentation	hsa04612	1	3.32	XXVII
Jak-STAT signaling pathway	hsa04630	21	3.08	XXVIII
T cell receptor signaling pathway	hsa04660	14	3.06	XXIX

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Table 4.2.2. miTGs targeted in different KEGG pathways by the five down regulated miRNAs. The genes shown in bold are reported in literature to be up regulated either in vivo or in vitro following HIV infection. Here, E = enrichment score; P = proximity; p = significance. The settings were as follows – Part A: E=1, p(E)=0.05; Part B: P=0.4, p(P)=0.05

Pathway	**Targets** [*]	**E**	***p*** **(E)**	**P**	***p*** **(P)**
Part A					
Insulin signaling pathway	**IRS2**[I] FOXO1 MKNK1 **ELK1**[II] RPS6KB1 PRKAR2A **PDPK1**[III] SORBS1 SOS1 **FASN**[IV] PRKACB TRIP10 AKT3 **PIK3R1**[II,V] MAP2K1 FLOT2 ACACA RAF1 IRS1 NRAS **CBLB**[VI] EIF4E **CRKL**[II] PRKAR1A IKBKB	1.13	0.0001	0.45	0.97
Prostate cancer	FGFR2 FGFR1 **E2F3**[VII] MAP2K1 **TP53**[VII] RAF1 FOXO1 NRAS IGF1R **CCND1**[VIII] **PDPK1**[III] **EP300**[X] BCL2 SOS1 IKBKB **PIK3R1**[IV,V] AKT3	1.07	0.0014	0.4	0.75
Melanoma	IGF1R FGFR1 NRAS **E2F3**[VII] **CCND1**[VIII] FGF7 MAP2K1 **TP53**[VII] RAF1 **PIK3R1**[II,IV] AKT3	1.28	0.0029	0.46	0.99
Progesterone-mediated oocyte maturation	GNAI3 ADCY7 MAP2K1 CDC23 RAF1 **CDC27**[XI] CDC25B IGF1R RPS6KA3 **MAPK13**[II,VII] PRKACB AKT3 **PIK3R1**[IV,VII]	1.07	0.0051	0.86	0.93
Non-small cell lung cancer	NRAS **E2F3**[VII] **CCND1**[VIII] **PDPK1**[III] MAP2K1 SOS1 RAF1 **FOXO3**[IX] **PIK3R1**[II,IV] AKT3	1.19	0.0076	0.46	0.76
Endometrial cancer	NRAS **CCND1**[VIII] **PDPK1**[III] MAP2K1 SOS1 RAF1 **ELK1**[II] **FOXO3**[IX] **PIK3R1**[II,IV] AKT3	1.19	0.0076	0.47	0.53
Chronic myeloid leukemia	NRAS **E2F3**[VII] BCR MAP2K1 SOS1 SMAD4 **TP53**[VII] RAF1 SMAD3 IKBKB **PIK3R1**[II,IV] AKT3	1.06	0.0078	0.67	1.00
mTOR signaling pathway	EIF4B **PDPK1**[III] RPS6KA3 EIF4E ULK2 **VEGFA**[XII] RPS6KB1 **PIK3R1**[II,IV] AKT3	1.23	0.0092	0.32	0.96
TGF-beta signaling pathway	PPP2R1B PPP2R1A ACVR2A SMAD5 SMAD4 SMAD3 **SMURF2**[XIII] RPS6KB1 SMURF1 BMPR1A	1.14	0.0097	0.76	0.47
Hedgehog signaling pathway	CSNK1G1 **BTRC**[XIV] **PTCH1**[XV] PRKACB FBXW11 WNT7A	1.45	0.0149	0.4	0.71
Aldosterone-regulated sodium reabsorption	**PDPK1**[III] **IRS2** [XVI] **SGK1** ATP1B4 IRS1 **PIK3R1**[II,IV]	1.28	0.0272	0.24	1.00

Part B					
MAPK signaling pathway	FGFR2 MEF2C **FGFR1**[XVII] FGF7 **ELK1**[II] MKNK1 **TAK1**[II] SOS1 PRKACB **RAPGEF2**[XVIII] RASA1 AKT3 MAP2K1 NF1 MAP2K4 **TP53**[VII] RAF1 CDC25B NRAS RPS6KA3 **CRKL**[II] **MAPK13**[II,VII] RRAS2 PLA2G6 IKBKB	0.50	0.0361	0.36	0.004 4
Natural killer cell mediated cytotoxicity	NRAS VAV3 MAP2K1 SOS1 RAF1 **PIK3R1**[II,IV]	- 0.76	0.9559	0.22	0.007 6
B cell receptor signaling pathway	NRAS VAV3 MAP2K1 SOS1 RAF1 **PIK3R1**[II,IV] AKT3	0.12	0.4712	0.34	0.023 7

[*]Targets

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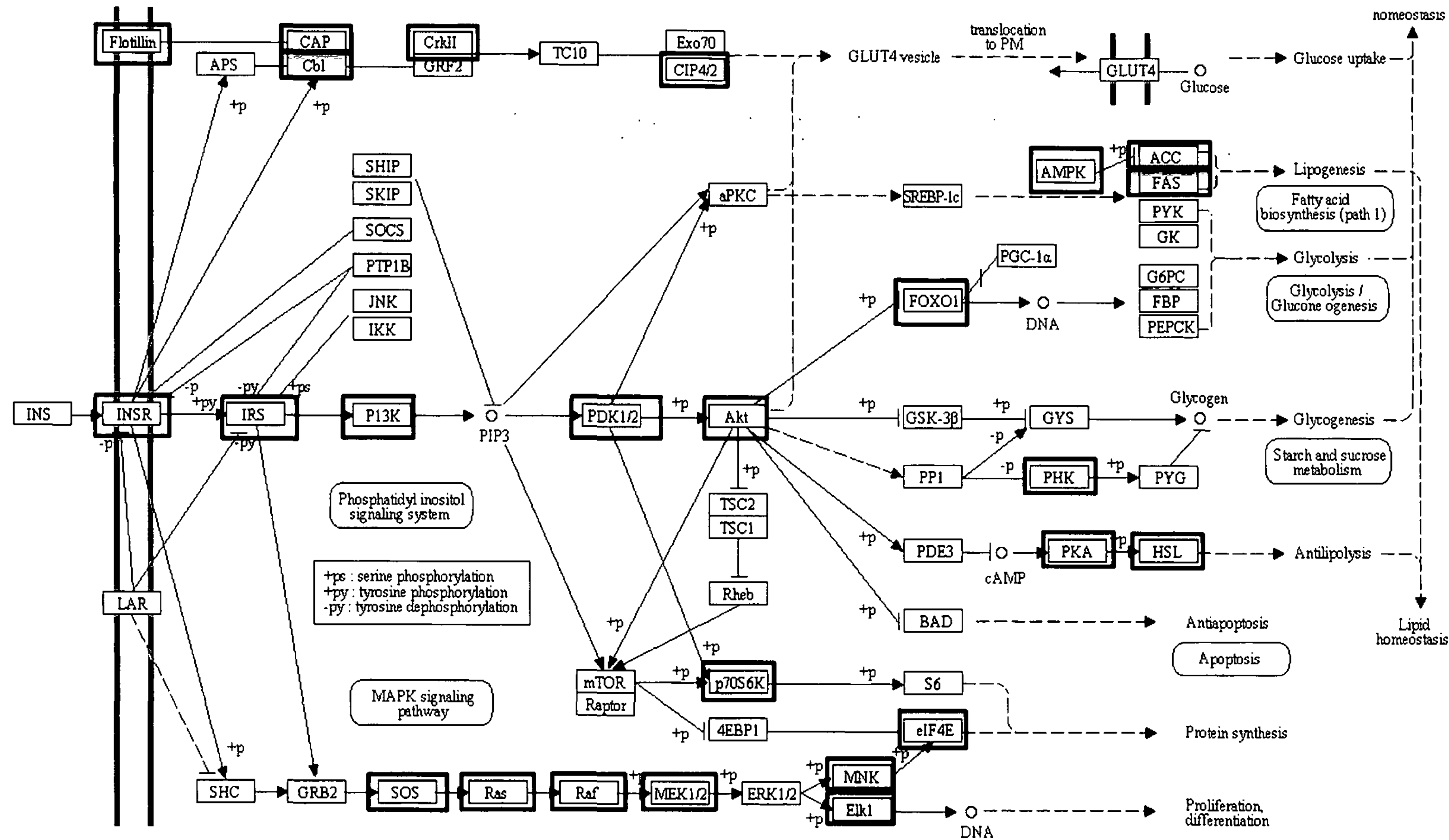


Figure 4.2.6: The insulin signaling pathway is targeted by the miRNAs in this study. The pathway visualization was carried out using KEGG from within the miTALOS application. The red boxes show 7 genes that are upregulated in this pathway during HIV infection either in vivo or in vitro. The black boxes show other miTGs for one or more of the miRNAs that are not yet reported to be upregulated in HIV infection. Here, miR-16 targets IRS2, MAP2K1, ACACA, MKNK1, FOXO1, RAF1, IRS1, PRKAR2A, CRKL, EIF4E, SOS1, FASN, IKBKB, TRIP10, AKT3, PIK3R1; miR-150 targets EIF4E, PRKAR1A, ELK1, PIK3R1, and miR-223 targets CBLB, PDPK1, SORBS1, FOXO1, RPS6KB1, PRKACB.

