

BIOCHEMICAL PARAMETERS IN HIV/TB CO- INFECTION

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1.3 LIST OF COMMONLY USED ABBREVIATIONS AND ACRONYMS

°C	: degree Celsius
5'HT	: Tryptophan
ABS	: Absorbance
ADE	: Antibody Dependent Enhancement
AG	: Arabinogalactan
AHR	: Aryl Hydrocarbon Receptor
AIDS	: Acquired immunodeficiency syndrome
ALT	: Alanine aminotransferase
APC	: Antigen presenting cells
ARDS	: Acute Respiratory Distress Syndrome
ARI	: Acute renal injury
ART	: anti retroviral therapy
ARV	: Anti Rabies Vaccine
BAL	: Bronoalveolar Lavage
BCG	: Bacillus Calmette-Guerin
BHK-21	: Baby Hamster Kidney Cells
BMI	: body mass index
CAT	: Catalase
CC50	: Median Cytotoxic Concentration
CD 4	: Cluster of differentiation 4
CD 8	: Cluster of differentiation 8
CDC	: Center for Disease Control & Prevention
CDNB	: 1-chloro-2,4-dinitrobenzene
CKD	: Chronic kidney disease
CMI	: Cell Mediated Immunity
CRP	: C-reactive Protein
CTL	: Cytotoxic T lymphocytes
CVS-11	: Challenge Virus Standard
DC	: Dendritic Cells
DNA	: Deoxyribonucleic Acid
DOTS	: Direct Observed therapy
dsDNA	: double stranded DNA
DTNB	: 5,5'-dithio-bis-[2-nitrobenzoic acid])
EBV	: Epstein Barr Virus
ECM	: Extracellular Matrix
EDTA	: Ethylene Diamine tetra acetic acid
EPTB	: Extrapulmonary tuberculosis
FBS	: Fetal Bovine serum
FCS	: Fetal calf serum
FITC	: Fluorescein Isothiocyanate
FMDV	: Foot and Mouth Disease Virus
F-SH	: free thiols
GALT	: Gut associated lymphoid tissue
GAP-31	: Gelonium Anti-HIV Protein 31
GAPDH	: Glyceraldehyde-3 phosphate dehydrogenase
GM-CSF	: Granulocyte macrophage colony stimulating factor
gp 120	: glycoprotein 120
gp 41	: glycoprotein 41
GPX	: Glutathione peroxidase
GR	: Glutathione reductase
GSH	: Glutathione

GST	: Glutathione S-transferase
HA	: Hemagglutination Assay
HAART	: Highly acquired anti retroviral therapy
HAND	: HIV associated neurological disorder
HCC	: Hepatic cell carcinoma
HCMV	: Human Cytomegalovirus
HCV	: Hepatitis C virus
HIV	: Human Immunodeficiency Virus
HIVAN	: HIV associated neuropathy
HIVICK	: HIV associated immune chronic kidney disease
HLA	: Human Leukocyte antigen
HSV	: Herpes Simplex Virus
HTLV	: Human T cell leukemia Virus
IC50	: Median Inhibitory Concentration
ICTV	: International Committee on Taxonomy of Viruses
IDO	: Indoleamine 2,3-Dioxygenase
IFN	: Interferon Gamma
Ig A	: Immunoglobulin A
Ig G	: Immunoglobulin G
IGRA	: interferon Gamma release assays
IL-4	: Interleukin 4
IL-6	: Interleukin 6
i-NOS	: Inducible Nitric Oxide Synthase
K ₂ HPO ₄	: di-potassium hydrogen phosphate
KCL	: Potassium Chloride
KH ₂ PO ₄	: potassium dihydrogen phosphate
KOH	: potassium hydroxide
KYN	: Kynurenin
LPS	: Lipopolysaccharide
LRIP	: Luffa Ribosomal Inhibitory Protein
LTBI	: Latent TB infection
LTR	: Long Terminal Repeat
MA	: mycolic acids
M-CSF	: Macrophage colony stimulating factor
MDCK	: Madin-Darby Canine Kidney Cells
MDM	: Monocyte derived macrophages
min	: Minutes
MIP-1 β	: Macrophage inflammatory protein 1- β
ml	: mili litre
mRNA	: messenger RNA
MTB	: Mycobacterium tuberculosis
MTT	: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
Na ₂ HPO ₄	: di-sodium hydrogen phosphate
NaCl	: Sodium Chloride
NADPH	: Nucleotide adenosine diphosphate
NAFLAD	: Non-alcoholic fatty liver disease
NaH ₂ PO ₄	: sodium dihydrogen phosphate
NaOH	: sodium hydroxide
NASH	: Non-alcoholic steatohepatitis
NF- κ B	: Nuclear Factor κ beta
NNRTI	: non-nucleoside reverse transcriptase inhibitor
NO	: Nitric Oxide
NOS	: Nitric oxide synthase

NRTI	: Nucleoside reverse transcriptase inhibitor
NSAIDs	: Non Steroidal Anti-Inflammatory Drugs
PAP	: Pokeweed Antiviral Protein
PBMCs	: Peripheral blood mononuclear cells
PBS	: phosphate buffered saline
PCR	: Polymerase Chain Reaction
PG	: Peptidoglycan
PI	: Protease inhibitor
PLHAs	: People living with HIV
P-SH	: protein thiols
qRT-PCR	: Real Time Reverse Transcriptase PCR
RABV	: Rabies Virus
RFFIT	: Rapid Fluorescence Foci Inhibition Test
RIG	: Rabies Immunoglobulin
RIP	: Ribosome Inhibitory Proteins
RNA	: Ribonucleic Acid
RNI	: Reactive Nitrogen Species
ROS	: Reactive Oxygen Species
RPMI 1640	: Rosewell memorial Park Institute
SI	: Selectivity Index
SIV	: Simian Immunodeficiency Virus
SOA	: Super oxide anion
SOC	: Suppressor of Cytokine signalling
SOD	: Super oxide dismutase
TBA	: thiobarbituric acid
TBARS	: thiobarbituric acid reactive species
Tc CELLS	: Cytotoxic T Cells
TCA	: tri chloro acetic acid
TGF- β	: Transforming growth factor -beta
TMP	: Tetra methoxy Propane
TMV	: Tobacco Mosaic Virus
TNF	: Tumor necrosis factor
t-RNA	: Transfer RNA
TRP	: Tryptophan
T-SH	: total thiols
TST	: Tubercilin skin tests
UNAIDS	: United nations AIDS
WHO	: World Health Organisation
μ M	: micro molar
μ L	: Micro litre

2 INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) has been a significant target for modern medical research from the time of its first description in 1981. The development of drugs against its equally elusive etiological agent—Human Immunodeficiency Virus (HIV) has almost been a biological wild goose chase. Since its discovery in 1983, HIV has been one of the biggest targets for biological, clinical and medical research. The fact that HIV is an evolving virus has made things far more difficult in the management of AIDS than perceived in the early eighties. The result of HIV infection is relentless destruction of the immune system leading to onset of AIDS and ultimately to the death of individuals. The AIDS epidemic has already resulted in the deaths of over half its victims. All HIV-infected persons are at risk for illness and death from opportunistic infections and neoplastic complications as a consequence of the inevitable manifestations of AIDS (1, 2). The AIDS pandemic has evolved over time, with four main phases of evolution:

1. Emergence of HIV from endemic rural areas to urban populations at an accelerating rate.
2. Dissemination and involvement of definable risk groups (sexual promiscuity and injection drug use).
3. Escalation through the 1980's of HIV infections at an alarming rate due to the behaviours of these risk groups, and
4. Stabilization in some regions such as Western Europe, North America, and Australia, where control measures appear to be having a positive effect. However, some regions such as central Africa and Asia continued to experience escalation of the pandemic through the 1990's and into the 21st century(3, 4)

In 1983, Francoise Barre-Sinoussi, Claude Chermann and Luc Montagnier at the Pasteur Institute isolated a retrovirus from the lymph node cells of a patient with lymphadenopathy and termed their virus lymphadenopathy-associated virus. The next year, Robert Gallo's group at the NIH confirmed and extended this finding, linking this virus to the immunodeficiency syndrome, AIDS. In 1986, HIV-2 was isolated from West Africa.

The frightening image of AIDS as a lethal plague sweeping across the globe without prospect of control belies the fact that the mechanisms and risks of transmission are clearly defined. Accordingly to World Health Organisation (WHO), at the end of year 2015 there were 36.7 million [34.0 million–39.8 million] people

living with HIV. Around 36.7 million [34.0 million–39.8 million] people globally were living with HIV; 2.1 million [1.8 million–2.4 million] people became newly infected with HIV; 1.1 million [940 000–1.3 million] people died from AIDS-related illnesses (5).

As per the Government of India, world's second most populous country is experiencing a highly-varied HIV epidemic (6). In India, approximately 2.5 million people are living with HIV year 2015. The prevalence rate is around 0.3 % adult prevalence rate. 86,000 new infections till 2015. around 68,000-70,000 death occurred . The spread of HIV within India is diverse as the societal patterns between its different regions, states and metropolitan cities. Although the per cent prevalence of PLHAs in India is not large, it has the third largest absolute numbers of PLHAs only after South Africa and Nigeria

This prevalence however, is not uniform throughout the globe. The global epidemic or pandemic of HIV-1 infection is growing most rapidly in the nations of South-East Asia and in the subcontinent of India, although the largest number of HIV-1 infected persons is still in sub-Saharan Africa. Recent data suggest that over 36 million adults and children are infected by HIV-1. It was observed that subtype B is most prevalent in the Americas and in Europe, while subtype C viruses are the most common viruses globally with subtype A being the next most prevalent. What this highlights is that there are regional epidemics of genetically distinct viruses, which may have important implications for vaccination.

HIV is the aetiological agent of AIDS. Its primary target is the activated CD4+ T helper lymphocyte but it can also infect several other types of cells. Role of oxidative stress has also been suggested in activation of HIV replication. HIV gene expression can be achieved *in-vitro* by oxidative stress. Furthermore, opportunistic infections can participate in AIDS progression by induction of oxidative stress mechanisms.

The alarming increase in the number of individuals being infected every day has given enough cause for research and development of anti-retroviral or specifically anti-HIV drugs. At present, nearly 23 individual antiretroviral (ARV) drugs have been licensed for antiretroviral therapy (ART) in the treatment of HIV-1: seven nucleoside reverse transcriptase inhibitors (NRTI), four non-nucleoside reverse transcriptase inhibitors (NNRTI), eight protease inhibitors (PI), one fusion inhibitor, one entry inhibitor, one CCR5 inhibitor, and three integrase inhibitor, one pharmacokinetic

enhancer drug. Also there are combination of drugs which form combination therapy drugs which is administered accordingly to the patients and the drug resistance pattern (7).

The chronic nature of the disease following infection with HIV-1, the rate of clinical disease progression varies between individuals. Factors such as host susceptibility, genetics and immune function, health care and co-infections as well as viral genetic variability may affect the rate of progression to AIDS. As an approach towards combating drug resistance, specific to single drugs, a treatment method comprising a combination of anti-retroviral drugs has resulted in the so-called HAART that is currently being used against HIV (8).

The use of combinations of ARV drugs has proven remarkably effective in controlling the progression of HIV disease and prolonging survival. Combination therapy or Highly Active Antiretroviral Therapy (HAART) was discovered in the mid- to late- 1990s when it was found that using three or more drugs in a combination, with a protease inhibitor thrown in, was much more effective than using them singly or in twos. This way, drugs show their effect for a longer time. Current first line HAART consists of zidovudine (AZT)/stavudine (d4T) + lamivudine (3TC) + nevirapine/efavirenz in resource limited settings including in India (8, 9)

With the raging, global HIV epidemic, there is an urgent need to exploit all potential interventions to halt its continued spread and to enhance the health, quality of life and survival of those already infected. The present study was taken to study the various oxidative stress parameters in the HIV patients and their role in the pathology of disease.

2.1 Tuberculosis

Tuberculosis is one of the most ancient diseases, caused by *Mycobacterium tuberculosis* (Mtb). *M. tuberculosis* may have killed more people than any other microbial infection. The earliest detection of MTB is in the remains of animal dated 18,000 years (10). However, origin of tuberculosis whether in cattle and then transfer to humans, or deviation from a common ancestor is still unclear. MTB is a slow growing bacterium with many unique characteristics which increases its chances of survival in body. They are non-motile, intracellular, obligate aerobic bacterium that grows in well aerated regions of the lungs. MTB have slow generation time (15-20 hours) which contributes to its chronicity. They are capable of multiplying and

surviving in the phagocytic cells particularly monocytes and macrophages (11). Before the Industrial Revolutions, tuberculosis may sometimes have been regarded as vampirism. When one member of a family died from TB infection, the other members of family that were infected would drop their health gradually. People believed that this was caused by original victim draining the life from the other family members. Furthermore, people who had TB presented symptoms such as red, swollen eyes, pale skin and coughing blood suggesting the idea that only way to for the afflicted is to replace this loss blood by sucking blood.

Robert Koch was the first to identify *M. tuberculosis* as the causative agent of TB in 1882 [4]. In 1890, Koch announced a glycerine extract of the tubercle bacilli called as ‘tuberculin’ as a “remedy” for tuberculosis. *M. tuberculosis* belongs to the genus *Mycobacterium* which was originally found in soil (12). It is believed that 10,000 to 25,000 years ago domestication of cattle enabled the passage of mycobacterial pathogen from the livestock to the humans. *M. bovis* causes tuberculosis like disease in cattle and is considered as the precursor in evolving into the pathogen *M. tuberculosis*. Members of tuberculosis complex ; *M. africanum*, *M. microti*, *M. cavetti*, *M. bovis* and *M. tuberculosis* have around 99.9% similarity in their DNA sequence, but the presence of rare synonymous single nucleotide polymorphism (sSNP) distinguishes them apart (12).

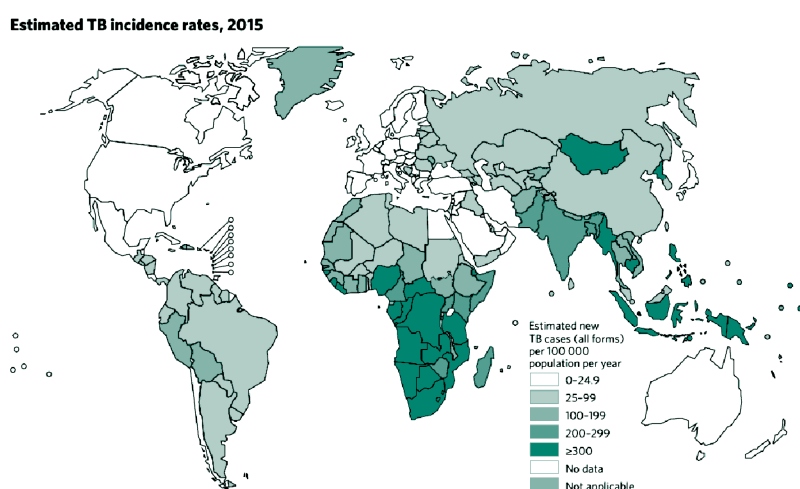
According to the WHO Global report 2017(13), TB was one on major cause of deaths across the globe. The best estimate is that there were 1.4 million TB deaths in 2015 and additional 0.4 million deaths results for the TB disease among the HIV seropositive people. There were 10.4 million new TB cases (including 1.2 million among HIV -positive people) of which 5.9 million were among men, 3.5 million among women and 1.0 million children. Overall 90% of cases were in adults and 10 % in children and the ratio of male: female was 1.6:1.

India is the country with the highest burden of TB. The World Health Organisation (WHO) TB statistics for India for 2015 give an estimated incidence figure of 2.2 million cases of TB for India out of a global incidence of 9.6 million. The TB incidence for India is the number of new cases of active TB disease in India during a certain time period (usually a year).The estimated TB prevalence figure for 2015 is given as 2.5 million. The TB prevalence is the number of people in India who are living with active TB. Prevalence is usually, but not always given as a percentage

of the population. It is estimated that about 40% of the Indian population is infected with TB bacteria, the vast majority of whom have latent TB rather than TB disease.

In developing countries and poor nations, tuberculosis remains a big threat. Poor economic status of the country limits the accessibility of the patients in reliable diagnostic tests that can identify the infection at an early stage. Most of the new technologies for rapid testing are expensive and are not routinely used here. Social status and the poor lifestyle of the population increases the probability of transmitting the disease, as it easily spreads from person to person via respiratory route. Unawareness and lack of proper guidelines to prevent the disease is major reason for spreading of the TB. Focus on research and awareness about the disease prevention and control is necessary in developing countries to reduce the morbidity which is constantly rising over the years. Development of antibiotics, widespread use of BCG vaccine and better public health services had reduced the tuberculosis morbidity rate in the 20th century. However it was short lived, due to emergence of AIDS which affects the immunity. Homelessness and poverty increased the number of new TB cases (14). Without treatment 60% patients died, this is the scenario in the developing countries reflecting the poor disease management, lack of healthy living conditions. Global burden is augmented by the emergence of Multidrug Resistant Tuberculosis (MDR TB) in countries where available antibiotics are of inferior quality and the patient's inability to adhere to the dose regime.

Figure 1: Global Distribution of Tuberculosis



Source: WHO Global TB report 2017

2.2 HIV + TB Co-infection

Tuberculosis (TB) and human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) constitute the main burden of infectious disease in resource-limited countries. The HIV/TB dual epidemic has affected a large number of populations across the globe. The best estimate is that there were 1.4 million TB deaths in 2015, and an additional 0.4 million deaths resulting from TB disease among HIV-positive people.² In terms of cases, the best estimates for 2015 are that there were 10.4 million new TB cases (including 1.2 million among HIV-positive people), of which 5.9 million were among men, 3.5 million among women and 1.0 million among children. Overall, 90% of cases were adults and 10% children, and the male: female ratio was 1.6:1.

3 AIMS AND OBJECTIVES

The primary aim of the study is to assess the biochemical parameters in HIV+TB co-infection study cohorts with the following objectives:

1. To assess pro- and antioxidant markers in HIV +ve, TB +ve and HIV/TB co-infected patients.
2. To assess the level of proinflammatory cytokines in the study cohort.
3. To track the activation of NF- κ B in the study cohort.
4. To correlate the prognosis of HIV and TB disease with the above mentioned markers.

4 REVIEW OF LITERATURE

4.1 Human Immunodeficiency Virus

As we have reached to the fourth decade of the HIV epidemic, still we are a way behind eliminating or killing the virus or finding possible cure. The research from last 30 odd years have only helped to understand pathology of the virus and to some what extent we have reach to develop the drugs which can be effective reducing the rate of infection. The HIV vaccine is still in the developmental phase since due to variation in the subtypes we are not able to find a vaccine. HIV/AIDS has been the Centre of attention for the medical, clinical and scientific community with inadequate solutions to prevent, treat or cure HIV/AIDS.

4.2 Epidemiology of HIV/AIDS

4.2.1 Global preview

According to the WHO-United Nations Programme on HIV/AIDS (UNAIDS) report on the Global AIDS Epidemic 2016 (5). There were 36.7 million people infected with HIV out that adults were around 34.9 million; women 17.8 million, children <15 years 1.8 million. Newly infected people in 2015 total number 2.1 million new case registered; Adults 1.9 million; children 1, 50,000 cases. Deaths caused to AIDS in 2015 1.9 millions, Adults 1.0 million, children 1, 10,000.

4.2.2 India specific epidemiology

In 2016, HIV prevalence in India was an estimated 0.3%. This figure is small compared to most other middle-income countries but because of India's huge population (1.324 billion) this equates to 2.1 million people living with HIV. In the same year, an estimated 62,000 people died from AIDS-related illnesses.

4.3 Virology of HIV

4.3.1 Origin and classification of HIV

4.3.1.1 Origin of HIV

The origin of HIV is believed to be a zoonotic transmission (a human disease caused by crossover of an animal pathogen) from African primates. It is also believed that HIV-1 was transmitted to humans from the chimpanzee, *Pan troglodytes*, which harbour a related simian immunodeficiency virus, Simian Immunodeficiency Virus (SIV). HIV-2 on the other hand is believed to have originated from West Africa, from

wild living sooty mangabeys (*Cercocebus atys*) infected with a virus known as SIV sm.

4.3.1.2 Classification of HIV

HIV is classified using the International Committee on Taxonomy of Viruses (ICTV). The system as follows:

- Group: Group VI (ssRNA-RT)
- Family: Retroviridae
- Genus: Lentivirus (long latency viruses)
- Species: HIV (Human Immunodeficiency Virus)

Since the outbreak of the AIDS pandemic, several genetic mutations in HIV have been accumulated for distinct types and subtypes to arise. Several distinct types (also known as clades or genotypes) of HIV-1 have been identified. Two types of HIV have been characterized: HIV-1 and HIV-2. HIV-1 is more virulent, more infective and is the cause of the majority of HIV infections globally. The lower infectivity of HIV-2 compared to HIV-1 implies that fewer of those exposed to HIV-2 will be infected per exposure. Because of its relatively poor capacity for transmission, HIV-2 is largely confined to West Africa.

4.3.1.3 Structure of HIV

4.3.1.3.1 Structure and genome

HIV is composed of two strands of RNA, 15 types of viral proteins and a few proteins from the last host cell it has infected which is surrounded by a lipid bilayer. Together these molecules allow the virus to infect the immune system and force them to build new copies of the virus. Each molecule in the virus plays a role in this process, from the first steps of viral attachment to the final process of budding. Twenty-five years of research on the structural biology of HIV have revealed the atomic details of these proteins and are publicly available in the protein database (15).

4.3.1.3.1.1 *Viral Genome:*

The genome is composed of two identical strands of RNA, each about 9700 nucleotides long. The genome encodes the information needed to synthesize viral

proteins. Portions of the genome also form hair-pin loops that interact with the regulatory proteins.

Cellular proteins: Several cellular proteins are captured when the virus buds from the cell surface, including cyclophilin A, which assists the folding of proteins. Two molecules of cellular transfer RNA are also captured, and are used to initiate the synthesis of viral genome in the next cell that the virus infects.

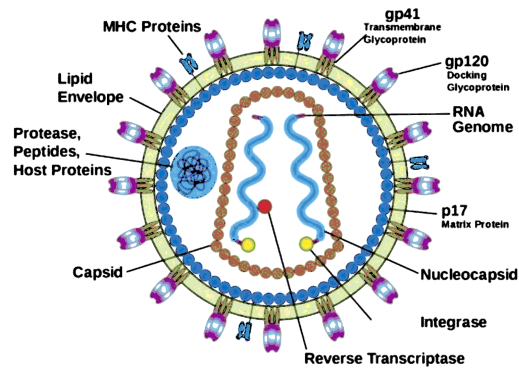
Viral proteins: 15 types of viral proteins are encoded by the HIV genome and are responsible for a variety of functions, some of which have yet to be fully understood. The viral proteins are of three types: viral enzymes, structural proteins and accessory proteins.

Lipid bilayer: HIV is enveloped in a lipid bilayer that is taken from the infected cell when the virus buds from the cell membrane.

4.3.1.3.1.2 Viral structure of HIV

HIV is enveloped icosahedral shaped sphere. The diameter of virus is around 80-100 nm. The two identical non-complimentary strands of RNA (viral genomes) and three enzymes (reverse transcriptase, integrase, protease) are packed in one sphere. This core is surrounded by a protein called capsid and the capsid along with the enclosed nucleic acid is called nucleocapsid that includes a layer of protein called p17, and an inner layer of protein called p24. The capsid comprises a large number of polypeptides known as 'A' unique two-layered envelope derived from the host cell membrane surrounds this core. The envelope is acquired by progeny viruses through budding. The viral envelope is derived from the host cell and contains some host-cell membrane proteins, such as class I and class II Human Leukocyte antigen (HLA) molecules. The outermost envelope is made of glycoprotein sub-units that are exposed as spikes (or knobs). These projecting structures are called peplomers. The surface glycoprotein 120 (gp120) is bound to the virus by a transmembrane protein (gp41). Each enveloped virus expresses 72 glycoprotein projections composed of gp120 and gp41. gp120 is associated with gp41 and serves as the viral receptor for CD4 on the host cell ('capsomeres'). A schematic diagram of the HIV virion is shown in figure

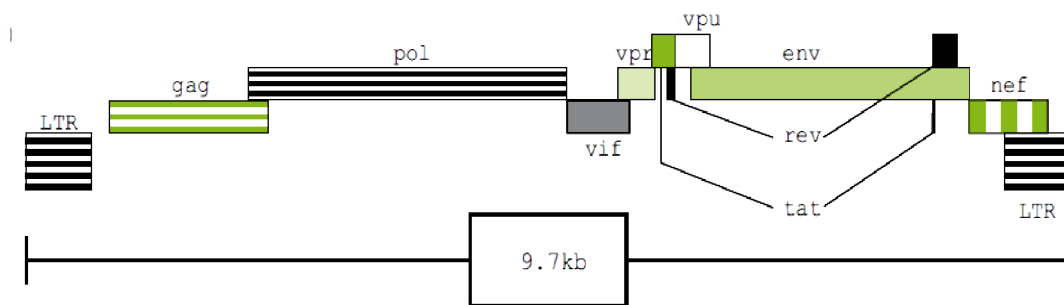
Figure 2: Structure of HIV(16)



Source: http://commons.wikimedia.org/wiki/File:HIV_Virion.svg

The HIV genome consists of at least seven structural proteins i.e. Long Terminal Repeat (LTR), Trans-activation response element (TAR), rev response element (RRE), pulmonary embolus (PE), Specific reverse transcriptase slippage (SLIP), *Cis*-acting repression sequence (CRS), INS and nine genes i.e. Group-specific antigen (*gag*), DNA polymerase (*pol*), envelope (*env*), trans-activator of transcription (*tat*), regulator of viral gene expression (*rev*), negative effector (*nef*), viral infectivity factor (*vif*), viral protein r (*vpr*), viral protein u (*vpu*) and sometimes a tenth *tev*, which is a fusion of *tat*, *env* and *rev*, encoding 19 proteins. Three of these genes, *gag*, *pol*, and *env*, contain information needed to make the structural proteins for new virus particles; *env* codes for a protein called gp160 that is broken down by a cellular protease to form gp120 and gp41. The six remaining genes *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu* (or *vpx* in the case of HIV-2), are regulatory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus or cause disease (15).

Figure 3: Structure of HIV genome (17)



Source: <http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html>

The two Tat proteins (p16 and p14) are transcriptional transactivators for the LTR promoter acting by binding the TAR RNA element. The TAR may also be processed into microRNAs that regulate the apoptosis genes ERCC1 and IER3. The Rev protein (p19) is involved in shuttling RNAs from the nucleus and the cytoplasm by binding to the RRE RNA element. The Vif protein (p23) prevents the action of APOBEC3G which is a cell protein that deaminates DNA: RNA hybrids and/or interferes with the Pol protein. The Vpr protein (p14) arrests cell division at G2/M. The Nef protein (p27) down-regulates CD4 which is the major viral receptor, as well as the major histocompatibility complex (MHC) class I and class II molecules [30] [55] [38]. Nef also interacts with SH3 domains.

The Vpu protein (p16) influences the release of new virus particles from infected cells. The ends of each strand of HIV RNA contain an RNA sequence called the LTR. Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell. The Psi element is involved in viral genome packaging and recognized by Gag and Rev proteins. The SLIP element (TTTTTT) is involved in the frameshift in the Gag- Pol reading frame required to make functional Pol (18, 19).

Table 1: The nine major genes of HIV and their associated gene products

Protein class	Gene	Gene products
Structural	<i>gag</i>	p17 (matrix), p24 (capsid)
	<i>env</i>	p7 (nucleocapsid), gp41
Catalytic	<i>pol</i>	Protease, Reverse transcriptase, Integrase
Regulatory	<i>tat</i>	Tat
	<i>rev</i>	Rev
Accessory	<i>vpu</i>	Vpu
	<i>vif</i>	Vif
	<i>vpr</i>	Vpr
	<i>nef</i>	Nef

4.3.1.4 HIV replication

The retroviruses generally are not able to replicate outside the body of living host cells as they do not contain DNA. The pathogenesis of HIV infection is a function of virus life cycle, host cellular environment, and quantity of viruses in infected individuals. HIV enters in the body via oropharyngeal, cervical, vaginal, and gastrointestinal mucosal surfaces (20)

After entering in the body virus particle is able to find the appropriate CD4 receptor molecules so that it can attach itself there by fusion to the susceptible cell membrane or by endocytosis mechanism and then enters the cell. The virus particle enters lymphocytes and monocytes through recognition of the viral glycoprotein gp120 with the CD4 molecule and a chemokine receptor they are named as CCR5 or CXCR4 (21-23). The interaction of these glycoprotein induces the binding of the another glycoprotein gp41 of the virus to heparan sulfate which is present on the host plasma membrane which helps in triggering the fusion of viral envelope and the release of the capsid in the cell cytoplasm (23, 24).

The HIV strains can be classified on the basis of the cell tropism macrophagic-tropic also known as M-tropic and T-cell tropic also known as T-tropic. The M-tropic cells utilize the CCR5 as the co-receptor for the infection to the lymphocytes and are always referred as R5 viruses. They primarily infect the macrophages and primary T-cells and infect poorly CD4⁺ T-cell lines. Also the M tropic viruses are tend to transmit via route of sexual transmission very easily (25). The T-tropic strains utilizes the CXCR4 coreceptor for the infection to the cells. They T-tropic viruses are referred as X4 viruses. These X4 viruses helps in the syncytia formation in the infected cells(26, 27) The HIV virus particle tend to use the CD4 molecules, co-receptors to infect the cells and then make entry into the cytoplasm.

After making the entry in the body, the viral capsid releases the viral RNA in the cytoplasm and also the viral proteins. The enzyme reverse transcriptase helps in transcribing the viral RNA into complementary DNA (cDNA) by using the cellular lysine tRNA molecule as primer (28). This newly synthesized HIV-1 cDNA is then transported to the nucleus. In the nucleus two HIV proteins Vpr and Vif take important role in the transportation. Vpr is able to enhance the HIV-1 preintegration complex transportation to the nucleus while the Vif associates with cytoskeletal elements and increase the infection potential (29, 30). Once the HIV-1 cDNA enters the nuclear membrane, the peptides from Vpr and the matrix protein provide a nuclear

localization signal to enter into the nucleus for further action. The viral cDNA then integrates randomly into the host cell genome with the help of viral enzyme integrase.

After the integration of viral DNA in the host genome, the viral particle requires some cellular transcription factors which are able to activate the viral gene transcription which in turn produces the low levels of short, multiplied spliced mRNA transcripts. These encode the regulatory proteins Tat, Rev, and Nef. Tat transactivates transcription by the binding the 5' end of the viral DNA sequence which helps in increasing the viral replication to 1000 fold (31). Rev binds an RNA structure in the env region and mediates export of incompletely spliced transcripts out of the nucleus. Rev favours the export of partially spliced mRNA transcripts encoding the structural proteins and full length mRNA transcripts which constitute the viral RNA genome (32).

After integration of the viral DNA in the host genome the further transcription and translation process takes place in the nucleus to make more virus assembly and the shredding of the virus into the system. For the assembly of virus, the HIV viral core components are initially translated in to pr55 which is a pre-protein resulting from a singly spliced gag mRNA which is then cleaved during the maturation process. Gag-Pol is another protein which is cleaved to produce the viral enzymes protease, integrase and reverse transcriptase. The HIV protease which mediates the specific cleavage of these pre-proteins, and its inhibition results which marks the suppression of viral replication (33).

Pr55 and Gag-Pol are the results of two reading frames that control the relative amount of the proteins to produce more number of structural components than viral enzymes. the first reading frame encode the pr55 which is more efficient than other one which is responsible for the Gal-Pol synthesis (34). An intermediate length mRNA which produces gp160 which migrates to the cell membrane which is cleaved during the virion formation to originate the regulatory proteins Vpr. Vpu, Vif and the envelope proteins gp41 and gp120. These proteins and other host membrane proteins which are incorporated on the plasma membrane to form the viral envelope around the capsid, containing the viral RNA genome. The entire virus assembly process is energy dependent process which involves several unidentified cellular factors (35). gp120 can be cytopathic by binding with the CD4 receptor and altering its protein trafficking in the cytoplasm. The proteins such as VPu induces the degradation of CD4 receptor and might a role in the gp120 processing for the process of functional

envelope and also in the downregulation of CD4 surface expression (36). The HIV virus particle matures in this way to more copies of the virus so that they can infect the host via suitable mechanism

4.3.2 Immunologic response to HIV

Once the HIV pro-viral DNA is with the host infected genome, it cannot be eliminated or destroyed from the body unless the host destroy itself. This proviral DNA then directs its replication by the infected cells. This replication process as described may first occur with the inflammatory cells at the site of infection or within the peripheral blood mononuclear cells but later the site of replication shifts to the lymphoid tissue of the body. The initial burst of viral replication that follows infection is followed by the replication at a lower level, which accounts for the clinically apparent latency of infection. But the viral replication is stimulated by a variety of cytokines such as interleukins and tumour necrosis factor, which activates CD4 lymphocytes and make them more susceptible to HIV infection (37, 38).

The activation of viral synthesis leads to release of new virus particles from the host cell surface budding. Replication also causes the cell lysis which in turn releases the additional virus particle which can be infective to the cells. Host cell death may be mediated via several mechanisms; direct viral cytopathic effects, fusion to multinucleated giant cells (syncytia formation), cytotoxic immune response by other lymphocytes (CD8⁺ cytotoxic lymphocytes), autoimmune mechanism, disruptive mechanism of HIV envelope proteins with cell membrane, immune clearance from alteration of antigenicity of the host cell, activation of apoptosis (programmed cell death) or toxic accumulation of the viral DNA, RNA or proteins (37-40).

Apoptosis plays a major role in the decline of T cell populations in HIV infection. The acute infection of HIV results in immune activation with apoptosis of infected lymphocytes. It has reported that the expression of tumour necrosis factor (TNF) related apoptosis inducing ligand (TRAIL) and FAS ligand have increased and they have a paracrine effect to promote the further apoptosis of bystander cells (41). Mechanism which contributes to the HIV associated lymphocyte apoptosis include chronic immunologic activation via gp120/160 of the CD4 receptor, enhanced production of cytotoxic ligands or viral proteins which are released by the monocytes, macrophages, B cells, and CD8 cells and direct infection of target cells of HIV

resulting in apoptosis. HIV envelope glycoprotein included chemokine receptor CXCR4-dependent autophagy of uninfected lymphocytes, which is required for caspase dependent apoptotic cell death and caspase -independent, non-apoptotic cell death. Apoptosis of lymphocytes is generally increased with progression of HIV and diminished with effective antiretroviral therapy (42, 43).

The TH1 or T helper cells 1 are majority responsible for CD4+ lymphocytes population which is important in determining the host response to infection. These cells are responsible for determining a cytotoxic CD8+ T-lymphocytes response while increasing the antibody response production. The individuals those with the HIV infection have a dominant TH1 response tends to survive longer. These CD8+ lymphocytes have ability to inhibit the HIV infection but it is affected by the HLA - restricted cytotoxicity as well as the suppressive activity mediated through a number of multiple suppressive factors called as CD8 antiviral factors (CAF) (44).

The switch from a TH 1 to TH 2 response is major factors in the development of AIDS. CD4+ lymphocytes produce IL-2 and IFN-gamma in TH 1 response and IL-4 and IL-10 as part of TH 2 response. Production of interleukins -5 (IL-5) and IFN-gamma by CD4+ and CD8+ T- lymphocytes expressing CD30 is associated with the promotion of B-lymphocyte immunoglobulin production. This soluble CD 30 is marker for detection of viremia (45, 46). There is possibility of imbalance in the T helper response to mainly the TH2 response which is mediated by the HIV proteins gp120 and Tat which in turns triggers the release of cytokines which are necessary for the TH2 response. These HIV proteins stimulate the mast cells and basophils. The Tat protein upregulates chemokine receptors CCR3 on mast cells and basophils and reduces them to susceptible for the infection by CCR3 tropic HIV. The various studies have shown the increase serum IgE levels indicated that a aTH2 response has occurred and its points to a poor prognosis of the disease (47).

The CD8+ lymphocyte response to early HIV infection is not sufficient to contain the continuous viral replication. In the process of making new variant of HIV many of the clones have been reduced or eliminated by the CD 8 response. The HIV mutation clones termed as “escape mutants” that helps in infection process. In an acute HIV infection, the CD8 + cell response occur to suppress viremia. This efficacy of early CD8 cell response determines the set point of plasma viremia that predicts the subsequent course of HIV infection. CD8 cell responses in acute HIV infection appear

to suppress the viral replication but these responses get diminished in the chronic phase of HIV infection (48).

The macrophages and dendritic cells in the epithelial tissues of the body are important as they both are reservoirs and vectors for the spread of HIV in the body. These cells are important to fight against the HIV infection. The macrophages originate from the blood monocytes and contribute to rise to the mononuclear phagocyte system. The Patients those and the antiretroviral therapy are healthy may show a demonstrable HIV-1 within their alveolar macrophages (49).