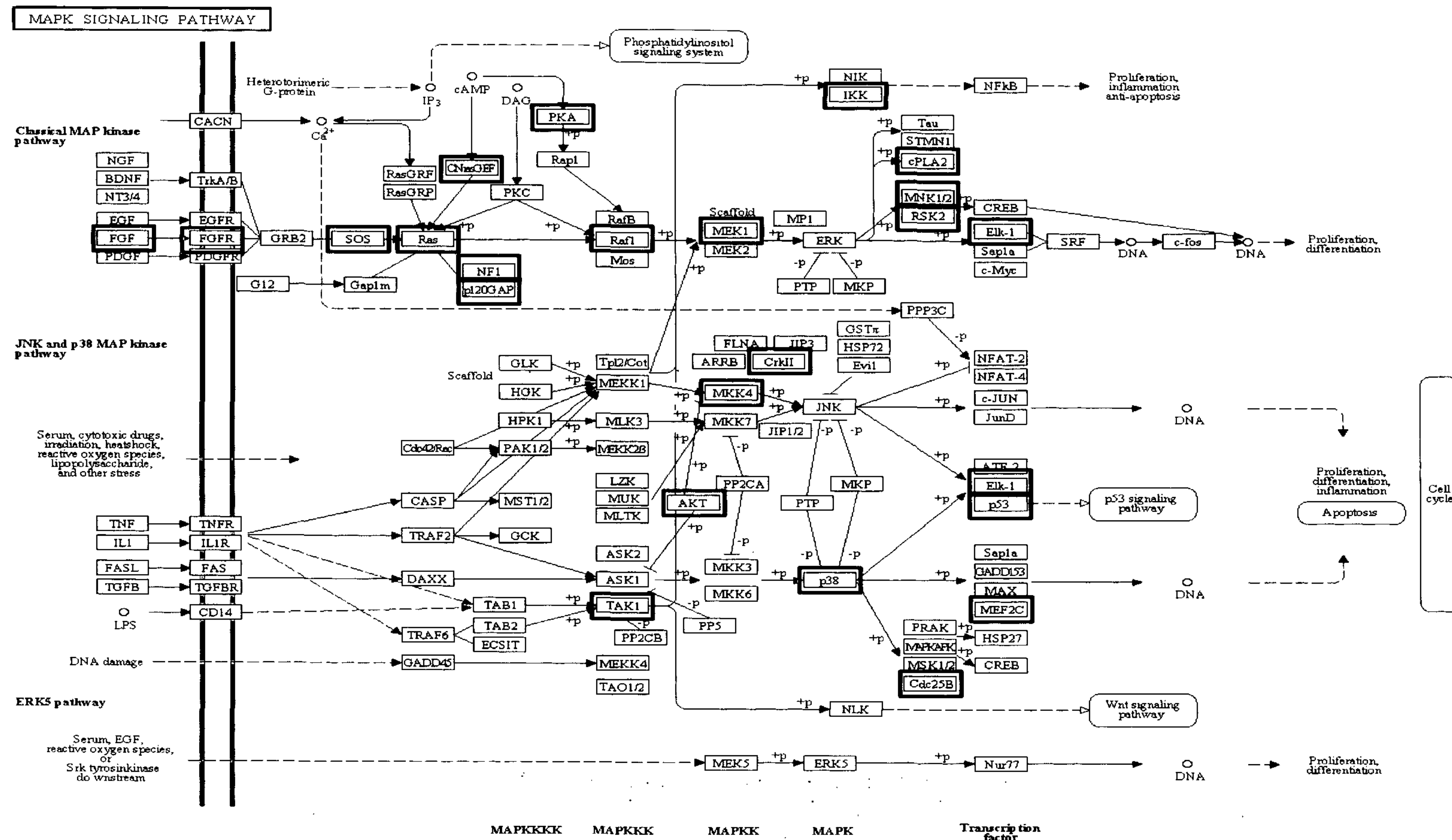


Figure 4.2.7: The MAPK signaling pathway is targeted by the miRNAs in this study. The pathway visualization was carried out using KEGG from within the miTALOS application. The red boxes show 7 genes that are upregulated in this pathway during HIV infection either in vivo or in vitro. The blue boxes show other miTGs for one or more of the miRNAs that are not yet reported to be upregulated in HIV infection. Here, miR-16 targets FGFR1, FGF7, MAP2K1, NF1, MAP2K4, MKNK1, RAF1, CDC25B, MAP3K7, RPS6KA3, CRKL, SOS1, IKBKB, RAPGEF2, AKT3; miR-150 targets MAPK13, TP53, ELK1; and miR-223 targets MEF2C, FGFR2, RRAS2, PLA2G6, PRKACB, RASA1.

Result and Discussion 3

Objective:

To characterize metabolite signatures in the plasma, urine and saliva of HIV/AIDS patients in different stages of disease and therapy.

Summary:

Though HIV primarily infects cells that do not have a major role in host metabolism, i.e. T lymphocytes and monocytes, clinical features of end-stage HIV/AIDS patients show gross metabolic changes. Moreover, there are many reports of metabolic syndromes that occur after starting ART in HIV/AIDS patients. But, there is inadequate information on global metabolic changes during HIV/AIDS, as well as before and after ART. We therefore carried out metabolic profiling of biofluids (plasma, urine and saliva) collected from asymptomatic and symptomatic HIV/AIDS patients as well as those on ART and compared these to biofluids from healthy persons.

The biofluids were profiled with ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy, followed by statistical analysis and annotation. Metabolites were identified by 2D ^{1}H-^{13}C HSQC NMR and from published literature. The modulated metabolic pathways were identified. We found that ^{1}H-NMR spectra of plasma, urine and saliva of HIV/AIDS patients and patients on ART showed clear separation from that of healthy individuals. A number of metabolites were differentially altered in the three types of samples. These included L-Glutamine, L-Methionine, L-Lysine, L-Isoleucine, L-Threonine, L-Threonine, L-Aspartic acid, Mannitol, Neopterin and Epi/Norepinephrine, etc. The association of these metabolites with pathways revealed the potential importance of amino acid (tyrosine, alanine, aspartate, glutamate, glycine, serine and threonine) metabolism, glycolysis or gluconeogenesis, nitrogen metabolism and butanoate metabolism following HIV infection and after introduction of ART. Further analysis and comparison with previously published data predicts the role of these pathways (metabolites) in the pathogenesis of HIV/AIDS and metabolic syndromes that develop following ART.

Results:

Multivariate analysis of metabonomics data:

The chemical shift and signal intensity in one-dimensional ^{1}H-NMR spectra of plasma, urine and saliva from patients in different stages of HIV/AIDS, including those on ART were compared with healthy controls. Figure 4.3.1 shows the 500 MHz ^{1}H-NMR spectra of plasma, saliva and urine of patients and controls. Since the spectra were complex and also showed variations between individuals, we used chemometric methods for spectral analysis. Principal Component Analysis (PCA) was performed with the first three principal components (PCs) on plasma, urine and saliva data sets of the study samples. Through these it was possible to distinguish between patients and controls on the basis of their NMR spectra, where PC1 alone explained 99.3%, 87.2% and 78.8% variations in plasma, urine and saliva respectively (Figure. 4.3.2).

Partial Least Squares Discrimination Analysis (PLS-DA) is a supervised method, which describes maximum separation between pre-defined classes in the data. Here samples were each classified into one of three groups – HIV/AIDS patients who are without ART, patients on ART and healthy controls and this was provided as a Y-table. A regression analysis of the original data against the Y-table was performed and in the PLS-DA scores plot, the first three partial least squares (PLS) components, PLS1, PLS2 and PLS3 showed improved separation between patients and controls, compared to the PCA analysis (Figure. 4.3.3). PLS-DA performed on variables collected from NMR spectra of plasma, urine and saliva gave a three-component model with an accuracy of 0.81 ($R^2 = 0.79$, $Q^2 = 0.70$), 0.86 ($R^2 = 0.80$, $Q^2 = 0.37$) and 0.75 ($R^2 = 0.63$, $Q^2 = -0.32$), respectively. To measure the robustness of the PLS-DA model, R^2 and Q^2 are used. The R^2 value is the fraction of variance explained by a model and Q^2 explains the fraction of the total variation predicted by the model. A model with $R^2 > 0.7$ and $Q^2 > 0.4$ is regarded as a good model with biological data (Lundstedt et al., 1998). It indicates that models generated from the changes of the metabolites of plasma and urine would build better models than that generated from saliva and these models could accurately predict 81%, 86% and 75% respectively, any stage of HIV/AIDS, i.e. asymptomatic, symptomatic or on ART.

Identification of metabolites:

As a supervised method, PLS-DA can perform both classification and selection of important features depending on the VIP (variable importance in projection) score. The VIP score is a weighted sum of squares of the PLS loadings. Higher the VIP score in PLS-DA, higher is the importance of a variable, in this case a metabolite or a group of metabolites. To identify the important features we set the VIP score at 0.8 (Tan et al., 2008). In MetaboAnalyst, PLS-DA analysis also provided the patterns of change for those variables in the plasma, urine and saliva data sets. The variables were identified as probable metabolites using MetaboAnalyst and later conformed through Metabominer (Xia et al., 2008) and published data from different sources (Lindon et al., 1999; Nicholson et al., 1996). The resonances corresponding to those variables are attributable to metabolites whose levels were significantly different either in the plasma, urine or saliva of HIV/AIDS patients without or with ART when compared with the control group (Table 4.3.1).

Changes in the level of metabolites:

The metabolite changes are summarized in Table 4.3.1 and the quantification of select ones is shown in Figure 4.3.4. There was increase in the level of MMA (Methyl Malonic acid), Choline, Sarcosine and L-Aspartic Acid in the plasma of HIV/AIDS patients, which decreased after the introduction of ART, but the levels still did not reach those in healthy persons (Figure 4.3.4). On the other hand, the levels of some metabolites like 5b-Cholestanol, L-Lysine, Acetoacetate and L-Threonine were lower in HIV/AIDS patients and after starting ART the levels increased but never reached those found in healthy controls. In urine, Glutamic Acid, Formic Acid levels were higher in HIV/AIDS patients compared to healthy controls and the trend continued even after the initiation of ART. On the other hand, the levels of Mannitol, Neopterin and Epi/Norepinephrine were higher in HIV/AIDS patients but decreased on ART administration. The levels of Methionine, 2-Methylglutaric Acid, L-Alanine in urine were lower in HIV infection and ART administration brought it back up to normal levels. The levels of Glycocholic Acid were lower in urine and it remained low even with ART. The metabolite changes in saliva were not significantly different between infected persons, those on ART and healthy controls. Only salivary L-Lactic acid levels

were higher following infection and remained so on therapy. Alternatively, there was an increase in the levels of Alpha-Tocopherol, Butyric acid, L-Lysine, N-acetyl glutamic acid and Propionic acid, and a decrease in testosterone and ethanol in saliva after ART introduction.

Pathway analysis and localization of metabolites:

The metabolites identified from our study were found to be associated with a number of metabolic pathways (Table 4.3.2). In this analysis we considered only those pathways that have at least two metabolite hits. The profile indicates significant changes in metabolic processes during HIV infection and after the introduction of ART. These changes are especially prominent in metabolic cycles, glucose metabolism, hormone biosynthesis and amino acid biosynthesis pathways. To get an idea about the cells, tissues and organs affected in HIV infection, localization analysis of metabolites present in biofluids was carried out (Table 4.3.3).

Discussion:

HIV primarily infects the CD4+ cells especially the T cells and monocytes. Though these cells have no major role in host metabolism, HIV/AIDS patients commonly suffer from weight loss and malnutrition (Green, 1995; Vorster et al., 2004). These clinical features indicate metabolic abnormalities in these patients. Several factors may contribute to malnutrition in patients with HIV/AIDS including insufficient intake, malabsorption and increased energy expenditure (Wilson et al., 1997). In addition, the significance of endocrine dysfunction and the metabolic cost of inflammation, including cytokine production, have been suggested as additional factors that contribute to weight loss and changes in body composition (Grinspoon & Mulligan, 2003). Therefore studying metabolic changes during the different stages of HIV infection is important. Moreover, there are many reports of metabolic syndromes that occur after starting ART in HIV/AIDS patients. Unfortunately, at present there is inadequate information present on global metabolic changes in patients with HIV/AIDS before and after ART. To address this, we have performed metabolic profiling of biofluids - plasma, urine and saliva, collected from HIV/AIDS patients and patients on ART. The NMR spectra were collected for these biofluids from patients and were compared to the same from healthy controls. Univariate and multivariate statistical analyses were then carried out. We found the NMR spectra of healthy individuals, HIV/AIDS patients and those on ART to be separable from each other. This suggests that the variables of the NMR spectra with different spectral intensity i.e. quantity of each metabolite, varied in each group of samples and there are infection/disease-specific changes in the biofluids. The changes are most visible in plasma and urine, and much less in saliva. Using a metabonomics approach, the metabolites differentially present in plasma, urine and saliva in HIV-infected patients and their changes on ART were identified and these were extrapolated to pathway networks and systemic effects following HIV infection and treatment.