Langerhans cells originate in bone marrow and migrate to the peripheral epithelial locations in skin and mucus membranes thus acting as the antigen presenting cells for the lymphocytes. These dendritic cells cross the endothelium and circulate freely in to both lymphoid and mucosal tissues. HIV can be replicated within dendritic cells for up to 45 days (50).

Both these macrophages and Langerhans cells are infected by the HIV but cannot be completely destroyed. Dendritic cell capture the HIV in their processes, providing a space for the infection of the other cells. In of the experimental cell culture study, there are two pathways of HIV-1 found 1) fluid -phase diffusion of cell free virion and (2) cell to cell spread of virions where observed. The cell to cell spread of virions is potentially more efficient. This transfer includes the budding with fusion of closed opposed cell membrane and also the cell to cell fusion give the syncytia formation. The viral particle undergoes the process of endocytosis which may be stored in the cell surface-accessible compartment, or some time these directly infect the cells via receptors. The long-term transmission of HIV from dendritic cells to CD4 cells results in the active infection of dendritic cells than just trapping and presenting the virion, along with this short term transmission occurs through the cell surface - HIV interaction, there are various pockets pf the plasma membrane which harbours the virions on the dendritic cells which provides the means to the virion to CD4 cells via a so called “infectious synapse” (51).

The T regulatory (T-regs) cells are involved in the immune response following the HIV infection. Persistent immune activation followed by the HIV infection leads to the Treg expansion. The population of Tregs may show suppress generalized Tcell activation to reduce the inflammatory response or weaken the specific immune response leading the detrimental condition. The Transforming growth factor -beta (TGF- β) is one of marker of chronic immune activation that is associated with the

HIV disease progression. The balance between the Tregs responses may help in modulating the course of HIV infection (52, 53).

When the primary infection occurs, most of the infected cells are CD4 lymphocytes, accounting for the 80% of all infected cells at the site of mucosal inoculation and 90 % of infected cells in lymphoid tissues. But the follicular dendritic cells (FDCs) within the lymphoid tissues are the greatest reservoir in well-established HIV-1 infections, particularly throughout the clinically latent period before the onset of AIDS harbouring an estimated 1011 copies of HIV-1 RNA. The pool of infected CD4 cells diminished rapidly and eventually leading to the failure of the immune system and onset of AIDS (54).

Since the HIV provirus becomes part of the infected host's cellular DNA, the hosts' cell may become infectious even in the absence of demonstrable HIV serum viremia or detectable HIV antibodies. However, the antibodies formed against HIV are not protective and a viremic state can persist despite the presence of even higher antibodies titres. Since the HIV has ability to mutate easily despite the error rate in the production of the reverse transcriptase enzyme, which introduces approximately once per 2000 incorporated nucleotides, such a mutation rate is millions of times faster than of mammalian DNA. This high mutation rate leads to emergence of HIV variants within the infected person's cell that can then resist the immune attack with exhibiting the higher cytotoxicity, generate syncytia more readily or can impart the drug resistance. Over the time various tissue of the infected host's body may harbour differing HIV variants (55, 56)

The primary target of HIV is the immune system itself which is gradually destroyed. The viral replication actively continues following initial HIV infection and the rate of CD4 lymphocyte destruction is very rapid and progressive. Clinically the HIV infection appears "latent" for the years during this period of ongoing destruction. During this time the enough of immune system remains intact to provide the immune surveillance and prevent most of infection. When a significant number of CD4 cells are destroyed and the new CD4 cells cannot match with the destruction then the failure of the immune system leads to the clinical AIDS (39, 40).

HIV infection is sustained through the continuous viral replication with reinfection of additional host cells. Both HIV in plasma and HIV -infected host cells appears to have a short span and further in the late progression of AIDS the half-life of plasma HIV is about 2 days. Thus, the persistent viremia requires continuous

reinfection of new CD4 lymphocytes followed by the viral replication and cell turnover. The rapid turnover of HIV and CD4 lymphocytes promotes the origin of new strains of HIV because of continuing mutation of HIV. The presence or emergence of different HIV subtypes also account for appearance of antiretroviral drug resistance as well as the variability in the pathologic as different cell types that are targeted or different cytopathic effects are elicited during course of infection (57, 58)

Active replication of HIV occurs at all stages of replication, but a month of the initial infection and peak viremia occur, there is an equilibrium which needs to be established between HIV replication and control of HIV by the host system. In general terms the clearance rates of HIV are similar among persons but the rate of HIV production determines the viral load in the steady state. This shows that there is clinically latent phase of HIV infection occurred. The presence of viremia as detected but by serum HIV-1 RNA suggests that the immune system is not capable of containing the virus. The increasing levels of serum HIV-1 RNA suggests that a loss of equilibrium and emergence of latency leads to a more rapid progression to AIDS. The absence of the detectable serum HIV-1 RNA suggests to the slower clinical progression to AIDS. Also the higher the values of HIV-1 RNA in the patients with the symptomatic acute HIV infection suggests that such persons may have rapidly progressed to AIDS stage (59). As the stage of AIDS advances the number of CD4 cells diminishes, but the macrophages serve as the key sites of the continuing viral replication (44). Even after years of infection, the viral suppression with antiretroviral drugs, there is a typically rebound of detectable peripheral blood viremia within 2 weeks of cessation of therapy which suggests that the reservoirs for persistent HIV infection remain (37).

4.3.3 Role of Cytokines in the HIV infection

Cytokines are a group of low molecular weight proteins which mediate communication between immune system cells. These contribute to chemical signalling pathways that regulate the mechanism of development of tissue repair, role in haematopoiesis, roles in regulation of inflammation and immune responses. Cytokines have pleiotropic activities and has the functional redundancy. These cytokines has a important roles in the complex cellular and immune network in which

one cytokine can influence the production of cytokine and then shows response to many other cytokines.

HIV infects the immune cells of the macrophage and the T-cell lineage. The entry into these cells requires the CD 4 as major receptor and in addition to that a co-receptor which is frequently is either chemokine receptor CCR5 or CXCR4 (60). The binding and entry into the human cells requires the two HIV glycoproteins gp120 and gp41. the gp41 has a transmembrane domain and is associated with the viral envelope while the gp120 which is present in association with gp41 does not insert or comes in contact with the viral membrane (61). Generally these two glycoprotein are present in HIV as tetramers, therefore three gp41 molecules associate with the viral membrane, while the three molecules of gp120 associate with gp41.

To facilitate the viral entry into the human cells, gp120 binds to the CD4 receptor with high affinity .this binding causes a conformational change in the gp120 that in turns reveals a co-receptor binding site. This receptor in then facilitated to turn a conformational change in the glycoprotein gp41 N-terminus (61).

HIV-1 viral variant generally use CCR5 or CXCR5 co-receptor for the entry into the human cells (60). The normal function of chemokine receptors is to bind the chemokine that target the immune cells to the areas of inflammation with the human body. Certain HIV-1 viruses has increased ability to either bind the CCR5 receptor and are known as R5 viruses while which bind to CXCR4 are known as X4 viruses. The macrophage tropic HIV-1 viruses are generally R5 and T-cell tropic are X4 viruses (62). Early during the infection R5 viruses predominate and there is mechanism which selects these viruses during the transmission process (63) for eg an HIV-1 naïve individual may be expose to the both R5 and X4 virus particles from an infected individual but it can only become infected with the R5 viral particles. There would be many multiple reasons for affecting these processes including the co-receptor availability and the pH at the sites of infection. The acidic PH may act to disrupt the cationic charge which is present in the gp120 proteins and they bind to the CXCR4 preferentially (64, 65). R5 viruses are prominent during the chronic infection. X4 viruses or R5X4 have mixed affinity which can arise later during the infection and their presence often is seen the disease progression and immune cell depletion (66).

4.3.4 Cytokine regulation in HIV

As hypothesized by Mossman the cytokines can be classified as Th1 (IL-2, IFN- γ) AND Th-2 (IL-4, IL-6, IL-10). TNF- α is categorized with the Th1 cytokines.

4.3.4.1 HIV and the Th1 to Th2 cytokine shift

Under the normal circumstances, the immune system utilizes a Th1 subset response to the viral infections. The activated antigen presenting cells (APC) secretes interleukin -12 (IL-12) which causes the Th cell differentiation into the Th1 subset of cells (66). These Th1 cells then secrete a characteristic Th1 profile of cytokines such as interleukin-2 (IL-2), interferon-gamma (IFN- γ), and tumor necrosis factor -beta (TNF- β). IL-12 induces the proliferation of naïve Th cells (T_0), amplifying the Th response. IFN- γ induces further IL-12 production in the activated APCs, amplifying the Th1 response, and then suppressing any Th2 response. IFN- γ also plays an important role in the activation of the cytotoxic Tc cells which destroys the virally infected cells. In the individuals infected with HIV, the normal Th1 response to the viral infection is shifted to a Th2 response (45, 67). The measurement of serum cytokine levels of HIV infected patients has shown an increase in Th2 cytokines as well as decreased in Th1 cytokines (45, 67).

4.3.4.2 TNF - α and HIV infection.

TNF- α is produced by many types of immune cells in response to the number of stimuli including antibody crosslinking, LPS stimulation, and numerous viral infections. The TNF- α has a proinflammatory effects when its presence is systematically and their expression is regulated at all the levels. TNF- α activity is mediated by the wither of the two receptors TNF receptor 1 (TNF-R1) or TNF receptor 2 (TNF R2) which are ubiquitously present on both nucleated surface and in the soluble form. These soluble forms of TNF-R1 and TNF-R2 act to attenuate the activity of TNF- α but it acts simultaneously as a reservoir for the available TNF- α . This TNF- α binds to membrane bound TNF-R receptor resulting in trimerization of membrane bound TNF-R and it initiates cell -type dependent intracellular cascades, which are NF- κ B dependent which results in the biological effects. TNF- α depending on the target cell type can mature and activate antigen presenting cells induce IL-1 β , IL-8, GM-CSF, M-CSF and IFN- γ from monocytes, activate the CTL and or induce the apoptosis of mature T -cells. Some of these actions results in the clinical

symptoms such as fever, hypotension, anorexia and capillary leakage among the other events. TNF- α act as the potent inhibitor of IL-12 (68).

The various studies have been shown that HIV infection induces increase production of TNF- α by macrophages. HIV -1 induces TNF- α expression and the exogenous TNF- α enhances a positive correlation between the increase levels of TNF α and the HIV -1 plasma viral load, which have been demonstrated both in vivo and in vitro (69, 70). Various studies performed on the transgenic mice which are deficient in TNF- α and IL-1 β have shown that these cytokines are the essential for HIV-1 replication in vivo. Various studies have shown that some HIV-1 infected slow progresses possess naturally occurring anti-TNF- α - α antibodies (71).

The mechanism by which TNF- α increase HIV-1 replication is incompletely understood, and there is a variation in this ability between strains of HIV-1 (72). one mechanism states that TNF- α increases HIV-1 replication by stimulating the proinflammatory signalling cascade via TNF-R1 activating NF- κ B, resulting in the induction of HIV-1 replication in previously latent cells (73). A circuit which has been described in vitro culture systems in which the HIV-1, NF- κ B and TNF- α each exert a positive feedback on each other, amplifying virus replication and inducing the changes in the cellular gene expression which is independent if HIV -1 replication (74). TNF- α produced by macrophages in the presence of HIV-1 is very important in inducing the T-cell anergy (75).

The TNF- α upregulation of receptor CXCR4 expression may represent mechanism where it increases the HIV replication. [64]. this is upregulation help in increasing the susceptibility to X4 tropic viruses while there is concurrent downregulation of CCR5 in some population of cells, which is partially mediated by TNF- α with association with IL-13 which subsequently results in the decreasing HIV replication (76, 77).

The various studies have proved that human TNF- α is more actively involved in the development of lymphoid follicles trapping early HIV-1 and host immune responses (78). Various studies showed that in addition to the role of TNF - α in increasing HIV-1 replication there is likely the macrophage dependent, nef mediated, anti-apoptotic effect and productively infected CD4 + T-lymphocytes which are physiologically targeted for the apoptosis in order to eliminate infection. These cells are maintained in circulation to contributing to the pool of latently infected T-cells (79, 80).

During the mycobacterial infection, the mycobacteria have proven that they are able to induce TNF- α expression both in-vivo and in-vitro. This resulting in increased of HIV-1 replication and suggesting a possible role of the co-infection with HIV-1 and contributing to acceleration of both HIV-1 and TB disease progression (81, 82).

An important mechanism for the stimulation of the APC effector function is to present appropriate antigens and provide stimulus to the T-cells. Vpu, one of the intrinsic HIV-1 protein which blocks TNF- α induced antigen processing, thus effectively blocks its physiologic antiviral activity against HIV-1 infection. This Vpu appears to inhibit NF-KB dependent antigen degradation and thus limiting the physiologic activity of TNF- α to stimulate antigen processing and presentation (83).

TNF- α is capable of increasing the HIV-1 replication both in vivo and in vitro by using the TNF-R1 receptor (84). Some cases showed that it inhibits HIV-1 replication in monocytes systems via the down regulation of CCR5 receptor and simultaneously upregulating CXCR4 receptors and it helps in increasing the susceptibility to X4 virus stains. With more evident finding TNF- α , a proinflammatory cytokine generally shows increase in the activity as the HIV-1 progresses until immunologic defects are more pronounced that cells are no longer capable of cytokine production.

4.3.4.3 Interleukin -6

IL-6 is a pleiotropic cytokine that is commonly found at local tissue sites and released into circulation in almost all situations of homeostatic alarm typically including the endotoxemia, endotoxic lung, trauma and acute infections. In addition to these function the IL-6 has critical participation in the generation of immunity against chronic intracellular infections, circulating IL-6 together with other alarm cytokines TNF- α and IL-1 which are known to be required for the induction of acute phase reactant proteins which composes of fever, corticosterone release and hepatic production of acute phase reactant proteins out of which many are protease inhibitors (85). They activate target genes involved in the differentiation, survival, apoptosis and proliferation. The members of this cytokine family show the pro-inflammatory as well as anti-inflammatory properties. These have a major role in haematopoiesis, as well as in acute phase and immune responses of the organism.

The induction of IL-6 by live HIV preparations occurred in the absence of T cells and could be neutralized by human anti-HIV serum indicating that HIV was responsible for the IL-6 inducing activity. Various studies have demonstrated that IL-6 can be produced by a variety of cells giving various kinds of stimulation. T-cells infected with HTV-1, fibroblasts stimulated with polyI:C IL-2, platelet derived growth factor, TNF- α , FCS, or LPS and monocyte/macrophage stimulated with LPS. Monocyte/macrophage is one of target cells of HIV, produced IL-6 upon stimulation with both live and inactivated HIV (86).

IL-6 both increase HIV-1 replication in latently infected macrophages and alters the function of infected macrophages and turning them into sub optimally functional (87, 88). IL-6 also plays a prominent role in immune reconstitution in the setting of HAART and via a complex system of neuroendocrine actions, which as a significant role in HIV-1 associated lipodystrophy syndrome (89, 90).

Also, overproduction of IL-6 results in HIV-1 related hypergammaglobulinemia seen in the early in infection (91, 92). IL-6 levels increased in HIV-1 are detrimental to infected hosts. The mechanism whereby the higher than normal IL-6 levels facilitate HIV-1 replication in macrophages remains unknown and further more study in to be taking ahead.

4.3.4.4 Interferon gamma (IFN- γ)

Interferon- γ (IFN γ) is a Type II interferon that is pivotal in the regulation of the host immune response against viral and intracellular bacterial pathogens. The effects of IFN γ are broad exhibiting polyfunctional effects on immune activation, proinflammatory responses, and immune modulation. High levels of IFN γ are secreted by Type 1 T helper cells (Th1 cells), CD8⁺ cytotoxic T lymphocytes (CD8⁺ CTLs), and NK cells during active infection (93).

IFN- γ has a major effect on the regulation of antigen presentation by macrophages and dendritic cells, and in induction of class switching of B cells (94, 95). As a pro-inflammatory cytokine, IFN- γ directly activates phagocytic cells and stimulates oxidative burst and the release of degradative enzymes, supporting the host defence responses against intracellular pathogens (96). IFN- γ also induces the production of proinflammatory cytokines and chemokines on endothelial cells, epithelial cells, and fibroblasts. Release of IFN- γ results in vasodilation and

upregulation of adhesion molecules, promoting diapedesis of neutrophils, and macrophages to the site of inflammation in the body.

In addition to upregulation of innate defence mechanisms, IFN- γ is also important role in immune modulation. IFN- γ upregulates the expression of MHC-I and -II molecules, activates antigen presenting cells and induces macrophage maturation toward a proinflammatory phenotype (97, 98). IFN- γ also works synergistically with other cytokines such as IL-2 and -4 to balance the T helper subset Th1/Th2, regulating the cytotoxic versus humoral T cell immune response (98).

4.3.5 Cytokines and regulation of cytokine synthesis

4.3.5.1 NF- κ B pathway

The nuclear factor κ B is a critical regulator of the immediate early pathogen response playing an important role in promoting inflammation and in the regulation of cell proliferation and survival (99). The NF- κ B/I κ B family promotes overall expression of over 2000 target genes which has role in the host immune response. These proteins includes a multiple number of cytokines and chemokines, and their receptors required for the immune recognition, proteins involved in antigen presentation and adhesion receptors involved in the transmigration across blood vessels walls .Due to this type of extensive role in immune action. NF- κ B has named as the central mediator of immune response. Gene knockout and other studies have established roles for NF- κ B in the ontogeny of immune system but it also shows that NF- κ B takes part in the process of the oncogenesis and as well as apoptosis (100, 101)

NF- κ B pathway provides an attractive target to all viral pathogens to express their pathogenicity into the host's organisms. The activation of NF- κ B is very rapid process with immediate early (IE) events which happens within minutes after the exposure to the viral pathogens /inducer which do not require the de novo protein synthesis pathway. This subsequently results in strong transcriptional stimulation of various viral and as well as the cellular genes. The various viruses have evolved with the different strategies to modulate the NF- κ B pathway to enhance their viral replication, host cell revival and evasion of immune responses. The activation of NF- κ B is main target for the virus to attack using various cytokines, growth factors and their receptors and proto-oncogenes which influence the host cell cycle. In addition to these factors some viruses exploit the anti-apoptotic properties of NF- κ B to evade the

host defence mechanism which limit replication by killing the infected cells or also leading to the apoptosis as a trigger mechanism to increase the spread of virus.

The persistent activation of the NF- κ B pathway is maintained by the certain viruses which contribute to oncogenic transformation (100). The classical example which shows the avian REV-T retrovirus which contains the v-REL oncoprotein which induces a rapid and fatal B -cell lymphoma in young birds(102) . Some studies have demonstrated that NF- κ B family members contribute to human oncogenesis process. In this process the localization of NF- κ B -encoding genes are at sites of chromosomal translocations and genomic rearrangement in cancer. Various studies showed that viral oncogene products such human T-cell leukaemia virus type - 1 (HTLV-1) Tax protein, Epstein Barr Virus latent infection membrane protein 1 (EBV LMP1) acts by unique mechanism to disrupt the NF- κ B regulation and initiate the viral transformation.

4.3.5.1.1 Biochemistry of NF- κ B

The latent cytoplasmic NF- κ B/I κ B complex generally activated in response to a variety of stimuli, including the viral and bacterial pathogens, different cytokines and stress inducing agents is activated by phosphorylation (103). In the case of I κ B α the phosphorylation modification occurs at serines 32 and 36 by I κ B kinase (IKK) complex.

The NF- κ B family consists of five structurally related proteins (c-REL, Rel A, Rel B, p50/p105 and p52/p100) which share an approximately 300 odd amino acids NH2 terminal Rel Homology domain which contains sequences essential of dimerization, DNA -binding, and nuclear transport mechanism. Family members such c-REL, Rel A, Rel B has COOH terminal domains, while p50/p105 and p52/p100 function as either short DNA binding proteins or larger inactive domains. Due to a huge potential for generating diverse homo-hetero dimers, these proteins allow the transcriptional specificity by forming the combinations that can interact with structural variants of the I κ B DNA binding sites.

The IKK kinase complex is major complex which had helped in understanding the NF- κ B regulation. The IKK kinase complex contains two catalytic subunits IKK- α and IKK- β and the regulatory subunit IKK- γ (NF- κ B essential modulator NEMO). The predominant form of the IKK complex is and IKK- α /IKK- β heterodimer associated with IKK- γ which is mediated by the interaction of IKK- β and IKK- γ . The

identification of IKK complex the attention has focused on the various upstream kinases of various signal transduction pathways and how these pathways converge. And further how the viral pathogens use to pathways to induce their replication

4.3.5.1.2 Viral activation of NF- κ B

NF- κ B is activated by different types of viruses such as HIV-1, HTLV-1, hepatitis B virus (HBV) Hepatitis C virus (HCV), EBV, and influenza virus. This activation has several different functions to promote the viral replication prevent virus induced apoptosis and mediate through the immune response to the invading pathogen. Although the molecular mechanism details remain unclear about viral products which activate the NF- κ B, tax protein, HIV-1 tat protein and EBV LMP1) ref5 appears to act through several other mechanisms to enhance the virus replication. With the HIV virus, the viral and cellular membrane fuses together and then activates the NF- κ B. For this process the CD4 receptor is subsequently required which is the primary target of the HIV-1 to invade the host body. The other viruses activate in similar way using the membrane proximal signalling cascades that activate NF- κ B. The NF- κ B is also activated using the synthetic dsRNA, suggesting that viruses that generate the dsRNA replicative intermediates employ a common mechanism to enhance the viral replication.

4.3.5.1.3 NF- κ B activation in HIV-1 infection

The HIV-1 virus genome had ability to co-opt host signalling pathways to activate viral transcription due to intracellular efficiency of gene expression and replication (104) The promoter -proximal region of the HIV-1 long terminal repeats (LTR) contains two adjacent NF- κ B binding sites (-109-79) that plays an important role in mediating inducible HIV-1 gene expression. Also, the trans dominant mutant of I κ B α blocks the NF- κ B induction and inhibit de novo HIV-1 Infection in T cells by interfering with the viral replication (105, 106). The molecular organization of the LTR region in different naturally occurring HIV-1 subtypes appears to have co-evolved with variations in the HIV-1 enhancer that influence the viral expression and replication for example the HIV-1 clade E which is predominant subtype in Asia contains a single NF- κ B binding site in their enhancer region which has a reduced response to the TNF- α , Whereas the subtype C which is predominant subtype is Asia contains three NF- κ B binding sites and it has a higher transactivational activity than

the HIV-1 LTR from other subtypes. The subtype E with one NF- κ B binding site shows higher transmission efficiency than the North American subtype b which carries two NF- κ B binding sites. The discrepancy appears to lie with the single nucleotide deletion in the upstream copy of the NF- κ B binding site in clade E LTR. This helps in creating the binding site for GABP α and GABP β 1 which is known as the transcription factor. When this GABP binding site is introduced into the LTR of the clade B virus it behaving better than the wild types virus (107).

The role of NF- κ B in activating HIV transcription has been studied. In normal human CD4⁺T lymphocytes, NF- κ B binding activity is low and consists of p50, but not p65, the DNA binding. T-cell activation results in formation of the p50-p65 NF- κ B complexes and also the formation of enhancer dependent HIV LTR transactivation, indicating that unstimulating CD4⁺ cells offer a cellular environment of low permissiveness to HIV LTR function. The studies have shown that NF- κ B dependent transactivation is essential for the HIV-LTR. The CD4⁺ T lymphocytes carrying an infectious HIV provirus with point mutation in these elements fail to transcribe viral RNA upon cell activation (108). Some studies have shown that NF- κ B sites are not required for viral growth since HIV-1 will grow slowly in absence of NF- κ B domain (109).

NF- κ B is has constitutively activated in primary monocytes and myeloid cell lines chronically infected with HIV (105). Increasing the intracellular levels of NF- κ B permits high levels of HIV-1 gene expression and thus it could provide a favourable condition environment for HIV replication. There is still no clear role how IKK is activated by HIV-1 infection although multiple mechanisms appear to be operating and several HIV-1 proteins can activate the NF- κ B.

Envelope gp120 has role to signal NF- κ B by engaging the CD4 receptor in pathway that involves p45 and activates the NF- κ B and HIV-1 LTR transcription (110). HIV regulatory or accessory proteins also stimulates the NF- κ B activity, the HIV Tat protein stimulates a wide variety of cellular responses including the NF- κ B induction. Various studies have reported that role of T-cell specific tyrosine in the different cell lines such as Jurkat T- cells and JCaM1 shown to activate NF- κ B and induce the NF- κ B dependent genes.

HIV-1 infection of the immune system is itself a potent and persistent cause of immune activation. Several endogenous cytokines are released by activated cells that profound affect HIV disease progression. The expression of the various cytokines is

dysregulated in HIV -infected individuals (104). Many of these factors including IL-1 α , IL-1 β , IL-2, -3, -6, -7, -11, -15, -18, TNF- α , β along with the growth factors such as M-CSF, GM-CSF induce the HIV expression. But the other cytokines such as IL-13, -15, IFN- α , β suppresses the viral replication. TGF- β , IL-4, IL-10, IL-13 and IFN- γ can either induce or suppress the HIV replication, depending on the duration of the treatment and the cell system. Various studies in PBMCs and the lymph node cells indicate the HIV transcription is intimately related to increase expression of cytokines such as TNF- α , IL-1 β , IL-6, some of these cytokines activate NF- κ B via IKK pathway (111). Furthermore, activation of this pathway would augment cytokine and LTR - directed gene expression. The dsRNA generated during this HIV-1 replication interact with the endogenous IFN to activate the dsRNA-activated kinase PKR and thus induce IKK activation and their subsequent degradation of I κ B α and I κ B β . Some studies have shown that HIV proteins activate NF- κ B via I κ B β to maintain its persistent state.

4.3.6 Pathogenesis of HIV infection

HIV infection occurs through oropharyngeal, cervical, vagina and gastrointestinal mucosal surfaces, even in the absence of mucosal disruption. Routes of HIV entry into mucosal lamina propria include dendritic cells, epithelia cells. The epithelium cells provide reservoir to the HIV virus into the host body. The virus can cross a tight epithelial barrier by the process of transcytosis during the contact between HIV infected dc cells and the apical surface of the epithelial cell.

Transcytosis of virions happens through the intact epithelium is favoured via surface of expression of syndecans and chemokine receptors by epithelial cells, however the efficiency of transcytosis is poor with only 0.02 % of original inoculum of HIV able to navigate across genital epithelium. Thus, the intact epithelium is a significant barrier to HIV infection, but the presence of antigen processing cells and inflammatory cells increases the HIV transmission (112). The exposure to HIV-1 can upregulate pro-inflammatory cytokine production by genital epithelial cells, including the tumor necrosis factor (TNF)- α that impairs the tight junction barrier which helps in allowing HIV-1 and luminal bacteria to translocate across the epithelium.

As HIV infects cells CD4⁺ cells to gain entry into host cells. These cells are susceptible to HIV infection include cells of mononuclear phagocyte system such as

blood monocytes and tissue macrophages, T lymphocytes, natural killer, dendritic cells, microglial cells in brain (112-114)

Apart from the CD4⁺ surface receptor the other receptor CCR5 and CXCR4 are chemokine receptor are involved by the viral gp120 for the entry into host cells. The gp41 and gp120 has the conformation changes in the host cells and allow the entry of HIV virus into cells.

Cellular localization of chemokines receptors helps in HIV infection. Macrophages and monocytes as well as subpopulations of lymphocytes, neuroglia, astrocytes, and microglia in central nervous system also express the CCR5 receptor. Areas of inflammation contain increased numbers of mononuclear cells with CCR5 and they helps in transmission of HIV at those sites (115)

The principal constituent of HIV-1 is gag, accounting for the entire virion mass. Viral lipid membrane composed of major of the mass along with other viral and cellular protein they contribute to additional 20 %. The Gag, gal-pro-pol,env, two copies of genomic RNA,the tRNA primer, and lipid envelope are all necessary for viral replication.

Viral maturation process is carried out by use of the gag, gal-pro-pol, env poly proteins. Along with these proteins the matrix (MA), Capsid(CA) and nucleocapsid (NC) helps in the process of maturation and subsequently release of new virus particles (116)

Macrophages plays important role in all stages of HIV infection, the acute infection macrophages become infected with HIV though not as readily as CD4 lymphocytes. Nevertheless, sufficient macrophages become infected to contribute to establishment of HIV infection. Once infection macrophages acts as antigen presenting cells which assists in the immune response that leads to antibody production and cytotoxic CD 8 lymphocyte response. Thus macrophages assist in the immune response that reduces the viral load. But the macrophages harbours HIV during latent phase of infection; although macrophages can elaborate viral restriction factors, the HIV protein and restriction factors leads to continuing HIV proliferation (117).

Dendritic cells play a key role in HIV infection. Two populations of these cells have been characterized and they have a role in the HIV. The convention dendritic cells such as Langerhans cells are found in epithelia and mark with CD11c. these CD11c are infected with HIV and they transport the virus via lymph and blood to

multiple sites within the body. They also secrete interleukin-12 that induces cytotoxic responses to infection. In contrast to this the plasmacytoid cells mainly circulate in blood but can migrate to many tissues and are important in infection. These cells are marked with CD123 positive and they secrete interleukins type I which helps in stimulation of conventional dendritic cells. DCs circulating in blood tend to decrease inversely in proportion to increase in HIV viremia.

Dendritic cells also express high amounts of HIV entry receptors CCR5 and CXCR4 but relatively low amounts of CD4 which allows the gp120 attachment of HIV virions. When these DCs mature they upregulate CXCR4 but also downregulate the CCR5 coreceptor. Although HIV poorly infects DCs, the virions carried by DCs can infect nearby CD4⁺ T cells. DCs may promote initial HIV infection and dissemination through chemokine secretion (118).

Both the conventional and plasmacytoid dendritic cells activate the NK cells, which provide an innate immune response. However, the HIV *nef* gene encodes a protein that downregulates HLA-A and B but not the C, expression of infected cells to evade cytotoxic lymphocyte responses and killing by NK cells that recognize the HLA-C. Also some studies have reported that HIV reduces cytokines which activate NK cells. Dendritic cells infected with HIV become resistant to NK cell destruction.

HIV virions are trapped in the processes of follicular dendritic cells, where they reside in endosomal compartments that are formed from invaginations of cell membrane in the lymph nodes. These compartmented virions in dendritic cells infect CD4 lymphocytes that are percolating through the lymph nodes, these DCs themselves are infected but are not destroyed (119). The stromal dendritic cells become infected via chemokine receptor pathways but also they have a surface protein called dendritic cell specific ICAM3-grabbing non-integrin (DC-SIGN) that captures HIV by binding to the HIV envelope. DC-SIGN-bound HIV is more infectious and has a longer half-life than free HIV (67). DCs can migrate in lymph and blood to carry HIV throughout the body. The presence of gp120 of HIV helps to reduce the capacity of dendritic cells to produce interleukin-12 by suppressing the cell-mediated immune responses (120).

Most of the HIV infection likely begins from the single virus which is known as “a founder” virus from which the subsequent clones develop. Host cells elaborate an antiviral Apo lipoprotein B mRNA-editing enzyme catalytic polypeptide like-3G (APOBEC3G) with cytidine deaminase activity that leads to defective viral

replication. In addition to these HIV gene for reverse transcriptase has a high mutation rate and a high rate of error for the reverse transcription. Thus most initial HIV interactions with host cells do not result in established infection (121).

After the initial entry of HIV into the host cells and establishment of infection, HIV virions are released from infected cells and then they enter the systematic circulation and are carried to the widespread sites within the body. Cells of mononuclear system phagocyte system including those lymph nodes, spleen, liver and bone marrow are generally infected but the HIV. Besides these cells the gut associated lymphoid tissue (GALT) in gastrointestinal mucosa provides a substantial reservoir for HIV. Primary HIV infection is followed by a burst of viremia in which virus is easily detected in PBMCs and plasma. In terms of clinical latency there is detectable virus in the peripheral blood but the viral replication is actively continued in lymphoid tissues (121).

Infection of the central nervous system by HIV requires that HIV-infected peripheral blood mononuclear cells cross the blood brain barrier. Then the infection of macrophages and microglial cells occurs. The immune activation leads to release of neurotoxic factors that further stimulate microglial activation along the neuronal apoptosis (122).

4.3.7 Clinical manifestation of HIV

The WHO and CDC classification system for the clinical staging of HIV infected individuals is made to classify the individuals on the basis of the CD 4 counts and the other different manifestation shown during the progression of the disease. The CDC disease staging system (most recently revised in 1993) assesses the severity of HIV disease by CD4 cell counts and by the presence of specific HIV related conditions. The definition of AIDS includes all HIV infected individuals with CD4 counts of <200 cells/ μL (or CD4 percentage $<14\%$) as well as those with certain HIV related conditions and symptoms. Although the fine points of the classification system rarely are used in the routine clinical management of HIV infected patients, a working knowledge of the staging criteria (in particular, the definition of AIDS) is useful in patient care. In addition, the CDC system is used in clinical and epidemiologic research.

In contrast to the CDC system, the WHO Clinical Staging and Disease Classification System (revised in 2007) can be used readily in resource constrained

settings without access to CD4 cell count measurements or other diagnostic and laboratory testing methods. The WHO system classifies HIV disease on the basis of clinical manifestations that can be recognized and treated by clinicians in diverse settings, including resource constrained settings, and by clinicians with varying levels of HIV expertise and training.

4.3.8 CDC Classification System for HIV Infection

The CDC categorization of HIV/AIDS is based on the lowest documented CD4 cell count and on previously diagnosed HIV related conditions. For example, if a patient had a condition that once met the criteria for category B but now is asymptomatic, the patient would remain in category B. Additionally, categorization is based on specific conditions, as indicated below. Patients in categories A3, B3, and C1, C3 are considered to have AIDS

Table 2: Clinical categorization of CD4 cell counts

CD4 cells counts categories	Clinical categories		
	A: symptomatic, acute HIV or PGL	B: symptomatic condition not A or C	C: AIDS defining conditions
(1) > 500 cells/ μ L	A1	B1	C1
(2) 200-499 cells/ μ L	A2	B1	C2
(3) <200 cells/ μ L	A3	B3	C3

*PGL-Persistent generalized lymphadenopathy

- **Category B Symptomatic Conditions:** Category B symptomatic conditions are defined as symptomatic conditions occurring in an HIV infected adolescent or adult that meet at least one of the following criteria:
 - They are attributed to HIV infection or indicate a defect in cell mediated immunity.
 - They are considered to have a clinical course or management that is complicated by HIV infection. Examples include, but are not limited to, the following:
 - Bacillary angiomatosis
 - Oropharyngeal candidiasis (thrush)
 - Vulvovaginal candidiasis, persistent or resistant

- Pelvic inflammatory disease (PID)
- Cervical dysplasia (moderate or severe)/cervical carcinoma in
- Hairy leukoplakia, oral
- Herpes zoster (shingles), involving two or more episodes or at least one dermatome
- Idiopathic thrombocytopenic purpura
- Constitutional symptoms, such as fever ($>38.5^{\circ}\text{C}$) or diarrhea lasting >1 month Peripheral neuropathy

- Category C AIDS-Indicator Conditions
- Bacterial pneumonia, recurrent (two or more episodes in 12 months)
- Candidiasis of the bronchi, trachea, or lungs
- Candidiasis, oesophageal
- Cervical carcinoma, invasive, confirmed by biopsy
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestinal (>1 month in duration)
- Cytomegalovirus disease (other than liver, spleen, or nodes)
- Encephalopathy, HIV related
- Herpes simplex: chronic ulcers (>1 month in duration), or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis, disseminated or extrapulmonary
- Isosporiasis, chronic intestinal (>1 month in duration)
- Kaposi sarcoma
- Lymphoma, Burkitt, immunoblastic, or primary central nervous system
- *Mycobacterium avium* complex (MAC) or *Mycobacterium kansasii*, disseminated or extrapulmonary
- *Mycobacterium tuberculosis*, pulmonary or extrapulmonary
- *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary
- *Pneumocystis jiroveci* (formerly *carinii*) pneumonia (PCP)
- Progressive multifocal leukoencephalopathy (PML)

- *Salmonella* septicemia, recurrent (nontyphoid)
- Toxoplasmosis of brain
- Wasting syndrome caused by HIV (involuntary weight loss >10% of baseline bodyweight) associated with either chronic diarrhoea (two or more loose stools per day for ≥ 1 month) or chronic weakness and documented fever for ≥ 1 month

4.3.9 WHO Clinical Staging of HIV/AIDS and case definition

The clinical staging and case definition of HIV for resource constrained settings were developed by the WHO in 1990 and revised in 2007. Staging is based on clinical findings that guide the diagnosis, evaluation, and management of HIV/AIDS and it does not require a CD4 cell count. This staging system is used in many countries to determine eligibility for antiretroviral therapy, particularly in settings in which CD4 testing is not available. Clinical stages are categorized as 1 through 4, progressing from primary HIV infection to advanced HIV/AIDS (see Table). These stages are defined by specific clinical conditions or symptoms. For the purpose of the WHO staging system, adolescents and adults are defined as individuals aged ≥ 15 years.