Changes in the amino acids

L-Aspartic acid is a non-essential amino acid that is synthesized from glutamic acid. It has important roles in the urea cycle and DNA metabolism. We found increased plasma L-Aspartic Acid in HIV/AIDS patients, which was reduced

after ART but still remained above the levels observed in uninfected healthy persons. The level of citrulline was also significantly higher in HIV/AIDS patients (Wanchu A et al., 2002). Increased levels of L-Aspartic Acid and citrulline from the urea cycle may also be a source of elevated levels of fumaric acid in the plasma of HIV patients and in patients with ART found in our study. Plasma L-lysine and L-threonine are the two essential amino acids, which cannot be synthesized by humans and are obtained through diet. These were lower in HIV patients. We also found higher levels of another essential amino acid methionine in urine. Low levels of plasma threonine and methionine were previously seen in HIV patients and both of these amino acids are regarded as rate-limiting for whole-body protein synthesis in AIDS patients (Hortinet al., 1994; Laurichesse et al., 1998). Lower levels of serum methionine were detected in AIDS patients with myelopathy (Di Rocco et al., 2002). Carnitine, a quaternary ammonium compound, synthesized from lysine and methionine, augments the production of acetoacetate (Steiber et al.,2004; Bressler R and Richard I,1965). We could not identify any changes in the levels of carnitine in present study, but found lower levels of methionine to be excreted in urine. We suggest that lower levels of acetoacetate found in our study may be due to lack of methionine and/or lack of total and free carnitine that was observed earlier in majority of the AIDS patients (De Simone et al., 1992). In this study elevated levels of sarcosine, a natural amino acid, were found in plasma and this was reduced after ART. Increased level of sarcosine in oral metabolites of HIV/AIDS patients were reported earlier, but this was not affected after ART (Ghannoum et al., 2010). Sarcosine is an intermediate and byproduct in glycine synthesis and degradation and is found in muscles and other tissues. Sarcosinemia may occur from severe folate deficiency associated with the requirement of folate for the conversion of sarcosine to glycine. Folate deficiency in HIV infection (Alani et al., 2010) may be the reason of sarcosinemia in HIV infection.

Reduced glycolysis

In the present study, plasma D-Glucose levels were elevated in HIV infected and ART groups. In various other studies glucose levels were also increased in HIV patients (Dominic et al., 2006) and patients on ART (Gelato, et al., 2003) due to high levels of pro-inflammatory cytokines (Limone et al., 2003), viral

proteins (Kino et al., 2002; Leow et al., 2003) and due to reduced insulin sensitivity and type 2 diabetes (Brown et al., 2005). Moreover, we also found that urinary excretion of sucrose increased in HIV patients and remained elevated even after the ART initiation.

Changes in metabolites related with the immune system

We found considerable changes in two important indicators of immune function - neopterin and vitamin E. There was increased urinary excretion of neopterin in HIV patients, which was reduced on ART. Neopterin is a catabolic product of guanosine triphosphate (GTP) synthesized by monocytes/macrophages, endothelial cells, B cells and dendritic cells. The measurement of neopterin in body fluids like plasma, cerebrospinal fluid or urine is indicative of a pro-inflammatory immune status (Murr et al., 2002). There is strong correlation between urinary and serum neopterin, which correlates inversely to CD4+ lymphocyte counts in HIV infection (Fuchs et al., 1988; Fahey e al., 1999; Breustedt et al., 1989). Our findings reaffirmed the earlier reports that there are significant changes of neopterin in different stages of HIV infection, which is reversed after ART. In addition, we also found that the level of α-Tocopherol in saliva was reduced in HIV patients and increased because of ART. Alpha-Tocopherol is a type of vitamin E, which is preferentially absorbed and accumulates in humans. It acts as an antioxidant and immunostimulator. Increased vitamin E excretion in the urine was earlier incriminated for the reduced plasma levels of vitamin E in HIV patients (Jordão et al., 1998). We also think that increased excretion of vitamin E though urine may be responsible for the reduced salivary vitamin E, which was reversed due to ART. This effect was more pronounced in patients on ART with protease inhibitors than those not taking these drugs (Tang et al., 2000).

Changes in metabolites related to the nervous system

In the present study we observed modulation of norepinephrine and choline which are precursors for the neurotransmitter, acetylcholine. We found higher urinary and salivary norepinephrine in HIV/AIDS patients, which reduced in patients on ART. Circulating neurotransmitters in blood are filtered through the kidneys and subsequently excreted in the urine. The urinary levels of

neurotransmitters represent their levels in the body (Alts et al., 2007). We think that the high levels of norepinephrine in urine indicate its increased systemic levels. High urinary concentrations of norepinephrine were also reported to be associated with high HIV viral load (Ironson et al., 2008). Previously it was suggested that norepinephrine enhanced adhesion of HIV-1-infected leukocytes to endothelial cells of the cardiac microvasculature (Sundstrom et al., 2003). In addition, high levels of urinary norepinephrine excretion also correlates with depression and anxiety (Hughes et al., 2004), which is frequently seen in HIV/AIDS patients (Lyketsos et al., 1995).

Choline is important precursor for acetylcholine and for membrane synthesis (Droz et al., 1978). It is also involved as a methyl donor in various metabolic processes, and in lipid metabolism. Elevated plasma levels of choline in HIV patients found in this study were also noticed earlier in plasma (Bogden et al., 2000) and serum (Philippeos et al., 2009), and in the brain of HIV/AIDS patients. It was proposed as a biomarker of the AIDS dementia complex (Meyerhoff et al., 1999). A rise in choline levels is associated with increased membrane breakdown or turnover, demyelination or inflammation and was observed in demyelinating diseases (Roser et al., 1995). In HIV infection, inflammatory demyelinating neuropathies are frequently diagnosed (de Ganset et al., 1989; Leger et al., 1989; Thornton et al., 1991). We found the levels of choline to be reduced after the introduction of ART, which signify its role as a biomarker of HIV/AIDS especially in the AIDS dementia complex. (Meyerhoff et al., 1999).

Changes in Sex Hormones

Several studies have shown that HIV can affect the ability to produce and maintain hormone levels, especially the sex hormones in infected humans (Meena et al., 1996; Teichmann et al., 1998). We observed higher levels of salivary testosterone in HIV patients, which were reduced after starting ART. Previously plasma levels of free, bioavailable and total testosterone were reported to be low (Dobs et al., 1996) in HIV patients; this was regarded as a reason for hypogonadism observed in male HIV patients. There was a positive correlation between serum testosterone and CD4 in HIV patients (Meena et al., 2011). Though in the ART group we found decreased salivary testosterone, HAART was earlier shown not to decrease plasma testosterone levels

(Rietschel et al., 2000). This discrepancy between salivary and plasma testosterone found in our study was noticed previously as well (Flyckt et al., 2009). Additionally, we found increased levels of salivary estradiol, which was increased even further after the introduction of ART. Higher levels of serum and urinary estradiol were earlier reported in HIV infection (Teichmann et al., 1998). It was suggested that the deregulation of sex hormones in HIV infection might be related with complaints of diminished libido and increased impotence often observed in HIV patients (Teichmann et al., 2003).

Metabolic changes in oral fluid: possible role of oral pathogens

We found significant changes in three metabolites in saliva - butyric acid, lactic acid and ethanol, which may be associated with opportunistic pathogens prevalent in HIV/AIDS patients. Higher levels of butyric acid were also observed in patients on ART. Butyric acid is a product of *P. gingivalis* and *F. nucleatum*, which cause peridontal disease observed in HIV/AIDS patients, including those on ART in India and elsewhere (Ranganathan et al., 2004; Mataftsi et al., 2011). The existence of these bacteria in the oral cavity of HIV patients was also confirmed in other studies (Gonçalves et al., 2004; Kesic et al., 2008). It was suggested that butyric acid causes histone acetylation and thus induces reactivation of the "repressed" provirus containing host chromatin *in vitro* (Imai et al., 2009). This reactivation may break the latency of HIV in host cells. Our study indicates that after the induction of ART, despite the improvement in general health, the butyric acid producing bacteria remain in the oral cavity. The role of higher butyric acid in oral fluid in reactivation of HIV latency remains to be elucidated *in vivo*.

In earlier studies the levels of lactic acid in blood were found to be significantly higher in patients on NRTI drugs (ter Hofstede et al., 2004; Carr, 2003). Interestingly we found higher levels of lactic acid in the saliva of HIV/AIDS patients and patients on ART. We think that this increase may not be due to plasma lactic acidosis, but due to lactic acid producing bacteria present in the oral cavity of HIV/AIDS patients (Madigan et al., 1996). Lactobacilli and mutans streptococci convert glucose, fructose and sucrose found in the oral cavity into lactic acid through glycolytic fermentation. We believe this to be the reason for higher levels of salivary lactic acid in HIV/AIDS patients in our study.

We also found elevated levels of ethanol in the oral fluid of HIV patients, which was reduced after ART. Oropharyngeal candidiasis caused by *C. albicans* is the most prevalent opportunistic infection in HIV/AIDS (Barchiesi, et al., 2002). Under anaerobic conditions *in vitro* and *in vivo*, *C. albicans* produces ethanol via fermentation (Ogasawara et al., 2006; Chang et al., 1989). However, in this study we do not have any direct evidence of ethanol production by *C. albicans* in the oral cavity of HIV/AIDS patients. Since HIV infection of primary oral epithelial cells in culture was increased significantly by alcohol (Chen et al., 2004), increased levels of ethanol in the oral cavity may increase the risk of continuous reinfection cycles of HIV in the mouth. This could be an interesting area of research.

Changes in other metabolites:

In plasma, the levels of methylmalonic acid (MMA) were increased. Elevated levels of MMA are related to vitamin B12 (cobalamine) deficiency (Lindenbaum et al., 1988). In that deficiency, lack of by 5'-deoxyadenosyl-cobalamin impairs the conversion of methylmalonyl-CoA to succinyl-CoA, which might lead to increased release of MMA in blood and its excretion in the urine. Several researchers have reported vitamin B12 deficiency in HIV infection (Meyer et al., 1996; Harriman et al., 1989; Rule et al., 1994), which may be related to elevated levels of MMA. In addition, fumaric acid was found to be increased in plasma and 2-methylglutaric acid was decreased in the urine of HIV patients and patients on ART. Fumaric acid and 2-methylglutaric acid are the metabolites of the citric acid cycle. Fumaric acid is formed by the oxidation of succinate by succinate dehydrogenase, and 2-methylglutaric acid is a metabolite of succinic acid. So the decreased excretion of 2-methylglutaric acid in urine may be due to increased formation of fumarate from succinate in HIV patients. Formic acid in urine was increased in HIV patients and increased further in patients on ART. Formic acid inhibits cytochrome oxidase, resulting in cell death due to depletion of ATP. Reduced activity of cytochrome C in HIV patients was observed in adipocytes after ART (Hammond et al., 2004). Increased levels of formic acid may be involved in the inhibition of cytochrome oxidase after induction of ART.

Metabolic pathway analysis

We observed differential production of metabolites in plasma, urine and saliva in response to HIV infection and their changes after the introduction of ART. Interestingly ART does not normalize increased levels of some metabolites such as MMA, Sarcosine, Acetoacetate in plasma, Neopterin in urine, testosterone in saliva, etc. There was a further increase in the levels of some other metabolites such as Formic acid in urine, Butyric acid and Ethanol in saliva, or a decrease in others such as L-Alanine, Methionine, 2-Methylglutaric acid in urine. On network analysis we found the presence of several metabolites representing amino acid biosynthesis, carbohydrate metabolism pathways, steroid synthesis, nitrogen metabolism, etc. Among these metabolites, some such as D-Glucose, Sucrose, L-Glutamine, L-Aspartic acid and L-Glutamic Acid are commonly involved in overlapping metabolic processes like Glucose and protein synthesis pathways, and may correspond to principal regulatory points of the human metabolome in HIV infection. There are several reports of dysregulation in glucose (Weis et al., 2004; Larson et al., 2006) and amino acid metabolism (Hortin et al., 1994; Yarasheski et al., 2005) during HIV infection. Undoubtedly these metabolic pathways are involved in general health and their dysregulation leads to gradual weight loss, muscle wasting, anorexia, malabsorption and malnutrition observed in HIV patients. The metabolites involved in amino acid metabolism may also play important roles in regulating host immunity. Therefore changes in their levels impair immune function and increase the susceptibility to other infections (Li et al., 2007). This warrants detailed investigations to better understand and manage HIVAIDS patients. The analysis for localization of metabolites showed that in HIV infection and after introduction of ART, many organs such as liver, intestine, muscle, brain, spleen, gonads, etc. are metabolically involved. The most metabolically affected cells appear to be fibroblasts, neurons and sperm. At the subcellular level, the altered metabolites are likely to be localized in the endoplasmic reticulum, mitochondria and peroxisomes.

Conclusion:

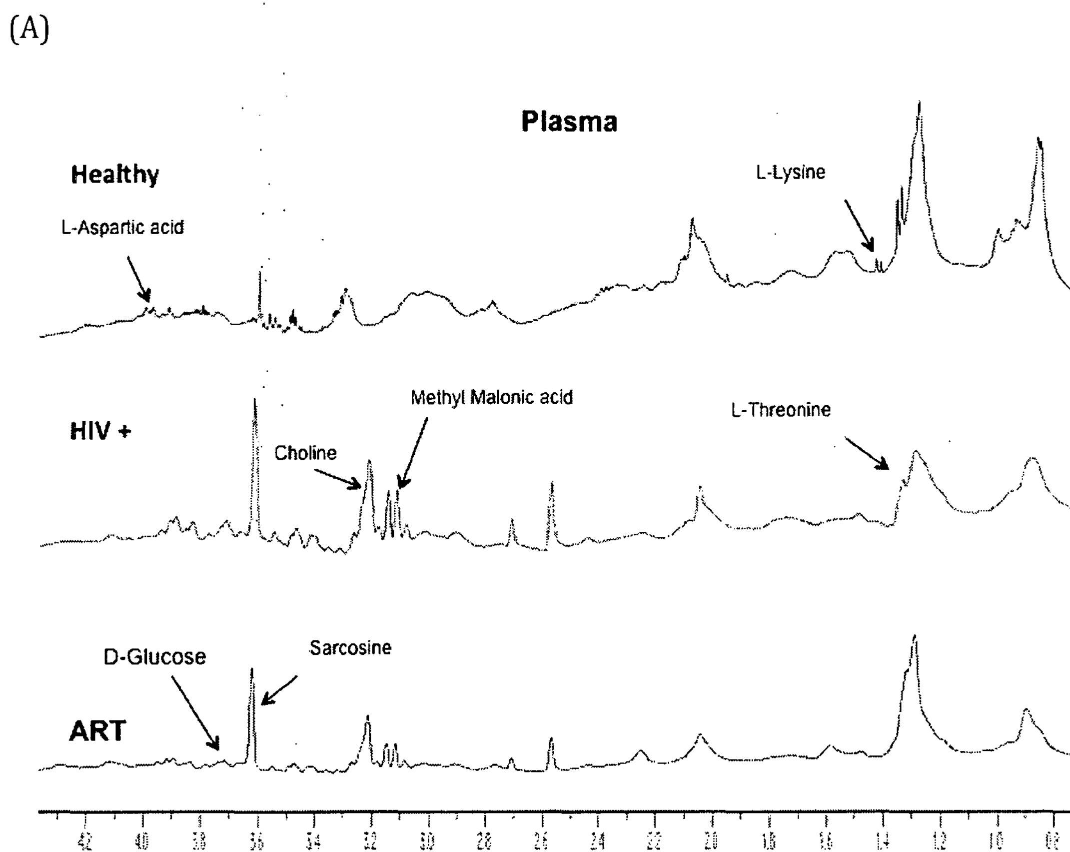
This metabolnomic study revealed that metabolites present in plasma, urine and saliva are differentially produced in response to HIV infection and there are changes in their levels after the introduction of ART. Furthermore, it also

established that a metabonomic approach using biological samples is one of the ways to differentiate between HIV/AIDS patients and patients on ART from healthy persons, and may offer diagnostic possibilities. The dysregulated metabolic pathways, which are involved in disease outcome in HIV infection, were also identified in this study. This study emphasizes the need to further study the consequences of HIV infection on host metabolism and their implications for antiviral immunity.

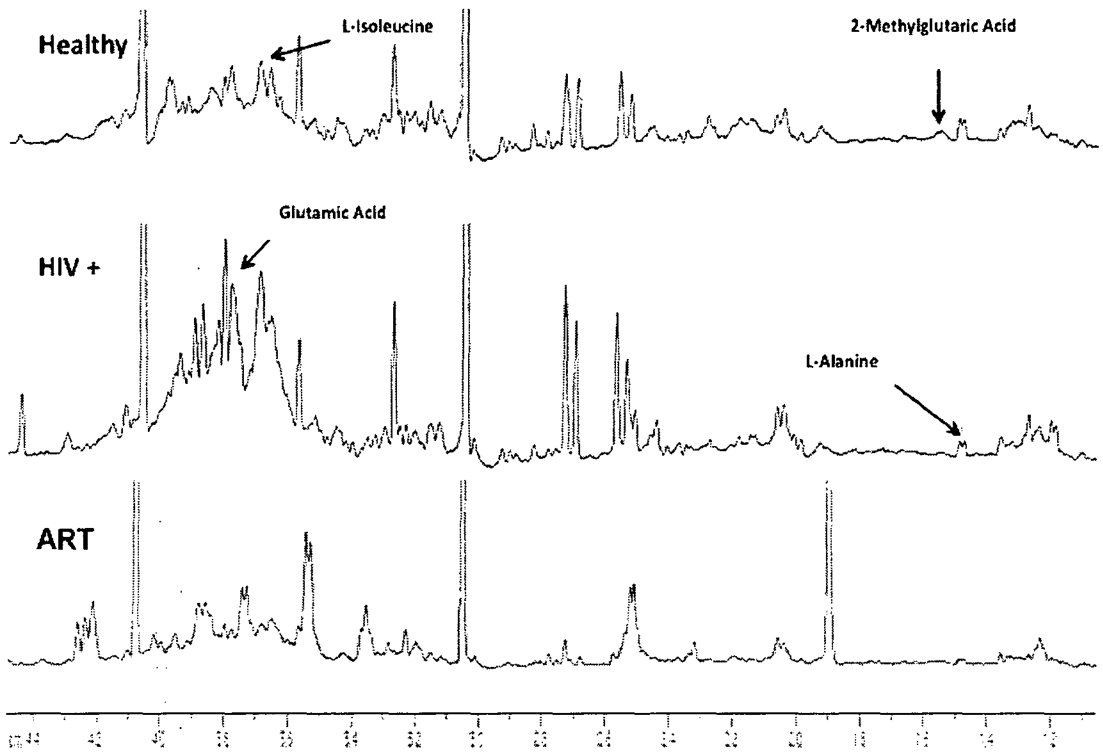
Figures and Tables

Figure 4.3.1: Representative one-dimensional 1^H NMR spectra of (A) plasma and (B) urine and (C) saliva obtained from a healthy control, patient with HIV, and HIV patient on ART.

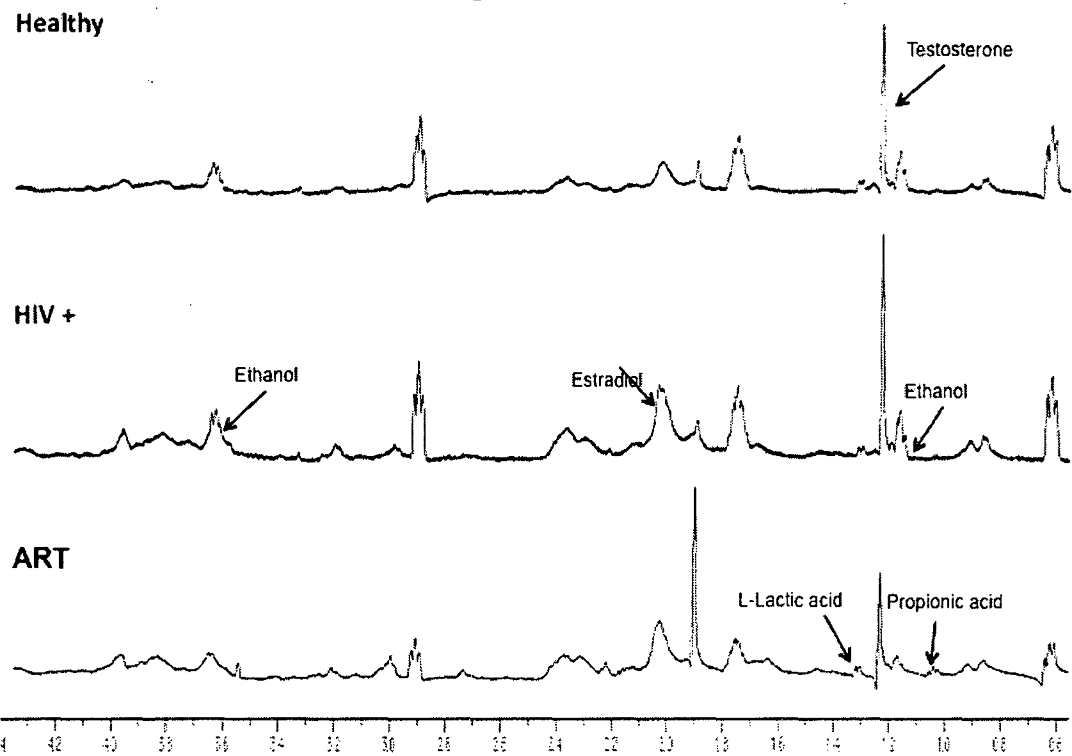
(A)