Table 3: Classification of HIV disease

Clinical stage	Clinical symptoms or conditions
Primary HIV Infection	• Asymptomatic • Acute retroviral syndrome
Clinical stage 1	• Asymptomatic • Persistent generalized lymphadenopathy • Moderate unexplained weight loss (<10% of presumed or measured body weight) • Recurrent respiratory infections (sinusitis, tonsillitis, otitis media, and pharyngitis) • Herpes zoster
Clinical Stage 2	• Angular cheilitis • Recurrent oral ulceration • Papular pruritic eruptions

	• Seborrheic dermatitis • Fungal nail infections • Unexplained severe weight loss (>10% of presumed or measured body weight) • Unexplained chronic diarrhea for >1 month • Unexplained persistent fever for >1 month (>37.6°C, intermittent or constant) • Persistent oral candidiasis (thrush) • Oral hairy leukoplakia
Clinical Stage 3	• Pulmonary tuberculosis (current) • Severe presumed bacterial infections (e.g., pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteremia) • Acute necrotizing ulcerative stomatitis, gingivitis, or periodontitis • Unexplained anemia (hemoglobin <8 g/dL) • Neutropenia (neutrophils <500 cells/μL) • Chronic thrombocytopenia (platelets <50,000 cells/μL) • HIV wasting syndrome • <i>Pneumocystis</i> pneumonia • Recurrent severe bacterial pneumonia • Chronic herpes simplex infection (orolabial, genital, or anorectal site for >1 month or visceral herpes at any site) • Esophageal candidiasis (or candidiasis of trachea, bronchi, or lungs) • Extrapulmonary tuberculosis • Kaposi sarcoma • Cytomegalovirus infection (retinitis or infection of other organs)

	• Central nervous system toxoplasmosis HIV encephalopathy • Cryptococcosis, extrapulmonary (including meningitis)
Clinical Stage 4	• Disseminated nontuberculosis mycobacteria infection • Progressive multifocal leukoencephalopathy • Candida of the trachea, bronchi, or lungs • Chronic cryptosporidiosis (with diarrhea) • Chronic isosporiasis • Disseminated mycosis (e.g., histoplasmosis, coccidioidomycosis, penicilliosis) • Recurrent nontyphoidal <i>Salmonella</i> bacteremia • Lymphoma (cerebral or Bcell nonHodgkin) • Invasive cervical carcinoma • Atypical disseminated leishmaniasis • Symptomatic HIV associated nephropathy • Symptomatic HIV associated cardiomyopathy • Reactivation of American trypanosomiasis (meningo encephalitis or myocarditis)

HIV has affected the human host and majority of organs are more infected are liver, kidney, gastrointestinal tract and brain leading to more complex problems. The injury to these organs can lead to various pathological conditions and it helps to infect the whole system of host and leading to AIDS conditions.

4.3.10 Effect of HIV on Liver

Liver disease in human immunodeficiency virus (HIV) infected individuals incorporates a wide range for abnormal liver function test, liver decompensation with, without evidence of cirrhosis, or biopsy, alcoholic to non-alcoholic liver diseases and its more severe forms HIV can infect multiple cells in liver leading to various conditions known as intrahepatic apoptosis, activation and fibrosis. HIV can also alters

the gastrointestinal tract permeability leading to more increased absorption of circulating lipopolysaccharides that have impact on liver.

4.3.10.1 Epidemiology of HIV and Liver disease

Liver disease, including liver-related mortality secondary to chronic liver disease and HCC, is an emerging management problem in HIV-infected individuals in high-income countries, where people are surviving longer on ART. The data shows more number of people with HIV is infected with the liver disease across the globe. The liver disease is major pathology cause due to the ART drugs, co-infection with hepatitis C and E, leading to increase levels of the liver enzymes, apoptosis. Chronic liver disease, as measured by increase/elevated levels of alanine aminotransferase (ALT), has been widely studied in HIV infected individuals, particularly in high income settings. Some studies in Swiss cohort population showed 2365 HIV-infected individuals not co-infected with either HBV or HCV, 385 (16%) had chronically elevated ALT (defined as $> 2 \times$ upper limit of normal) (123). Risk factors associated with increased levels of ALT were high HIV RNA and patients were on ART therapy. These study subjects were having high body mass index (BMI), alcohol abuse and increasing age (123). Various studies in the Chinese study populations have reported to over 3500 cases where hepatic cell carcinoma is major cause of mortality and morbidity with the co-infection of viral hepatitis (124). In French study database the low CD4count was a major risk factor for HCC with ratio, or risk increasing from 2 to 7.6 when comparing patients with a high CD 4 counts (> 500 cells/mL) to those with a low CD4 count (< 50 cells/mL) respectively (125).

4.3.10.2 Pathogenesis of HIV and Liver Disease

HIV affects the liver function by two ways either direct or indirect mechanism. Various studies have reported that HIV infection spread in to a wide range of non-hemopoietic cells in the liver. In addition to this HIV affects the gastrointestinal tract (GI) permeability via depletion of GI tract associated CD4 + T cells. This change can have a deleterious effect on the immune activation and liver disease.

4.3.10.3 Direct effects of HIV on liver

There are numerous studies showing the HIV infection happens in liver. Hepatic cells, Kupffer cells and differentiated tissue macrophages are major cells that are affected by HIV. Some In Vitro studies have reported that HIV infection of Kupffer cells leads to productive infection (126, 127). HIV RNA has been detected in sinusoidal cells and hepatocytes in vivo (128, 129). Out of this the primary sinusoidal cells have been reported to be permissive to HIV infection in vitro (129). A number of studies have proved the hepatocyte cell lines are largely infected by HIV. It is thought that infection of hepatocyte is CD4- dependent as most of hepatic cells and primary hepatocytes do not express CD4 cells (130). HIV infection occurs via receptor mediated endocytosis or with help of alternative co-receptors (131). These hepatocytes then act as a transient reservoir for HIV and promote the CD 4+ T cell infection by cell-cell contact.

HIV can also induce hepatocyte apoptosis in vitro via the gp120 signalling through CXCR4 in the absence of infection. Hepatocyte apoptosis can trigger the pro-fibrotic activity of hepatic stellate cells (HSC) activity and has shown in both HIV-HBV co-infection and HIV-HCV co-infection (132, 133)

4.3.10.4 Indirect effects of HIV on the Liver

HIV infection of the GI tract associated CD4+ T-cells leads to increased permeability to bacterial endotoxins such as Lipopolysaccharide (LPS). Increased systemic levels are shown to contribute to chronic immune activation in HIV -infected patients via the process of activation of monocytes (134). Kupffer cells are main cell type in the liver that's respond to LPS. When stimulated with LPS these cells produce pro-inflammatory cytokines such TNF-a, TGF-B, IL-6, IL-12 and IL-18(135). Elevated LPS levels have shown to contribute to liver disease progression in alcoholic liver disease NAFLAD and NASH (136, 137), also chronic HCV (138) and HIV-HCV co-infection. In HIV, infected individuals, increase in the systemic LPS levels has a potential to contribute to liver disease progression.

4.3.10.5 Clinical presentation of Liver disease in HIV co-infection.

4.3.10.5.1 Norcirrohotic portal hypertension

Norcirrohotic portal hypertension has been described recently in the HIV mono-infected individuals without the other known risk factors for the liver disease.

The individual with HIV infection shows characteristic of decompensated portal hypertension, such as ascites or bleeding esophageal varices without showing the histological symptoms of cirrhosis on liver biopsy. This condition is clinically termed as the “Nor-cirrhotic portal hypertension” (NCPH) (139) or idiopathic portal hypertension or cryptogenic pseudo cirrhosis to differentiate it from other cirrhosis from different etiologies.

4.3.10.5.2 ART and Liver disease

Hepatotoxicity due to ART drugs is more likely due to the NRTIs and NNRTIs and protease inhibitors. The severity of hepatotoxicity differs from sharp increase in the transaminase enzyme levels to hepatic failure and death via various mechanisms. NNRTI such as nevirapine and efavirenz may cause hypersensitivity (140, 141). NRTI such as ddI causes direct mitochondrial toxicity which leads to abnormal liver function. The other mechanism of ART can cause liver related toxicity which leads to direct cell stress and disturbances in lipid/sugar metabolism and steatosis due to use of protease inhibitors. The protease inhibitor such as ritonavir and tipranavir and darunavir have shown to increase the increased in ALT enzyme in liver.

4.3.10.6 Fatty liver disease in HIV

NAFLD or non-alcoholic fatty liver Disease commonly found in high income countries in presence or absence of HIV infection. The ranges of NAFLD range from mild steatosis to NASH which may lead to severe fibrosis and cirrhosis. NAFLD may be associated with other symptoms of metabolic syndrome including mainly obesity, insulin resistance, diabetes mellitus, and dyslipidaemia such as raised triglyceride, LDL. The ART drugs such as stavudine/ddI have affected the insulin resistance and lead to advanced fibrosis.

4.3.10.6.1 Co-infections other than HBV or HCV

The major co-infection with HIV is of Mycobacterium tuberculosis and its treatment is also one of the important causes of liver diseases in low and middle income countries. The bio-burden of tuberculosis is more so the treatment of this AKT or DOTS therapy is used. The AKT drugs are also known to be hepatotoxic and can lead to liver diseases. Concomitant anti-tb therapy was associated with 5 fold

increased risk of abnormal aminotransferase levels in Ugandan individuals who are administered NNRTI based ART.

4.3.10.6.2 Effect of HIV on Kidney

Kidney is major organs in the human body to perform all the vital functions of metabolism and release of toxic waste during the metabolic processes. Kidney being main organs is readily infected by HIV due to epithelial cells both glomerular and epithelial cells. Renal problems related to HIV can be caused directly by the HIV virus when it enters the kidneys and multiplies or by the medicines patients must take to manage HIV. Highly active antiretroviral therapy (HAART) and other HIV treatments have side effects that can sometimes be toxic to the kidneys, including:

- lactic acidosis — a build-up of lactic acid in the body
- crystal-induced obstruction — a build-up of crystals in the kidneys
- interstitial nephritis — a disorder in which tissues surrounding the kidneys become inflamed
- electrolyte abnormalities — abnormalities in the body's levels of sodium, potassium or calcium

HIV-associated renal diseases, like most chronic kidney diseases, are caused by a combination of genetic and environmental factors. The two most common HIV-associated renal conditions generally found are a collapsing glomerulopathy known as HIV-associated nephropathy (HIVAN) and a collection of immunoglobulin-related glomerulonephritides collectively known as HIV immune-complex kidney disease (HIVICK). HIVAN is the most common renal complication among patients with HIV-1 and is predominantly seen in patients of African descent (142, 143).

Kidney disease is a frequent complication of HIV infection, occurring in 3.5 – 48.5% (144), and occurs as a complication of HIV infection, other co-morbid disease, and infections and as a consequence of therapy of HIV infection and its complications, although various reports have shown the use of ART therapy have some deleterious effects on kidney function and subsequently pathologies associated with HIV, Hepatitis C.

4.3.10.6.3 Epidemiology of HIV and Kidney disease

The HIV associated Nephropathy (HIVAN) occurs mainly in the Africa and American study populations. Also some studies have shown the African -American descendants and the Haitian people have advanced glomerularitis.

HIVAN is observed majority in African Americans and is the third leading cause of ESRD among blacks aged 20-64 years. Most patients with HIVAN are young black men, and around 50% of patients with HIVAN are intravenous drug abusers. Overall, HIVAN is observed more often in men than in women, with a male-to-female ratio of 10:1. pathogenesis of HIV and Kidney disease.

Despite the success of the combination of new anti-retroviral drugs in improving the mortality rate, the persons living HIV have a greater risk death and acute renal injury (ARI) and acute chronic kidney disease (CKD) remains a cause of mortality in these patients. CKD is mediated by number of factors such as host-virus interaction, environmental factors and genetic predispositions.

Numerous studies have established that HIV infect glomerular epithelial cells and tubular epithelial cells simultaneously in vitro and in vivo suggesting that renal expression of HIV genes has a central role in promoting HIVAN pathogenesis (145). The importance of the HIV infection of kidney goes beyond in role in causing the kidney disease since it has effort to cure the patients from HIV infection. The studies have shown as the kidney may act as the compartment for the virus to harbour its quasispecies which are different from the virus that are commonly found in leukocytes(146). As the renal epithelial cells do not contain the CD4 + T -cells which express the HIV receptors are co-receptors, there is no exact mechanism known how the HIV virus enters renal epithelium cells. One of study done by Ray et.al (147)showed that incubation of HIV -infected macrophages with the tubular epithelial cells resulted in CD4-independent infection of tubular cells leading to infection of tubular cells can occur even when cells were separated by using specialized trans well filter suggesting that cell-cell fusion is not required for the infection.

One of study by Hubner et.al (148) showed that HIV infection of lymphocytes occurs more efficiently when the virus is delivered directly from the infected lymphocytes to uninfected lymphocytes rather than by the cell free virus. More studies by the same group demonstrated that the transfer of viral RNA and subsequent expression of HIV genes were increase in tubular epithelial cells exposed to infected lymphocytes (147). The transfer of viral material from T lymphocytes to tubular

epithelial cells required stable cell-cell contact and occurs independently of CD 4 expression (149) therefore it is likely that the renal tubular epithelial cell HIV infection is initiated via transfer from leukocytes.

4.3.10.6.4 Pathogenesis of HIV and kidney disease

Some studies on rats and mice that express an HIV transgene lacking the gag and pol genes under the control of endogenous viral LTR promoter develops the kidney disease which then phenocopies the clinical and histopathological findings of HIVAN (145, 150).

Various proteins of HIV such as Tat, Vpr, Nef are non-structural proteins that have important role in the HIV life cycle. Tat protein increase efficiency of HIV transcription and can induce expression of many cellular genes including the proinflammatory cytokines (151).

- Vpr promotes the nuclear import of the viral reintegration complex which is key step in integration of HIV into the host genome and can arrest the cells in G2/M phase leading to the promotion of viral replication.
- Nef protein has different effects in the circa life cycle and also on host cells which in turn activates the T cells helping in the viral transcription; this reduces the cell surface CD4 expression and activation of cell signalling pathways.

4.3.10.6.5 Role of inflammation in promoting HIVAN

Several studies have reported a role for inflammation in the pathogenesis of HIV induced kidney injury. When tubular epithelial cells are infected by HIV-1 in vitro, the most prominent transcriptional response is that there is a upregulation of nuclear factor kappa B. NF- κ B is found to be activated in podocytes in HIVAN models such as transgenic mice (152)

4.3.10.6.6 Direct effects of HIV on Kidney

HIV associated immune complex kidney disease (HIVICK) is a term that is used to describe a group of immune complex related kidney diseases occurring in hit HIV positive patients. HIVICK includes post-infection glomerulonephritis, IgA, nephropathy, membranous nephropathy, membrane proliferative glomerulonephritis another histological pattern of kidney disease (153, 154). The studies have found that

not always the HIV infection can cause the glomerular lesion in non-infective HIV patients. This may be due to the infection of hepatitis B and C. one of study showed that the four HIV positive patients showing the proliferative glomerulonephritis were found with the circulating IgA or IgG-HIV immune complexes suggesting that anti HIV antibodies may form immune complexes that promotes glomerulonephritis in some patients (155).

4.3.10.6.7 HIV-associated thrombotic microangiopathy

Thrombotic microangiopathy has been recognized as a complication of HIV infection since the epidemic of HIV/AIDS epidemic. HIV-TMA occurs late in HIV infection but due to the ART therapy the incidence has been decreased (156). Although the pathogenesis mechanism is not known, there is likely that TMA is due to endothelial injury caused by viral proteins and many other factors including the proinflammatory molecules, circulating phospholipids antibodies etc. there are no reports for HIV-TMA for clinical trials. And it is more likely to occur in patients with advance HIV disease and active viral replication; these patients should be treated with ART.

4.3.10.7 Antiretroviral nephrotoxicity

Persons living with HIV are exposed to wide variety of nephrotoxic agents including the anti -microbial medicines. Also the antiretroviral therapy is a one of major cause of nephrotoxicity in the HIV patients.

4.3.10.7.1 Reverse transcriptase inhibitor

The NRTI and NNRTI are two main categories of these groups. NRTI don't have direct nephrotoxic effect but they have been reported to be capable causing the systemic lactic acidosis when subject to treatment of stavudine and didanosine (157). These medicines can cause type B lactic acidosis by inhibiting the function of DNA polymerase- γ , which often leads to the reduced cellular mitochondrial DNA content, reduced expression of proteins which are necessary for oxidative phosphorylation and thus it increases the oxidative stress due to mitochondrial injury (158, 159)

The NNRTI such as tenofovir is approved for use against HIV, which has a similar mechanism of action against HIV as NRTI medication as except is does not need to be phosphorylated in order to become active against the reverse transcriptase.

The other drugs in NNRTI such adefovir and cidofovir are also used against hepatitis b and cytomegalovirus. The adefovir is not been as anti HIV now since it has proven to be nephrotoxic when used at high dose. Tenofovir, adefovir and cidofovir are all capable of inducing renal tubular injury.

4.3.10.7.2 HIV protease Inhibitors

HIV protease inhibitors are functioned to inhibit the ability of the viral protease to process Gag structural polyproteins, thereby inhibiting viral maturation rendering non-infectious virions. As these medications are largely excreted via the hepatic metabolism mode, dosage for these drugs is not necessary with the glomerular filtration rate. Also it is seen that the most of the protease inhibitors are not soluble in aqueous solutions leading to promote crystal Luria or nephrolithiasis when found to be concentrated in urine. The drug named indinavir has been reported to be associated with several acute or chronic renal syndromes including nephrolithiasis.(160)

Due to the toxicity indinavir is not widely used and now atazanavir is prescribed. This drug has more or less effect on the kidney function and also somewhat reduced the CKD in some patients. The drugs can affect the oxidative stress mechanism and leads to the generation of free radicals.

4.3.10.8 Effect of HIV on nervous system

HIV virus has effect on the central nervous system subsequently showing the HIV associated neurological disorders (HAND), HIV-associated dementia as other pathogenesis of brain and nervous system (161). HIV dementia refers to a series of conditions such as mental slowness, forgetfulness, poor concentration and changes in behaviour, discombobulation, speech problem and decrease in spontaneity. Neurologic disease is the first manifestation of symptomatic HIV infection in roughly 10 % of persons, while about 60% of patients with advanced HIV disease will have clinically evident neurologic dysfunction during the course of their illness. The incidence of subclinical neurologic disease is even higher: autopsy studies of patients with advanced HIV disease have demonstrated pathologic abnormalities of the nervous system in 75-90% of cases.

4.3.10.8.1 Epidemiology of HIV on central nervous system

In developing countries, opportunistic infections of the central nervous system (CNS) account for most of the reported neurologic morbidity and mortality in AIDS. Cryptococcal meningitis, fulminant bacterial meningitis, neurotuberculosis, toxoplasmosis, and neurosyphilis are common among HIV infected individuals in Asia and Africa. The epidemiology of HIV-associated neurological disease in the industrialized world has changed significantly in the era of HAART. These changes have occurred concurrently with changes in the treatment patterns for HIV infection over the past 10 years.

4.3.10.8.2 Pathogenesis of HIV on CNS

HIV crosses the blood brain barrier and enters the nervous system early, probably concomitant with initial systemic infection (162). The virus has been cultured from different sources of the body such as brain tissues, nerve, and cerebrospinal fluid (CSF) from persons at different stages of HIV disease, including those without neurologic signs or symptoms (163, 164). Positive HIV-1 cultures obtained in the CSF does not predict the presence or absence of the neurologic signs or symptoms; but later on it can predict if there is any infection associated with HIV. The development of neuroAIDS (condition due neurologic manifestations of AIDS) depends on various number of factors, such as antiretroviral treatment history, degree of immunosuppression, and the molecular biology of the viral strain, particularly its neurovirulence (165). Host factors, such genetic makeup, certainly play a role in selective vulnerability to neuroAIDS, but are not yet well characterized. Despite its potential to cause disease at all levels of the neuraxis, HIV-1 does not directly infect central or peripheral neurons, astrocytes, or cells. Latent or low-level of HIV infection in the CNS is maintained by virus infected cells of the monocyte/macrophage lineage. "Indirect effects" of macrophage activation such as dysregulation of cytokines and chemokines, free radical (oxidative stress) injury, and secretion of soluble factors that are effectively neurotoxic have been implicated as effectors of nervous system injury in HIV.

4.3.10.8.3 Pathogenesis of AIDS dementia complex

The role of HIV-1 in the proliferation of AIDS associated dementia complex is bit controversial. The viral strains have been able to replicate inside the brain

macrophages it may play a role in pathogenesis of brain injury. Some studies have reported that the increased HIV-1 proliferation in brain have resulted in the development of ADC complex formation. Some investigators proposed that a macrophage associated cascade of events which can lead to brain dysfunction and clinical dementia, even there is absence of the viral load in brain. Activated macrophages, whether infected with HIV or not, are capable of secreting potent neurotoxins which mainly are inducing proinflammatory cytokines, and thus generating oxygen free radicals that can would eventually damage cells and lead to neuronal dysfunction or death (166, 167) A particular subtype of monocyte/macrophages derived from the peripheral blood were found to be importantly increased among patients who are infected with AIDS dementia compared with both HIV-1 infected and uninfected controls. Soluble factors from these macrophages were found to be highly neurotoxic, as they killed human brain cells in culture.

4.3.10.8.4 Direct effects on HIV on central nervous system

Viral and, rarely, fungal and parasitic opportunistic infections can affect the spinal cord. Systemic lymphoma can penetrate nerve roots and meninges, sporadically causing a mass lesion within the spinal cord. In addition this, the HIV itself is associated with a spastic paraparesis a condition which is similar to that seen with vitamin B12 deficiency. In this condition the Peripheral nerve injury is very common, a painful distal neuropathy is observed at a very late stage in HIV infection. A study showed that about 35% of hospitalized patients with advanced HIV disease have shown to have peripheral neuropathy (168). Among 272 HI infected outpatient subjects studied over time, showed that 55% had signs, or both signs and symptoms, of distal neuropathy. Although there are typical myalgias or muscle pains are a frequent complaint. Both inflammatory myopathies and a toxic myopathy secondary to zidovudine (AZT) have been observed. More recently, a syndrome of acute neuromuscular weakness, which is often associated with lactic acidosis, has been described in association with several nucleoside analogue reverse transcriptase inhibitors(NRTIs) such as zidovudine (AZT), stavudine (d4T), didanosine (ddI), and lamivudine (3TC), either alone or administered in combination(169). Although the pathophysiology of this potentially fatal syndrome is not yet fully understood, the presence of lactic acidosis may suggests that there is an acute mitochondrial toxicity,

or "mitochondropathy," possibly caused by the inhibition of mitochondrial DNA synthesis by nucleoside analogues. Among the HIV patients with neurological disease have shown some management and trivial symptoms. More efficiently the management of neurological symptoms is based on management of pain and as symptoms.

4.3.10.8.5 Effect of Antiretroviral therapy on Brain

The recent studies have showed ART may also have neurotoxic effect that adversely affects the cognition. NRTIs were demonstrated to induce toxicity in peripheral tissues through mitochondrial dysfunction and PIs were shown to damage proteasome function. (170) MRI studies on some patients showed that subject on didanosine and stavudine regime exhibits decreased N-acetylaspartate (NAA) concentration in frontal white matter and the longer treatment correlated with lower NAA levels (171).

4.3.11 Oxidative stress in HIV

Oxidative stress is defined as an imbalance between the antioxidant and pro-oxidant system with shift towards the pro-oxidant system. The oxidative stress is also defined as the modification and accumulation of biological molecules which are altered by different kinds of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both these ROS and RNS affect gene transcription and cell growth/cell proliferation

ROS and RNS are highly reactive toxic oxygen or nitrogen moieties, such as hydroxyl radical, peroxy radical, superoxide anion, hydrogen peroxide, nitric oxide (NO) and peroxynitrite. The half-life of ROS species varies from nanoseconds from the hydroxyl radicals to second for NO and peroxy radicals. Because of the differences in half lives, the reactivity of ROS differs from aqueous environment in which they were formed to reacting deep with the biological membranes.

In biological systems, the cellular membrane constitutes in a main target of the ROS and RNS. In addition to the cellular membrane the other intracellular membrane are important target of the oxidative stress such as mitochondrial, nuclear and endoplasmic reticulum membranes that can suffer the elusive actions of ROS and RNS by changing their form and functions.

The Pro-oxidant and anti-oxidant are the two major species generated by the ROS and RNS. The pro-oxidants are chemicals that induce the oxidative stress, either by generating the reactive oxygen species or inhibiting the antioxidant systems.

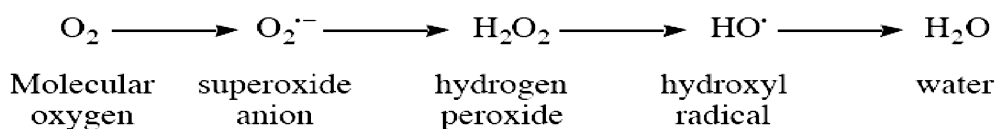
Free radicals, ROS and RNS are derived either from normal essential metabolic processes in the human body i.e. endogenous systems or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals. Reactive oxygen species and reactive nitrogen species e.g. nitric oxide, NO• are well recognized for playing a dual role as both deleterious and beneficial species (172). A harmony between free radicals and cancer prevention agents is fundamental for appropriate physiological capacity (173). The generation of free radicals in cells is closely linked with the participation of redox-active metals, which in itself is largely linked to an iron in part to copper redox couple and is maintained within strict physiological limits.

A free radical can be characterized as any sub-atomic species equipped for autonomous presence that contains an unpaired electron/s in a nuclear orbital. This unpaired electron/s as a rule gives an impressive level of reactivity to the free radical. The nearness of an unpaired electron brings about certain basic properties that are shared by generally radicals. Numerous radicals are insecure and exceptionally responsive. They can either give an electron to or acknowledge an electron from different atoms, subsequently carrying on as oxidants or reductants. Oxygen is a generally inert intensify that can be metabolized in vivo to shape very receptive oxidants known as oxygen free radicals. The most imperative oxygen-containing free radicals in numerous malady states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical (174, 175).

4.3.11.1 Formation of ROS by Electron Transfer Reactions

The following scheme shows how the sequential univalent reduction of oxygen to water with formation of different intermediates: superoxide anion radical, hydrogen peroxide and hydroxyl radical:

Figure 4: Reduction of oxygen to water (176)



Superoxide anion, arising either through metabolic processes or following oxygen “activation” by any physical process, is considered the “primary” ROS, and can further interact with other molecules to generate “secondary” ROS, either directly or prevalently through enzyme- or metal-catalyzed processes

4.3.11.2 Superoxide anion radical

The production of superoxide occurs mostly within the mitochondria. The mitochondrial electron transport chain is the main source of ATP generation in the mammalian cell and it is vital process. Superoxide is produced from both Complexes I and III of the electron transport chain, and once in its anionic form it is too strongly charged to readily cross the inner mitochondrial membrane. As of late, it has been shown that Complex I-subordinate superoxide is solely discharged into the lattice and that no recognizable levels escape from in place mitochondria. The generation of superoxide within mitochondria is approximately 2–3 nmole/min/mg of protein.

The addition of one electron to molecular oxygen results in the formation of $\text{O}_2^{\cdot-}$. Superoxide anion radical is not only very reactive species but also chemical reactivity will depend on its site of generation in the cell. This increases the possibility of formation of proton to a stronger oxidant i.e. perhydroxyl radical and subsequently lead to the collision with suitable substrates. Two reactions are important in a cellular setting, which change the chemical reactivity of superoxide anion.

1. Reactivity of superoxide anion with itself: Superoxide anion is short lived and tends to react with itself, a process known as dis-mutation or disproportionation and which yields molecular oxygen and H_2O_2 [10].
2. Protonation of superoxide anion: This process may take place in the vicinity of membranes with an increased proton gradient. The protonation of superoxide anion yields perhydroxyl radical which is an extremely reactive species [10].

4.3.11.3 Hydrogen peroxide

Peroxisomes are known to produce H_2O_2 , under physiologic conditions. Peroxisomes are major sites of oxygen consumption in the cell and participate in several metabolic functions that use oxygen. Oxygen consumption in the peroxisome leads to H_2O_2 production, which is then used to oxidize a variety of molecules (177).

H_2O_2 can be formed upon two-electron reduction of molecular oxygen or one-electron reduction of superoxide anion. H_2O_2 is not a free radical, but it may be considered as an oxidant. H_2O_2 is little reactive (178).

Its reactivity in biological systems depends on two properties:

- First, it can diffuse long distances crossing membranes.
- Second, it reacts with transition metals by a homolytic cleavage yielding the highly reactive hydroxyl radical

4.3.11.4 Hydroxyl radical

Hydroxyl radical is the most reactive oxygen species originating from a reaction between superoxide anion radical and hydrogen peroxide H_2O_2 making it a very dangerous radical with a very short *in vivo* half-life of approximately 10^{-9} sec.

Chemically, one-electron reduction of hydrogen peroxide yields hydroxyl radical and water. $\text{HO}^\cdot$ is the most powerful oxidant and unlike superoxide anion and H_2O_2 , it indiscriminately reacts with almost all biological compounds. The extremely reactive nature of $\text{HO}^\cdot$ suggests that it will only mediate direct effects close to its site of generation i.e. it cannot diffuse long distances; once generated $\text{HO}^\cdot$ will not diffuse large distances within the cell, but it will damage the first molecule it collides with (179). When peroxisomes are damaged and H_2O_2 their consuming enzymes down regulated, H_2O_2 releases into the cytosol which is significantly contributing to oxidative stress (180).

4.4 Tuberculosis

4.4.1 Tubercle bacillus

Mycobacteria are known to include a large group of acid-fast, alcohol-fast, aerobic or micro aerobic, non-spore forming, non-motile bacilli (181). Understanding of the pathogenesis of tuberculosis began with the work of Theophile Laennec at the beginning of the 19th century. This work further advanced by the demonstration of the transmissibility of *Mycobacterium tuberculosis* infection by Jean Antoine Villemin in 1865 and the identification of the tubercle bacillus as the etiologic agent by Robert Koch in 1882. Clemens von Pirquet developed tuberculin skin test in 1907 and 3 years later used it to demonstrate latent tuberculosis infection in asymptomatic children. In the late 19th and 20th centuries sanatoria developed for the treatment of patients with tuberculosis. The modern era of tuberculosis treatment and control was heralded by the discovery of streptomycin in 1944 and isoniazid in 1952. Systematic classification of tuberculosis is mentioned below (182).

Classification of the organism

Classification of *Mycobacterium tuberculosis*

Domain : Bacteria

Phylum : Actinobacteria

Class: Actinobacteria

Order: Actinomycetales

Family: Mycobacteriaceae

Genus: Mycobacterium

Species: *M.tuberculosis*

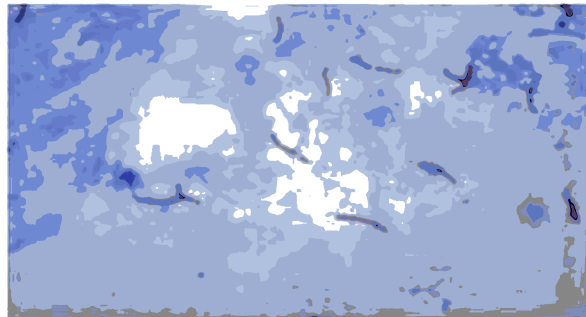
4.4.2 Global Epidemiology of Tuberculosis

In 2015, there were an estimated 10.4 million new (incident) TB cases worldwide, of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children. People living with HIV accounted for 1.2 million (11%) of all new TB cases (183).

Of the many different mycobacterium, only *M. tuberculosis*, *M. bovis* and *M. africanum* are recognized as tubercle bacilli, these all being suspect of a single species. *M. tuberculosis* is an obligate parasite that is infectious to human, other primate and other mammals. It produces progressive tuberculosis disease in guinea pigs (184) .

Tubercle bacilli are difficult to stain but once stained they strongly retain the dye, which is not removed by acid-alcohol solution. This acid and alcohol fastness can be demonstrated by the ZN (Ziehl-Neelsen) staining procedure, of which there are various modifications. Tubercle bacilli can also be stained by fluorescent dyes such as auramine and rhodamine, and acid fast staining procedures have been developed with these fluorescent dyes in which the bacilli fluoresce strongly (185).

Figure 5: Microscopic view of Tubercle bacilli (186)



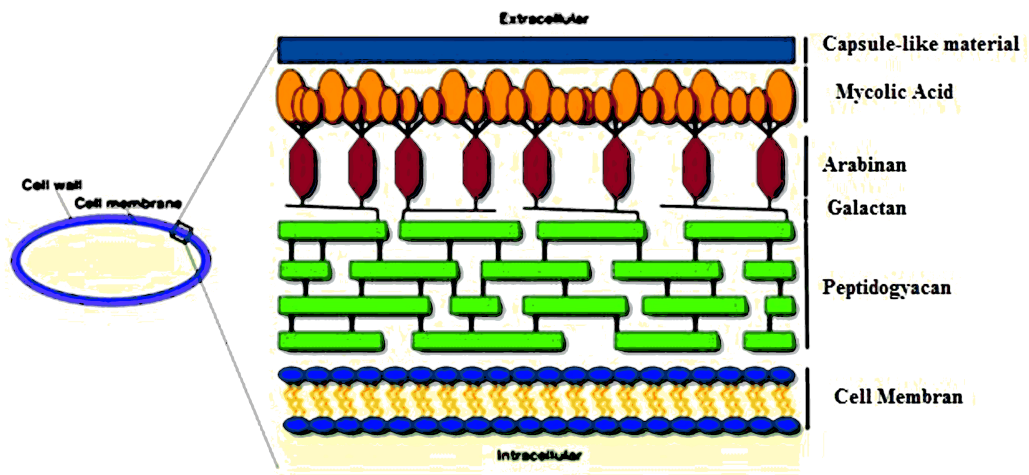
Source: https://cs.wikipedia.org/wiki/Ziehl-Neelsenovo_barven%C3%AD

In stained smears of pathological material *M. tuberculosis* is slightly bent rod, 2-4 μm long and 0.2-0.5 μm wide, which may be evenly stained or beaded and granular (Fig 5). In liquid media, the bacteria tend to be parallel and form long threads or cords.

Culture-tubercle bacilli grow slowly, taking 2-6 weeks to form colonies on Lowenstein-Jensen (LJ) medium and oleic acid-albumin agar media. Growth is optimal at temperature of 33-39 C, pH 6.5-6.8 in an atmosphere of 5-10% CO_2 . An identification test includes production of niacin, of which *M. tuberculosis* produces the most. A supportive test for *M tuberculosis* includes strongly positive nitrate reduction and loss of catalase activity at 68°C.

4.4.3 *M. tuberculosis* - Cell Structure

Figure 6: Cell structure of *M. tuberculosis* (184)



Mycobacterium tuberculosis has a tough cell wall that limits the penetrability of substances from outside to inside the organism. The cell wall is divided into 2 layers, the outer layer surrounding plasma membrane and inner layer. Outer layer comprises of both lipids and proteins, the lipid polysaccharide complexes includes lipomannan, lipoarabinomannan (LAM), phthiocerol-containing lipids such as phthiocerol dimycocerosate, dimycolyl trehalose (cord factor), phosphatidylinositol mannosides and sulfolipids. The LAMs are capped at the terminal β -Ara residue with mannose residues and are referred to as Man LAMs. These outer proteins and lipids interact with the immune system and are soluble components of the cell wall (187, 188).

The inner layer is comprised of peptidoglycan (PG), arabinogalactan (AG), and mycolic acids (MA) which are covalently linked together to form a complex, the MA-AG-PG complex that extends outwards from the plasma membrane. This is the core of the cell wall and is insoluble in nature (189).

The most common primary route of entry for Mtb is via the respirator route. The bacteria could spread from its prime initial location in lungs to other parts of body like lymph nodes.

Tuberculosis is spread from person to person through aerosols. Activities like coughing, sneezing, speaking, singing, spitting by an active TB infected patient releases infectious aerosol droplets containing the etiological agent *Mycobacterium tuberculosis*. A single sneeze could release upto 40,000 infectious droplets. Inhaling fewer than 10 bacteria has the ability to transmit the disease (190) People in close

contact with the active TB infected patient have 22% chances of acquiring the disease and are at high risk.

Mycobacterium tuberculosis is a facultative intracellular organism which is ingested by phagocytes and is resistant to intracellular killing. Human TB strain is responsible for the vast majority of cases. The bovine strain affects mainly cattle and other animals. Regarding virulence, the Indian tubercle bacillus is said to be less virulent than European bacillus. In recent years, a number of “atypical” mycobacteria have been isolated from human. These have been classified into four groups

1. Photochromogens
2. Scotochromogens
3. Non-Photochromogens
4. Rapid growers

All these are mainly saprophytic. Diseases attributed to them have resembled pulmonary tuberculosis and chronic cervical lymphadenitis. Non-specific infections have been reported to be widely prevalent in the southern part of India

4.4.3.1 Source of Infection (191)

Humans and cattle's are two sources of infection. For Man, most common source of infection is the human case whose sputum contains tubercle bacilli and who has not taken treatment or not been treated completely. Such sources can discharge the bacilli in their sputum for years.

The tubercle bacilli in a human case are usually a mixed group containing strains with rapid and slow growth rate. The more rapidly a bacillary strain multiplies the more susceptible it is to the bactericidal action of chemotherapeutics drugs. The slow multiplier are the sources of persisters or dormant bacilli; they can remain alive for years without causing harm to the host, but when conditions are favourable, they may start multiplying again and cause active disease. That is, they are the seeds of a future relapse.

The bovine source of infection is usually infected milk. Because of the practice of boiling milk before consumption, chances of bovine tuberculosis infection is less problematic in India

4.4.3.2 Route of Transmission

Tuberculosis is spread from person to person through aerosols. Activities like coughing, sneezing, speaking, singing, spitting by an active TB infected patient releases infectious aerosol droplets (0.5-5.0 μm in diameter) containing the etiological agent *Mycobacterium tuberculosis* (192). A single sneeze could release upto 40,000 infectious droplets. The frequency and vigour of cough and the ventilation of the environment effect on the transmission of infection. Patients with extra-pulmonary tuberculosis have less chance for transmission of infection. Inhaling fewer than 10 bacteria has the ability to transmit the disease (193). People in close contact with the active TB infected patient have 22% chances of acquiring the disease and are at high risk (194)

The most common primary route of entry for tubercle bacilli is via the respirator route. The bacteria could spread from its prime initial location in lungs to other parts of body like lymph nodes (195).

The likelihood of transmission of bacterium depends on 4 factors (195) :

1. Number of organisms expelled by the TB infected person.
2. The bacterial load i.e. concentration of organisms present in the air.
3. Exposure time of the contaminated air by the healthy person.
4. Immune condition of the exposed individual.

4.4.3.3 Host Factors

4.4.3.3.1 Age

Tuberculosis affects all the ages. Developing countries show a sharp rise in infection rates from infancy to adolescence. In India, from an average of 1% in the “under 5 age group”, the infection index climbs to about 30% at the age of 15 years in the developing countries; the disease is now more common in the elderly.

4.4.3.3.2 Gender

TB is more prevalent in males than in females.

4.4.3.3.3 Nutrition

Malnutrition is widely believed to predispose to tuberculosis, but the available evidences are indirect. Pioneering studies in India at the tuberculosis chemotherapy

center, Chennai showed that diet had no discernible influence on the recovery of the patients in the context of potent chemotherapeutics drugs (196).

4.4.3.3.4 Immunity

Human has no inherited immunity against tuberculosis. It is acquired as a result of natural infection of BCG vaccination. Past infection with atypical mycobacteria is also credited with certain amount of naturally acquired immunity. It is now known that both delayed hypersensitivity and acquired resistance to tuberculosis are cell mediated responses. In most cases, the cellular immunity proves adequate to limit further multiplication and spread of bacilli.

4.4.3.3.5 Social Factors

Tuberculosis is a social disease with medical aspect. It is described as a indicator of social welfare. The social factors include many non-medical factors such as deprived quality of life, poor housing and overcrowding, Population outburst, under nourishment, lack of education, large families, early marriage etc. These factors are interrelated and contribute to the incidence and spread of tuberculosis. In fact, tuberculosis began to drop in the western world before the advent of chemotherapeutics drugs. This has been attributed to improvements in the quality of life.

4.4.3.4 Tuberculosis - Classical symptoms

Tuberculosis at any site may first produce a systemic effect which is non-organ specific symptoms. Generally 35-80% of patients present with fever, night sweats along with loss of weight, loss of appetite and malaise (197). 10% of the patients suffering with localized tuberculosis show hematological symptoms including increase in the polymorphonuclear leucocyte count and anaemia(198).

After the disease progresses into an active tuberculosis infection, cough is the most common symptom shown by the patient. Initially the cough is limited, but as the disease progresses, tubercles grow, causes necrosis and inflammation in the tissue and sputum formation it develops into chronic cough. Blood could also be coughed along with sputum. Haemoptysis is one of the presenting symptoms, but it may reflect certain underlying conditions like rupture of dilated vessel in cavity and tubercle rupture. Inflammation of lung parenchyma cells of the pleural surface may cause

pleural pain and chest pain in the patients. Crackles may be heard in the area affected and may cause bronchial breathing (199). Lung lesions can be seen in chest X-rays.

4.4.3.5 Pathology of Tuberculosis

As described above the route of transmission of the bacterium is via inhalation via respiratory droplets and are deposited in the distal alveoli, *M. tuberculosis* is presumed to first encounter and be ingested by alveolar macrophages. The majority of TB cases are attributed to *M. tuberculosis* or the closely related organism *Mycobacterium africanum*; a minority of cases are due to zoonotic members of the *M. tuberculosis* complex such as *Mycobacterium bovis* or *caprae* (200). As far as studies done with the MTB is has no known environmental reservoir (201) humans are only best known reservoirs. Thus, making the MTB as both pathogen and a symbiont which has a possible understanding of the host-parasite interactions.

4.4.3.6 Host -Pathogen interactions

Due to the new cutting edge techniques in the field of genomics have proven substantial genetic variability among the isolates of the *Mycobacterium* around the world. The genetic variability has shown either the accumulated drift is associated with the human migration have led to the variable pathogenicity of different lineages. It has been postulated that the hyper virulent strains exist, as based on the previous epidemiological studies. The study of such hyper virulent strains would uncover lineage -specific virulent factors (201) could ultimately use to prioritize patient care and infection control decisions. Although the several attributes of *Mtb* including the increased transmissibility in humans, drug resistance and mortality in an experimental model have been linked with specific strains (202). Furthermore, the interactions between the host and the pathogen are very complex. Thus, studying *M. tuberculosis* virulence factors in the absence of host factors of vulnerability can obscure synergistic interactions. For example, a specific host-pathogen interaction might explain why strains of East-Asian origin are highly communicable and pathogenic in Asian population (203) but they populations have a normal clinical and epidemiological presentation when imported into Canada or Switzerland (204, 205). Conversely, strains that are otherwise average are associated with outbreaks given the appropriate social and epidemiological settings (206).

4.4.3.7 Virulence factors associated with Tuberculosis

The risk of progression from latent TB infection to active TB disease is different magnitude of higher than the risk of developing disease from the live vaccine strain, *M. bovis* Bacillus Calmette-Guerin (BCG). There is genomic difference between the *M. tuberculosis* and the BCG which can be used to search basis for attenuated virulence. The genomic comparison s showed revealed some difference such of region of difference 1(RD 1) (207, 208), this helped a lot to understand why the BCG vaccine is given to millions of new-born infants each year with a low risk of progression to disease.

Region of difference or RD 1 contains genes that encode a bacterial system which is known as the ESX-1 secretion system (209). Once the bacteria have been adopted in a phagosome by the host macrophages the ESX-1 secretion system facilitates the delivery of certain bacterial products into the macrophage cytoplasm. The absence of RD 1 in the BCG strains has enabled the development of immunological assays to keep it different from host immune response to the MTB infection from the responses caused by the BCG vaccine (BCG osis).The majority of non -tuberculous mycobacteria lack the RD 1 so this makes them to identify the infection caused by Mtb from the infection caused by environmental bacteria such as *Mycobacterium avium* (210).

The ESX-1 plays major role in the pathogenesis of active TB disease but it has proved that the ESX-1 antigens are conserved in a few non-tuberculous mycobacteria (211) such as *Mycobacterium kansasii* and *Mycobacterium marinum* has provoked a reassessment of the importance of ESX-1 in MTB virulence (212). This has shown the ESX-1 is necessary but not always responsible for the virulence caused by MTB . A better understanding of what's set MTB apart from other mycobacteria might provide different insight into the pathogenic mechanism of active TB disease and the finding new targets for new diagnostics and improvement of vaccines.

4.4.3.8 Latent TB infection

The exposure to *M. tuberculosis* leads to two broad out i) elimination of the pathogen and ii) persistence of the pathogen. In the first cade the pathogen is eliminated either because of innate immune response such tuberculin skin tests (TSTs) or interferon γ (INF- γ) release assays (IGRA) which might be showing negative results and second reason might be due adaptive immune response which

shows results with TST and IGRA either positive or negative depending on whether the memory T cell response have been reduced. This is case the patients will not get the benefit of the LTBI therapy regardless of how the pathogen is eliminated from the host body. The studies done previously have recognized that even among the person coming with contact with Tb infected persons the half of exposed individuals have a negative TST results. There must be a genetic predisposition to remaining persistently TST negative despite there is an ample exposure of the MTB and it also provides one potential explanation why some people are naturally resistant to TB (213).

However, if the MTB infection is not quickly eliminated and the pathogen cans still persist in quiescent or latent stage the individual will develop positive for TST and IGRA results. Some individuals would be benefitted from the LBTI therapy. But unfortunately, a positive TST or IGRA results does not imply LTBI, although the infection have might been completely eliminated there is still a chance of TST or IGRA showing positive results due to the memory T cell responses (214) this finding explains hoe the TSTs and IGRA are not good markers to detect the MTB infection as they have a low predictive values (215, 216).

4.4.3.9 Immunology of Tuberculosis

Understanding the mycobacterium tuberculosis infection was limited when the infection came to humans but the studies done with laboratory animals such as mice, rabbits, guinea pigs and non-human primates has greatly helped to understand the importance of early events happening during the primary infection (217). The route of transmission for the MTB is via respiratory tract following inhalation; MTB is translocated to the lower respiratory tract where it comes across alveolar macrophages. These macrophages are the dominant cell that that MTB infects these cells adopts the bacteria by the process of receptor mediated phagocytosis, with numerous other receptors contributing to this process. The phagocytosis process has been extensively studied without knowing about the microenvironment present in the alveolus which has proteins namely surfactants that are present in abundant quantity in the fluid that lines the epithelium. These surfactants have an important role in the initial host-pathogen interactions (218).

Once the MTB adopts into the respiratory system and the alveolar cavity; it actively blocks the phagosome fusion with lysosome ensuring its own survival(219). Then through activity of the ESX-1 secretion system MTB disrupts the phagosomal

membrane causing the release of the bacterial products including the mycobacterial DNA into the macrophage cytosol. Leading to finding of a few bacteria in the cytosol in the subsequent days of infection. The one of possibility in the activation of the cytosolic surveillance pathway results in the induction of type I IFN response which promotes the growth of intracellular bacterial pathogens (220, 221). Besides various experimental studies have shown that the type of cell death whether apoptotic or necrotic is experienced by the infected macrophages is crucial for the both innate and adaptive immune responses (222, 223).

After infecting the alveolar macrophages in the airways, MTB gains access to the lung interstitium, where the process of infection evolves. However, it is still not known how the MTB accesses the lung parenchyma is not known. There might be two possible mechanism; one involving the MTB directly infecting the epithelial cells and second would be migration of MTB-infected macrophages across the epithelium. This might be the route for the MTB to infect the parenchyma and lead to the recruitment of increasing number of cells to the site of infection and generating a multicellular host response called a granuloma.

As the primary infection is established the MTB is not transported by either infected dendritic cells or inflammatory monocytes to the lymph nodes for the T cell priming (224, 225). MTB had been shown to actively delay the initial T cell priming as well as T cell trafficking into lung (224, 226). HIV infection substantially reduces the number of CD4 + T cells and is at greater risk factor for the progression from MTB infection to active TB disease. However, some studies have reported that risk of active TB disease is increased during the early HIV infection when the number of CD4 T cells is normal in range. This might suggest that the other T cell-independent immune responses are also impaired (227). In addition, for vaccination it is not yet clear how the enhanced T cell responses provide better protection. A recent study on the mice model of TB had shown that the increasing the total CD4 + T cell response in the programmed death 1 (PD 1) leads to the reduced protection and enhanced mortality (228, 229).

4.4.3.10 The granuloma

The granulomas are very important in the MTB since they are involved in control of infection and also in the persistence of the pathogen (230). The granuloma illustrates the duality of MTB infection: from the host's perspective, the granuloma

acts as bacterial prison with the potential to ‘wall off’ infection from the rest of body; however, this differs from the bacterial perspective; the granuloma formation is a growing collection of the phagocytic cell to infect and replicate within. For example, ESX-1 secretion system can initiate a type I INF response which has been directly linked to the recruitment to the nascent granuloma of the myeloid population (CD 11 b⁺/80⁺GrIⁱⁿ) which is highly permissible to MTB infection (231). A study has reported that generally the immune response is geographically surrounded around the granulomas with the centre containing pro-inflammatory components whereas the surrounding tissue has anti-inflammatory components. The granulomas have a maximal burden /carrying capacity beyond which the infection would continue to progress (232). If the granuloma contains the infection with inducing substantial tissue pathology, then the person would be infected with latent TB infection.

4.4.3.11 Progression to Active TB disease

The individuals who are having the LTBI, the combination of macrophages, dendritic cells and T cells is sufficient to maintain a controlled, asymptomatic infection. This however in hosts the reasons are not clear how the infection can lead to clinical disease as early in some weeks or as it stays as long as in decade. Certain experiments in human immunology have provided clues as why some individuals with LTBI are unable to contain the infection and thus lead to active TB disease.

The important contributor to the progression to TB disease is presenting intact antigenic proteins. The genomic studies of clinical isolates have shown that MTB genes that are predicated to be involved in the production of the immunodominant CD4 + T cell activation in humans. The CD4 +T cells do not vary across the strains and lineages suggesting a possibility that the MTB might have benefitted from the antigen-specific CD4+ T cells on humans (233). This hypothesis derives an indirect support from the HIV-TB syndemic; although it is known that the HIV is clearly risk factor for the progression of LTBI to active TB disease in an individual, as HIV/AIDS is negatively associated with contagion (234). The importance of these immunodominant antigens extends their role beyond understanding the pathogenesis of active disease to a translational goal of defining a strategy for making vaccines. Traditionally, this identification of immunodominant MTB antigens are generating the repertoire of *M. tuberculosis* -specific T-cells which is considered the foundation for T cell mediated protective immunity and thus making it an effective vaccine-based

strategy. But despite inducing a modest level of enhance T -cell mediated responses, a vaccine that was generated using an immunodominant M-tuberculosis antigen failed to improve protection in human trial (235). We are still unaware of exactly how the century old BCG vaccination provides the protection against TB and also we don't know exactly to what extent the protection is mediated by CD4 + T cells or through the innate immunity.

From the host viewpoint, the three-natural epidemiological experiment have formed the risk of active TB disease, hence there are some crucial pathways which would lead to knowledge about controlling the infection such as HIV, tumor necrosis factor, neutralizing antibodies and inborn errors in immunity. The role of TNF in containing the MTB infection was demonstrated in mice in early 1990 and it showed that an increased risk of active TB disease in patients recovering from the anti-TNF treatments. However further investigation showed that TNF being very complex the TNF can be protective with the anti-TNF being a risk factor for the disease an emerging interpretation suggested that there would be an ideal set point for TNF in controlling M. tuberculosis infection. The excessive activation worsens the existing immunopathology and insufficient activation leads to lack of immune containment (236, 237). This model is supported by the studies such as adjunctive use of anti-inflammatory agents such as steroids to address the inflammatory pathology of TB in confined anatomical spaces for e.g. in brain.

The inborn errors in immunity leads to some findings on mechanism of immune response to TB (238). Over 100 million infants are vaccinated with BCG each year and only a small number of people develop the disseminated BCG disease which helped in possibility of having a map mutations in gene encoding proteins that are crucial for mycobacterial containment. Many of these proteins are involved in the IL-12, IFN- γ axis. Although some of these defects were originally identified in patients due to BCG vaccine or non-tuberculous mycobacteria, also in some cases the mutations had linked to active TB disease. Several other genes had been linked to experimental TB animal models which were linked to TB and/or leprosy in human genetic conditions. Still there is no exact mechanism why some people with LTBI leads to active TB disease.

4.4.3.12 Cytokine in tuberculosis

Cytokine are the molecules that mediate mainly intercellular communication in the immune system which is being produced by the different cell types in the body. These cytokines are pleiotropic in nature and they actively participate in the host defence process and in the inflammatory and tissue compensation process (239).

In tuberculosis, an effective and coordinated participation of different cytokine was already identified such as interleukin -12, (IL-2), IL-23, IL-27, IL-18, IL-1, IL-7 and IL-15. One of the important aspect connected with the production of different cytokines in MTB infections is the activation of macrophages in response to the INF- γ and TNF- α signalling (240). The non-activated macrophages are part of the usual habitat of the MTB, which generally resists in the intracellular environment such as blocking of the phagosome fusion with lysosome and thus avoiding its exposure to low pH and to the reactive nitrogen intermediates (RNIs) which are important to its destruction (241) out of these cytokines the INF- γ activated macrophages alter this blockage and helps in formation of phagosomes which will express the RNIs which would be enabled to eliminate MTB in the various infection sites (242). There is one cord factor protein known as 19 KD a lipoprotein along with other MTB components which induces the production of IL-12 by macrophages. These processes help in mobilizing the Th1 cytokine pathway.

The cytokines such as IL-12, IL-17 and IL-23 contribute to the host response of mycobacteria. Thus improving the development of Th1 cells (243). From this Th1 cytokines and TNF- α are most important agents of the antimycobacterial cytokine cascade. The reasons is due to formation as well as the maintenance of the granuloma, which is mediated by the TNF- α always acting synergistically with INF- γ leading to the activation of macrophages to produce effector molecules (244).

4.4.3.13 Role of Tumor Necrosis Factor (TNF- α)

The Tumor Necrosis Factor (TNF- α) was originally characterized as necrosis inductor in sarcomas in vivo (245). TNF- α is known as the proinflammatory cytokine which has multiple biological effects. TNF- α expression is strictly controlled since its overproduction lead to damaging effects found in different conditions in arterial hypotension, disseminated vascular coagulation and lethal hypotension.

In the process of infection control of mycobacteria, TNF- α seems to have a primordial role upon a wide variety of cells. The activated macrophages,

T-lymphocytes, dendritic cell are main producing cells (246). This TNF- α acts in synergy with INF- γ stimulating the production of reactive nitrogen intermediates (RNIs), thus mediating the tuberculostatic function of macrophages (247). TNF- α also helps in stimulation of migration of immune cells to the site of infection which further leads to granuloma formation which are in turn capable of controlling the disease progression (248).

The TNF- α blocking has dramatic effects on the progression on tuberculosis in experimental models. Neutralization of TNF- α in murine model results in tuberculosis aggravation or reactivation in these animal models. Studies have shown that the TNF- α is expressed in MTB infected tissues during the whole latent phase of infection (249) suggesting that there is the contribution of IFN-gamma in the control of the bacillus multiplication.

Increased levels of TNF-alpha are most commonly detected in culture supernatants of peripheral blood mononucleated cells (PBMCS) from patients with pulmonary tuberculosis which are stimulated by the mycobacterial antigens (250, 251). Some studies evaluated Immune response of patients prior to and after treatment observed that the patients with active tuberculosis produced increased levels of TNF-a ;but they did not observe a significant difference in these levels after treatment. This may conclude that cytokines has role in both pathophysiology and the protective immunity of the disease.

There are some studies showing that TNF-alpha may be associated with immunopathological responses in TB which also is main mediator of the destruction of the pulmonary tissue. The elevated levels may indicate to an excessive inflammation with necrosis and cachexia (252, 253).

The role of TNF- α in MTB has been subject of controversy. It has been described that the mycobacteria decrease the production of TNF in human PBMCs which probably contributes to its ability to establish chronic infections. The TNF- α is produced by macrophages, lymphocytes, neutrophils, and some endothelial cells. This tnf -alpha coordinates the inflammatory response via induction of other cytokines such as IL-1 and IL-6 and helps in recruitment of immune and inflammatory cells through the induction of chemokine and supraregulation of adhesion molecules. Some experimental models have shown the TNF- α plays an important role in host response against MTB as well as in the immunopathology of tuberculosis.

The other main function of TNF- α is to increase the capacity of macrophages to phagocytose and kill mycobacteria and stimulates apoptosis of macrophages depriving bacilli of host cells and leading to death and presentation by dendritic cells of mycobacterial antigens (254). TNF- α is required for the formation and maintenance of granulomas in in vivo Neutralization of TNF- α produced by mice chronically infected with *M. tuberculosis* specific monoclonal antibodies which disrupts the integrity of granulomas, which exacerbates the infection and increase the mortality .

MTB has evolved and has developed mechanisms which interact and modulate the host immune response. MTB expresses many surface antigens which can induce the production of IL-10, IL-4 which have anti-inflammatory effects (255). TNF- α is one of the major controlling factors for the recruitment of monocytes and is a powerful inducer of cell death by apoptosis (256). While necrosis on the other hand is linked with the lysis of the infected cell.

4.4.3.14 Role of Interferon gamma (IFN- γ)

The Interferons are substances originally identified as the cell culture supernatants infected by virus and they interfere directly in the viral replication. These Interferons are of two major types type I Interferons are induced as they act effectively in response against viruses: INF- α secreted by leucocytes and IFN- β is produced by fibroblasts (256)

The type II Interferons are now referred as IFN- γ are synthesized mostly by T lymphocytes and NK cells after this cell activation with immune and inflammatory stimuli rather than the viral infection. IFN- γ is the main cytokine which is involved in the protective immune response against the mycobacterial infection (239, 257). It is primarily produced by the CD4 and CD 8 T lymphocytes and the NK cells. Also, it is known that the natural killer T cells (NKT) and $\gamma\delta$ T lymphocytes produce the IFN- γ in response to mycobacterial stimulation thus displaying the protection against M>tuberculosis infection both in vivo and in vitro (258) .

The main function of IFN- γ is activation of macrophages which helps them to exert its microbicidal functions. It also enhancing the antigen presentation through the induction of the expression molecules from the MHC class I and II which is promoting the differentiation of CD4 + T lymphocytes to the Th1 subpopulation (259, 260). IFN- γ induces the transcription of around 200 genes in macrophages including

the genes those are to produce antimicrobial molecules such as oxygen free radicals and nitric oxide. These free radicals represent the effector mechanism MTB(261).

Studies done by Morosini and colleagues (260) emphasize through their finding in human subject that at least at certain stages of pulmonary tuberculosis there is differential compartmentalization of IFN- γ and of the other regulatory cytokine IL-12 and IL-10. In this case the protection factor associated with secretion of IL-12 which is present in lungs and the component is associates with immunosuppressive cytokines IL-10 whose secretion is predominant in peripheral blood.

Some other studies have reported that the patients with less severe forms of pulmonary tuberculosis has a predominance of Th1 cytokines such as IFN- γ whereas the increase in IL-4 levels, is a Th2 response which is related to the disease severity (262, 263)

After inhalation and subsequent infection with MTB in the lungs, dendritic cells infected with bacilli migrate to the regional lymph node, which averagely occurs around 14 days after infection thus helps in initiating the activation of T cells (264). A model study had used the dendritic cells infected with MTB was inoculated intratracheally in the lung. As result it is found that dendritic cells exposed to MTB prior to inoculation were better in migrating to the lymph node and in T cell activation (265). These findings are with information about the secretion of cytokines and their activation of CD4 +T cells. This indicates that there are different subtypes of CD4 +T cells involved in the protection in tuberculosis which are activated in the initiated phases of infection and produce cytokines classically considered immune protectors such as IL-2,IFN- γ , and TNF- α (266).

There is evidence that suggest the CD4 + T cells contribute both to the control of MTB as well as of immunopathology, contributing to morbidity and mortality in tuberculosis disease.

4.4.3.15 Role of IL-6 in Tuberculosis

IL-6 has also implicated in the host response to M. tuberculosis. This cytokine has multiple roles in the immune responses, including inflammation, haematopoiesis, and differentiation of T cells. IL-6 is produced in large amounts in the pleural fluid and bronchoalveolar lavage cells from TB patients.

This cytokine tends to antagonize the effects of $\text{TNF-}\alpha$, another proinflammatory cytokine by preventing its binding of $\text{TNF-}\alpha$ to macrophages and antagonizing its antimicrobial activity (267, 268)

A potential role for IL-6 in suspension of T cell was reported. This study showed that the BCG -infected macrophages were inefficient in stimulating the T cell responses to an unrelated antigen, and this effect could be partially retracted by the neutralization of IL-6. The study also showed that the low dose of aerosol infection increase early lung burden, which also leads to decrease $\text{IFN-}\gamma$ production, which was observed in IL-6 $-/-$ mice when compared with control mice . This study suggested that the IL-6 is important in the initial innate response to the pathogen (269)

Once the acquired immunity is developed the IL-6 $-/-$ mice has controlled the infection and they have survived. The memory response to the virulent aerosol challenge was also found to be intact in IL-6 $-/-$ mice. The IL-6 $-/-$ mice succumbs to the infection with a high dose intravenous of Tuberculosis (270)

The IL-6 has been known to enhance mycobacterial growth both intra and extracellularly while it is inhibiting the $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ at the transcriptional level in-vitro (271). IL-6 also suppresses T cell proliferation and the IL-2 production in response to the antigenic stimulation (272)

4.4.3.16 NF- κ B regulation in the Tuberculosis

The transcription factor NF- κ B is a central inflammatory mediatory which is essential for the induction of various inflammatory genes in response to various pathogens and inflammatory cytokines. One of important cytokine is tumor necrosis factor- α (TNF), is a key regulator of host responses to the infection in response to *Mycobacterium tuberculosis*. TNF affects the immune response to MTB through several mechanisms such as induction of macrophage activation to kill the bacteria efficiently (273) leading to induction of chemokine and cytokine and apoptosis . All these important processes are regulated by the NF- κ B signalling pathways thus making the TNF a key factor for restricting the bacterial growth in the granulomas and the aggregation of bacteria and immune cells within the lungs that forms as result of the immune response. Hence the TNF-induced NF- κ B signalling pathway is the core pathway to the MTB immune response and regulation of intracellular NF- κ B signalling dynamics leading to the possible controlling of the MTB infection.

Granulomas are the very important part of MTB. These granulomas can contain mycobacterial growth and it further helps to develop a clinically latent infection. If these granulomas are not functional they can lead to impairment of infection progression, furthermore granulomas enlarge and then bacteria seed new granulomas. This process results in disease progression and the pathology of disease leads to the active TB disease. In clinical latency, immunologic perturbation at the level of the granuloma can result in reactivation of infection (270). Various studies have provided an input on the principal role of TNF in containment of bacteria within TB granulomas (274).

NF- κ B is found to be resting in the cell which is generally bound to the I κ B proteins which hold them latent in cytoplasm. Binding of TNF to TNF receptor type 1 results in the activation of I κ B kinase (IKK) and IKK mediated phosphorylation of I κ B proteins while eventually leads to the ubiquitination and proteasome mediated degradation of I κ B. Later, the free NF- κ B accumulates in the nucleus and it helps in transcription of the target genes (275). These target genes include the extracellular signalling molecules such as TNF and different chemokines; while the intracellular proteins such as macrophage-activating and inhibitor of apoptosis proteins known as (IAP), as well as the negative regulators of NF- κ B such as I κ B α and A20 (275, 276). The inhibitory effect of A20 on NF- κ B which results in attenuating the TNFR1 activity and thus inhibiting the IKK activation (103). The regulation of NF- κ B via multiple critical intracellular feedback mechanism is crucial for the control of inflammation and human activation (277, 278).

The structural characteristic of the inflammatory genes induced by NF- κ B requires a stability of their corresponding mRNA transcripts which in turn controls the dynamics of NF- κ B mediated responses in cells (279). But the significance of intracellular molecular mechanisms controlling these dynamics of TNF-induced NF- κ B signalling in regulation of the long term immune response to MTB is poorly characterized (280).

The inhibition of NF- κ B activation impacts the viability of the intracellular MTB in human macrophages including the differentiated THP-1 monocytes, primary monocyte derived macrophages (MDM) and alveolar macrophages (AM). Some studies had shown that inhibiting the NF- κ B activation reduces the viability of intracellular MTB in all three human macrophages. Some studies revealed the impact of inhibiting NF- κ B activation on apoptosis and autophagy process (281, 282), two

cellular processes known to facilitate the mycobacterial killings (283, 284). The inhibition of NF- κ B activation reduces the intracellular survival of MTB by enhancing both host-protective apoptosis and autophagy of the infected macrophages (285).

4.4.3.17 Effects of tuberculosis on different organs

The Mycobacterium tuberculosis has major pathological effect on lungs as its is main organ of the respiratory system and possible route of infection.

4.4.3.17.1 Effects of Tuberculosis on lungs

Lungs are most important organs in the TB disease. The Mycobacterium enters the air inhalation pathway. A tiny drop of TB bacilli enters through the environ into the oral cavity and then it attaches itself to the cavity of lung tissues. Macrophages are the preferred intracellular location for *M. tuberculosis in vivo*. Infected individuals cough and expel droplet nuclei which contain *M. tuberculosis* bacilli and they remain suspended in the air. After inhalation and within the body, the bacilli are transported to the small alveoli in the lungs where they encounter alveolar macrophages which are relatively non-activated (286).

These non-activated macrophages are not efficient at killing or retarding growth of invading microbes. Initially bacilli are taken up into the phagosomal compartments and the replicated. As the immune system becomes activated, macrophages are stimulated by cytokine INF- γ to increase their efficiency to kill the mycobacteria and thus becoming more efficient for producing reactive oxygen intermediates and acidic stress. In response, to these, *M. tuberculosis* pushes itself back against the macrophages and differentially regulates key genes (287, 288).

The primary site of infection in the lungs, known as the "Ghon focus" or Ghon complex. This complex is located in either the upper part of the lower lobe of the lung (289). When other macrophages attack the infected macrophage, they fuse together to form a giant multinucleated cell called as granuloma, in the alveolar lumen. This granuloma may prevent dissemination of the mycobacteria and provide a perfect local environment for interaction of cells of the immune system (290). However, more recent evidence suggests that the bacteria use the granuloma formation to avoid destruction which can be caused by the host's immune system in response to any attack by bacteria. Macrophages and dendritic cells in the granulomas are unable to

produce the antigen to lymphocytes; which results in the suppression of the immune response. Bacteria inside the granuloma become dormant, and thus resulting in latent infection (291).

Another important feature of the granulomas is that there is a development of abnormal cell death, termed as cell necrosis in the centre of tubercles. With the granuloma, both the T cells and macrophages secrete the cytokine TNF- α and lymphotoxin $\alpha 3$. The TNF- α is not only crucial for the host defence (292) but also it facilitates the structural integrity of the granuloma by intermediating the formation of (293, 294) transforming growth factor [TGF- β] (295, 296). The macrophages present also secrete some growth factors such as growth factor 1, fibroblast, GF, fibronectin and platelet derived GF (297). All these growth factors are chemotactic in nature and these support the macrophage proliferation and also help in arranging the ECM which comprises of collagen, fibronectins and glycosaminoglycan (298). The caseous necrosis is important symptoms which are occurring in 2-3 weeks in the animal model such as rabbit (299) which is associated with the low oxygen content which creates the unfavourable conditions for the mycobacterial multiplication. Thus, together with macrophage activation and CD3 effector cell mechanism leads to conclusion in mycobacterial sterilization.

The controlled dissolution of the granuloma follows the mycobacterial containment incomplete if there had been a massive production of caseum. The protease enzyme cleaves components of ECM (300) the macrophages phagocytose are degraded ECM components which are terminally degraded within the lysosome or they are transported out the lung (301).

4.4.3.17.2 Effects of Tuberculosis on Liver

Tuberculosis and the liver can be related to each other with either of direct hepatic involvement by the disease itself, which is common but rarely does it cause the marked damage of the hepatic functions.

Hepatic tuberculosis presents in three forms (302). The most common is the diffuse hepatic involvement which is seen with the pulmonary or military tuberculosis in 50 to 80 % of patients who die due to pulmonary tuberculosis. Despite the diffuse involvement of the liver symptoms of the liver disease are often absent. The second form is a diffuse hepatic infiltration observed without recognizable pulmonary

involvement which is also known as granulomatous hepatitis. The third form is much rarer form which presents as a focal/local tuberculoma or abscess observed.

Tubercle bacilli reach the liver by way of haematogenous dissemination. This portal of entry in the case of miliary tuberculosis which is through the hepatic artery whereas in the case of focal liver tuberculosis the entry is via the hepatic portal vein. Irrespective of the mode of entry, the liver responds by granulomas formation.

And these tuberculous granulomata are most frequently found in the periportal areas (303). Both caseating and non-caseating granulomas are seen frequently. In focal tuberculosis, various granulomas may amalgamate to form a large tumour like tuberculoma. This tuberculoma which has undergone extensive caseation and liquefactive necrosis forms a tubercular abscess.

Treatment for tuberculosis leads to hepatic injury by various hepatotoxic anti-tubercular drugs. The drugs like Rifampicin, pyrazinamide, isoniazid, ethionamide and PAS are all proven to be hepatotoxic drugs (304). Clinically these drugs have reported several symptoms such as jaundice, nausea, vomiting and abdominal pain or as elevated levels of bilirubin and hepatic transaminases. Hepatotoxicity is the most common adverse effect leading to interruption of therapy (305). The hepatotoxicity is associated with a mortality rate around 6–12% if the AKT drugs are still continued after the onset of above mentioned symptoms (306). The risk of hepatotoxicity is increased when the drugs are combined that in case of drug resistance of the Tb strains MDR/ XDR strains. However, the risk is comparatively lower among patients receiving intermittent, thrice-weekly treatment from DOTS Centre.

The patients with the chronic liver disease develop the tuberculosis and they pose a special management problem. These include the potent antitubercular drugs which are also known to be hepatotoxic leads to underlying disease process. Thus drug regimens need to be modified to prevent the further damage to the hepatic system. Besides this the chronic liver disease is also an important risk factor for the development of ATT induced hepatitis (307). It is also observed that there is elevation of liver enzymes during the anti-tubercular therapy most commonly found in HBsAg carriers than in healthy control subjects (308). One study showed that the antitubercular drug induced liver injury is more common in hepatitis C virus (HCV) infected patients(309) . Hepatic dysfunction alters the absorption and distribution of drugs that are metabolized or excreted in the liver such as isoniazid and rifampicin

(310). Therefore it is advisable to include fewer hepatotoxic drugs and subsequently increased the period of treatment (311).

4.4.3.17.3 Effect of Tuberculosis in Central Nervous System

Central nervous system (CNS) disease caused by *Mycobacterium tuberculosis* is an rare case but yet it is highly devastating manifestation of tuberculosis, which was universally fatal in the era before the antituberculosis therapy had been initiated. CNS tuberculosis accounts for around 1% of all cases of tuberculosis which carries a high mortality and a distressing level of neurological morbidity and it afflicts the children and HIV infected individuals. Due to its relative rarity and the symptoms, the tuberculosis of the CNS remains a formidable diagnostic challenge.

For the CNS tuberculosis, the manifestation of disease beings with development of small tuberculous foci (Rich Foci) in the brain or spinal cord or meninges. The location of these foci and the capacity to control them later determines which form of CNS tuberculosis occurs in the patients.

CNS tuberculosis manifests itself as tuberculosis meningitis (TBM). The less commonly forms are known as tubercular encephalitis, intracranial tuberculoma or just as brain abscess (312).

The cytokine tumor necrosis factor- α (TNF- α) is very important in the cytokine in the neuropathogenesis of M. tuberculosis (313, 314) Although the TNF- α had a definite role in granuloma formation of different mycobacterial infection (315), local CNS production of TNF- α has shown in the experimental bacterial meningitis which leads to altered blood-brain barrier (BBB leading to permeability and cerebrospinal fluid (CSF) leucocytosis (316, 317). It has various implications in nurturing the disease progression of TBM in the murine models (318).

A distinctive characteristic of M. tuberculosis is its capacity to enter and replicate within the macrophages. In CNS, the microglial cells are the resident macrophages as such these human microglial cells are readily infected with M. tuberculosis and they are main target in the CNS. *M. tuberculosis* infected microglia produces a robust amount of several important cytokines and chemokines such as TNF- α , IL-6, IL-1, CCL-2, CCL-5 and CXCL 10 (319).

One study proved that human microglia is more efficient at ingesting *M. tuberculosis* than the virulent and avirulent strains of *M. avium*. This follows the infection caused by the strains and there is a lasting inhibition of both IL-1 and IL-10

production (320). The above study suggested that mycobacterial infection induces immunosuppressive effects on microglial cells, which are more evident with more virulent strains. Another observation showed the microglia has emerged as key cells for understanding the neuropathogenesis of tuberculosis.