B)



(c)



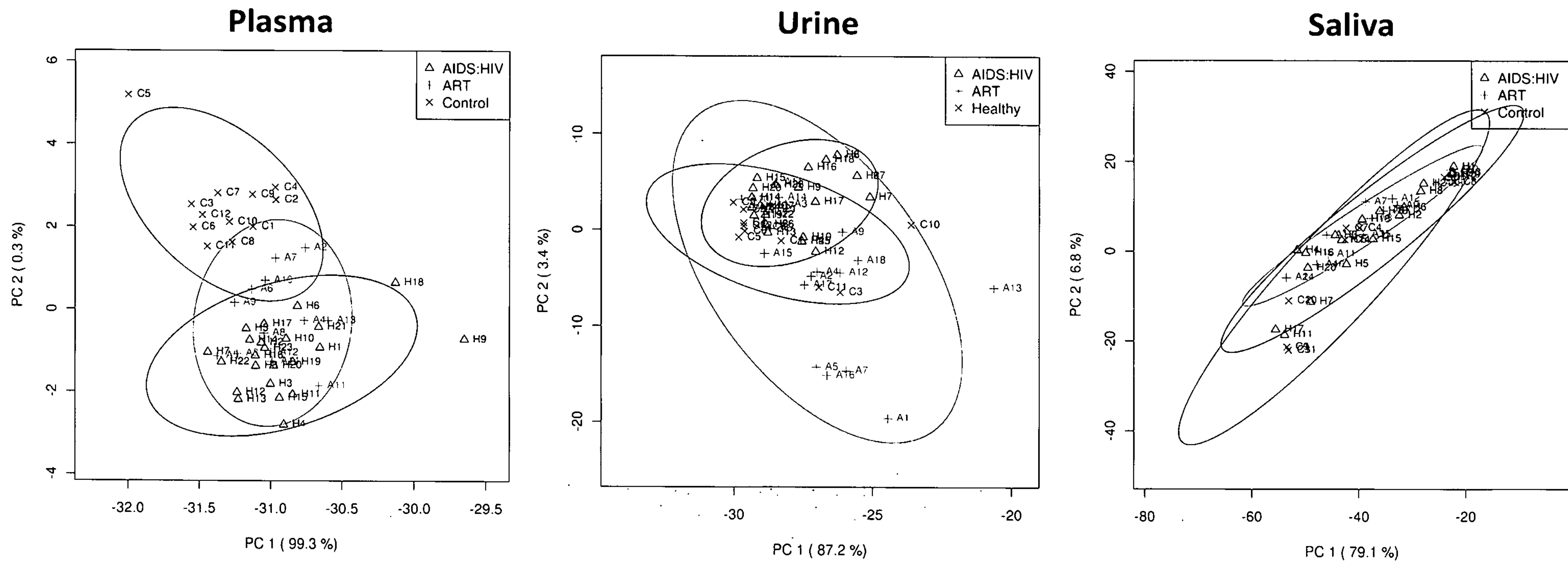


Figure 4.3.2: 2D score plots of of principal component 1 (PC1) versus principal component 2 (PC2) developed from Principle Component Analysis (PCA) of ^{1}H NMR spectra of plasma, urine and saliva collected from healthy control (X), HIV-infected patients (Δ) and patients on ART (+).

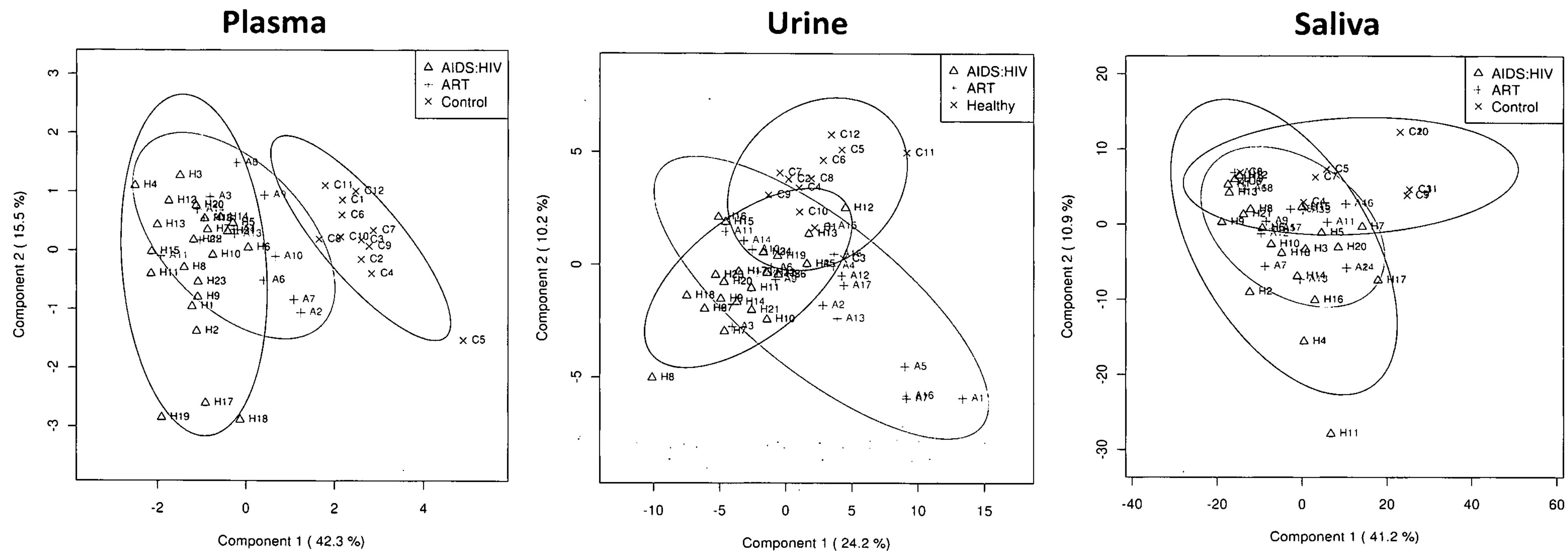


Figure 4.3.3: Parital least squares discrimination assay (PLS-DA) 2 D score plot derveloped from the 1H NMR spectra of plasma, urine and saliva collected from healthy control (X) and HIV-infected patients (Δ) and patients on ART (+). There is clear separation between these groups observed in all the sample types resulting in three individual separate clusters in the plot.

Figure 4.3.4: The box and whisker plots showing the levels of representative metabolites in plasma, urine and saliva collected from healthy (red) and HIV-infected patients (green) and patients on ART (blue). The y-axis shows the normalized concentrations of the metabolites

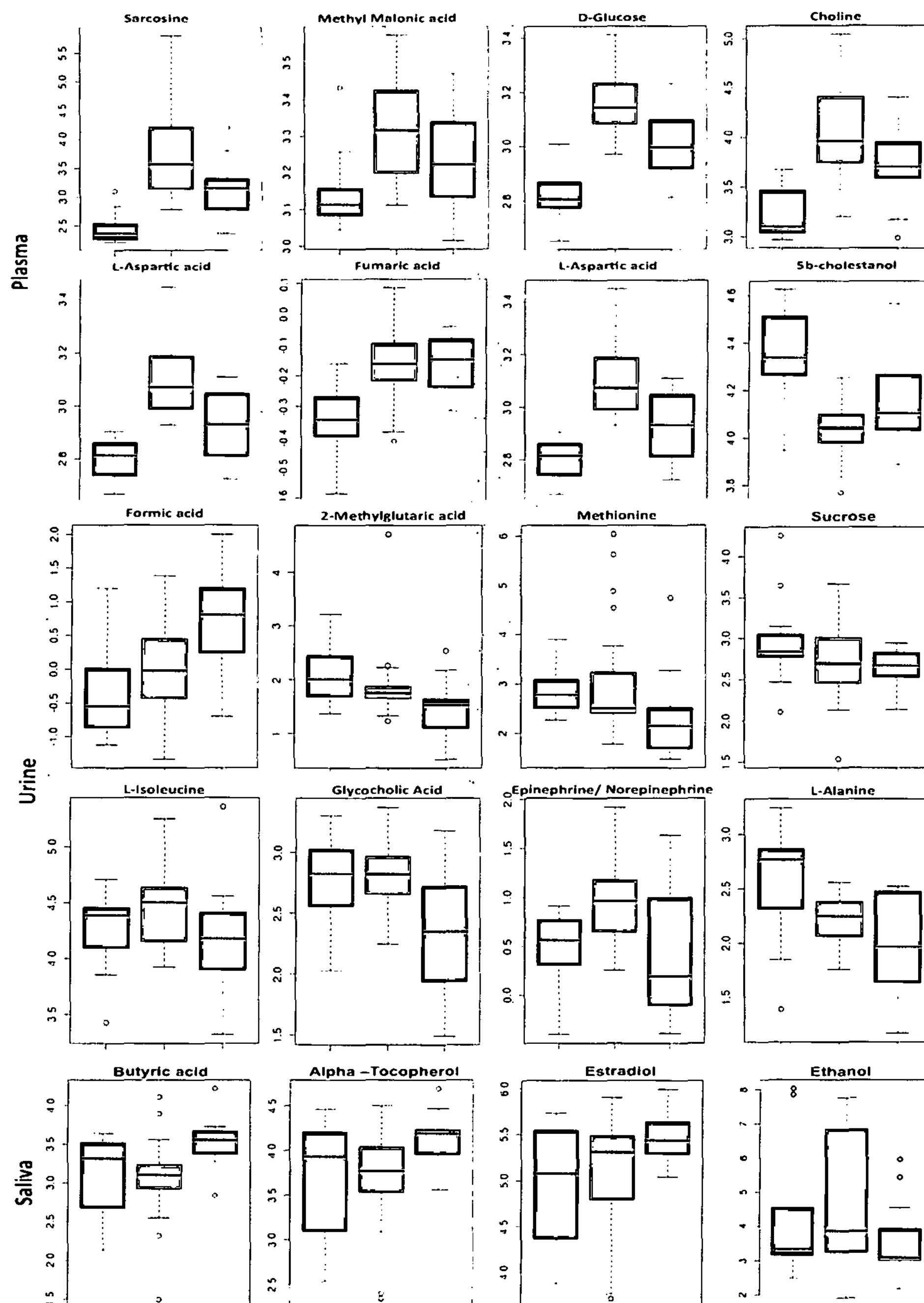


Table: 4.3.1. Name of the metabolites differentially regulated in serum (p), Urine (u) and saliva (s) in HIV/ AIDS patients and patients on ART comparing with healthy control. Arrow indicates lower (↓) or higher (↑) arbitrary level of metabolites.