The diagnosis of CNS TB is done by different methods such as traditional CSF analysis, followed by routine microbiological detection. More advanced techniques are followed by Molecular and biochemical detection using PCR as diagnostic tool; followed by antigen detection, antibody detection and chemical method such as adenosine deaminase and tuberculostearic acid measurements. The regular radiologic detection CT scan can help in the detection of CNS TB.

Treatment for the CNS TB is generally same as the prescribed AKT drugs such as isoniazid, rifampicin and ethambutol. The drug regime can be altered with the response to the therapy. Drug resistance for the AKT is also important and MDR/XDR strains of TB can lead to alteration of therapy using 2nd line drugs for the management of TB. Fluoroquinolones, capreomycin, kanamycin, amikacin can be given to treat the patients; cycloserine have good CNS penetration and may be among the only agents available to begin building a treatment regimen for XDR TBM.

4.4.3.18 Oxidative stress in Tuberculosis

The unique ability of *M. tuberculosis* to persist in immunocompetent infected individuals for decades in a drug-unresponsive, sometime latent form and then reactivate into active disease is the vital function to its success. The ability to endure the host-generated oxidative stress is important for the MTB survival during the latent state. MTB encounters various oxidoreductive stresses during infection and transmission. For example during the transmission of the bacteria it is enclosed in the aerosols, the bacterium experiences hyperoxic stress since MTB resides primarily in the alveolar macrophages where the normal oxygen levels are lowered as compared to the atmospheric oxygen and there is a case of low nutritional levels, when inside the naïve macrophages where it actively replicates, the MTB encounters the reactive oxygen intermediates (ROI), inside the activated macrophages, ROI as well as reactive nitrogen intermediates (RNI) are encountered leading to the hypoxic stress in human granulomas and can lead to possible calcification of granulomas in humans.

There are two predominantly sources of redox stress for mycobacteria known as endogenous and exogenous oxidative stress. The endogenous oxidative stress is

face by the bacteria using the oxygen as main terminal electron acceptor, due to its univalent transfer of electron from the ETC to O_2 . Exogenous oxidative is generated by some of the specialized pathways generated by the host in response to eliminate the pathogen from the host body. For these intracellular pathogens, the survival to upon exposure to exogenous stress is critical.

MTB is generally exposed to a number of exogenous stresses generated by the immune responses of the host. After the process of phagocytosis of MTB, the macrophages and the neutrophils produce considerable amounts of ROI and RNI. ROI formation is initiated via the production of moderately reactive superoxide radical (O_2^-), which is considered to be the precursor of RNI (321, 322). The generation superoxide radical is catalysed by the NADPH oxidase (NOX) through the one electron reduction of O_2 via NADPH as an active electron donor.

The NOX is a multiprotein enzyme complex which is composed of the core components such as $p40^{phox}$, $p67^{phox}$ and $gp91^{phox}$ and other regulatory components. The bacteria undergoing the phagocytosis upon response to the stimuli helps to initiate the assembly and activation of the membrane bound and cytosolic components of NOX. Out of this enzyme complex there is one important component which has a low potential, b-type cytochrome capable of transferring electrons to oxygen (323). Genetic defects in the cytochrome b subunits are one of causes for chronic granulomatous disease which is strongly characterized by failure to generate the superoxide radical which results in the severe and recurrent infections (324). The superoxide radical can react with itself in dismutation reaction which is catalysed by superoxide dismutase (SOD), leading to the production of H_2O_2 .

The H_2O_2 generated from the dismutation reaction reacts with the metal ions such as Fe^{3+} and cu^{2+} under the physiological conditions generate the hydroxyl radical. This hydroxyl radical instantaneously reacts with cellular components and causes the death of the bacteria. This phenomenon has helped number of bactericidal antibiotics function via the higher levels of the hydroxyl radicals (325). The further reaction of H_2O_2 leads to highly reactive hypochlorite by enzyme myeloperoxidase catalysed oxidation of chlorine. This hypochlorite is an oxidizing halogen that leads to oxidation of proteins, lipids and DNA of the pathogen. Since being a primary halogen, it also leads to formation of number of microbicidal halogen derivatives.

ROI and RNI are effective in killing several types of bacteria including various species of mycobacteria (326). H_2O_2 and O_2^- are two most common forms of

ROI encountered by MTB inside the macrophages. Most of mycobacterial species such as *M. tuberculosis*, *M. bovis* BCG, *M. microti*, *M. intracellulare*, and *M. leprae* are susceptible to killing by H_2O_2 . The mycobacterial infection of macrophages leads to the activation of NOX and production of superoxide radical. A very important role of superoxide radical is that the patients with chronic granulomatous disease are susceptible to infection by different species of mycobacteria.

Mycobacterial infection of macrophages initiates another major anti mycobacterial pathway of NO generation through inducible nitric oxide synthase (NOS2) (327, 328). NO is produced in parallel with superoxide radical and reacts rapidly with it and thus resulting in the production of another highly reactive peroxynitrite ($OONO^-$).

This peroxynitrite can lead to further generation of highly reactive oxidants in the form of NO^- , NO_2 , N_2O_3 , N_2O_4 and S-nitrosothiols and dinitroyl-iron complex (329, 330). The role of RNI in TB is more defined than the role of superoxide radical. At higher concentrations, these RNI efficiently kill the MTB in vitro, whereas at lower concentration they show a bacteriostatic (331) or hormetic effects. Some studies performed by Orme and colleagues suggest that NOS2 is the important enzyme to produce NO in macrophages, which is not essential for the early control of MTB in murine models (332). But the studies further supported that human alveolar macrophages and peripheral blood monocytes can equally control the growth of intracellular MTB in the evidence of NOS inhibitors (333) however there is more evidence suggesting that the important role of NO in TB control in humans and mouse model. The murine macrophages as well as the macrophages derived from the patient's lung express NOS2 and produce significantly higher levels of NO which can kill the mycobacteria.

4.5 HIV-TB coinfection

4.5.1 Global statistics

The global HIV/TB 2016 report will shed information about the dual epidemic. In the individual host the two pathogens, *M. tuberculosis* and HIV, help each other symbiotically so that they accelerate the deterioration of the immune system and thus leading to the death of the host if left untreated (334).

Both HIV and Tb can have their effects on the immune system, as they can disarm the host's immune system through mechanisms which are not fully

understood. HIV co-infection is most powerful known risk factors for the progression of *M. tuberculosis* infection is the most powerful known risk factor for the progression of *M. tuberculosis* infection to active disease, increasing risk of latent TB reactivation (335)

4.5.2 Role of HIV in disease progression of *M. tuberculosis* infection

The HIV related dysfunction of local immune responses in HIV/TB co-infected individuals reduces the ability of the granulomas, by edge of lymphocytes to confine the tubercle bacilli leading to their multiplication and dissemination leading to severe pathology (336).

The exacerbation of tuberculosis pathology is linked to the increase replication of HIV at the sites where the infection of *M. tuberculosis* occurs. HIV replicates within the activated CD 4 + cells and macrophages; these cells are known to be sites of accumulation of granuloma. The elevated levels of p24 antigen are viral load in bronchoalveolar lavage (BAL) fluid are detected in patients with tuberculosis when they are compared with patients having no tuberculosis infection (337). Also studies showed that there was an increase in viral loads in the pleural fluid when compared with plasma among patients with pleural tuberculosis and it has greater HIV replication in activated macrophages co-infected with *M. tuberculosis* and HIV compared with HIV-infected macrophages, suggesting that HIV replication is increased at the sites where *M. tuberculosis* infection occurs

The killing of CD4 + cells by HIV within the granuloma is commonly associated with the primary tuberculosis or also with the reactivation of the tuberculosis. Some animal studies have demonstrated that certain monkey were coinfecting with simian immunodeficiency virus strain mac251 (SIV -mac251) and *M. tuberculosis* had fewer cells CD 4 + T cells in granulomatous tissue than those with active tuberculosis alone (338). The studies shown that HIV-infected patients with lower CD4 + T cells are more susceptible to tuberculosis than those with higher counts. Also there are reports stating that HIV/TB coinfecting patients have lower CD 4 + T cells detected in their BAL fluid than those infected with tuberculosis alone. (339).

The functionality of the different immune cells such as macrophages and T cells are generally found to be altered in the HIV/TB coinfecting individuals (340). Macrophages from these coinfecting patients release lower levels of TNF- α and they

induced less TNF-dependent apoptosis than those infected with tuberculosis alone (341, 342). There is an evidence for the negative impact of HIV on the ability of tuberculosis -specific T cells to contain the bacterial formation. For example there are less interferon γ (IFN- γ) producing the *M. tuberculosis* specific memory T cell following the HIV infection in patients with latent tuberculosis. Some studies have revealed that lower levels of INF- γ and IL-2 production and proliferation by the *M. tuberculosis* specific memory T cells in HIV infected individuals are found than the patients with active tuberculosis (343, 344).Recent findings showed that IL-10 production was significantly higher in HIV-TB co-infected individuals after the *M. tuberculosis* stimulation was given. This may suggest that the chronic HIV infection could render the cell mediated immunity (CMI) which is ineffective against *M. tuberculosis* further the studies showed that the levels of IL-2 were less and levels of macrophage inflammatory protein -1 β (MIP-1 β) were produce by the M-tuberculosis specific CD4+ T cells in HIV infected vs HIV-uninfected patients. Supporting the fact that HIV preferentially infects and depleted IL-2 producing CD4+ T cells and it is partially inhibited from depleting the MIP-1 β producing CD 4 +T cells.HIV-1 promotes the secretion of indoleamine 2,3 dioxygenase (IDO) a tryptophan -catabolizing enzyme which is required for the T cell suppression (345, 346). Recent studies have shown the role of co-inhibitory molecules play an important role in the activation of T cell in HIV/TB coinfection. The study showed that when there is higher expression of PD-1 on T cells from HIV-TB co-infected individuals than on the T cells which were from the tuberculosis patients and healthy controls (347) further this indicated that HIV may contribute to the lower CMI responses against the TB bacilli by regulating the expression of the co-inhibitory molecules on the effector immunes cells (348, 349). Thus HIV helps in promoting the TB bacilli to invade the immune system.

4.5.3 Role of the TB in the HIV-1 disease progression

As we have seen how the HIV-1 exacerbate the TB infection and leads to pathways leading to the infection of both in to the host. Co-infection with *M. tuberculosis* negatively impacts the HIV immune responses and this is demonstrated by the higher case of new AIDS-defining opportunistic infection in HIV/TB co-infected individuals. The plasma viral load is higher and HIV replication is upregulated in blood and pulmonary lymphocytes and in alveolar macrophages ex

vivo in the HIV infected patients with the active tuberculosis disease. The exacerbated HIV infection in alveolar macrophages are the primary targets for *M. tuberculosis* which are also been detected in the BAL from the co-infected patients (350).

HIV uses its co-receptor CCR5 for the early course of infection, whereas as the disease progression precedes the other co-receptor CXCR4 is used for the infection. *M. tuberculosis* creates a suitable environment for the accommodation of CXCR4 -tropic HIV by increasing the expression of the CXCR4 in alveolar macrophages and subsequently suppressing the entry of the CCR5 -tropic HIV, thus creating a selective pressure for the replication of CXCR4-tropic HIV to survive (351)

It has been established that HIV and *M. tuberculosis* infection co-stimulate the TNF- α production which in in turns enhances the HIV replication in the macrophages (352). *M. tuberculosis* infection then activates the suppressor of cytokine signalling 1 (SOC 1) which I known as the inducible host factor. This SOC 1 is required during the HIV-1 infection which facilitates viral replication by inhibiting type-1 IFN antiviral signalling. Apart from this effect on HIV replication, *M. tuberculosis* has ability to down regulate the proinflammatory cytokines and antigen presenting abilities and then it activates the a anti-inflammatory functions of dendritic cells (353). This DC-mediated activation of T cells is impaired in HIV infection, and it leads to migration of DCs along with HIV contributes efficient viral distribution. Wax D complex has also been implicated in the activation of HIV replication by enhancing the production of IL-2 and TNF- α by the dendritic cells which could give rise to increased HIV-1 replication leading to the immunosuppression. In the coinfection condition HIV infected T cells becomes dysfunctional leading to loss of intracellular killing abilities of macrophages which are concealing *M. tuberculosis*. Simultaneously, these *M. tuberculosis*- infected macrophages contains the LAM (Wax-D) to produce the increase levels of TNF- α , IL-6 and IL-1 which leads to enhanced viral replication and HIV persistence with macrophages (354). One theory support that the HIV helps in *M. tuberculosis* to persevere with the macrophages while enabling the increased synthesis of IFN- γ which in turns promote the secretion of IDO, a tryptophan-catabolizing enzyme, leading to the T -cell inhibition. If the IFN- γ levels have no association with IDO secreted by the DCs, the role of prostaglandin E2 (PGE2) could be considered for the investigation of the theory above mentioned. Also by decreasing the available tryptophan and producing the

tryptophan metabolites such as kynurenine (kyn), these IDO inhibits T cell functions and leads into immune suppression

4.5.4 Effects of HIV and *M. tuberculosis* on each other

Several in vitro studies demonstrated that phagocytosis of *M. tuberculosis* induces the macrophages activation and also the production of proinflammatory cytokines such as TNF- α , IL-1 β and IL-6 by macrophages which are known to enhance the replication of HIV-1 (355, 356). Growth of *M. tuberculosis* was observed in HIV-1 infected monocyte-derived macrophages (MDM) when compared with HIV-1 negative cells. Also, adding TNF- α to these HIV-1 infected MDM induces a significant increase of *M. tuberculosis* growth when compared with cultures performed in the absence of the cytokine, whereas addition of TNF- α has no effect on *M. tuberculosis* growth in uninfected MDMs (357). In latently HIV-1 infected promonocytic cells, phagocytosis of *M. tuberculosis* induced viral production and acutely HIV-1 infected MDMs, and *M. tuberculosis* growth was seen increased. Also, HIV-1 infection induced a greater bacilli burden in co-infected cell cultures and specifically induced accelerated growth of *M. tuberculosis* which in turn bacteria induced the replication of HIV-1(358). The co-infection of HIV/TB synergistically decreased the viability of macrophages and increased levels of proinflammatory cytokine and it is specific to *M. tuberculosis* rather than other species of mycobacteria (359).

4.5.5 Clinical Presentation and diagnosis of tuberculosis in HIV/TB co-infection

The clinical presentation of tuberculosis in HIV/TB coinfecting individuals is known to vary according to the immune status and certain atypical characteristics is observed such as involvement of lower lob with trend towards diffusion of the infection rather than to create cavitation (360) in the severely immunocompromised HIV -infected individuals, the pulmonary basal involvement, hilar or mediastinal lymphadenopathies, and miliary tuberculosis are most commonly observed(361). The diagnosis of active tuberculosis in HIV-infected individuals remains challenging because of fewer bacilli in the sputum samples from the HIV/TB infected individuals rather than in just TB positive HIV-seronegative individuals which is due to the significantly reduced transfer of bacilli in respiratory secretions (359).

4.5.6 Extra pulmonary tuberculosis in HIV positive Individuals

The incidence of extra pulmonary tuberculosis has been reported to be higher in HIV -infected individuals than among the immunocompetent population (362). EPTB is high significant in HIV being designated and AIDS defining illness and occurs frequently and earlier than the other opportunistic infections with a high rate of mortality. Various studies have showed that the lymph nodes are the most common site of EPTB among the HIV-infected individuals. While one study revealed that the EPTB is most common form of tuberculosis in HIV patients whose CD4+ T -cell counts are lower and they have a common symptoms such as fever, weight loss and cough (363).

4.5.7 Cytokines during the HIV/TB dual infection

During the M. tuberculosis infection the excess of proinflammatory cytokines such as TNF- α may be very important to the expansion of virus burden. The interaction of M. tuberculosis with a mononuclear phagocyte induces the expression of TNF- α before and at the phagocytosis of the bacilli due to the exposure of the proteins release by the organism. In the HIV/TB coinfectd patients the levels of circulating TNF- α activity correlate with HIV activity (364) TB/HIV co-infection also results wasting conditions. This condition in turn affects the inflammatory response, suppresses the cellular immunity and aggravates the severity and outcome of disease.

5 MATERIALS AND METHODS

The study included groups HIV sero-positive, HIV-TB sero-positive, TB positive, HIV sero-negative and control study subjects. 50 individuals were selected in each study cohort and 50 healthy individuals were selected for the study. All the individuals who were participating in the present study were explained in brief about the project aims and objectives as well as the possible benefit for the study. The consent form was taken from each of the individuals before taking any samples from them. The study was approved by the Institutional Ethics Committee EC/67/2011 dated 27th June 2011.

5.1 Study groups:

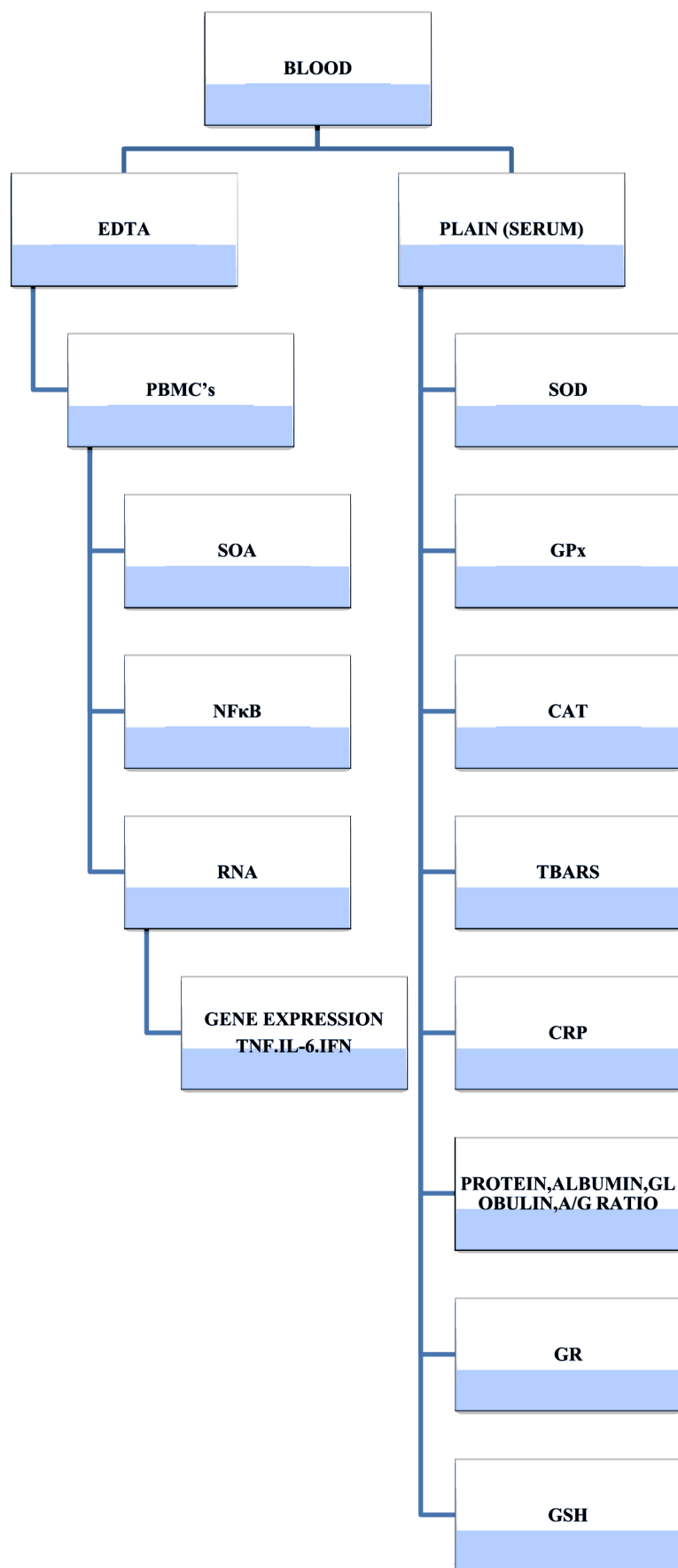
5.1.1 Inclusion criteria

- a. A minimum of 50 HIV sero-positive individuals, as determined by case history taking, were included in this group. The individuals, whose age was greater than 18 years, belonging to both sexes and either attending outdoor patient department or admitted to government and municipal hospitals. The individuals on ART were included in the study.
- b. A minimum of 50 TB individuals sero-positive for HIV as determined by case history taking will be included in this group. The individuals, whose age was greater than 18 years, belonging to both sexes and either attending outdoor patient department or admitted to government and municipal hospitals. The individuals on AKT/ART were included in the study
- c. A minimum of 50 TB culture positive individuals, as determined by case history taking, will be included in this group. The individuals, whose age was greater than 18 years, belonging to both sexes and either attending outdoor patient department or admitted to government and municipal hospitals. The individuals on AKT were included for the study
- d. A minimum of 50 healthy individuals were included in the study as healthy control volunteers.
- e. A written informed consent has been acquired from all individuals that were included in the study.

5.1.2 Exclusion Criteria

- a. The patients belonging to age group below 18 years of age were excluded from the study.
- b. The pregnant women, lactating women have been excluded from the study.

5.1.3 Flow chart



5.1.4 Blood Collection

Ten millilitres of blood was collected by trained technicians from all study groups individuals by venipuncture into vacuum collection tubes (Vacutainers; Becton, Dickinson and Company, Gurgaon, India). The blood was aliquoted appropriately in K3-EDTA and plain bulb. The serum was separated from the plain bulb aliquoted and stored at -80°C. An EDTA tube (Purple capped; K3 EDTA) was used for whole blood and separation of plasma and PBMCs. Appropriate biosafety precautions were used during the collection of blood samples, destruction and discard of needles and transport of samples to the laboratory.

5.1.5 Separation of Whole Blood, Plasma and PBMCs

Whole blood from HIV/HIV TB /TB/Control study individuals collected in EDTA vacutainers was used immediately for the determination of CD4/CD8 counts. For the separation of plasma and PBMCs, from all study groups and for the donor PBMC group, a modified protocol from National Institute of Allergy & Infectious Diseases (NIAID), NIH was utilized (365).

- a. Whole blood in EDTA vacutainers was centrifuged and clear plasma separated above the blood cell pellet was carefully pipetted out into 2 appropriately-sized aliquots and stored in a -80°C freezer until further processing.
- b. To the remaining blood, RPMI-1640 medium (GIBCO®, Invitrogen Bio Services India Pvt. Ltd, Bengaluru, India) was added at a ratio of 1:1.
- c. This whole blood was mixed properly and over layered on Histopaque®-1077 (Sigma-Aldrich Corporation, Bangalore, India) with the ration of 2:2. The blood was added with care so that the blood was not mixed with the Ficoll.
- d. The tube was centrifuged at 3000 rpm for 30 min. The PBMCs formed a white buffy coat on top of the clear Ficoll layer and was washed once with RPMI-1640 medium.
- e. To remove any residual red blood cells (RBCs), the cells were treated with ACK buffer (GIBCO).
- f. The cells were then re-suspended in 10.0 mL complete RPMI-1640 medium (cRPMI) consisting of RPMI-1640 medium with 10% Foetal Bovine Serum (FBS; GIBCO) and Penicillin-Streptomycin solution (100 units Penicillin, 0.1 mg/mL Streptomycin; HiMedia Laboratories Pvt. Ltd., Mumbai, India).

- g. The flask was kept overnight in a humidified CO₂ incubator (New Brunswick Scientific, Connecticut, USA) at 37°C and 5% CO₂.

5.1.6 Extraction of Viral RNA from PBMCs

Extraction of RNA from PBMCs from all the study groups was carried out manually using a QIAamp® Viral RNA mini kit (Qiagen India Pvt. Ltd., New Delhi, India) as per the manufacturer's instructions (366, 367)

- a. Briefly, buffer AVL containing carrier RNA (1 µg/µL) was added to PBMCs sample.
- b. 560 µL of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube was added.
- c. Around 140-200 µL PBMCs was added to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.
- d. Incubated at room temperature (15–25°C) for 10 min.
- e. Around 560 µL of ethanol (96–100%) was added to the sample, and mix by pulse-vortexing for 15 s.
- f. 630 µL of the solution from step 4 was added 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.
- g. Carefully QIAamp Mini column was opened and 500 µL of Buffer AW1 was added to the column. It was centrifuge at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini Column was placed on a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded.
- h. The QIAamp Mini column was carefully opened, and 500 µL of Buffer AW2 was added. The tube was centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.
- i. The QIAamp Mini column was placed on a new 2 ml collection tube, and the old collection tube with the filtrate was discarded. Centrifuged at full speed for 1 min.
- j. The QIAamp Mini column was placed on a clean 1.5 ml microcentrifuge tube. The old collection tube containing the filtrate was discarded.

- k. The QIAamp Mini column was opened carefully and 60 μL of Buffer AVE was added and equilibrated to room temperature. The cap was closed and incubated at room temperature for 1 min. Centrifuge at 6000 $\times g$ (8000 rpm) for 1 min.
- l. The RNA eluted was stored at $-80\text{ }^{\circ}\text{C}$ till further use.

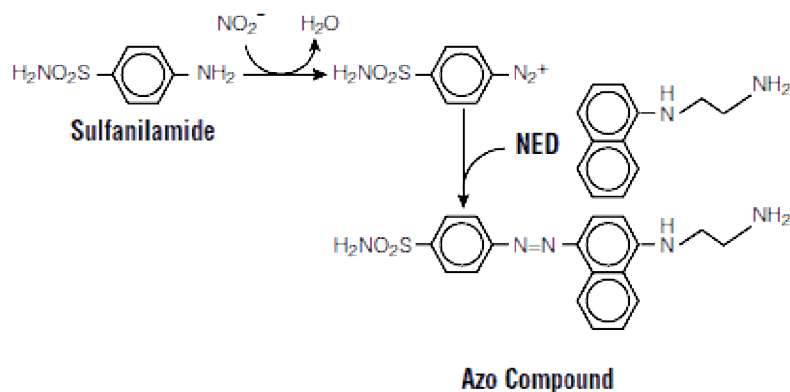
5.1.7 Oxidative stress markers

The oxidative stress parameters were performed using the serum separated from the Patients and healthy controls.

5.1.7.1 Determination of NOS (Nitric Oxide)

The Griess Reagent System is based on the chemical reaction, which uses sulphanilamide and *N*-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This assay relies on a diazotization reaction that was originally described by Griess in 1879 (368, 369).

Figure 7: Mechanism of Griess Reagent System



5.1.7.1.1 Method:

- i. 1% Sulphanilamide Solution in 5% OPA and 0.1 % NED Solution was allowed to equilibrate to room temperature (15–30 min).
- ii. 50 μL of serum sample was added to wells.
- iii. Sodium Nitrite was used as the standard.
- iv. Then, 50 μL of the 1% Sulphanilamide Solution was added to all wells.
- v. Plate was incubated 5–10 min at room temperature, protected from light.
- vi. 50 μL of 0.1 % NED Solution was added to all wells.

- vii. And, again plate was incubated at room temperature for 5–10 min, protected from light. A purple/magenta colour of Azo - compound will begin to form immediately.
- viii. Readings were taken on Multi-mode reader at 520 nm within 30 min.

5.1.7.1.2 Standard Curve

0.1 M of Sodium Nitrite Standard was prepared and diluted with distilled water (i.e. 1:1000) to give working standard of 100 μ M. Using the working stock standards of 100 μ M, 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, 3.13 μ M and 1.56 μ M concentration were prepared.

5.1.7.2 Estimation of TBARS

Malonyldaldehyde (MDA) is an endogenous genotoxic product of enzymatic & oxygen induced lipid peroxidation.

5.1.7.2.1 Principle:

MDA reacts with Thiobarbituric acid (TBA) under acidic conditions to form an adduct which can be measured colorimetrically (370).

5.1.7.2.2 Method:

- a. 50 μ L of serum sample was diluted with 950 μ L of TRIS- Base buffer (pH- 7.2) and added in amber glass vials containing 250 μ L of 1.22 M ortho-phosphoric acid, 450 μ L distilled water & 250 μ L of TBA reagent.
- b. Tetramethoxypropane (TMP) was used as the standard as it yields equimolar amounts of MDA under the conditions of the assay.
- c. The mixture was incubated at 95°C in a water-bath for 60 min.
- d. The samples were then cooled on ice followed by addition of 360 μ L methanol and 40 μ L of 1M NaOH to neutralize the sample.
- e. Readings were taken on a Multimode Reader at 532 nm.
- f. The standard curve of TMP concentration v/s Absorbance was plotted using which concentration of MDA in serum samples was calculated.

5.1.7.2.3 Standard curve

A standard TMP stock solution of 1.75 μL was diluted with 10 ml of TRIS- Base buffer (pH-7.2) to give a working standard of 20 mM. Using the working stock standards, 1 nmol/ml to 50 nmol/ml concentrations was prepared along with an appropriate reagent blank.

5.1.7.3 C-Reactive Protein

Serum CRP was estimated using the commercially available kit (Tulip Diagnostics).

- a. According to the manufacturer instructions, 3 μL of calibrator and samples with 450 μL of buffer were added to tubes.
- b. Optical density (O.D. 1) of standards, controls and samples was read at 546 nm.
- c. To this 50 μL of CRP antiserum was added; the solutions were mixed and incubated for 5 min at room temperature.
- d. Optical density (O.D 2) of calibrator and samples at 546 nm again measured. O.D. was calculated using (i.e. O.D 2- O.D 1), CRP activity was measured in g/dL.

5.1.7.4 Superoxide Anion (371)

- a. Superoxide anion was measured from PBMCs extracted from the patient's blood using the Nitro Blue Tetrazolium salt to an insoluble blue formazan.
- b. To 100 μL PBMC's (cell count $=1 \times 10^6$ cells), 100 μL of 1mg/ml of NBT solution was added.
- c. The cells were incubated for overnight. Following incubation, the tubes were centrifuged at 1500 RPM for 15 min and the supernatant was discarded.
- d. The pellet was with PBS to remove the excess of medium. The cells were then treated with 120 μL of 2N Potassium hydroxide and 140 μL of DMSO.
- e. The supernatant was measured at 650 nm.

5.1.7.5 Total sulfhydryl groups (Total-SH), Protein bound sulfhydryl groups (Protein-SH) and free sulfhydryl groups (Free-SH)

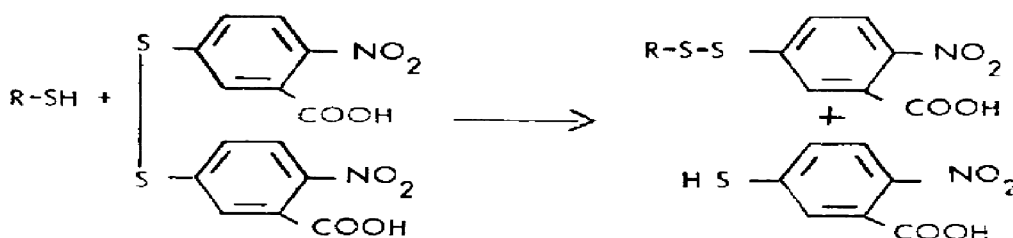
Total-SH, Protein-SH and Free-SH was determined by method described by Ellman and Sedlak and Lindsay (372).

5.1.7.5.1 Principle

DTNB is reduced by thiol groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of thiol. This mercaptobenzoic acid has intense yellow colour which is used to measure thiol groups at 412 nm.

5.1.7.5.2 Reaction

Figure 8: Reaction of DTNB with thiols



Source:

5.1.7.5.3 Procedure

5.1.7.5.3.1 GSH standard

Add 950 μL of TEA to 50 μL of glutathione stock to make it 1mM.

Prepare following concentration series:

Table 4: Dilution table for standard GSH

Concentration (μM)	0	50	100	250	500	1000
GSH (μL)	0	25	50	125	250	500
TEA (μL)	500	475	450	375	250	0

5.1.7.5.3.2 Total-SH

- Add the following components to micro-centrifuge tubes:
 - 50 μL of standard or sample
 - 25 μL of 2mM DTNB
 - 445 μL of PBS
- Vortex mix above components well and centrifuge at 4°C, 15000 rpm for 15minutes.
- Transfer the supernatant to 96 well micro-titre plate and read under multimode reader at 412 nm.

5.1.7.5.3.3 Free-SH / Reduced GSH

- i. Add following components to micro-centrifuge tubes:
 - a. 50 μ L of sample or standard
 - b. 50 μ L of 50%TCA
- ii. Vortex mix above components well and centrifuge at 4°C, 15000 rpm for 15 min.
- iii. Take the supernatant of 50 μ L to this add 50 μ L of 50% TCA
- iv. Vortex mix above components well and centrifuge at 4°C, 15000 rpm for 15 min
- v. Set up the following reaction in 96 well micro-titre plate
 - a. 50 μ L of supernatant from second time TCA precipitation
 - b. 195 μ L of PBS
 - c. 25 μ L of DTNB
- vi. Read the above reaction under multimode reader at 412 nm.

5.1.7.5.3.4 Protein-SH

Calculate the difference between Total-SH and free-SH.

5.1.7.5.3.5 Calculation for total sulfhydryl groups (Total-SH), free sulfhydryl groups (Free-SH) and protein bound sulfhydryl groups (Protein-SH):

1. Estimation of Total-SH or Free-SH (μ M) =
$$\frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{slope of the line (from standard graph)}}$$
2. Estimation of Protein-SH (μ M) = Total-SH – Free-SH

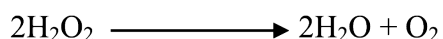
5.1.7.6 Catalase activity

Catalase activity was carried out by the method described by Abei (373).

5.1.7.6.1 Principle

In the UV range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by decrease in absorbance at 240nm ($E_{240} = 40\text{cm}^2/\mu\text{mole}$). The difference in absorbance (ΔE_{240}) per unit time is measure of catalase activity.

5.1.7.6.2 Reaction



5.1.7.6.3 Procedure

- i. Reagents were pipette out as per table given below in respective cuvette.

Reagents	Blank cuvette	Test cuvette
30mM H ₂ O ₂	3 ml	2.99 ml
Sample	--	10 µL

Table 5: Preparation of Catalase assay reaction mixture

- ii. Spectral scan was taken on UV spectrophotometer at 240 nm for 3 min.

5.1.7.6.3.1 *Calculation for Catalase activity:*

1. Calculate average per minute value for samples and blank.
2. Calculate enzyme activity by using formula of :

$$\text{Catalase unit activity (Units/mg of proteins)} = \frac{(A_t - A_s) \times \text{Total volume}}{0.0436 \times \text{Volume of sample in ml}}$$

Where, A_t: Average of sample per minute

A_s: Average of blank per minute

H₂O₂ solution in system: 3 ml

Extinction coefficient of H₂O₂ at 240 nm: 0.0436 µM⁻¹cm⁻¹

5.1.7.7 Glutathione S-transferase activity:

GST activity was carried out by the method described by Habig et al.(374).

5.1.7.7.1 Principle:

The enzyme is assayed by its ability to conjugate GSH and CDNB; the extent of conjugation causing proportionate change in absorbance at 340 nm. One unit of GST activity is given by amount of enzyme producing 1nmole of GSH-CDNB conjugate per minute per ml under the given conditions.

5.1.7.7.2 Procedure:

- i. Blank tube was prepared by adding 2.8ml of phosphate buffer and 100 μ L of 30 mM CDNB while sample test tubes were prepared by adding 2.7 ml of phosphate buffer and 100 μ L of 30 mM CDNB.
- ii. Incubate the blank tube and sample test tubes at 37 °C for 5 min.
- iii. Take the spectral scan of blank tube immediately after 5 min of incubation against plain phosphate buffer for time interval of 5 min.
- iv. Take the spectral scan of sample test tubes were taken by adding 100 μ L of GSH and 100 μ L of sample for time interval of 5 min.

5.1.7.7.3 Calculation for Glutathione S-transferase activity:

- i. Calculate ΔA_{340} of blank and samples.
- ii. Calculate enzyme activity by using formula of:

GST activity (nmole/min/ml) =

$$\frac{\Delta A_{340} \text{ of sample} - \Delta A_{340} \text{ of blank}}{0.0096} \times \frac{\text{Total volume of system}}{\text{Volume of sample used}} \times \text{sample dilution}$$

Where, Extinction coefficient of CDNB at 340nm is $0.0096 \mu\text{M}^{-1} \text{cm}^{-1}$

Sample dilution factor 10

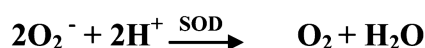
5.1.7.8 Superoxide dismutase activity:

Superoxide dismutase activity was carried out by the method described by Marklund and Marklund (375).

5.1.7.8.1 Principle:

Pyrogallol (1,2,3- benzenetriol) auto-oxidizes very rapidly especially in presence of alkaline solution. The autoxidation primarily involves a free radical chain involving superoxide as chain mediating species. SOD univalently reduces superoxide anion radical.

5.1.7.8.2 Reaction:



5.1.7.8.3 Procedure:

- i. The assay was directly set in disposable cuvettes as per following table:

Reagents	Blank Cuvette	Pyrogallol Cuvette	Test cuvette
TCB	2 ml	2 ml	2 ml
Sample	---	---	250 μ L
Pyrogallol	---	250 μ L	250 μ L

Table 6: Preparation of SOD assay reaction mixture

- ii. The reagents were added in successive order. Reaction was started by addition of pyrogallol at the end.
- iii. The solutions were mixed well with a plastic paddle.
- iv. The rate of auto-oxidation was measured as the incremental difference in A_{420} ($\Delta O/D$) for 3 minutes on UV spectrophotometer.

5.1.7.8.4 Calculation of superoxide dismutase activity:

- i. Unit activity of SOD was calculated by following formula:

$$\text{SOD activity (Units)} = \frac{\Delta OD \text{ of pyrogallol} - \Delta OD \text{ of sample}}{\Delta OD \text{ of pyrogallol}} \times 100$$

- ii. One unit of enzyme corresponds to 50% inhibition of the $\Delta O/D$.

$$\text{Hence, SOD unit activity} = \frac{\text{SOD activity}}{50} \times \text{sample dilution}$$

- iii. Calculate specific activity of enzyme as follows:

$$\text{Specific activity of SOD (Units/mg of proteins)} = \frac{\text{SOD unit activity}}{\text{mg of proteins}}$$

5.1.7.9 Measurement of glutathione peroxidase (GPx) activity:

5.1.7.9.1 Principle:

The main reaction that GPX catalyses is $2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS-SG} + \text{H}_2\text{O}$.

Glutathione reductase then reduces the GS-SG to complete the cycle:



5.1.7.9.2 Methodology:

- i) The glutathione peroxidase activity was measured by the method of Mohandas et al. (1984) (376).

- ii) The reaction mixture consists of 1.44 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml of EDTA (1 mM), 0.1 ml of sodium azide (1.0 mM), 0.05 ml of GR (1 IU / ml), 0.05 ml of GSH (1.0 mM), 0.1 ml of NADPH (0.2 mM), and 0.01 ml of H₂O₂ (0.25 mM) and 0.1 ml Serum sample (10%) in a total volume of 2.0 ml.
- iii) Disappearance of NADPH at 340 nm was recorded.
- iv) The enzyme activity was calculated for 10 min as nmol NADPH oxidized / min / mg protein using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.
- v) Calculation for the activity

$$\text{a. } \frac{\Delta A_{340} \text{ of sample} - \Delta A_{340} \text{ of blank}}{0.00602} \times \frac{\text{Total volume of system}}{\text{Volume of sample used}} \times \text{sample dilution}$$

- vi) Calculate specific activity of enzyme as follows:

$$\text{Specific activity of GPX (Units/mg of proteins)} = \frac{\text{GPX unit activity}}{\text{mg of proteins}}$$

5.1.7.10 Measurement of glutathione reductase (GR) activity

5.1.7.10.1 Principle:

This assay is based on the oxidation of NADPH to NADP⁺ catalyzed by a limiting concentration of glutathione reductase. One GR activity unit is defined as the amount of enzyme catalyzing the reduction of one micromole of GSSG per minute at pH 7.6 and 25°C. One molecule of NADPH is consumed for each molecule of GSSG reduced. Therefore, the reduction of GSSG is determined indirectly by the measurement of the consumption of NADPH, as demonstrated by a decrease in absorbance at 340 nm (A₃₄₀) as a function of time.

5.1.7.10.2 Methodology

- i. The GR activity was measured by the method of Carlberg and Mannervik, (1975) (377).
- ii. The assay mixture consists of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1.0 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml PMS (10%) in a total volume of 2.0 ml.
- iii. The enzyme activity was recorded for 3 min at 25 °C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized / min / mg protein using molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.
- iv. Calculation of the assay

$$v. \quad \frac{\Delta A_{340} \text{ of sample} - \Delta A_{340} \text{ of blank}}{0.00602} \times \frac{\text{Total volume of system}}{\text{Volume of sample used}} \times \text{sample dilution}$$

vi. Calculate specific activity of enzyme as follows:

$$\text{Specific activity of GR (Units/mg of proteins)} = \frac{\text{GR unit activity}}{\text{mg of proteins}}$$

5.1.7.11 Total Serum Proteins

- i. The serum total protein was using Biuret method endpoint using commercially available kits (Transasia Biomedicals Ltd.).
- ii. The assay was carried into tubes containing the 1000 µL of biuret reagent added into Blank, standard and test tubes.
- iii. To this standard 20 µL was added in the tube, while 20 µL of test samples was added. Incubate the tubes at 10 min at 37 °C.
- iv. The absorbance of standard, and each test was measured at 546 nm.
- v. The serum total protein was expressed in g/dL

5.1.7.12 Serum albumin

- i. The serum albumin was measure by the BCG Dye method using the commercially available kit (Transasia Biomedicals Ltd.).
- ii. The assay was carried into tubes containing the 1000 µL of albumin reagent added into Blank, standard and test tubes.
- iii. To this standard 10 µL was added in the tube, while 10 µL of test samples was added. Incubate the tubes at 1 min at 37 °C.
- iv. The absorbance of standard, and each test was measured at 630 nm.
- v. The serum albumin was expressed in g/dL.

5.1.7.13 Serum Globulin

Serum Globulin was calculated using following formula

$$\text{Total globulin} = \text{total protein} - \text{total albumin}$$

5.1.7.14 A/G Ratio

A/G ratio was calculated using the formula concentration of albumin/concentration of globulin.

5.1.8 Cytokine gene expression

For the real time, PCR Primers: the primers for the three cytokines: TNF- α , IL-6, IFN- γ and one house keeping gene GAPDH were purchased from Sigma-Aldrich Company.

Table 7: Primers for Molecular characterization

Sr.no	Primer name	Sequence (5'-3')	Amplicon size (bp)
1	TNF ALPHA (FORWARD)	AGCAGTGAAAGCATGATCCGG	~271
	TNF ALPHA (REVERSE)	CATGGGCTACAGGCTTGTCAC	
2	IL-6 (FORWARD)	TGGTCTTTTGGAGTTTGAGGTA	~346
	IL-6 (REVERSE)	AGGTTTCTGACCAGAAGAAGGA	
3	IFN –GAMMA (FORWARD)	TGAACCACCTGGAGAGGATGT	~655
	IFN-GAMMA (REVERSE)	CATGCCCCCACGCTATTTTG	
4	GAPDH FORWARD	ATC TTC CAG GAG CGA GAT CC	~500
	GAPDH REVERSE	ACC ACT GAC ACG TTG GCA GT	

5.1.8.1 SYBR Real-Time PCR: One-Step SYBR Prime Script RT-PCR Kit II (Perfect Real Time)

The PCR reaction was set as per the manufacturer instructions

The 10 μ L reaction was set using the above kit for all the cytokines and housekeeping gene. The reaction was run on the M/s Applied Biosystems Step One Plus real time PCR instrument (378, 379).

TNF- α Cytokine gene expression –PCR master mix preparation

Reagents	Volume (10 μ L) (1X)	Primer concentration
2X One Step SYBR RT-PCR Buffer 4	5.0	0.6 μM
PrimeScript 1 step Enzyme Mix 2	0.4	
PCR Forward Primer (0.6 μ M)	0.6	
PCR Reverse Primer (0.6 μ M)	0.6	
RNase Free dH ₂ O	1.4	
total RNA	2	

CONDITIONS FOR PCR REACTION TNF- α

STEP -1 Reverse Transcription	42 ° C for 5 min	
	95 ° C for 10 sec	
STEP-2 PCR Reaction	95 ° C FOR 30 SEC	40 CYCLES
	64 ° C FOR 45 SEC	
	72 ° C FOR 45	
Melt curve stage	AS PER THE INSTRUMENT	

IFN- γ PCR REACTION

Reagents	Volume (10 μ L) (1X)	Primer Concentration
2X One Step SYBR RT-PCR Buffer 4	5.0	0.4 μM
PrimeScript 1 step Enzyme Mix 2	0.4	
PCR Forward Primer (0.4 μ M)	0.4	
PCR Reverse Primer (0.4 μ M)	0.4	
RNase Free dH ₂ O	1.8	
total RNA	2	

CONDITIONS FOR PCR REACTION FOR IFN- γ

STEP-1 Reverse Transcription	42 ° C for 5 min	
	95 ° C for 10 sec	
STEP-2 PCR reaction	95 ° C FOR 30 SEC	40 CYCLES
	61 ° C FOR 1 Min	
	72 ° C FOR 1 Min	
Melt curve stage	AS PER THE INSTRUMENT	

GAPDH (HOUSE KEEPING GENE) PCR REACTION

Reagents	Volume (10 μ L) (1X)	Primer Concentration
2X One Step SYBR RT-PCR Buffer 4	5.0	0.2 μM
PrimeScript 1 step Enzyme Mix 2	0.4	
PCR Forward Primer (0.2 μ M)	0.2	
PCR Reverse Primer (0.2 μ M)	0.2	
RNase Free dH ₂ O	2.2	

total RNA	2.0	
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CONDITIONS FOR PCR REACTION FOR GAPDH

STEP -1 Reverse Transcription	42 ° C for 5 min	
	95 ° C for 15 sec	
STEP-2 PCR Reaction	95 ° C FOR 15 sec	40 CYCLES
	55 ° C FOR 1 Min	
	72 ° C FOR 1 Min	
Melt curve stage	AS PER THE INSTRUMENT	

IL-6 GENE PCR REACTION

Reagents	Volume (10 µL) (1X)	Primer concentration
2X One Step SYBR RT-PCR Buffer 4	5.0	0.4 µM
PrimeScript 1 step Enzyme Mix 2	0.4	
PCR Forward Primer (0.4 µM)	0.4	
PCR Reverse Primer (0.4 µM)	0.4	
RNase Free dH2O	1.8	
total RNA	2.0	

CONDITIONS FOR PCR REACTION FOR IL-6

STEP-1 Reverse Transcription	42 ° C for 5 min	
	95 ° C for 15 sec	
STEP-2 PCR reaction	95 ° C FOR 30 sec	40 CYCLES
	59 ° C FOR 1 Min	
	72 ° C FOR 1 Min	
Melt curve stage	AS PER THE INSTRUMENT	

5.1.9 Extraction of cytoplasmic and nuclear proteins (380).

- i. PBMCs Cell) the cell counts were adjusted to the given cell population $1 \times 5 \times 10^6$ cells to extract the cytoplasmic and nuclear protein.
- ii. PBS
- iii. Nuclear Extract (NE) buffer* (HEPES [20 mM] pH 7.9, NaCl [0.4 M], EDTA [1 mM], Glycerol 25%, Protease Inhibitors 1x (add just before use)
- iv. Cytoplasmic Extract (CE) buffer* (HEPES [10 mM] pH 7.9, KCl [10 mM], EDTA [0.1 mM], NP-40 0.3% (add just before use), protease inhibitors 1x (add just before use))

* for 50 mL of buffers:

NE BUFFER	VOLUME	CE BUFFER	VOLUME
1M HEPES	1 ml	1M HEPES	0.5 ml
5 M NaCl	4 ml	2 M KCL	0.25 ml
0.5 M EDTA	100 μ L	0.5 M EDTA	10 μ L
Glycerol	12.5 ml	NP-40	Sufficient for
Protease	Sufficient for	Protease	Sufficient for

B) Procedure

- i. Cell suspension was centrifuged at 14000 rpm for 5 min to remove the supernatant.
- ii. Around 1 ml of ice cold PBS was added and the pellet was resuspended in PBS
- iii. The cells were again Centrifuged at 6000 rpm for 5 min in cold room
- iv. All supernatant was removed without disturbing the cell pellet.
- v. The tubes were kept on ice and 5X more CE buffer was added to the pellet (i.e., if the pellet was 20 μ L, 100 μ L of CE buffer was added to the tube)
- vi. The pellet was resuspended in this CE buffer and incubated on ice for 5 min. the tube was vortexed from time to time

- vii. Centrifugation was done in cold room at 3000 rpm for 5 min.
- viii. The supernatant was harvested (this is the cytoplasmic extract. Note the pellet is not dense)
- ix. Again, the pellet was resuspended in 100 μ L of CE buffer with NP-40
- x. Cells were centrifuged in cold room at 300 rpm for 5 min. supernatant was removed. To obtain the better yield of nuclear extract, washing step no 8 & 9 was again performed.
- xi. An equal volume of NE buffer to this pellet (i.e., if the pellet was 40 μ L, 40 μ L of NE buffer was added)
- xii. The cells were resuspended and incubated on ice for 10 min. vortexed time to time.
- xiii. The cells were again centrifuged at 14000 rpm for 5 min at cold temperature.
- xiv. The supernatant was harvested; this is the nuclear protein extract.
- xv. The extracted protein was stored at -80 °C till further use.

5.1.10 NF- κ B ELISA

The commercially available Human NF- κ B ELISA Kit was used (Cayman Chemicals USA). The nuclear protein Extracted from the patient PBMCs were used to perform the ELISA accordingly to the manufacturer protocol.

Brief assay procedure:

- i. CTFB was prepared as described in the manual.
- ii. 90 μ L CTFB was added per sample well (80 μ L if adding competitor dsDNA), 100 μ L to Blank and NSB wells).
- iii. 10 μ L of competitor dsDNA was added (optional) to appropriate wells.
- iv. 10 μ L of positive control was added to appropriate wells.
- v. 10 μ L of sample containing NF- κ B was added to appropriate wells.
- vi. The Plate was incubated overnight at 4 °C or one hour at room temperature without agitation.
- vii. Washing step was carried out as per the instruction using the 200 μ L of 1X Wash buffer.
- viii. 100 μ L of diluted NF- κ B (p65) Antibody was added per well (except Blank wells).
- ix. Incubate one hour at room temperature without agitation.

- x. Washing step was followed for each well five times with 200 μ L of 1X Wash buffer.
- xi. 100 μ L of diluted secondary antibody was added to the wells (except Blank wells).
- xii. The plate was incubated for one hour at room temperature without agitation.
- xiii. Washing step was followed for each well 5 times with 200 μ L of 1X wash buffer.
- xiv. 100 μ L of developing solution was added per well.
- xv. Plate was incubated for 15 to 45 min with gentle agitation.
- xvi. 100 μ L of stop solution was added per well.
- xvii. Absorbance was measured at 450 nm.

Statistical analysis

All the presented data were statistically analysed using GRAPHPAD PRISM™ 5 software (GraphPad software, Inc. La Jolla, CA). The results obtained in this work were performed in duplicates by identical methods whenever indicated.

For the demographic results were expressed in Mean $\pm$ Standard Deviation (S.D) was used.

Results were expressed as Mean $\pm$ Standard error of mean (S.E.M.) for each group of study groups. 95% confidence interval (CI) was considered while calculating the data. Statistical significance of differences between experimental groups, whenever as indicated, was assessed by ANOVA- Tukey multiple Comparison test- compare selected pairs of columns .In all cases, significant difference was defined as follows: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.0001$ (***).

Differences in the fold change of cytokine gene expression and NF- κ B levels between the cohorts were assessed ANOVA (Kruskal Wallis) test with Dunn's post hoc multiple comparison test. $p < 0.05$ was considered statistically significant.

6 RESULTS

The present study was carried out to study the biochemical /oxidative stress in HIV, TB and HIV +TB co-infected patients. Total 211 samples were included in the study cohorts with 50 patients comprising of each study cohort and controls. 57 HIV infected individuals, 54 TB positive HIV sero –negative individuals, 50 HIV+TB both sero-positive individuals and 50 healthy volunteers.

6.1 AGE DEMOGRAPHY

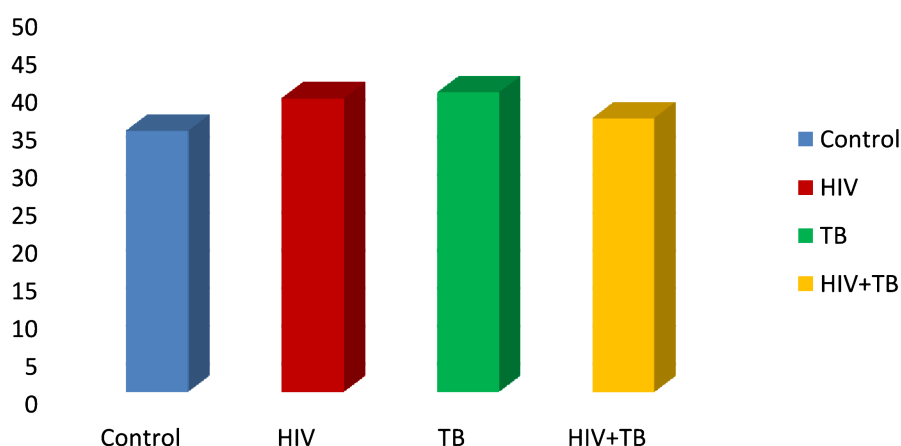
The present study was carried out to study the biochemical /oxidative stress in HIV, TB and HIV +TB co-infected patients. 211 samples were included in the study. 50 patients comprising of each study cohort and controls. 57 HIV patients, 54 TB patients, 50 HIV+TB patients. All the patients enrolled in the study were on the Anti-retroviral therapy and Anti-tubercular therapy. They patients were OPD attendees and some were admitted in the Hospital.

Table 8: Data Result- Age Demography

PARAMETERS	CONTROL (n=50)	HIV (n=57)	TB (n=54)	HIV+TB (n=50*)
AGE ($\pm$ SD)	34.60 $\pm$ 10.46	38.91 $\pm$ 9.09	39.65 $\pm$ 13.62	36.25 $\pm$ 8.76
MEDIAN	32.00	40.00	36.00	35.00
RANGE	21-59	18-59	19-72	23-55

The mean age for control cohorts is 34.60 ± 10.46 , for HIV cohort is 39.91 ± 0.09 , for TB cohort is 39.65 ± 13.62 , HIV+TB cohort 36.25 ± 8.76 respectively. Data is comparable in all four cohorts based on the age demography.

Figure 9: Mean Age of study cohorts across various groups



6.2 Distribution by Sex

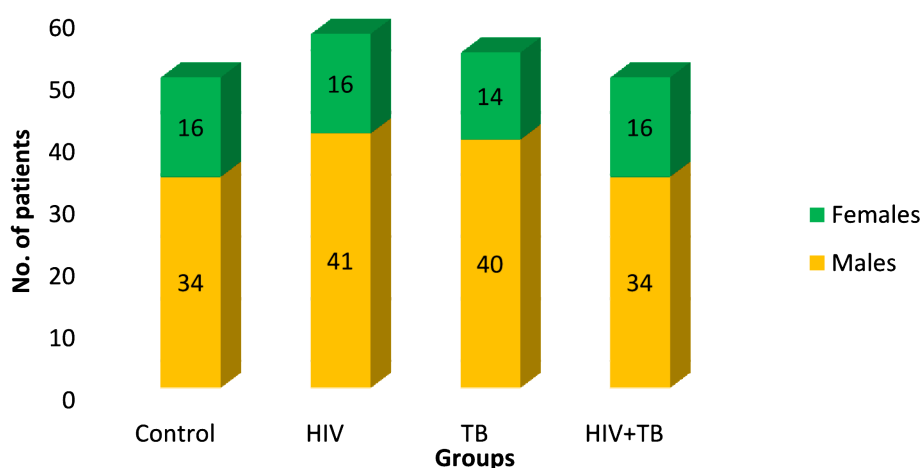
The samples were collected from the patients from both the sexes. The total number of males and females are described below in table -2. Number of males were higher in all the study cohorts, while the number of females were less as compared to males.

Table 9: Data Result- Gender Demography

PARAMETERS	CONTROL (n=50)	HIV (n=57)	TB (n=54)	HIV+TB (n=50*)
MALES	34	41	40	34
FEMALES	16	16	14	16

From the above table the control cohort has 34 males and 16 females, while in HIV cohort 41 males and 16 females, TB cohort 40 males and 14 females, and in HIV+TB 34 males and 16 females respectively. The number of males were higher in all study cohorts. All the data comparable with all study cohort based on gender demography. The Chi-square test for the results showed p -value 0.87.

Figure 10: Sex wise distribution of study cohorts



6.3 Marital status

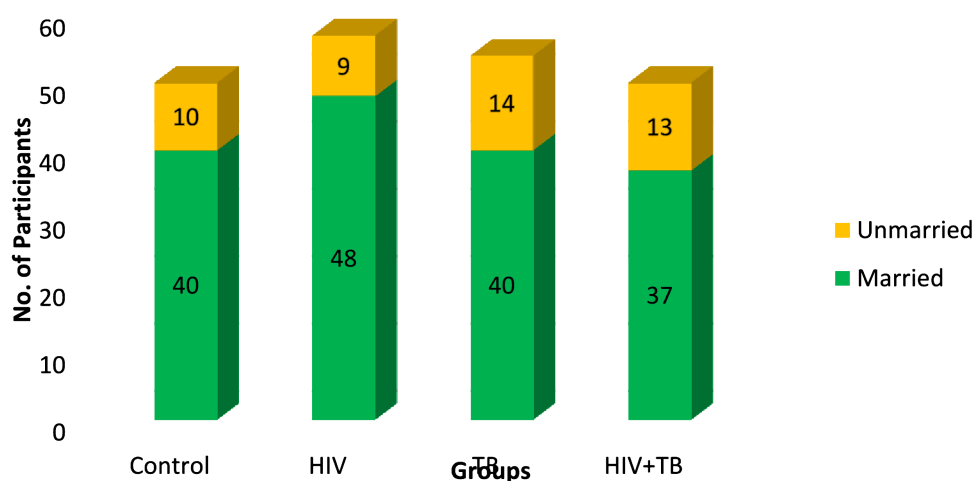
All the patients and control group marital status was taken in while taking the consent during the sample collection day. Most of patients are married in all the study groups. Few of them where unmarried.

Table 10: Data Result- Marital Status

PARAMETERS	CONTROL (n=50)	HIV (n=57)	TB (n=54)	HIV+TB(n=50*)
MARRIED	40	48	40	37
UNMARRIED	10	9	14	13

The number of married individuals were 40 in control, while 48 in HIV cohort, 40 in TB cohort and 37 in HIV+TB cohort. The unmarried individuals were 10 in control cohort, 9 in HIV and 14 and 13 in TB and HIV+TB respectively. The married individuals were more in HIV cohort. All the data is comparable with the all four groups based on the marital status. The chi-square test was applied and it showed the *p* value is 0.50

Figure 11: Martial status of study cohorts



6.4 Age of Onset of disease

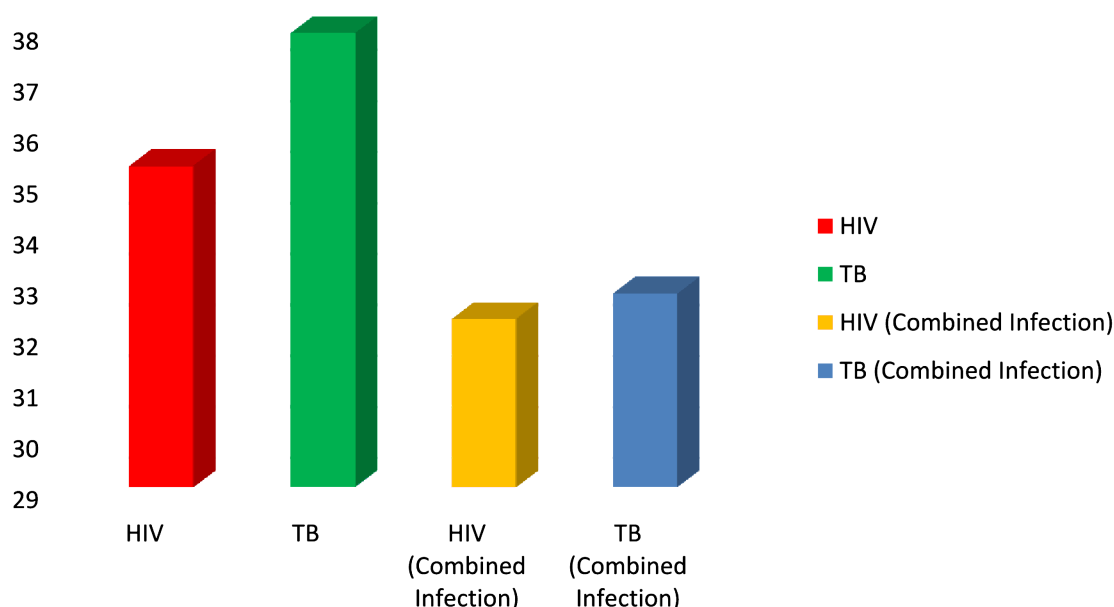
The age of onset of HIV, TB alone and HIV+TB co-infection was calculated using the clinical history from the patients. The median age of onset is described in the table below.

Table 11: Data Result- Age During Onset Of Disease

PARAMETERS	HIV (n=57)	TB (n=54)	HIV+TB (HIV) (n=50)	HIV+TB (TB) (n=50)
AGE ($\pm$ SD)	35.28 $\pm$ 9.22	37.89 $\pm$ 13.49	32.31 $\pm$ 8.15	32.78 $\pm$ 8.12
MEDIAN	37.00	35.00	31.00	31.00
RANGE	10.00-54	18-72	19-53	20-53

The onset of disease age for the all study cohort was done using the date of sample collection and clinical history of patients. The mean age for the onset of HIV disease was found to be 35.28 ± 9.22 , while in TB it was found to be 37.89 ± 13.49 , while in HIV+ TB coinfectd the mean age of HIV infection was found the 32.31 ± 8.15 , TB infection as found 32.78 ± 8.12 . The data was comparable with all the four groups based on the age of onset of disease.

Figure 12: Mean Age of onset of disease of the study cohorts



6.5 Pro-oxidant results

Table 12: Comparison of Mean Nitric oxide (NO) levels in controls & cases

Sr. No.	Groups	Mean NO levels ($\pm$ SEM) (μ M)	95% CI
1	Control	6.44 $\pm$ 0.53	5.38 to 7.51
2	HIV	17.40 $\pm$ 3.18*	11.40 to 23.71
3	TB	3.28 $\pm$ 0.39#	2.5 to 4.07
4	HIV + TB	6.40 $\pm$ 1.37\$	3.66 to 9.13

Legend: Values of Nitric oxide (NO) levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

* Levels of Nitric oxide (NO) in HIV group is significantly higher compared to the HIV +TB, TB and Control group ($p<0.0001$).

\$ Levels of Nitric oxide (NO) in HIV+TB group is significantly higher compared to the TB group ($p<0.0001$).

The mean serum levels of Nitric oxide showed significant increase in the HIV study cohort 17.40 $\pm$ 3.18 μ M when compared with all the study cohorts. The nitric oxide levels in HIV study cohort when compared with HIV+TB and TB showed increased. While the means NO levels in TB cohort were significantly decreased 3.28 $\pm$ 0.39 μ M with respect to control cohort 6.44 $\pm$ 0.53. The inter comparison between HIV and TB study cohort showed increase levels in HIV group while means NO levels in HIV+TB were significantly increased as compared with TB study cohort. All data was comparable in all study groups based on mean Nitric oxide levels.

Figure 13: Comparison of Mean Nitric Oxide levels in controls & cases

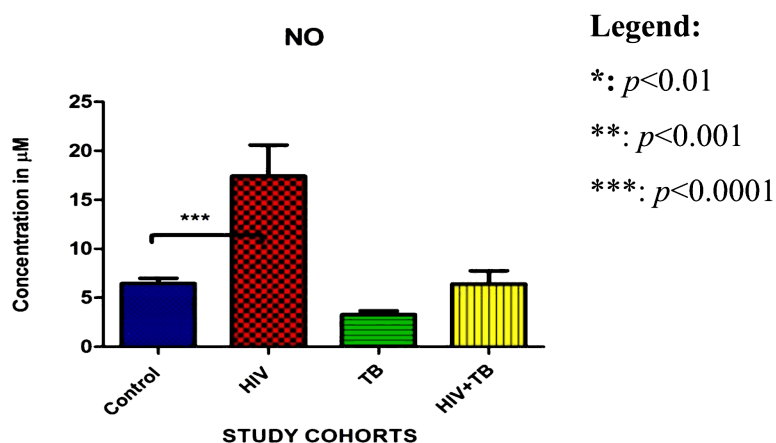


Table 13: Comparison of Mean Serum TBARS levels in controls & cases

Sr. No.	Groups	Mean TBARS levels ($\pm$ SEM)(nmoles/ml)	95% CI
1	Control	0.70 ± 0.06	0.57 to 0.83
2	HIV	$4.7 \pm 0.93^*$	2.92 to 6.53
3	TB	$14.9 \pm 1.44^{**\#\$}$	12.09 to 17.88
4	HIV + TB	$5.9 \pm 1.68^{***}$	3.5 to 8.22

Legend: Values of Serum TBARS levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

*, **, *** Serum TBARS levels in HIV group, TB group & HIV + TB group are significantly higher compared to the control group ($p < 0.0001$).

#, \$ Serum TBARS levels in TB group was significantly higher when compared to HIV group & HIV + TB group ($p < 0.0001$).

Levels of Serum TBARS in HIV group & HIV + TB group are comparable to each other ($p > 0.05$). The mean serum TBARS levels were increased in TB study cohort 14.9 ± 1.44 nmoles/ml when it was compared with respect to control study cohort 0.70 ± 0.06 nmoles/ml. The mean levels in TB group were significantly increased when compared with HIV 4.7 ± 0.93 nmoles/ml and HIV+TB 5.9 ± 1.68 nmoles/ml study cohorts.

Figure 14: Comparison of Mean Serum TBARS levels in controls & cases

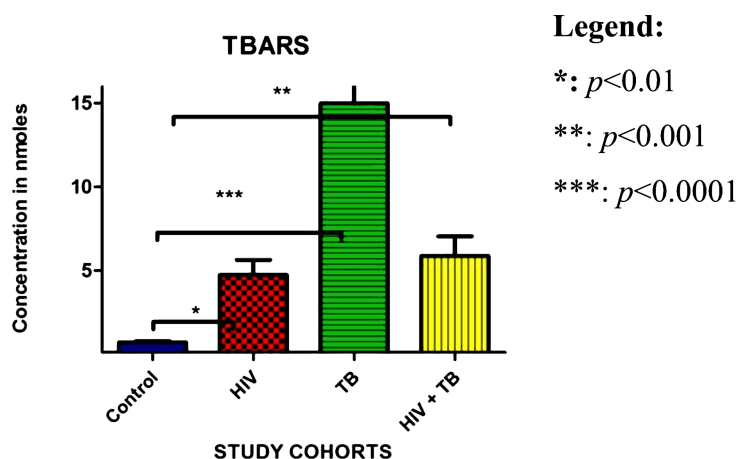


Table 14: Comparison of Mean Serum CRP levels in controls & cases

Sr. No.	Groups	Mean CRP levels ($\pm$ SEM)	95% CI
1	Control	0.14 $\pm$ 0.06	0.01 to 0.28
2	HIV	0.35 $\pm$ 0.09*	0.15 to 0.55
3	TB	3.67 $\pm$ 0.41##	2.84 to 4.49
4	HIV + TB	4.47 $\pm$ 0.63#,\$	3.18 to 5.76

Legend: Values of Serum CRP levels have been expressed as Mean $\pm$ SEM & 95% CI. $p < 0.05$ was considered significant.

#, \$ Serum CRP levels in HIV+ TB group was significantly higher when compared to HIV group & TB group and control groups ($p < 0.0001$).

Levels of Serum CRP in HIV group & HIV + TB group are comparable to each other ($p > 0.05$). The means serum CRP levels in HIV+TB group were significantly increased 4.47 $\pm$ 0.63 mg/dL when compared to control cohort 0.14 $\pm$ 0.06 and TB study cohort 3.67 $\pm$ 0.41 mg/dL. While when compared between study cohorts the CRP levels were increased in HIV+TB when compared with HIV study cohort 0.35 $\pm$ 0.09 mg/dL. While the means CRP levels in TB 3.67 $\pm$ 0.41 mg/dL increased in HIV study cohort.

Figure 15: Comparison of Mean Serum CRP levels in controls & cases

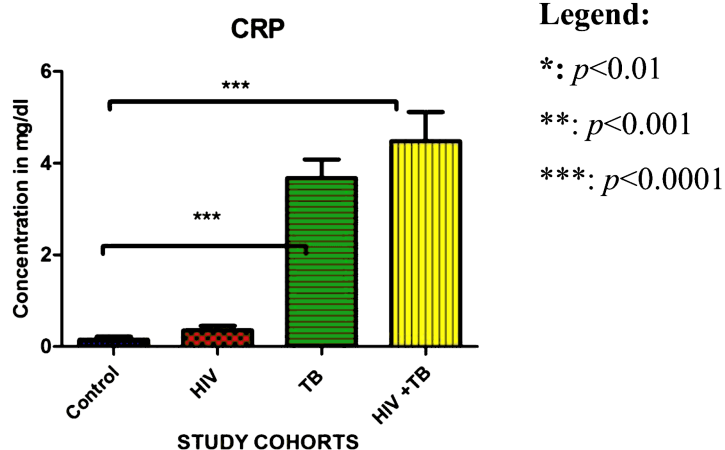


Table 15: Comparison of Mean Superoxide Anion (SOA) levels in controls & cases

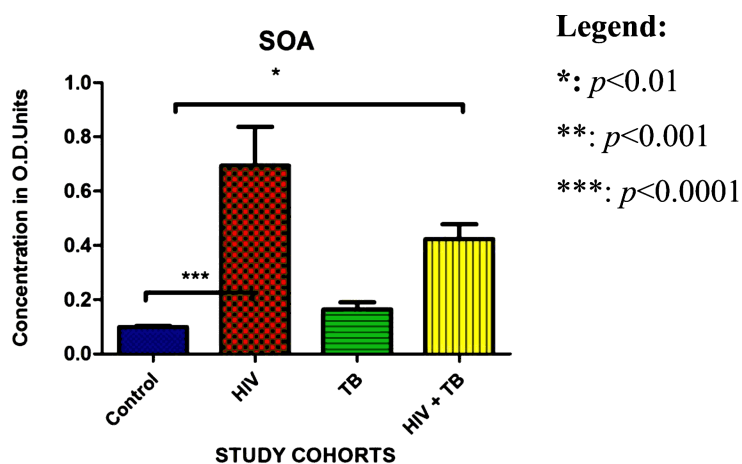
Sr. No.	Groups	Mean SOA levels ($\pm$ SEM)	95% CI
1	Control	0.09 ± 0.003	0.09 to 0.10
2	HIV	$0.69 \pm 0.14^*$	0.40 to 0.98
3	TB	0.16 ± 0.32^s	0.11 to 0.21
4	HIV + TB	0.42 ± 0.05	0.31 ± 0.53

Legend: Values of Superoxide Anion (SOA) levels have been expressed as Mean $\pm$ SEM & 95% CI. $p < 0.05$ was considered significant.

^{*}, ^s Levels of SOA in HIV group is significantly higher compared to the HIV +TB and Control group ($p < 0.0001$).

Levels of SOA in HIV group are significantly higher compared to the TB group. ($p < 0.0001$). The mean SOA levels in HIV group $0.69 \pm 0.14^*$ O/D units were significantly increased with respect to control cohort 0.09 ± 0.003 O/D units, while the levels of HIV+TB cohorts 0.42 ± 0.5 O/D units was significantly increased when compared with control cohort. There was no significance observed when compared between control and TB cohorts. The intra comparison between HIV and Tb cohort showed a significantly increased in HIV cohort.

Figure 16: Comparison of Mean SOA levels in controls & cases



6.6 Antioxidant results

Table 16: Comparison of Mean Serum Total Thiols levels in controls & cases

Sr. No.	Groups	Mean T-SH levels ($\pm$ SEM) (μ M)	95% CI
1	Control	715.2 $\pm$ 21.37	629.6 to 705.5
2	HIV	582.8 $\pm$ 45.42 ^{*, \$}	466.1 to 641.5
3	TB	362.9 $\pm$ 26.92 ^{#\$}	296.2 to 405.7
4	HIV + TB	294.0 $\pm$ 24.63 ^{#, \$}	217.1 to 315.8

Legend: Values of Serum total thiols levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

^{*}, Serum total thiols levels in HIV+TB group are significantly lowered when compared to the Control, HIV and TB group. ($p<0.0001$).

[#], ^{\$} Serum total thiols levels in HIV+ TB group was significantly lower when compared to HIV group & TB group ($p<0.0001$).