Metabolites	***ppm/multipliciy***	**HIV**	**On ART**
2-Methylglutaric acid/ u	1.52/m	↓	↓↓
Acetoacetate / p	2.28/s	↓↓	↓
Alpha –Tocopherol / s	2.32/m	↓	↑
Butyric acid / s	2.16/m	↓	↑
Choline / p	3.2/ s	↑↑	↑
D-Glucose /p	3.72 / m	↑↑	↑
Epinephrine/u/ Norepinephrine / s,u	6.94/m	↑	↓
Estradiol/ s	2.04/ br.s	↑	↓
Ethanol / s	1.16/t 3.64/q	↑	↑↑
Formic acid / u	8.44/s)	↑	↑↑
Fumaric Acid / p	8.52/s	↑	↑
Glutamic Acid / u	3.72 /dd	↑	↑
Glycocholic Acid/ u	2.08/m	-	↓
L-Isoleucine / u	3.66 / d	↑	↓
L-Alanine/ u	1.44 /d	↓	↓↓
L-Aspartic acid/ p	3.88/ dd	↑↑	↑
L-Lactic acid / s	1.32/ d	↑	↑
L-Lysine / p	1.4/m	↓	↓
L-Threonine / p	1.32/d	↓	-
Methionine / u	2.16/ m	↑	↓↓
Methyl Malonic acid/ p	3.12/ q	↑↑	↑
Neopterin / u	8.68 /s	↑↑	↑
Propionic acid / s	1.04/t	-	↑
Sarcosine / p	3.6/ s	↑↑	↑
Sucrose / u	3.48/dd	↑	↑
Testosterone / s	1.2/s	↑	↓

Table: 4.3.2. Metabolic Pathways modulated in HIV/AIDS and on ART patients. [The pathways which has at lest 2 metabolites in hit list are considered]

Pathway Name	Total Compounds	Raw p	Hits	Name of the Hits
Aminoacyl-tRNA biosynthesis	75	7.18E-05	7	L-Glutamine,L-Methionine, L-Lysine, L-Isoleucine, L-Threonine, L-Threonine, L-Aspartic acid
Glycine, serine and threonine metabolism	48	5.28E-04	4	Choline,Sarcosine, L-Threonine, L-Aspartic acid
Arginine and proline metabolism	77	0.0044999	4	L-Glutamine, L-Aspartic acid, Fumaric acid, Sarcosine
Propanoate metabolism	35	0.0014254	4	Methylmalonic acid, Propionic acid, L-Lactic acid, Acetoacetic acid
Nicotinate and nicotinamide metabolism	44	0.0033674	3	L-Aspartic acid, Fumaric acid, Propionic acid
Tyrosine metabolism	76	0.022891	3	Fumaric acid, Acetoacetic acid, Norepinephrine,
Alanine, aspartate and glutamate metabolism	24	0.004626	4	L-Glutamic Acid , Fumaric acid, L-Aspartic acid,L-Alanine
Glycolysis or Gluconeogenesis	31	0.0095828	3	D-Glucose, L-Lactic acid, Ethanol
Nitrogen metabolism	39	0.017998	3	L-Aspartic acid, L-Glutamic Acid, Formic Acid
Butanoate metabolism	40	0.019265	4	Butyric acid, Fumaric acid, Acetoacetic acid, L-Glutamic Acid
Starch and sucrose metabolism	50	0.034627	2	Sucrose, D-Glucose,
Steroid hormone biosynthesis	99	0.17207	2	Testosterone, Estradiol
Valine, leucine and isoleucine biosynthesis	40	0.057459	2	L-Threonine, L-Isoleucine
Lysine biosynthesis	32	0.077635	2	L-Aspartic acid, L-Lysine
Galactose metabolism	41	0.11846	2	Sucrose, D-Glucose
Cysteine and methionine metabolism	56	0.19497	3	L-Aspartic acid, L-Methionine, L-Alanine
Pyrimidine metabolism	60	0.21642	2	L-Glutamine, Methylmalonic acid
Pyruvate metabolism	32	0.04564	2	L-Lactic acid, Formic Acid
Histidine metabolism	44	0.085932	2	L-Aspartic acid, , L-Glutamic Acid,

Table 4.3.3: Localization of the metabolites. This library contains 57 metabolite sets based on organ, tissue, and subcellular localizations.

Metabolite Set	Total	Hits	Expect	P value	Name of the metabolites
Muscle	101	4	4.31	0.646	Butyric acid, L-Methionine; Sarcosine, Testosterone
Endoplasmic reticulum	53	3	2.26	0.397	Testosterone, Estradiol;
Mitochondria	98	3	4.18	0.813	Sarcosine, L-Glutamine;
Fibroblasts	183	3	7.81	0.993	Butyric acid, Glycocholic acid; Acetoacetic acid; L-Methionine; LTryptophan
Liver	234	3	9.98	0.999	Methylmalonic acid, Glycocholic acid; Acetoacetic acid
Brain	122	1	5.21	0.977	Testosterone
Intestine	145	2	6.19	0.992	Butyric acid, Methylmalonic acid
Kidney	164	2	7	0.997	Butyric acid, Methylmalonic acid;
Sperm	8	1	0.341	0.296	Testosterone
Gonads	9	1	0.384	0.326	Testosterone
Hypothalamus	9	1	0.384	0.326	Testosterone
Thalamus	10	1	0.427	0.355	Testosterone
Pancreas	32	1	1.37	0.76	L-Methionine
Erythrocyte	34	1	1.45	0.781	D-Xylose
Peroxisome	37	1	1.58	0.809	Sarcosine
Prostate	39	1	1.66	0.826	Butyric acid
Skeletal muscle	45	1	1.92	0.868	Sarcosine
Spleen	68	1	2.9	0.955	L-Methionine
Neuron	69	1	2.94	0.957	Butyric acid
Testes	72	1	3.07	0.963	Testosterone

Chapter 5: References

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Chapter 6: Appendix

Appendix 1

Table 3.1: Reagents and their sources:

Name of the reagents	Sources
Sample collection	
Tempus™ Blood RNA Tubes Part Number 4379233	Applied Biosystems, Foster City, CA, USA
K+EDTA-coated vacutainers	Becton Dickinson, Franklin Lakes, NJ, USA
PBMC isolation and preservation	
Ficoll-Paque PLUS	GE Healthcare, Uppsala Sweden
HBSS - Hank's Balanced Salt Solution	Invitrogen, Carlsbad, California
Dimethyl sulfoxide	Sigma-Aldrich Chemie GmbH, Germany
RNA isolation and quantification	
miRNeasy Mini Kit	QIAGEN, USA
Tempus™ Spin RNA Isolation Kit	Applied Biosystems, Foster City, CA, USA
NanoDrop 1000	(Thermo Scientific, Wimington, DE, USA
Agilent 2100 Bioanalyzer	Agilent Technologies, Inc, Santa Clara, CA
PCR array	
PCR array plates Format A (96-well) appropriate for StepOne Plus real time machine of Applied Biosystems. PA-010-24	SuperArray Biosciences, Frederick, MD, USA
RT First Strand Kit (C-03)	SuperArray Biosciences, Frederick, MD, USA
RT2 SYBR Green / ROX qPCR Master Mix	SuperArray Biosciences, Frederick, MD, USA
Cytokine Bead Assay	
Human Cytokine / Chemokine panel *MPXHCYTO-60K-13* Human Chemokine/Cytokine panel, 13-plex consisting of (GRO, PDGF-AB/BB, IL-13, IL-6, IL-8, MIP-15, MCP-1, MIP-13, TNF-3, GM-CSF, IFN-7, IL-10, IL-15) *MPXHCYP2-62K-03* Human Chemokine/Cytokine panel, 3 plex consiting of (MCP-2, TRAIL, Eotaxin –2)	Millipore SAS, BP 116 67124, Molsheim Cedex, France.
miRNA assay	
Taqman MicroRNA® Assays	Applied Biosystems, Foster City, CA, USA
MultiScribe™ Reverse Transcriptase	Applied Biosystems, Foster City, CA, USA
TaqMan 2X Universal PCR Master Mix No AmpErase UNG	
ABI StepOne Plus cycler	Applied Biosystems, Foster City, CA, USA

miRNA Primers hsa-miR-16 hsa-miR-146-5b hsa-miR-150 hsa-miR-191 hsa-miR-223 RNA-44	Applied Biosystems, Foster City, CA, USA
cDNA synthesis and real time PCR	
M-MLV reverse transcriptase	Promega Corporation, Madison, WI, USA).
dNTPs	Applied Biosystems, Foster City, CA, USA
Oligo dT	Applied Biosystems, Foster City, CA, USA
Nuclease free H_2O	QIAGEN, USA
5x Hot FirePol EvaGreen® qPCR Mix Plus	Solis BioDyne, Tartu, Estonia
Viral Load Measurement:	
QIAamp Viral RNA Mini Kit	QIAGEN, USA
SuperScript® III Reverse Transcriptase	Invitrogen, Carlsbad, California
dNTPs	Applied Biosystems, Foster City, CA, USA
Oligo dT	Applied Biosystems, Foster City, CA, USA
Nuclease free H_2O	QIAGEN, USA
RNA standard	NIH AIDS Reagent Bank
^{1}H NMR	
D_2O, Deuterium oxide, 99.8% atom D;	Sigma Aldrich Chemical Company, USA
DSS, 2,2-Dimethyl-2-silapentane-5-sulfonate sodium salt, DSS sodium salt, Sodium 3-(trimethylsilyl)-1-propanesulfonate	Sigma Aldrich Chemical Company, USA
NMR tubes	Wilmad, Sigma Aldrich, USA
Bruker Avance III spectrometer	Bruker, Germany

Table -3.2 Gene list of RT² Profiler™ PCR Array Human HIV Infection and Host Response (PAHS-051A)

Position	Unigene	GeneBank	Symbol	Description
A01	Hs.73722	NM_080649	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1
A02	Hs.659809	NM_145298	APOBEC3F	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F
A03	Hs.660143	NM_021822	APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
A04	Hs.370254	NM_004322	BAD	BCL2-associated agonist of cell death
A05	Hs.433759	NM_003860	BANF1	Barrier to autointegration factor 1
A06	Hs.624291	NM_004324	BAX	BCL2-associated X protein
A07	Hs.709690	NM_022898	BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein)
A08	Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2
A09	Hs.643802	NM_033637	BTRC	Beta-transducin repeat containing
A10	Hs.141125	NM_004346	CASP3	Caspase 3, apoptosis-related cysteine peptidase
A11	Hs.599762	NM_001228	CASP8	Caspase 8, apoptosis-related cysteine peptidase
A12	Hs.349283	NM_012117	CBX5	Chromobox homolog 5
B01	Hs.303649	NM_002982	CCL2	Chemokine (C-C motif) ligand 2
B02	Hs.75703	NM_002984	CCL4	Chemokine (C-C motif) ligand 4
B03	Hs.514821	NM_002985	CCL5	Chemokine (C-C motif) ligand 5
B04	Hs.271387	NM_005623	CCL8	Chemokine (C-C motif) ligand 8
B05	Hs.279906	NM_001240	CCNT1	Cyclin T1
B06	Hs.511794	NM_001123396	CCR2	Chemokine (C-C motif) receptor 2
B07	Hs.506190	NM_001837	CCR3	Chemokine (C-C motif) receptor 3
B08	Hs.184926	NM_005508	CCR4	Chemokine (C-C motif) receptor 4
B09	Hs.450802	NM_000579	CCR5	Chemokine (C-C motif) receptor 5
B10	Hs.278694	NM_021155	CD209	CD209 molecule
B11	Hs.156445	NM_000734	CD247	CD247 molecule
B12	Hs.631659	NM_000616	CD4	CD4 molecule
C01	Hs.502328	NM_000610	CD44	CD44 molecule (Indian blood group)
C02	Hs.208854	NM_001781	CD69	CD69 molecule
C03	Hs.436568	NM_004355	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain
C04	Hs.184298	NM_001799	CDK7	Cyclin-dependent kinase 7
C05	Hs.150423	NM_001261	CDK9	Cyclin-dependent kinase 9
C06	Hs.370771	NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
C07	Hs.517106	NM_005194	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta

C08	Hs.15591	NM_006833	COPS6	COP9 constitutive photomorphogenic homolog subunit 6 (Arabidopsis)
C09	Hs.445757	NM_001877	CR2	Complement component (3d/Epstein Barr virus) receptor 2
C10	Hs.459759	NM_004380	CREBBP	CREB binding protein
C11	Hs.531668	NM_002996	CX3CL1	Chemokine (C-X3-C motif) ligand 1
C12	Hs.522891	NM_000609	CXCL12	Chemokine (C-X-C motif) ligand 12
D01	Hs.593413	NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4
D02	Hs.99863	NM_001972	ELANE	Elastase, neutrophil expressed
D03	Hs.517517	NM_001429	EP300	E1A binding protein p300
D04	Hs.659872	NM_002000	FCAR	Fc fragment of IgA, receptor for
D05	Hs.728789	NM_005252	FOS	FBJ murine osteosarcoma viral oncogene homolog
D06	Hs.80409	NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, alpha
D07	Hs.655210	NM_002110	HCK	Hemopoietic cell kinase
D08	Hs.518805	NM_002131	HMGA1	High mobility group AT-hook 1
D09	Hs.204475	NM_014500	HTATSF1	HIV-1 Tat specific factor 1
D10	Hs.37026	NM_024013	IFNA1	Interferon, alpha 1
D11	Hs.93177	NM_002176	IFNB1	Interferon, beta 1, fibroblast
D12	Hs.856	NM_000619	IFNG	Interferon, gamma
E01	Hs.193717	NM_000572	IL10	Interleukin 10
E02	Hs.674	NM_002187	IL12B	Interleukin 12B (natural killer cell stimulatory factor 2,
E03	Hs.459095	NM_004513	IL16	Interleukin 16
E04	Hs.126256	NM_000576	IL1 ß	Interleukin 1, beta
E05	Hs.89679	NM_000586	IL2	Interleukin 2
E06	Hs.624	NM_000584	IL8	Interleukin 8
E07	Hs.436061	NM_002198	IRF1	Interferon regulatory factor 1
E08	Hs.654566	NM_002199	IRF2	Interferon regulatory factor 2
E09	Hs.562457	NM_002262	KLRD1	Killer cell lectin-like receptor subfamily D, member 1
E10	Hs.1116	NM_002342	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)
E11	Hs.186486	NM_005923	MAP3K5	Mitogen-activated protein kinase kinase kinase 5
E12	Hs.499674	NM_000242	MBL2	Mannose-binding lectin (protein C) 2, soluble
F01	Hs.534074	NM_172390	NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
F02	Hs.81328	NM_020529	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
F03	Hs.356331	NM_021130	PPIA	Peptidylprolyl isomerase A (cyclophilin A)
F04	Hs.180909	NM_002574	PRDX1	Peroxiredoxin 1

F05	Hs.491322	NM_004103	PTK2B	PTK2B protein tyrosine kinase 2 beta
F06	Hs.513609	NM_005611	RBL2	Retinoblastoma-like 2 (p130)
F07	Hs.728756	NM_000655	SELL	Selectin L
F08	Hs.525557	NM_000295	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
F09	Hs.75599	NM_000488	SERPINC1	Serpin peptidase inhibitor, clade C (antithrombin), member 1
F10	Hs.517070	NM_003064	SLPI	Secretory leukocyte peptidase inhibitor
F11	Hs.534350	NM_003073	SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
F12	Hs.642990	NM_007315	STAT1	Signal transducer and activator of transcription 1, 91kDa
G01	Hs.463059	NM_003150	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
G02	Hs.48849	NM_005653	TFCP2	Transcription factor CP2
G03	Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1
G04	Hs.241570	NM_000594	TNF	Tumor necrosis factor
G05	Hs.256278	NM_001066	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B
G06	Hs.478275	NM_003810	TNFSF-10	Tumor necrosis factor (ligand) superfamily, member 10
G07	Hs.370515	NM_033093	TRIM5	Tripartite motif containing 5
G08	Hs.523512	NM_006292	TSG101	Tumor susceptibility gene 101
G09	Hs.128420	NM_013245	VPS4A	Vacuolar protein sorting 4 homolog A (S. cerevisiae)
G10	Hs.546295	NM_002995	XCL1	Chemokine (C motif) ligand 1
G11	Hs.370770	NM_003400	XPO1	Exportin 1 (CRM1 homolog, yeast)
G12	Hs.388927	NM_003403	YY1	YY1 transcription factor
H01	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H02	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H03	Hs.728776	NM_012423	RPL13A	Ribosomal protein L13a
H04	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H05	Hs.520640	NM_001101	ACTB	Actin, beta
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

Table-3.3. Primer pairs and PCR conditions for real-time PCR. FP = Forward Primer; RP = Reverse Primer; s = seconds; m = minutes

Gene Target*	Primer sequences	Amplicon (bp)	PCR conditions
CASP3- FP CASP3- RP	ATGGACCACGCAGGAAGGG GGCAGCATCATCCACACATACC	68	95°C,10s; 63°C, 15s; 72°C,10s
CASP8- FP CASP8- RP	GAAAAGCAAACCTCGGGGATAC CCAAGTGTGTTCCATTCCTGTC	113	95°C,10s; 60°C, 15 s; 72°C,20s
TNFSF-10-FP TNFSF-10-RP	GCTGAAGCAGATGCAGGACAAG CTGACGGAGTTGCCACTTGAC	136	95°C,10s; 63°C, 15s; 72°C,20s
HMGA1-FP HMGA1-RP	CAGCGAAGTGCCAACACCTAAG CCTTGGTTTCCTTCCTGGAGTT	109	95°C,10s; 60°C, 15s; 72°C,20s
SERPINC1-FP SERPINC1-RP	TTTGTCCTTGCTGCTCATTG CGGGATCTTCTGTTCTGAGC	166	95°C,10s; 55°C, 15s; 72°C,20s
STAT1-FP STAT1-RP	CCCAGAATGCCCTGATTAATG CTGCAGCTGATCCAAGCAAG	101	95°C,10s; 55°C, 15s; 72°C,20 s
STAT3-FP STAT3-RP	CAGTCCGTGGAACCATACACA GACCAGTGGAGACACCAGGATA	111	95°C,10s; 60°C, 15s; 72°C,20s
DDX3-FP DDX3-RP	CATCGTATTGGTCGTACGG GCACCGCTACTTTGTCGGTA	245	95°C,10s; 58°C, 15s; 72°C,30s
MAP4-FP MAP4-RP	TGGCCACCAATACTTCTGCTCCTGAT GGGCCGGCTGTTTTAGTGACTGC	172	95°C,10s; 65°C, 15s; 72°C,20s
STMN1-FP STMN1-RP	TAGAATCTTGAGATTCTCTCTC CAGGTCAGCTGTTACTTT	133	95°C,10s; 68°C, 15s; 72°C,20s
UGP2 -FP UGP2 -RP	GGGGCTCAAGGTGTGCATGTG AATTGGGGGCGTTTATGGCGC	110	95°C,10s; 58°C, 15s; 72°C,20s
MDN1-FP MDN1-RP	TGCGGTTAATCGCAGCCAA GCAACTGTGCTAAGGTACTCAG	117	95°C,10s;56.5° C,15s; 72°C,20s
FOXO3-FP FOXO3-RP	TCTACGAGTGGATGGTGCGTT CGACTATGCAGTGACAGGTTGTG	109	95°C,10s; 65°C, 15s; 72°C,20s
CD43-FP CD43-RP	GCTGGTGGTAAGCCCAGAC GTTGGCTCAGGTAAAGGGGAA	249	95°C,10s; 58°C, 15s; 72°C,30s
LDLR-FP LDLR-RP	GCACCGTCAAGGCTGAGAAC ATGGTGGTGAAGACGCCAGT	142	95°C,10s; 57°C, 15s; 72°C,20s
AGO1-FP AGO1-RP	GATGTTGCACTTAGCAGCCATGT TTCCCCATCCATGCTGGACTT	103	95°C,10s; 60°C, 15s; 72°C,20s
AGO2-FP AGO2-RP	TGAGAACCCAATGTCATAGGAAA GCCCTGAGTTCATAGACTGGTTT	112	95°C,10s; 62°C, 15s; 72°C,20s

DICER-FP DICER-RP	TGGGTCCTTTCTTTGGACTG CTGGTTTGCAGAGTTGACCA	245	95°C,10s; 63°C, 15s; 72°C,30s
DROSHA-FP DROSHA-RP	CCCCTTCTCCCATTAGCCAAT TTCATCCGGTGGCTGTTCATC	167	95°C,10s; 60°C, 15s; 72°C,20s
GAPDH-FP GAPDH-RP	GCACCGTCAAGGCTGAGAAC ATGGTGGTGAAGACGCCAGT	142	95°C,10s; 55°C, 15s; 72°C,20s
HIV-C-FP HIV-C-RP Probe	ACCCATGTTTACAGCATTATCAGAAG GCTTGATGTCCCCCTACTGTATTT 5′ FAM – AGCCACCCCACAAGATTTA AACACCATGT- MGB 3′	80	cDNA synthesis: 25°C,10m; 42°C,50m; 70°C,15m PCR:Hold 50°C,2m; 95°C,10m; Cycle 95°C,15s; 60°C,1m

*Gene Target. CASP3, apoptosis-related cysteine peptidase-3; CASP8, apoptosis-related cysteine peptidase-8; TNFSF-10, tumor necrosis factor superfamily, member 10; HMGA1, high mobility group AT-hook 1; SERPINC1, Serine peptidase Inhibitor clade C member 1; STAT1, signal transducer and activator of transcription 1, STAT3, signal transducer and activator of transcription 3; DDX3, DEAD box polypeptide 3; MAP4, microtubule-associated protein 4; STMN1, Stathmin 1; UGP2, glucose-1-phosphate uridylyltransferase 2; FOXO3, forkhead box O3; CD43, Sialophorin; LDLR, low density lipoprotein receptor ; AGO1, Argonaute 1; AGO2, Argonaute 2; DICER 1, dicer1 ribonuclease type III; DROSHA, drosha ribonuclease type III; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIV-C - HIV-1 subtype C Indian strain.