In our study, we found out the total thiol decreased significantly in HIV study cohorts 582.8 $\pm$ 45.42 μ M when compared with control cohorts 715.2 $\pm$ 21.37 μ M; while the total thiols levels in TB study cohort were 362.9 $\pm$ 26.92 μ M and in HIV+TB cohorts 294.0 $\pm$ 24.63 μ M were decreased significantly when compared with control cohorts

Figure 17: Comparison of Mean Total Thiols levels in controls & cases

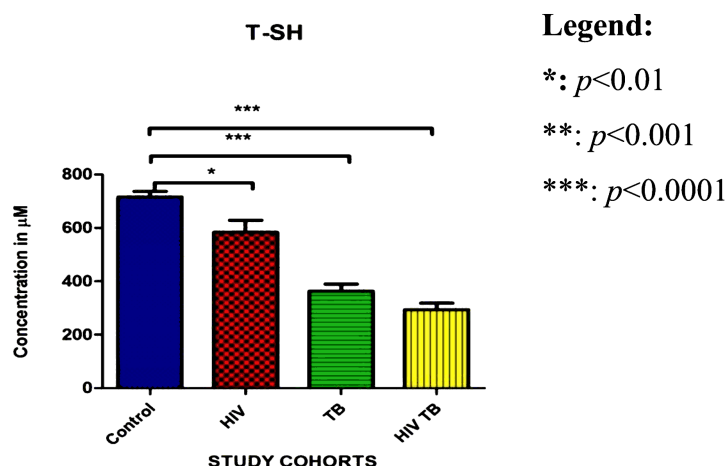


Table 17: Comparison of Mean Serum Free Thiols levels in controls & cases

Sr. No.	Groups	Mean F-SH levels ($\pm$ SEM) (μ M)	95% CI
1	Control	397.7 $\pm$ 6.52	384.5 to 410.8
2	HIV	29.10 $\pm$ 3.25*	22.57 to 35.63
3	TB	11.95 $\pm$ 1.53***	8.87 to 15.02
4	HIV + TB	27.50 $\pm$ 4.51**	18.40 To 36.61

Legend: Values of Serum Free thiols levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

*, Serum free thiols levels in TB group were significantly lowered compared to the Control, HIV and HIV+TB groups ($p<0.0001$).

#, \$ Serum free thiols levels in TB group was significantly lowered when compared to HIV group & HIV + TB group ($p<0.0001$).

In our study, we found out the free thiol decreased significantly in HIV study cohorts 29.10 $\pm$ 3.25 μ M when compared with control cohorts 397.7 $\pm$ 6.52 μ M ; while the total thiols levels in TB study cohort were 11.95 $\pm$ 1.53 μ M and in HIV+TB cohorts 27.50 $\pm$ 4.51 μ M were significantly decreased when compared with control cohorts

Figure 18: Comparison of Mean Free Thiols levels in controls & cases

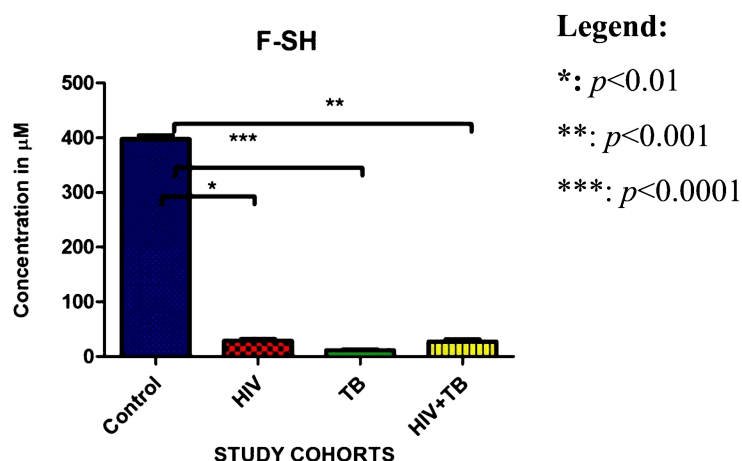


Table 18: Comparison of Mean Serum P-SH levels in controls & cases

Sr. No.	Groups	Mean P-SH levels ($\pm$ SEM) (μ M)	95% CI
1	Control	667.5 $\pm$ 18.88	629.6 to 705.5
2	HIV	553.7 $\pm$ 43.76 ^{*, \$}	466.1 to 641.5
3	TB	350.9 $\pm$ 27.3 ^{# \$}	296.2 to 405.7
4	HIV + TB	266.5 $\pm$ 24.5 ^{#, \$}	217.1 to 315.8

Legend: Values of Serum P-SH levels have been expressed as Mean $\pm$ SEM & 95% CI. *p*-value <0.05 was considered significant.

^{*}, Serum P-SH levels in HIV+B group is significantly lowered compared to the Control, HIV and TB group (*p*<0.0001).

^{#, \$} Serum P-SH levels in HIV group was significantly higher when compared to TB group & HIV + TB group (*p*<0.0001).

In our study, we found out the protein thiol decreased significantly in HIV study cohorts 553.7 $\pm$ 43.76 μ M when compared with control cohorts 667.5 $\pm$ 18.88 μ M ; while the total thiols levels in TB study cohort were 350.9 $\pm$ 27.3 μ M and in HIV+TB cohorts 266.5 $\pm$ 24.5 μ M were significantly decreased when compared with control cohorts

Figure 19: Comparison of Mean Protein Thiols levels in controls & cases

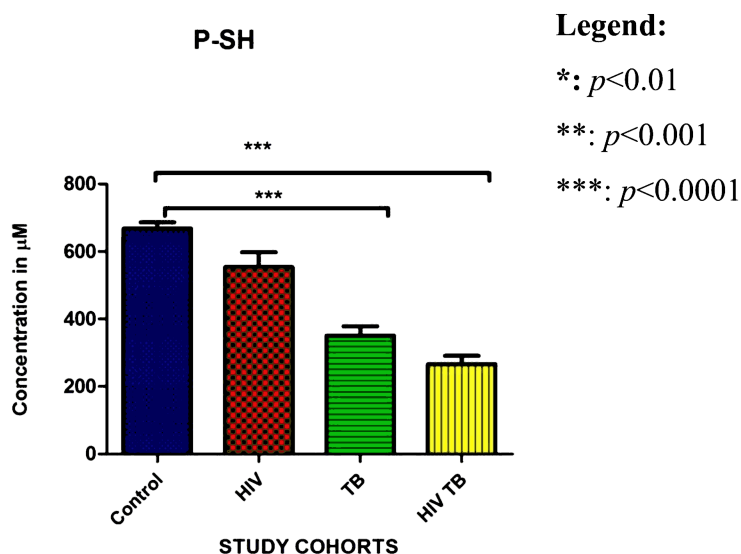


Table 19: Comparison of Mean Serum Catalase activity levels in controls & cases

Sr. No.	Groups	Mean CAT activity ($\pm$ SEM) (U/mg of protein)	95% CI
1	Control	3.01 ± 0.48	2.03 to 3.99
2	HIV	$1.74 \pm 0.27^*$	1.19 to 2.28
3	TB	$1.40 \pm 0.20^{#, \$}$	1.00 to 1.81
4	HIV + TB	2.96 ± 0.40	2.14 to 3.79

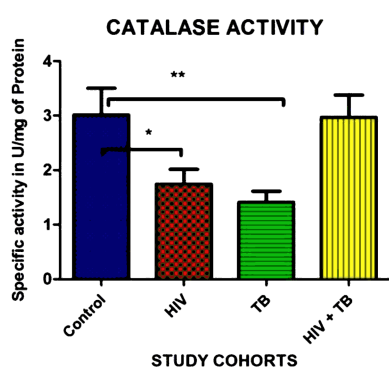
Legend: Values of Serum Catalase activity have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

^{*} Levels of Serum Catalase activity in TB group were significantly lowered compared to the Control, HIV and HIV+TB groups ($p<0.0001$).

^{#, \$} Levels of Serum Catalase activity in TB group was significantly lowered when compared to HIV group & HIV + TB group ($p<0.0001$).

The mean serum Catalase activity showed significantly decrease in HIV study cohort 1.74 ± 0.27 specific activity in U/mg of Protein as well as in TB study cohort 1.40 ± 0.20 specific activity in U/mg of Protein when compared to the control study cohort 3.01 ± 0.48 specific activity in U/mg of Protein. The inter comparison between the TB study cohort showed a significantly decrease 1.40 ± 0.20 specific activity in U/mg of protein while in HIV+TB study cohort it showed 2.96 ± 0.40 specific activity in U/mg of protein.

Figure 20: Comparison of Mean Catalase activity in controls & cases



Legend:

^{*}: $p<0.01$

^{**}: $p<0.001$

^{***}: $p<0.0001$

Table 20: Comparison of Mean Serum Glutathione S-Transferase Activity in controls & cases

Sr. No.	Groups	Mean GST activity ($\pm$ SEM) (U/mg of protein)	95% CI
1	Control	3.67 ± 0.13	3.41 to 3.94
2	HIV	$1.73 \pm 0.12^*$	1.48 to 1.99
3	TB	$1.33 \pm 0.13^{*,\#}$	1.06 to 1.61
4	HIV + TB	2.09 ± 0.23^s	1.62 to 2.56

Legend: Values of Serum GST Activity levels have been expressed as Mean $\pm$ SEM & 95% CI. *P*-value <0.05 was considered significant.

****,\$** Levels of Serum GST Activity levels in HIV+TB, group is significantly higher compared to the TB and HIV group (*p*<0.0001).

The serum GST activity in TB study cohort was significantly decreased 1.33 ± 0.13 Units/mg of protein when compared with control 3.67 ± 0.13 units/mg of protein; and also in HIV study cohort 1.73 ± 0.12 Units/mg of protein, while in HIV+TB cohort the levels were 2.09 ± 0.23 Units/mg of Protein. All the data was comparable with in all four groups.

Figure 21: Comparison of Mean Glutathione S-transferase Activity in controls & cases

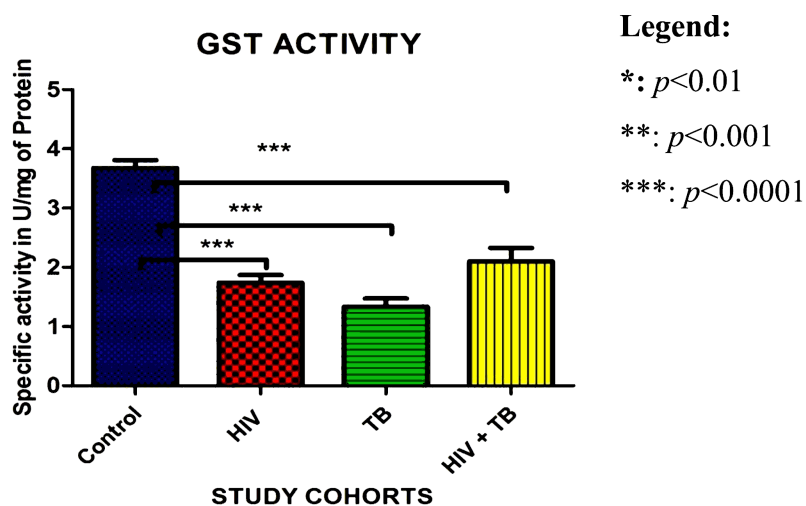


Table 21: Comparison of Mean Serum SOD Activity in controls & cases

Sr. No.	Groups	Mean SOD activity ($\pm$ SEM) (U/mg of protein)	95% CI
1	Control	0.06 ± 0.004	0.06 to 0.07
2	HIV	0.06 ± 0.005	0.05 to 0.07
3	TB	$0.51 \pm 0.16^{**}$	0.18 to 0.84
4	HIV + TB	0.1 ± 0.01	0.08 to 0.15

Legend: Values of Serum SOD Activity levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

*, #, \$ Levels of Serum SOD Activity levels in TB group was significantly higher when compared to HIV group & HIV + TB group ($p < 0.0001$).

Levels of Serum SOD Activity in HIV group & HIV + TB group are comparable to each other ($p > 0.05$).

The serum SOD levels were increased in TB study cohort 0.51 ± 0.16 U/mg of Protein when compared with Control study group, HIV and HIV+TB 0.06 ± 0.005 and 0.1 ± 0.01 U/mg of protein respectively. All the data was comparable between the four study cohorts with each other

Figure 22: Comparison of Mean SOD Activity in controls & cases

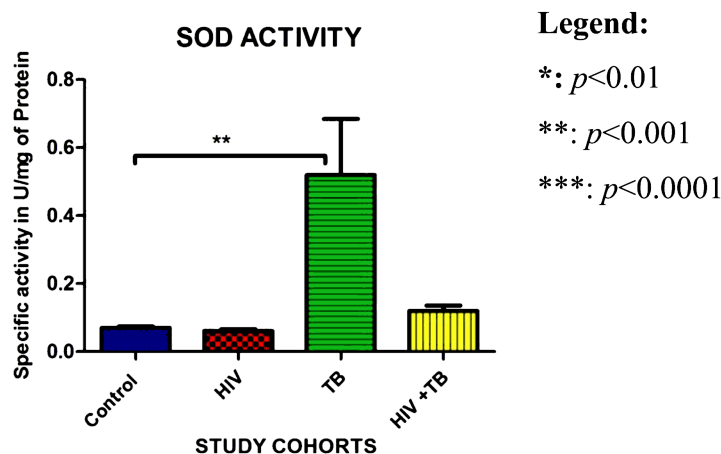


Table 22: Comparison of Mean Serum Glutathione Peroxidase Activity in controls & cases

Sr. No.	Groups	Mean GPX activity ($\pm$ SEM) (U/mg of protein)	95% CI
1	Control	0.068 $\pm$ 0.09	0.05 to 0.08
2	HIV	0.17 $\pm$ 0.04	0.07 to 0.26
3	TB	0.18 $\pm$ 0.07	0.02 to 0.34
4	HIV + TB	0.14 $\pm$ 0.03	0.07 to 0.20

Legend: Values of Serum GPX Activity levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant. The means serum GPX activity was showing no significant increase in either the study cohort compared with controls. The TB study cohort showed elevation but no significance was observed 0.18 ± 0.07 U/mg of protein with respect to control cohort showing 0.068 ± 0.09 U/mg of protein. While HIV+TB showed 0.14 ± 0.03 U/mg of protein.

Figure 23: Comparison of Mean Glutathione Peroxidase in controls & cases

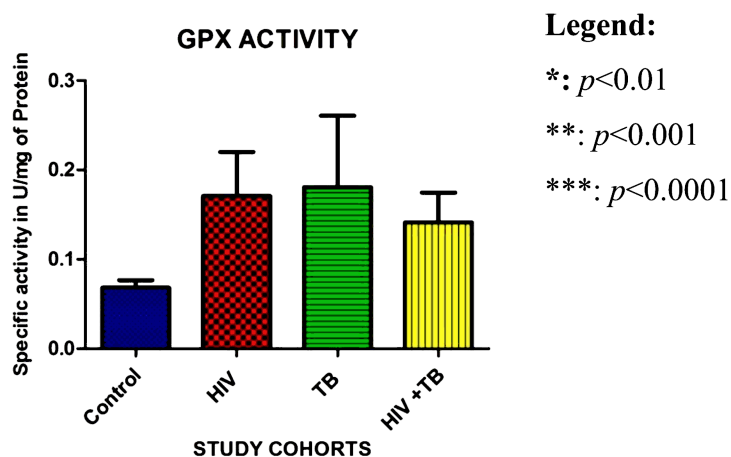


Table 23: Comparison of Mean Serum Glutathione Reductase Activity in controls & cases

Sr. No.	Groups	Mean GR levels ($\pm$ SEM) (U/mg of protein)	95% CI
1	Control	0.38 ± 0.06	0.24 to 0.51
2	HIV	$1.15 \pm 0.28^*$	0.57 to 1.73
3	TB	$0.48 \pm 0.09^\#$	0.28 to 0.67
4	HIV + TB	0.51 ± 0.12	0.26 to 0.76

Legend: Values of Serum Glutathione Reductase Activity levels have been expressed as Mean $\pm$ SEM & 95% CI. p -Value <0.05 was considered significant.

*, Levels of Serum GR Activity levels in HIV group is significantly higher compared to the Control group ($p < 0.0001$).

Levels of Serum GR Activity levels in HIV group is significantly higher compared to the TB group ($p < 0.0001$).

The mean serum GR activity was significantly increase in the HIV cohort 1.15 ± 0.28 U/mg of protein when compared with control 0.38 ± 0.06 U/mg of protein and in TB as well as HIV+TB study cohort 0.48 ± 0.09 U/mg of protein and 0.51 ± 0.12 U/mg of protein. The intercomparing between HIV and TB study cohort there was significant increase in HIV cohort. While no significance was observed in TB and HIV+TB cohort when compared with control cohort.

Figure 24: Comparison of Mean Glutathione Reductase activity in controls & cases

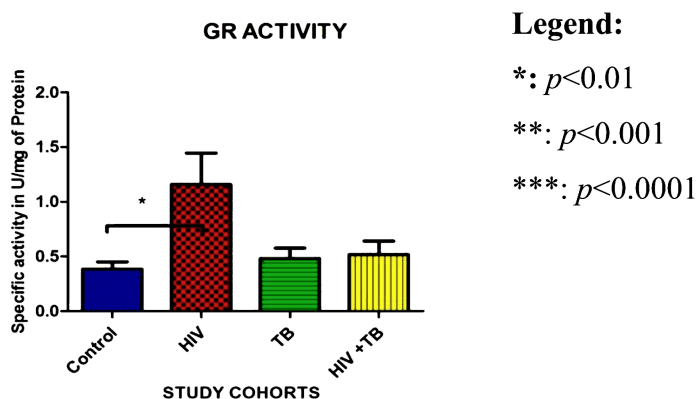


Table 24: Comparison of Mean Serum Protein levels in controls & cases

Sr. No.	Groups	Mean Protein levels ($\pm$ SEM) (mg/dL)	95% CI
1	Control	8.15 $\pm$ 0.14	7.86 to 8.44
2	HIV	12.23 $\pm$ 0.53 ^{*,##}	11.16 to 13.30
3	TB	16.02 $\pm$ 0.47 ^{#**}	15.07 to 16.97
4	HIV + TB	11.07 $\pm$ 0.54 ^{\$}	9.96 to 12.17

Legend: Values of Serum Protein levels have been expressed as Mean $\pm$ SEM & 95% CI. *p*-value <0.05 was considered significant.

^{*,#}, ^{\$} Serum Protein levels in HIV,TB, HIV+TB group is significantly higher compared to the Control group (*p*<0.0001).

^{##} Serum Protein levels in TB group was significantly higher when compared to group HIV+TB (*p*<0.0001).

^{**} Serum Protein levels in TB group was significantly higher when compared to HIV group (*p*<0.0001).

The mean total serum protein was found to significantly increased in TB study cohort 16.02 $\pm$ 0.47 mg/dl when compared to control cohort 8.15 $\pm$ 0.14 mg/dl While there was significantly increased in HIV and HIV+TB study cohort respectively 12.23 $\pm$ 0.53 mg/dl and 11.07 $\pm$ 0.54 mg/dl with respect to control cohort. There was significant increase when compared between HIV and TB study cohort.

Figure 25: Comparison of Mean Serum Protein Levels in controls & cases

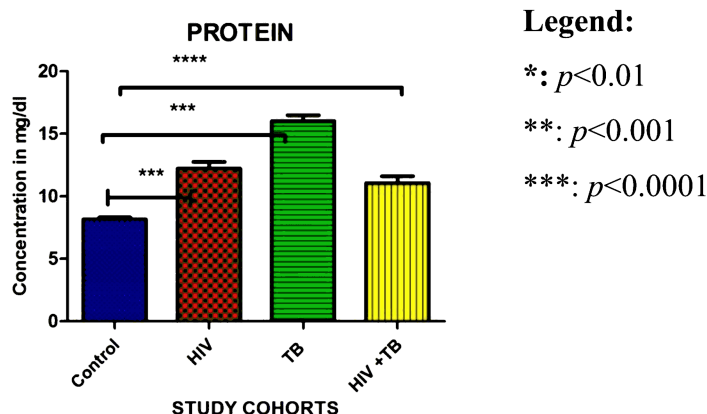


Table 25: Comparison of Mean Serum Albumin levels in controls & cases

Sr. No.	Groups	Mean Albumin levels ($\pm$ SEM) (mg/dL)	95% CI
1	Control	5.03 ± 0.09	4.83 to 5.22
2	HIV	4.55 ± 0.19	4.16 to 4.94
3	TB	$6.2 \pm 0.17^{*,\#,\$}$	5.85 to 6.56
4	HIV + TB	4.64 ± 0.18	4.27 to 5.01

Legend: Values of Serum albumin levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

*, #, \$ Serum albumin levels in TB group is significantly higher compared to the Control, HIV+TB, HIV group ($p<0.0001$).

The mean Serum albumin was significantly increased in the TB study cohort 6.2 ± 0.17 mg/dl when compared with control cohort 5.03 ± 0.09 mg/dl. While there was significant increase in TB cohort when compared with HIV cohort 4.55 ± 0.19 mg/dl and also when compared with HIV+TB study cohort 4.64 ± 0.18 mg/dl.

Figure 26: Comparison of Mean Serum albumin levels in controls & cases

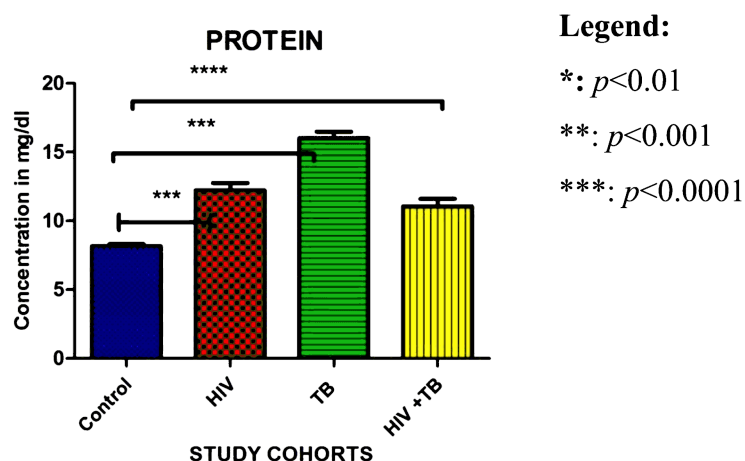


Table 26: Comparison of Mean Serum Globulin levels in controls & cases

Sr. No.	Groups	Mean Serum Globulin levels (±SEM) (mg/dL)	95% CI
1	Control	3.12±0.12	2.87 to 3.37
2	HIV	7.67± 0.37 ^{*,##}	6.91 to 8.43
3	TB	9.63± 0.34 ^{\$\$,#**}	8.93 to 10.33
4	HIV + TB	6.42 ± 0.42 ^{\$}	5.96 to 7.27

Legend: Values of Serum globulin levels have been expressed as Mean $\pm$ SEM & 95% CI. *p*-value <0.05 was considered significant.

*, #, \$ Serum globulin levels in TB group is significantly higher compared to the Control, HIV+TB, HIV group ($p<0.0001$).

The mean serum levels was significantly higher in TB study cohort 9.63 ± 0.34 mg/dl when compared with control cohort 3.12 ± 0.12 mg/dl, HIV study cohort 7.67 ± 0.37 mg/dl and HIV+TB study cohort 6.42 ± 0.42 mg/dl. While the serum globulin levels were increased significantly in TB when compared with HIV and HIV+TB study cohort.

Figure 27: Comparison of Mean Serum globulin levels in controls & cases

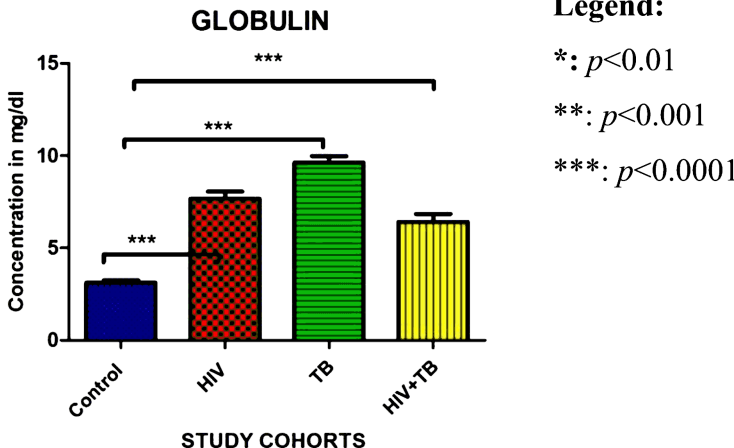


Table 27: Comparison of Mean Serum A/G ratio levels in controls & cases

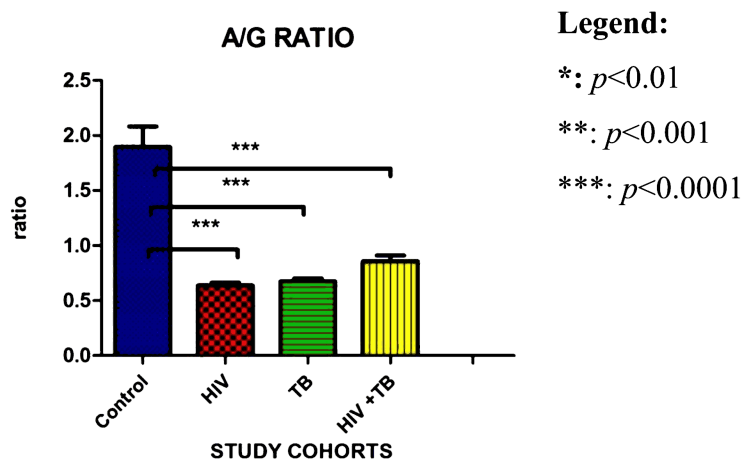
Sr. No.	Groups	Mean A/G levels ($\pm$ SEM)	95% CI
1	Control	1.89 ± 0.18	1.51 to 2.27
2	HIV	$0.63 \pm 0.02^*$	0.57 to 0.69
3	TB	$0.67 \pm 0.02^\#$	0.62 to 0.72
4	HIV + TB	$0.85 \pm 0.05^\$$	0.75 to 0.96

Legend: Values of Serum A/G ratio levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

*#\\$ Serum A/G ratio levels in Control group is significantly higher compared to the HIV, TB HIV+TB group ($p<0.0001$).

The mean serum A/G ratio levels were significantly decreased in HIV study cohort 0.63 ± 0.02 when compared with control study cohort 1.89 ± 0.18 and TB 0.67 ± 0.02 and in HIV+TB study cohort 0.85 ± 0.05 .

Figure 28: Comparison of Mean Serum A/G ratio levels in controls & cases



6.7 TNF- α

Table 28: Comparison of gene expression levels of TNF- α in controls & cases

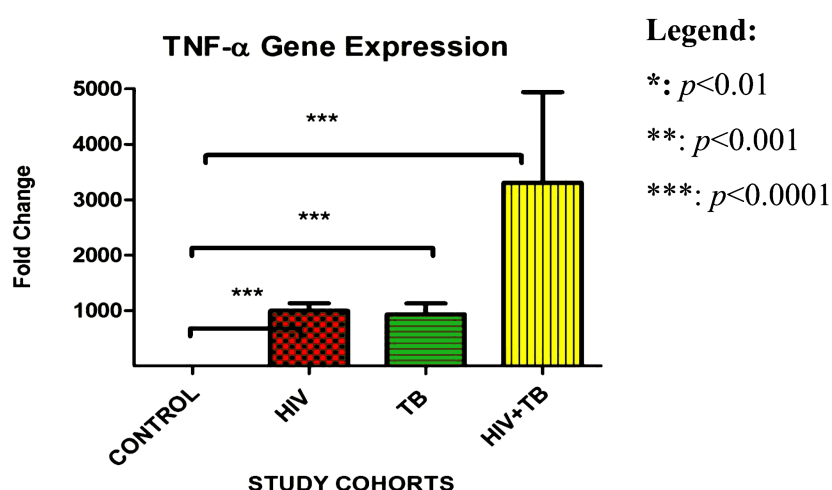
Sr. No.	Groups	Mean mRNA TNF- α levels ($\pm$ SEM)	95% CI
1	Control	1.585 $\pm$ 0.2206	1.13 $\pm$ 2.034
2	HIV	994.4 $\pm$ 135.8*	721.7 $\pm$ 1267
3	TB	931.8 $\pm$ 194.5 [#]	541.6 $\pm$ 1322
4	HIV + TB	3309 $\pm$ 1630 ^{\$}	5.84 $\pm$ 6611

TNF- α mRNA expression levels were expressed in Mean $\pm$ SEM & 95% CI.

\$ TNF- α mRNA expression was found to be higher in HIV+TB study groups when compared with the HIV, TB and control study cohorts.

There was increase in fold change in gene expression in all three study groups when compared with the control cohorts. There was 3309-fold increase of TNF- α gene expression in HIV+TB cohorts when compared with control cohort. Also we found 994 -fold increase of TNF- α gene expression in HIV study cohorts, while the TB study cohort showed 931-fold increase in TNF- α gene expression when compared with control cohorts.

Figure 29: Comparison of gene expression levels of TNF- α in controls & cases



6.8 IFN- γ

Table 29: Comparison of gene expression levels of IFN- γ in controls & cases

Sr. No.	Groups	Mean mRNA IFN- γ levels ($\pm$ SEM)	95% CI
1	Control	0.98 $\pm$ 0.21	0.50 $\pm$ 1.36
2	HIV	6.70 $\pm$ 1.69*	3.32 $\pm$ 10.10
3	TB	11.46 $\pm$ 1.83 [#]	7.71 $\pm$ 15.15
4	HIV + TB	20.16 $\pm$ 6.021 ^{\$}	7.93 $\pm$ 32.35

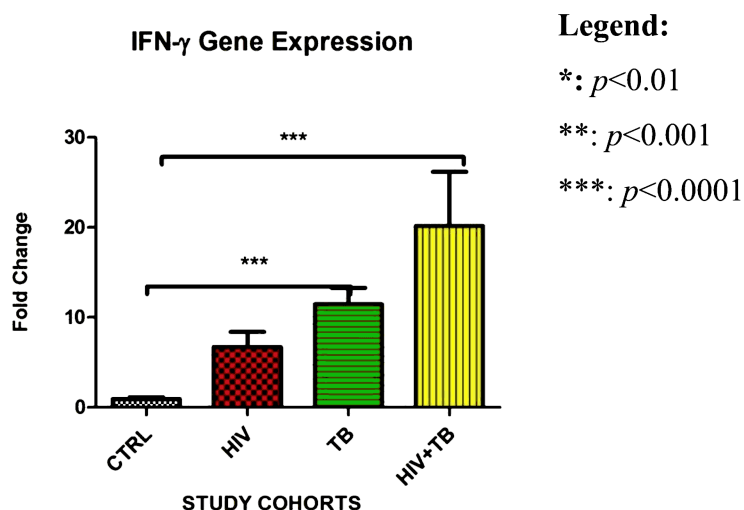
Legend: The IFN- γ mRNA expression value levels have been expressed as Mean $\pm$ SEM & 95% CI. p -value <0.05 was considered significant.

All expression levels were significantly increased when compared with control group.

^{\$}. Levels of IFN- γ in HIV+TB group is significantly higher compared to the HIV, TB and Control group ($p<0.0001$).

There was 20 –fold increase of gene expression in HIV+TB study cohorts, 11-fold increase TB and 6- fold increase in gene expression in HIV study cohort when they were compared with control cohorts.

Figure 30: Comparison of gene expression levels of IFN- γ in controls & cases



6.9 IL-6

Table 30: Comparison of gene expression levels of IL-6 in controls & cases

Sr. No.	Groups	Mean mRNA IL-6 levels ($\pm$ SEM)	95% CI
1	Control	1.240 ± 0.22	0.78 ± 1.69
2	HIV	$43.68 \pm 26.15^*$	-8.80 ± 96.15
3	TB	$758.3 \pm 149.5^\#$	456.4 ± 1060
4	HIV + TB	$784.9 \pm 225.8^\$$	325.4 ± 1244

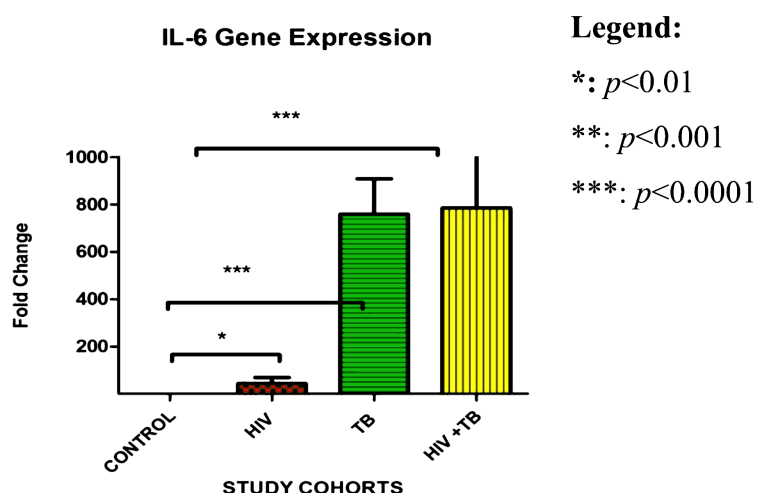
Values of IL-6 gene expression were expressed in Mean $\pm$ SEM and 95% CI.

The mRNA expression of IL-6 was significantly increased in all study cohorts when compared with control cohorts.

$\#$, levels of IL-6 gene expression was increased in HIV+TB study cohorts when compared with TB, HIV and control cohort.

There was 784-fold increase in the gene expression in HIV=TB study cohort when compared with control cohort. Similarly in HIV study cohort there was 43-fold increase in gene expression levels and in TB 758 -fold increase in gene expression when compared with control cohorts

Figure 31: Comparison of gene expression levels of IL-6 in controls & cases



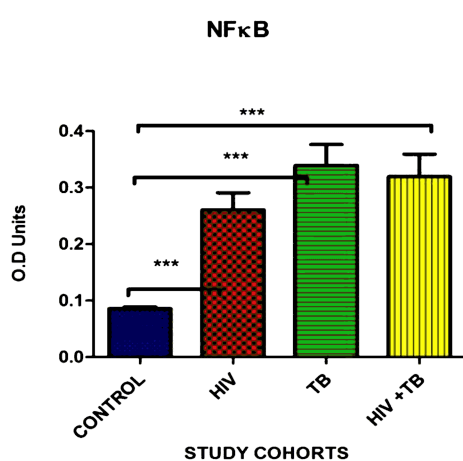
6.10 NF-κB

Table 31: Comparison of NF-κB levels in controls & cases

Sr. No.	Groups	Mean NF-κB levels ($\pm$ SEM) (O.D units)	95% CI
1	Control	0.08 ± 0.00	0.07 ± 0.09
2	HIV	$0.26 \pm 0.03^*$	0.19 ± 0.32
3	TB	$0.33 \pm 0.03^\#$	0.26 ± 0.41
4	HIV + TB	0.31 ± 0.04^s	0.23 ± 0.40

Legend: The activity of NF-κB levels were significantly increased all study cohorts when compared with control cohort. The levels of NF-κB activity were found to be increased in TB study cohort 0.33 ± 0.03 O.D units when compared with HIV 0.26 ± 0.03 O.D units and HIV+TB 0.31 ± 0.04 and control study cohorts.

Figure 32: Comparison of NF-κB levels in controls & cases



Legend:

*: $p < 0.01$

**: $p < 0.001$

***: $p < 0.0001$

7 DISCUSSION

Even after the 36 years of its known existence, the pathophysiology of HIV/AIDS still remains enigmatic. The multifactorial pathological changes which are manifested as a course of the infection, makes HIV/AIDS a multisystemic disease. Blood is a systemic fluid and changes in blood parameters are central to understanding the overall impact of the disease.

One of the key features of most viral infections including the HIV/AIDS is the fact that it causes a long term disease with disruption of redox balance. Redox balance being control to the maintenance of homeostasis in the host if disturb causes pathological outcomes.

During the course of infection of HIV multiple are reported. However the one that stands out is tuberculosis. Tuberculosis is known to mankind since around long time. *Mycobacterium tuberculosis* (Mtb) is one of the most successful human pathogen. Yet it remains a challenge to defeat. The MTB infection has faster rate causing a huge socio economical problem across the globe and pathophysiology is critical to be understand. The long term cause is Redox balance which has affected the human host. Mtb is persistently exposed to numerous oxidoreductive stresses during its pathogenic cycle of infection and transmission. The distinctive ability of Mtb, not only to survive the redox stress manifested by the host but also to use it for synchronizing the metabolic pathways and expression of virulence factors, is central to its success as a pathogen.

The dual infection of HIV and TB is more dangerous leading to the world wide causalities. The viral and bacterial go in synchronising to affect the host and leading to immunological, biochemical system failure and thus leads to death. The global challenge yet remains to control the dual epidemic cause by these two pathogens

Serum Nitric oxide levels in study cohorts

In present study we have assessed the levels of serum nitric oxide in the all study cohorts. The serum Nitric oxide levels in HIV study cohort were significantly increased when compared with control cohort, while in TB study cohort NO levels were non-significantly decreased when compared to control study cohort. And then in HIV+TB cohort the serum nitric oxide levels were comparable with control cohort.