Appendix-2

Data Sheet

ART Centre

a. Patient Identifier Number:

b. Gender: 1.M 2.F **c. Age:** **d. Height** **e. Weight**

f. HIV transmission category

1. Homosexual
2. Injecting drug user
3. Heterosexual
4. Multiple routes
5. Not known

g. Marital Status

1. Married
2. Divorced
3. Widowed
4. Separated
5. Bachelor

h. First detection of HIV infection (date):

i. CD4 count: **j. Viral load:**

Date	cells/mm3	Date	Copies /ml

k. Clinical stage of HIV infection (WHO): I II III IV

l. Presence or H/O any Opportunistic Infection:

1. Cryptococcal meningi
2. Gasteroenteropathy
3. *Pneumocystis Carinii* pneumonia
4. CMV Retinitis
5. Recurrent Bacterial Infection
6. Papular pruritic eruption
7. Extrapulmonary TB
8. Pulmonary TB
9. Oral Candidiasis
10. Molluscum contagiosum
11. Toxoplasmosis
12. Oral hairy leukoplakia
13. Dermatophyte infection
14. Cryptosporidial diarrhoea
15. Herpes zoster
16. Herpes Simplex 1/ 2
17. Oral Ulcers
18. Others

m. Antiretroviral therapy

1. Treatment naïve

2. First line Drugs: Starting date:

3. Second line Drugs: Starting date:

4. Treatment failure history:

n. H/O smoking or any other oral substances: 1. No 2. Yes

o. H/O Alcoholism: 1. No 2. Yes

p. H/O current medication other then ART: 1. No 2. Yes

q. H/O Hospitalization :

r. Vital signs : *BP -* mmHg *Pulse -* /min , *Ht -* *Wt-*

s. Dietary habit: 1. Vegetarian 2. Non-Vegetarian

t. AIDS-related Dementia: 1. No 2. Yes

u. Current STD / viral infection: 1. No 2. Yes

v. Metabolic / immunological disorders: 1. No 2. Yes

w. Results of Hematological / Biochemical tests:

x. Time of Diagnosis: Month / Year

y. Duration of Therapy:

z. H/O toxicity:

Appendix-3

Informed consent for Patients

Purpose of the study

I, Dr. Shahid Jameel, as Principal Investigator is going to conduct a study on "Identification of new HIV/AIDS Biomarkers From Body Fluids and Peripheral Blood Mononuclear Cells " to learn more about the health problems of HIV- positive persons before and after receiving treatment and will identify related biomarkers to facilitate the on going treatment strategy. Your participation in this study will help us to improve the quality of treatment for you and other people who need treatment for HIV infection.

Procedure

You will be asked questions on your history of illness, life style and treatment with ART or other drugs. You have to donate your blood, urine and saliva for this study.

Benefits and Risks

If you agree, your participation will help the researchers to address the health and ART related issues of PLHA's more accurately and efficiently. Your participation in the study may not benefit you directly. Whether you agree or not, both you and your family will receive all of the health care services that are normally provided through the government health system. Some of the questions that you will be asked may be sensitive and perhaps cause make you uncomfortable. The interviewer will not insist on answers to all questions.

Compensation

You will not receive any compensation for your participation in this interview.

Offer to answer questions and freedom to withdraw from the study

You can refuse to respond to any question and can stop the interview at any time. There will not be any consequences if you decide to drop out of the study. The interview will take approximately 15 minutes. Participation is completely voluntary.

Confidentiality

Everything that you report during the interview will be kept strictly confidential and your name will not be recorded on the questionnaire. This information will only be used by the researchers. All records of your interviews and data collected from medical records will be kept in a safe place under lock and key.

Subject's Statement

I have read the consent document regarding my participation in the study/the consent document regarding my participation in the study has been read out to me. I have been given a chance to ask questions and my questions have been answered to my satisfaction.

I understand that all records will be kept private and that I can leave the study at any time. I have also understood that my decision not to be in this study or to leave the study will not affect the services I will receive.

I agree to be in this study as a volunteer.

Name of the subject_______________

Signature of the subject:_______________ Date:_____________________

Signature of a witness:_______________ Date:_____________________

Informed consent for Patients

सहमति–पत्र

अध्ययन का उद्देश्य :–

मैं डॉ0 भरत रेवाड़ी, (प्रमुख जांचकर्ता) HIV + व्यक्तियों के स्वास्थ्य सम्बन्धित समस्याओं के विषय में अधिक जानने के लिए"Identification of HIV/AIDS Biomarkers From Body Fluids & Peripheral Blood Mononuclear Cells"नामक एक शोध आरम्भ करने वाला हूँ।

आपका सहयोग हमें आप व आप जैसे अनेक HIV + व्यक्तियों के लिए बेहतर उपचार ढूढंने में मद्द करेगा।

कार्यविधि :– आपसे आपके रोग, रहन–सहन एवं उपचार (ART व अन्य दवाओं) के विषय में प्रश्न पूछे जायेंगे। आपको अपना रक्त, पेशाब व लार दान करना होगा।

लाभ एवं हानि :–आपकी सहमति व योगदान, शोधकर्ता को HIV + व्यक्तियों के स्वास्थ सम्बन्धित समस्याओं के सटीक्र व सक्षम हल ढूंढने में मद्द करेगा। आपके योगदान से आपको किसी प्रकार का प्रत्यक्ष लाभ नहीं होगा। आप व आपके परिवार को स्वास्थ सम्बन्धित सुविधाऐं प्रदान की जायेगी, जोकि सरकारी स्वास्थ तंत्र द्वारा प्रदान की जाती हैं।

आपसे पूछे जाने वाले कुछ प्रश्न संवेदनशील हो सकते हैं और आप असहजता का अनुभव कर सकते है। इन्टरव्यूकर्ता आपसे सभी प्रश्नों का उत्तर देने का आग्रह नहीं करेगा।

मुआवज़ा :–अध्ययन में सम्मिलित होने के लिए आपको किसी प्रकार का मुआवज़ा नहीं दिया जायेगा।

प्रश्न पूछने व अध्ययन छोड़ने की स्वतंत्रता :– आप किसी भी प्रश्न का उत्तर न देने एवं किसी भी समय इन्टरव्यू समप्ति करने के लिए स्वतंत्र है। इन्टरव्यू की अवधि अधिकतम 15 मिनट होगी। इस कार्यक्रम में आपका योगदान पूर्णतः स्वैच्छिक होगा।

गोपनियता :–इन्टरव्यू के दौरान आपके द्वारा दी गयी समस्त जानकारी गोपनीय रखी जायेगी। प्रश्नावली पर आपका नाम रिकार्ड नही किया जायेगा। आपके द्वारा दी गयी जानकारी केवल शोधकर्ता तक सीमित रहेगी। आपके इन्टरव्यू के सभी रिकार्ड (मेडिकल रिकार्ड) तालाबंद सुरक्षित स्थान पर रखे जायेंगे।

रोगी की सहमति :–मैंने अध्ययन में सम्मिलित होने सम्बन्धित सहमति पत्र पढ़ लिया है/अध्ययन में योगदान सम्बन्धित सहमति पत्र मुझे पढ़ कर सुना दिया गया है। मुझे प्रश्न पूछने का अवसर प्रदान किया गया तथा मेरे द्वारा पूछे गये प्रश्नों का संतोषजनक उत्तर दिया गया है।

मुझे ज्ञात है कि सभी रिकार्ड गोपनीय रखे जायेंगे और मैं किसी भी समय इस अध्ययन को छोड़ सकता हूँ। मुझे यह भी बात है कि मेरे अध्ययन में भाग न लेने या मेरे द्वारा अध्ययन छोड़ने के कारण मुझे प्रदान की जाने वाली सुविधाओं पर कोई प्रभाव नहीं पड़ेगा।

मैं इस अध्ययन में योगदान के लिए सहमत हूँ।

रोगी का नाम ....

रोगी के हस्ताक्षर .... दिनांक....................

साक्षी के हस्ताक्षर .... दिनांक....................

Appendix 4

Softwares used in this study

Web-based PCR Array Data Analysis Software

http://sabiosciences.com/pcrarraydataanalysis.php

The PCR Array Data Analysis Web Portal is an integrated web-based software package for the PCR Array System, which automatically performs all ΔΔCt based fold-change calculations from the uploaded raw threshold cycle data.

DIANA-microT 3.0 algorithm (http://diana.cslab.ece.ntua.gr/microT/)

DIANA-microT 3.0 is an algorithm for microRNA target prediction, which is established on a number of parameters, calculated individually for each microRNA and combines conserved and non-conserved microRNA recognition elements into a final prediction score, which correlates with protein production fold change.

miRecords: http://mirecords.biolead.org/

miRecords is an integrated resource for animal miRNA–target interactions. The Validated Targets component of this resource hosts a large, high-quality manually curated database of experimentally validated miRNA–target interactions with systematic documentation of experimental support for each interaction.

miRWalk (http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/)

miRWalk is a wide-ranging database used to produce the predicted miRNA binding sites on the complete sequence of all genes based on a comparison of identified miRNA binding sites with the 8 established miRNA-target prediction program is presented. Moreover, it gives predicted miRNA binding sites on genes related with 449 human biological pathways.

DIANA-mirPath (http://diana.cslab.ece.ntua.gr/pathways/)

DIANA-mirPath is a web-based computational means developed to identify molecular pathways possibly altered by the expression of single or multiple microRNAs. The software executes an enrichment analysis of multiple microRNA target genes comparing each set of microRNA targets to all known KEGG pathways.

miTALOS (http://mips.helmholtz-muenchen.de/mitalos/index.jsp),

miTALOS considers the tissue-specific expression patterns of miRNAs and target transcripts for a more meaningful analysis of miRNA-regulated biological pathways. The algorithm recognizes the potential biological pathways by assigning an enrichment (E) score, which quantifies the proportion of target proteins in a specific pathway compared to all targets in all

pathways; it is represented as a log2 value. A proximity (P) score is calculated, which compares the minimal distance between the protein targets compared to randomly sampled targets. The value of P ranges between 0 and 1, with smaller values indicating better proximity. The significance (p-value) in both cases is calculated using Fisher's exact test.

MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp)
'MetaboAnalyst' is a data analysis module containing a collection of established statistical and machine learning algorithms, including chemometric analysis (PCA, PLS-DA) and feature selection path (SAM, EBAM, etc.). We analyzed the extracted data from ^{1}H-NMR spectra by Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA) and Significance Analysis of Microarrays (and Metabolites) (SAM).

Human Metabolome Data Bank (HMDB) http://www.hmdb.ca
The Human Metabolome Database (HMDB) is the most complete and comprehensive curated collection of human metabolite and human metabolism data in the world. It contains records for more than 2180 endogenous metabolites with information gathered from thousands of books, journal articles and electronic databases.

MetPA (Metabolomic Pathway Analysis) http://metpa.metabolomics.ca
MetPA (Metabolomics Pathway Analysis) is a user- friendly, web-based tool dedicated to the analysis and visualization of metabolomic data within the biological context of metabolic pathways. MetPA combines several advanced pathway enrichment analysis procedures along with the analysis of pathway topological characteristics to help identify the most relevant metabolic pathways involved in a given metabolomic study.