NO is a ubiquitous signalling molecule involved in both physiological and pathological processes. At low concentration, NO exerts vasodilatory and anti-inflammatory effects by contrast the elevated nitric oxide concentrations can exert indirect harmful effects through interactions with superoxide, forming a potent, highly reactive nitrogen species known as peroxynitrite. Higher concentrations of NO are generated by the inducible form of NOS (iNOS) and the expression of this enzyme is increased in HIV infection(381) consequently this might be reflected in increased expression of iNOS is reduced in serum and the PBMCs of HIV infected patients Earlier studies have reported the association between high viral load and increased production of NO in the serum of HIV-infected patients.(382) also in addition to this there is increased NO production in symptomatic and asymptomatic HIV-infected patients (250, 383). Also, HIV infection is associated with the accumulation of NO metabolites in patients with the CNS complications (384). Some studies have shown the persistently high levels of iNOS,nitrite and peroxynitrite are thought to contribute to the etiology of AIDS related dementia and persistent immunosuppression (385) Several studies have shown the role of NO in contributing to the pathogenesis of AIDS by enhancing viral replication in lymphocytes and monocytes (386) by increasing lymphocyte apoptosis (387).

NO has been shown to modulate several immune functions. There have, however, been some contrasting effects attributed to NO. some reports suggests that it upregulates proliferation and increases glucose uptake by T lymphocytes, while other reports have suggested that it inhibits T cell activation (388). HIV infected patients are known to have elevated levels of proinflammatory cytokines, especially tumor necrosis factor (TNF)- α (382) These are powerful activators of HIV replication. (389).Cytokines produced in response to cell activation can stimulate iNOS in an autocrine or paracrine manner.

The significance of NO production in HIV infection has been well established. There is an accumulation of NO metabolites, nitrite and nitrate, in individuals who have neurological complications (390). Nitrite and citrulline levels are elevated among those HIV infected individuals who have no opportunistic infection. There is also evidence to show an association between high levels of viral load and increased NO production in the serum of HIV infected patients (390) Consequently, production of large amounts of NO by macrophages could be a leading cause of lymphocyte inactivation and induction of persistent immunosuppression (391).

The immune activation and enhancement of oxidative stress may be accountable for the interaction and destruction of ingested microorganisms contributing to inflammatory injury of the host tissue. MacMicking *et al* (1997) (392) had demonstrated that among mice with the genetic inability to produce inducible nitric oxide synthase (i-NOS) replication of *Mycobacteria tuberculosis* is faster than in wild type animals. Serum levels of nitrite (NO₂) and nitrate (NO₃⁻) are used to estimate the level of NO formation, since NO is highly unstable and has a very short half-life. All the patients included in our study were on the antitubercular therapy so there would be possibility of the drugs reducing the bacterial load owing to which the lower levels of nitrite is observed. Elevated levels of NO among patients with HIV/TB co-infection have also been reported. The relevance of NO production in HIV infection lies in the ability of the former to modulate the replication of the latter (393, 394). Further, *Mycobacterium tuberculosis* (MTB) enhances the replication of HIV. In the presence of elevated NO production and MTB infection among HIV infected the disease tends to progress more rapidly. It is imperative to treat tuberculosis energetically and remove one of the variables that affect HIV replication. Use of antitubercular medications has been shown to reduce the level of immune activation in the HIV/TB coinfecteds (395). There has been one previous study looking at the level of NO metabolites after chemotherapy in patients with HIV/TB co-infection. In that study untreated HIV positive patients with pulmonary TB did not have increased urinary nitrite/nitrate levels as compared with controls. As opposed to this HIV negative patients with pulmonary TB had higher urinary metabolite levels and when some of them were followed after chemotherapy there was a significant reduction in these levels.

This concludes that the higher levels of NO are found in HIV patients while in TB patients it decreases /increases according to the rate of infection while in the co-infection patients the

TBARS (THIOBARBITURIC ACID REACTIVE SPECIES)

Lipid peroxidation is an indicator of oxidative stress in cells and tissues. Lipid peroxidation in serum or plasma have been measured by a variety of chemical tests and the most frequently used test is the measurement of malondialdehyde (MDA) by thiobarbituric acid (TBA) reaction. In the present study the TBARS were measured as the lipid peroxidation marker. There was a significant increase in the

TBARS levels in TB study cohorts when compared with control and HIV and HIV+TB study cohort. There was an increased TBARS level in HIV cohort when compared with the control cohort. Also the TBARS levels in HIV+TB cohort were significantly increased when compared with control cohorts.

Earlier studies have reported that there is a significant increase in levels of TBARS in HIV patients. Previous studies have shown HIV infected patients are under chronic immune activation which characterized by increased generation of ROS and perturbations of the antioxidant defence system (396)

In early stages of HIV infection a deleterious reductive-oxidative (redox) imbalance may occur this indicates that increased reactive oxygen intermediates are generated at the same time that stores of naturally occurring reducing agents are depleted (397) This leads to the uncontrolled presence of oxygen-containing molecules which may overwhelm the antioxidant capacity and cause damage to cell membranes, protein and nucleic acids and causes the alterations in the intra and inter-cellular environment.

The role of oxidative stress in the pathogenesis of TB suggests lower antioxidant capacity and higher free radical levels in TB patients than in control subjects (398). Significantly increased MDA in TB patients than in non-TB individuals has been previously reported by some authors (399) reported that; increased production of reactive oxygen species / reactive nitrogen intermediates (RNI) secondary to phagocyte respiratory burst occurs in pulmonary TB. Evidence suggest that increased circulating levels (400) of free radical activity are found in pathogenesis of active pulmonary TB and hence play a role in resultant fibrosis . Free radicals have been implicated in the development of lung fibrosis which may be a long term sequel of pulmonary tuberculosis (401). Free radicals attack the cell membrane causing tissue damage and wasting disease in pulmonary TB patients

The fact that, both HIV and TB infections alone produce high MDA(TBARS) value in the present study, which support the role of oxidative stress in the pathogenesis of these both conditions. The correlation between high MDA concentration and low concentrations of the anti-oxidant in this study suggest increased utilization of vitamin E by ROS as documented in earlier study (402). HIV-infected individuals have been reported to show decreased antioxidant concentrations, disruption in glutathione metabolism, and enhanced spontaneous generation of ROS (403). Apart from the HIV/TB induced oxidative stress, malnutrition may have

influenced the anti-oxidant status of the subjects used in this study as reported in a similar study by (Hemilia, 1994)

In this study HIV/TB co-infected cohort showed higher levels when compared to controls and other infected groups may be due to possible interaction between oxidative stress and pathobiology of mycobacterial infections. Mycobacteria are intracellular pathogens that grow and replicate in the host macrophages. In an attempt to kill mycobacterium, host cells namely macrophages, neutrophils and monocytes generate huge amount of reactive oxygen species which contribute to inflammatory injury to host tissues and may further lead to immunosuppression particularly in those with impaired antioxidant capacity such as HIV infected individuals (404). Moreover the malnutrition which is commonly present in patients with tuberculosis can add to the impaired antioxidant capacity in these patients. Therefore TB and HIV infections lead to continuous production of free radicals that may cause a resultant low level of antioxidants.

C-reactive protein in study cohorts

In present study, we assessed the levels of CRP in the study cohorts. The CRP levels were significantly increased in HIV study cohort followed by TB and then followed by HIV+TB study cohort.

CRP is an inflammatory marker whose concentration increases with acute or chronic inflammation. It is produced by the liver in response to stimulation by cytokines such as interleukin-1 beta, interleukin-6, and tumor necrosis factor alpha (405). High-sensitivity measurement of CRP has been shown to add prognostic information at all levels of Framingham risk,(406) but CRP has not been consistently shown to be an independent risk factor across all studies (407). C-reactive protein (CRP) is an acute-phase protein and nonspecific marker of systemic inflammation. The erythrocyte sedimentation rate (ESR) also provides a measure of inflammation, both elevated in the presence of inflammation, although, CRP response appears sooner than the rise in ESR. The CRP level falls to normal if treatment is successful but the ESR levels may still be abnormal for a longer period (408).

CRP levels in TB study cohort were significantly increased when compared with control. The patient enrolled in our study has been on anti-tuberculosis drug therapy for their confirmed diagnosis. CRP has been reported to be significantly elevated in patients with active TB. Higher CRP concentrations have also been

associated with more severe tuberculosis TB and poor prognosis. This observation is supported by reports that patients with more severe lung dysfunction, as evidenced by lung cavitation, had significantly higher CRP levels than patients without cavitation. *Mycobacterium tuberculosis* and its components have been shown to stimulate mononuclear phagocytes *in vitro* to release IL-6, which are regarded as inflammatory mediators. IL-6 is known to induce hepatic acute phase reactants (including CRP). Several clinical and laboratory findings support the significant relation between IL-6 and CRP. Bekker *et al* (409) suggested that the decrease in CRP concentration in patients treated for pulmonary tuberculosis is paralleled by a similar reduction in plasma cytokines such as IL-6, the principal cytokine that induces CRP synthesis, and that the fall in serum CRP concentration during treatment of pulmonary tuberculosis might be correlated with the resolution of the systemic inflammatory process.

CRP also serves as a biomarker for prognosis of HIV positive patients at a high likelihood of TB infection. As some studies have reported that CRP is known to have prognostic values among patients with HIV and HIV associated opportunistic infection (410, 411). There is connection in patients with HIV-associated TB showing a very strong correlation between high CRP concentrations, poor prognostic features and risk of death. CRP synthesis in the liver is immunologically mediated via interleukin-6 (IL-6) production by macrophages (412). Thus, theoretically, high CRP concentrations could arise from an intense immune response, regardless of pathogen load or alternatively might correlate with high mycobacterial load.

Superoxide anion levels in study cohorts.

The cellular superoxide is an indicator for the oxidative stress in the living system. In our study we measured intracellular synthesis SOA in PBMCs of the study cohort. The levels of Superoxide anion in HIV patients has significantly increased when compared with the control cohorts. While in TB study cohort there was increased but not significant when compared with the control cohort. The levels of intracellular SOA were significantly increased in HIV+TB cohorts when compared with the control cohort.

The intracellular SOA which is formed due to oxidative metabolism in cellular processes has also been found to play a significant role in the augmentation of HIV disease. The SOA, has been implicated in the cell to cell transfer of HIV in cell cultures (413). Role of oxidative stress has been pointed out in many

pathophysiological conditions associated with HIV/AIDS(414) have demonstrated that amplification of superoxide generation in cells of mononuclear phagocytic lineage by HIV-1 infection. Moreover role of elevated SOA had been pointed out in the cell to cell transmission of HIV in cultured human cells.

Molecules like SOA directly or indirectly damage many important biomolecule. One of the significant event is lipid peroxidation products such as Malondialdehyde (MDA),4-hydroxy-2-nonenal (4HNE),F2-isoprostanes etc. have been associated with cell and tissue damage and are indicated in conditions such as atherosclerosis, hepatorenal syndrome, *H.pylori* infection (415). MDA in particular has found to be mutagenic and forms DNA adducts. Elevated levels of MDA have been reported in HIV and tuberculosis by (416) This co-relates with the finding in our study that MDA levels are elevated in HIV patients as compared with control cohort. Previous studies have reported that SOA levels in early stage tuberculosis doesn't vary much as compared to healthy controls (417) however chronic tuberculosis leads to increase in neutrophils superoxide levels (418).

In HIV+TB co-infection SOA levels were increased. One study reported that at a higher concentration, nitric oxide combines with superoxide anion to form peroxynitrite (ONOO⁻). The synthesis of even a moderate flux of these molecules will result in the considerable oxidation and potential damage to the host cellular constituents, leading to the dysfunction of cellular processes, interruption of signalling pathways and induction of cell death through apoptosis and necrosis. Also, there could be a possibility of the generation of free radicals in the HIV patients along with TB infection would make the ROS formation more. There is a possible phagocytic burst of macrophages in the TB patients with HIV infection leading to the increased SOA generation.

Thiols in study groups

In our study Protein thiols, Total thiols, and free thiols were studied. The protein thiols levels in HIV study cohort were found to be non-significant decrease while TB and HIV+TB cohort there was significant decrease when compared with the control cohort. Also the total thiols levels in all three study cohorts where found to be significantly decreased when compared with control cohorts. The Free-thiols levels were decreased significantly when compared with control cohorts

Reduced Glutathione is central molecule for maintaining the redox homeostasis in cells. Decreased levels of G-SH has been associated with the many a conditions of Redox stress. Increased Oxidative stress consumes the GSH in orders to quench the increased pro-oxidants

Reduced glutathione is the most prevalent non-protein thiol in animal cells. Its de novo and salvage synthesis serves to maintain a reduced cellular environment and the tripeptide is a co-factor for many cytoplasmic enzymes and may also act as an important post-translational modification in a number of cellular proteins. The cysteine thiol acts as a nucleophile in reactions with both exogenous and endogenous electrophilic species. As a consequence, ROS are frequently targeted by GSH in both spontaneous and catalytic reactions. Since ROS have defined roles in cell signalling events as well as in human disease pathologies, an imbalance in expression of GSH and associated enzymes has been implicated in a variety of circumstances.

GSH levels have an impact on many immune functions, including activation of lymphocytes. Consequently, it was postulated that GSH deficiency could lead to the progression of immune dysfunction, a hallmark of AIDS. GSH levels are depleted in plasma, epithelial lining fluid, peripheral blood mononuclear cells and monocytes in asymptomatic HIV-infected individuals and in AIDS patients. *In vitro* have shown that low GSH levels both promote HIV expression and impair T cell function suggested a link between GSH depletion and HIV disease progression.

Human immunodeficiency virus itself plays an important role in activation of free radical oxidation and LPO and in pathogenesis of impaired activity of antioxidant enzymes For example, in reported studies (419) it has been found that highly specific immune antibody-antigen interaction in vitro is accompanied by significant imbalance in the thiol disulfide system. These reactions caused reduction of low molecular disulfides (both symmetric and mixed ones) accompanied by release of low molecular thiols (R-SH forms)

These processes may be associated with activation of free radical oxidation and lipid peroxidation (LPO) here in our case we measured TBARS as a part of lipid peroxidation product and also impairments in antioxidant enzymes observed in HIV infection. First, peroxide processes involve non enzymatic redox reactions recruiting reduced forms of low molecular thiols (420). It should be noted that low molecular thiols regulated free radical processes due to their “paradoxical” capacity to act under certain conditions as pro- and antioxidants. Second, antioxidant enzymes belong to

the group of thiol enzymes or enzymes requiring presence of thiols for manifestation of their catalytic activity (SOD, catalase, GPx, GR, etc.) (421). These enzymes actively use free glutathione –SH groups for catalysis. Glutathione is also utilized in redox reactions as the supplier of –SH groups, which protect cells against hydroxyl radical and other reactive oxygen species (422).

Several factors such as low food intake, nutrient malabsorption and inadequate nutrient release from the liver, acute infection and inadequate availability of carrier molecules may influence circulating antioxidant concentrations. Lower concentration of antioxidants suggest increased utilization by reactive oxygen species (ROS) as an important contributing factor to the lower concentration of anti-oxidants in TB (422). Reduced glutathione levels indicate the potential of oxidative damage to erythrocyte and erythrocyte membrane of pulmonary TB patients. GSH levels were decreased in patients with HIV–1 infection, and this decreased GSH causes increased risk of developing tuberculosis in HIV infected patients. This decrease would be associated with reduced capacity of monocytes to kill intracellular M.TB (420). These thiols represent the major intracellular redox buffering compounds.

Free radicals have been implicated in the development of lung fibrosis which may be a long term sequel of pulmonary tuberculosis (401). Oxidative stress could play a role in the pathogenesis of antitubercular drug (ATD) induced hepatotoxicity and lower levels of plasma glutathione and higher levels of MDA may be due to oxidative stress-resulting from ATD therapy (423).

Co-infection of tuberculosis in HIV positive individuals exacerbates the synthesis of free radicals. This has been showed by multiple studies including this study. Serum glutathione (P-SH, T-SH, and F-SH) were therefore found be the lowest in individuals co-manifesting HIV and TB diseases.

Catalase activity in study cohorts

In our study we studied the antioxidant enzymatic and non-enzymatic assay to see the level of oxidative damage in all study cohorts. To defend the oxidative stress cell have enzymatic anti-oxidants like SOD, G-PX, Catalase.

In present study we found out the catalase activity in HIV and TB study cohort are significantly decreased when compared with the control cohort. While in there was a non-significant decrease in catalase activity in HIV+TB study cohort when compared with control cohorts.

From our own previously studies where we found there is decrease in the catalase activity in HIV population when they were categorized on their CD 4 counts. The decrease in catalase activities in HIV infected patient's accounts for the production of high reactive oxygen species suggested that increased oxidative stress might be attributed to the deficiency of antioxidant defense system. This deficiency in HIV infected patients may be due to increased utilization of catalase.

Human monocyte-derived macrophages are involved in a variety of pathological events in HIV infection, the hallmark of which is immunodeficiency with progressive CD4⁺ T lymphocyte depletion. HIV infections cause a chronic inflammation as shown by high plasma levels of inflammatory cytokines and production of ROS in seropositive individuals.

Catalase is an enzyme present in animal tissue, characterized by its power to decompose hydrogen peroxide into water and oxygen. Catalase is constitutively expressed especially in type II pneumocytes along with SOD in the alveolar regions suggesting their role protective role against damage caused by free radicals (424, 425). In our study there was decreased activity of catalase suggesting that mycobacteria are resistant to hydrogen peroxide and organic peroxide because mycobacteria also release catalase which neutralizes the hydrogen peroxide and hence increase the infectivity of mycobacteria especially in immunocompromised patients. Low levels of this enzyme may be due to heavy load of free radicals which damage the membranes leading to oxidative stress. Also malnutrition due to lower BMI may enhance the oxidative stress.

One study has reported that the mycobacteria are resistant to hydrogen peroxide. In co-infection patients where there is amount of oxidative stress generated due to the free radical generation.

The correlation between high MDA concentrations and low concentrations of some anti-oxidants suggests increased utilization by ROS as an important contributing factor to the lower concentrations of anti-oxidants. HIV-infected individuals have been reported to show decreased antioxidant concentrations, disruption in glutathione metabolism, and enhanced spontaneous generation of ROS (426). Apart from the HIV-TB induced oxidative stress, malnutrition may also influence anti-oxidant status and oxidative stress in patients (427).

This observation is an indication of the level of depletion of the host immune system that is often associated with HIV infection. Hematological abnormalities are among the most common complications of infection with HIV(428).

Glutathione S-transferase Activity in Study cohorts

In present study the GST activity was significantly reduced in HIV, TB and HIV+TB study cohorts when compared with control cohorts.

GSTs can be induced by structurally unrelated compounds known to result in chemical stress and carcinogenesis including, phenobarbital, planar aromatic compounds, ethoxyquin, and *trans*-stilbene oxide. Some of the compounds known to induce GSTs are themselves substrates of the enzyme, suggesting that induction is an adaptive response mechanism.

The decreased activity might be due low levels of GSH and as GSH is substrate for the conjugation reactions which are catalysed by GST (429).

These results are evidently indicative of oxidative stress in the HIV sero-positive study population. However, apart from the HIV-host interactions, opportunistic infections also have considerable role to play in the pathogenesis and hence the prognosis of HIV disease (430).

In case of TB patients the GST activity has significantly decreased. The results correlate with the previous studies where the GST activity was decreased. This clearly indicates that there is oxidative stress and the antioxidant enzymes are lowered down. All the patients included in our study were on the anti-tubercular therapy; which can help to control infection rate and helps in curing of the disease. Also it is seen that the lipid peroxidation in the TB study cohort has remarkably increased this support the oxidative stress in the patient's cohorts. Also the glutathione metabolism has affected due to TB infection. The lowering GST value is good marker for the detoxification of toxic products from the body.

SOD Activity in Study cohorts

In present study the SOD activity was assessed in all study cohorts. We found that in HIV study cohort SOD activity was decreased but not significantly. While in TB study cohort the SOD activity was significantly increased and then in HIV+TB study cohort, there was increased activity when compared with the control cohorts.

Superoxide dismutase, is an antioxidant enzyme that dismutase the O_2^- anion to form O_2 and H_2O_2 . Animal cells contain two intracellular forms of SOD: the cytoplasmic or copper-zinc form (Cu-Zn SOD) and the mitochondrial or manganese form (Mn-SOD). This enzyme is the first line of defence against superoxide anion radicals.

The oxidative stress in major pathological condition found in the HIV patients leading to the lowered antioxidant enzymes. The various other studies have reported that the SOD levels are lowered due to release of free radicals. SOD scavenges the SOA via a dismutation reaction leading to the production of hydrogen peroxide. Previous studies showed that there were lowered levels of SOD in peripheral blood cells isolated from children infected with HIV(431). Pasupathi et al. showed the lower levels of HIV and AIDS patients(175). The HIV Tat protein has been shown to down regulate synthesis and override the induction of manganese-dependent form of SOD which is induced by oxidative stress in cultured cells.

In HIV/AIDS infected patients, the autooxidation results in the formation of hydrogen peroxide, which inactivates SOD. Therefore, the accumulation of hydrogen peroxide may be one of the explanations for decreased activity of SOD in these patients. The primary catalytic cellular defence that protects cells and tissues against potentially destructive reactions of superoxide radicals and their derivatives is the Cu/Zn-SOD. The co-relation between thiols and SOD was also stated in past studies indicating that auto-oxidation of thiols leads to formation of superoxide anion.

SOD activity observed in our study is contradictory to the most data available in this domain. This increase in SOD activity may however contribute to an increase in oxidative stress levels which in the event of significantly decreased catalase activity in the TB study cohort will build up the hydroxy radical $\cdot OH$. The excessive $\cdot OH$ radical generation gives rise to the peroxidation by products including MDA, 4-HNE etc. Thus, an increase in SOD activity and unchanged/decreased, catalase activity will be detrimental for maintaining for the redox balance of the cell.

The other studies showed decrease in their SOD activity with respect to TB study cohort. One of the study done in lungs cancer (432) showed decrease in the SOD activity as well as increase in Serum MDA levels. The serum MDA levels in our study are also significantly increased. The lipid peroxidation used as the marker for the oxidative stress. It also states the higher is ROS can cause detrimental effect on the individuals damaging the defense mechanisms.

An increase in activity of SOD is contradictory to the most data available in this domain. This increase in SOD activity may however contribute to an increase in oxidative stress levels which in the event of an unchanged catalase activity in the HIV+TB group and significantly decreased activity in the TB study cohort will build up the hydroxyl radical $\cdot\text{OH}$. The excessive $\cdot\text{OH}$ radical generation gives rise to the peroxidation by products including MDA, 4-HNE etc. Thus, an increase in SOD activity and unchanged/decreased, catalase activity will be detrimental for maintaining for the redox balance of the cell (433).

As described in case of HIV and TB alone the SOD activity in HIV+TB co-infection cohort showed increased. This may be due to the oxidative stress levels increased particularly catalase activity which in turns the SOD activity. The dual infection may help to exacerbate the free radical generation and thus utilizes the antioxidant system to protect itself from any damage.

Glutathione activity in Study cohorts

In present study the GPX activity was found to be increased non-significantly in all three study cohorts HIV, TB and HIV+TB co-infection study cohort when compared with control cohorts. The other studies have shown that there are increased values of glutathione peroxidase in HIV infected individuals.

Glutathione peroxidase essentially utilizes glutathione as a reductant in disposing the free radicals. It is a metallozyme associated with selenium as a metal prosthetic group. Glutathione peroxidase activity spares the requirement for vitamin E in the cytosol GPx is a selenoprotein consisting of four subunits, each containing a selenium atom (434). The importance of selenium and selenoproteins to human health has been well documented.

This explain that HIV infection causes increased oxidative stress during erythropoiesis, which, in turn, increases the level of GPX-1 expression, the predominant form of the enzyme found in erythrocytes. There is such hypothesis that the consistent higher concentration of plasma catalase (429) and erythrocytes superoxide dismutase (427) have also been seen during HIV infection. Also some study have shown that increased in the GPX activity in whole blood could be due to a presumed knowledge that HIV encoded GPX are expressed in HIV infected cells (435) but there is no such evidence which proves that in vivo expression of such enzyme and explains this phenomenon.

Higher GPX activity was associated with use of ART in HIV positive subjects. In our study all the HIV patients were on the ART therapy. One other study has reported a similar observation (436). ART use has also been associated with mitochondrial toxicity and cellular oxidative stress in cell culture (437) and with increased oxidative stress (i.e., higher plasma F2 isoprostane concentrations) in patients (430). On the other hand, decreased Malondialdehyde concentrations have also been seen after the initiation of ART in patients(438).These different results could be due to differences in the stage of HIV disease and the level of oxidative stress at the time of initiation of ART.

In case of TB patients we found the increased activity of GPX. Our result contradicts with some of the studies done in patients with TB and rifampicin resistant TB. In this study the GPX levels were significantly reduced. This is due to the possible factors such as depletion of antioxidant status happening due to inflammatory process of disease, lowered nutritional intake due and poor socio-economic circumstances of the patients (439).

Our result contradicts with the earlier studies done with the HIV+TB co-infection subjects where they found lowered levels of GPX activity. The SOD and GPX levels are generally lowered in the HIV+TB study groups due the free radical activity increased leading to the oxidative stress (440). One of study safarian et al(441) showed there is increase in the SOD and GPX activity showing the combined deficiency of these antioxidants may markedly increase oxidative stress, possibly adversely affecting the immune response and predisposition to drug toxicity. All the patients in the study are enrolled on the ART and AKT therapy which may improve their immune status.

Glutathione Reductase activity in study cohorts

In present study,the glutathione reductase activity in were found to be significantly increased in HIV study cohort while in TB and HIV+TB co-infection cohort there was non-significant increase when compared with control cohorts Glutathione is major molecule which is important in all physiological process. The Glutathione reductase is an important antioxidant enzymes required in oxidative stress pathways (442). Our results contradict with the some studies whereas they have got the decrease in the GR activity in the HIV cohort.

Since these antioxidant enzymes have activity to fight against the free radicals generated due to the oxidative stress. The various studies have reported that decrease in CD 4⁺ counts is associated with the decrease in the glutathione reductase activity (443, 444).

Our results contradicts the previous studies where they have found the GR levels in the TB patients lowered due to the antioxidant mechanism helping to clear the free radicals. Also the patients enrolled where on the ATT which can also have some effects of the metabolism of liver and generally leading to the toxicity in them. These elevated levels could be due to increasing the proinflammatory cytokines which are increased in TB patients.

We found out there were less studies in carried out in the HIV+TB co-infection. Although the glutathione metabolism is important in these patients where decreased glutathione levels are associated with the N-acetyl cysteine

Serum total Protein in Study cohorts.

Total serum proteins were analysed for all the study cohorts. we had analysed the serum albumin, globulin and A/G ratio for same study cohort. There was significant increase in levels serum total protein in all study cohorts when compared with control cohorts

Some studies have reported that there is a significant increase in serum protein levels in HIV patients suggesting all HIV patients who are on HAART therapy respond to the therapy and thus elevates the biochemical parameters (445). Since the total serum protein increased could be lead to muscle wasting syndrome in HIV patients were commonly seen. Also the oxidative stress is more important which leads to hyperproteinemia in the HIV patients.

In HIV patients there could be protein catabolism happening which is also lead to accumulation of systemic inflammation and leading to the higher levels of protein (446).

The levels of serum protein in TB study patients were very high indicating the hyperproteinemia in the study cohort (447). The results contradict the some studies where the serum protein levels are relatively lowered down as compared with the control study cohort (448).

The serum protein levels in HIV+TB co-infection have significantly increased when compared with the control cohort. Various studies have reported that there is decrease found in the patients

Serum Albumin levels in study groups

In our study we found serum albumin levels were showing a non-significant decrease in HIV study cohort. While in TB study cohort there was significant increase in serum albumin levels and in HIV+TB cohort there was decrease albumin levels when compared with control cohort.

The serum albumin levels are reported to be used as prognostic markers in HIV disease

The presence of an acute phase response among HIV-infected individuals is typically associated with a decrease in serum albumin and other plasma proteins (412). The underlying reason could be an associated fatty change of liver due to poor nutrition, loss of microproteins in the urine, and associated drug toxicities

Another possible explanation could be the low levels of albumin associated with chronic inflammation. Albumin is a negative acute-phase reactive protein (APP) and the level of albumin is depressed due to elevated cytokine levels (viz. tumor necrosis factor and interleukin 1, during chronic inflammation) during HIV infection. Further evidence for this hypothesis comes from other studies that have suggested that other APPs, including C-reactive protein are also associated with HIV disease progression (449). Several studies have suggested that albumin could be used as a surrogate marker of disease progression among HIV-infected individuals (450). Recent studies have shown that serum albumin level could increase in patients receiving HAART and might be used as a better prognostic indicator of clinical outcome. Previous longitudinal studies have shown an independent effect of albumin measured shortly after HIV seroconversion and could account for considerable mortality and (451) of lower albumin levels in the HIV patients.

In TB patients our results are contradictory to the literature found where the serum albumin is found to be decreased in TB patients (447).

One of the reasons for elevated levels of albumin in the TB patients would be due to their anti-tubercular drugs therapy. All the patients were on the AKT therapy, so due to drugs there is a possibility that these drugs can improve the immune status gain the increase in the albumin levels.

One study reported that there is decrease in the levels of albumin in the patients' population stating the reason. The possible causes for the low serum zinc and albumin in PTB patients were considered to be nutritional factors, enteropathy and acute phase reactant proteins (452). The hepatic synthesis of acute phase reactant proteins is induced by cytokines such as interleukin-6 and tumor necrosis factor (448), which inhibit the production of serum albumin and cause dramatic shifts in the plasma concentration of certain essential micro nutrients and albumin.

Globulin levels in study cohorts

In our study we found that there was significant increase in globulin levels in all three study cohorts HIV, TB, and HIV+TB study cohorts when compared with control cohorts.

The increased globulin levels indicate the hyperglobulinemia is observed in the HIV patients with increased levels of IgG.

Hyperglobulinemia in HIV patients was demonstrated by studies by Quensel A et al., (1994) (453) & Miguez – Burban M.J. et al (1995) (454) ($p=0.003$). Hyperglobulinemia seen in HIV positive patients may be due to increased levels of IgA and IgG in this disease. Ball S.G. (1994) stated that AIDS diarrhea is often associated with reduced serum albumin, as low as 10 g/L in severe cases, probably due to hypercatabolism, though other factors may contribute including malabsorption and protein losing enteropathy. He also states that polyclonal hyperglobulinemia may be sufficient to raise the total protein typically by some 10 to 15gm/L

Since the serum protein levels are increased in HIV patients due to metabolic alteration and possibly from the increased globulin fraction. This support the work done by which documented significant higher serum level of total protein when compared to control (455). There was also increase in globulin fraction which is essentially a compensatory mechanism for decreased albumin synthesis as seen in the HIV subjects, on the hand other the increased in globulin fraction in the HIV subjects may be attributed to humoral immune responses in the HIV subjects which is more vigorous in Non-ART group. Some studies supports that (455) and (456) where they reported high level of globulin fraction in HIV subjects compared to controls. This study have shown that abnormally high globulin fractions in the HIV subjects is associated with effective control of HIV disease progression and that humoral responses may have indirect effects on viral replication.

All the patients enrolled in our study were on the ART therapy. These drugs have the effects on the overall immunity depending on the immune status of the each patient.

Serum globulin levels in TB study cohort were increased significantly when compared with control. There is documented work stating that the globulin levels were increased in the patients in Nigeria (447). In our study serum protein are increased this may indicate the hyperglobulinemia in the patients due to the globulin fraction present. The other studies have reported globulin is increased in the TB patients.

A significant high level of globulin has arisen from the combination of elevation of various globulin factors observed in the different studies. The protein levels in our study have also been remarkably increased that may also support the globulin fraction increase. Arinola and Igbi have reported the high levels of IgM and IgG in pulmonary tuberculosis. Nagayama et al have stated that hyperglobulinemia in tuberculosis is one of the predictive factors for the development of residual pleural thickening in tuberculosis pleurisy. Higher levels of globulin in this study support the fact that humoral immune response is less affected and raises the possibility of polyclonal B cell activation in tuberculosis patients.

The mean serum protein has remarkably increased in HIV+TB indication the high globulin fraction present in the patients and also the patients are on both ART and ART so that can help in improving the immune status and somewhat extent benefit the patients.

A/G ratio in study cohorts

In our study, A/G ration in all the study groups was found to be significantly decreased in all three study cohorts when compared with the control cohorts.

In our study we have encountered the lower levels of albumin and higher levels of globulin could be the reason decrease in A/G ratio. This support the various other studies carried out the HIV patients where they have encountered lower levels of A/G ratio (455)

Higher the serum protein levels and lowers the albumin levels are major reason for the decrease in the A/G ratio.

Decreased A/G ratio was found in TB cohorts. The previous studies have shown there is decrease in the A/G ratio (457). Since there is an increase in the total protein levels in the TB study cohort there would be decrease in the A/G ratio. All the

patients were on the antitubercular therapy suggesting one study showed that due to the therapy the immunity has increased and this would support the decrease in the serum A/G ratio (458).

In our study there was increased in the serum protein and a decrease in the albumin levels in HIV+TB patients. This indicates there is hyperproteinemia and muscle wasting syndrome in the patients. Also the patients were on the drug therapy (AKT) which may cause the hepatic toxicity leading decrease in albumin values. One study shows that shows there are decrease in A/G ratio in HIV+TB patients (458).

Cytokines gene expression levels in the study cohorts.

In present study we studied three cytokines TNF- α , IL-6 and IFN- γ .

TNF- α in STUDY COHORT

In present study TNF- α mRNA levels were assessed in all three study cohort and compared with control cohorts. In present study the TNF- α mRNA levels in HIV were found to be significantly increased when compared to the control cohort showing there was 994 fold increases in levels when compared with the control cohort. The TNF- α mRNA levels in TB study cohort were found to be significantly increased when compared with control cohort showing there was 931 fold increased in the TB study cohorts when compared with the controls. There was a significant rise of 3309 fold in HIV+TB study cohorts when compared with control cohort.

TNF- α is major proinflammatory cytokine during HIV infection. It has been shown that HIV infection induces increased production of TNF- α by macrophages. TNF- α stimulates the production of free radicals or it is seen that the free radicals produced more likely to increase the TNF- α in different cells. TNF- α is considered one of most highly studied pro-inflammatory cytokine because it plays a critical role in the origin and progression of the disease such as HIV-1 (459). The immunoregulatory response of the host influences the pathogenesis of HIV-1 infection leading to the triggering of monocytes, macrophages and natural killer cells to produce more amounts of TNF- α (460). As a result there is a positive correlation between HIV-1 viremia and TNF- α levels in the serum of HIV-1 infected patients. This suggests that reducing the levels of TNF- α cause the severe inflammatory damage and toxicity making control of its production and secretion highly important. Regulation of release

of TNF- α serves as a potential means of therapy for HIV-1 and other disease. TNF- α also helps in inducing the pro-inflammatory cytokine such as IL-6 and IL-8 which helps in the upregulation of viral replication (461). TNF- α is secreted during the early phase of acute inflammatory diseases. Its pathogenic role in HIV-1 infection involves activation of nuclear factor κ B (NF- κ B), stimulating apoptosis of T-lymphocytes. Tissue and plasma samples of hosts express high levels of TNF- α , contributing to fever, anorexia, and other symptoms of HIV/AIDS. Thus TNF- α must be targeted at an appropriate time during the disease progression to the chronic stage. The ART therapy has helped to reduce the mortality among the HIV patients and new development of potent antiretroviral drug blocking HIV transcription to be successful. But the drug resistance and toxicity remains a major concern to treat the infection.

In some other studies showing that the TNF- α is increased in the muscle wasting syndrome particularly seen in HIV patients (462). The HIV muscle wasting syndrome is majority cause by the cytokines such as TNF- α and IL-6 (463). The other studies have shown that PBMCs TNF- α levels were elevated in HIV wasting. The cause for having loss of food intake and drug toxicity is major cause of wasting. Also, the metabolism changes such as protein turnover and catabolism also contribute to increase in the cytokine levels.

The nutritional status of an individual can be one of the major determining factors in resistance to infection. HIV infection, on the other hand, often produces malnutrition leading to wasting and cachexia (464). Wasting syndrome in AIDS is multifactorial, HIV infection of gastrointestinal tissue and lymphoid tissue particularly; anorexia, inadequate nutrient intake and malabsorption, and catabolic effects on intermediary metabolism play important roles along with similar effects of repeated secondary infections (465). Adequate antiretroviral therapy might be considered during early stages of HIV infection as part of the wasting preventive measures, because successful treatment of the primary infection with HIV or secondary infections is the most potent of anabolic therapies.

In the process of mycobacterial infection control the TNF- α has primordial role acting upon a variety of cells. The cells which primarily produce TNF- α are activated macrophages, T-lymphocytes and dendritic cells (243, 244). This cytokine is found to be acting in synergy with IFN- γ leading to the production of reactive nitrogen species intermediates (RNIs) leading to the mediating of the tuberculostatic

function of macrophages (247). TNF- α also stimulates the migration of immune cells to the infection site, contributing to the granuloma formation, capable of controlling the disease progression. TNF- α increases the capacity of macrophages to phagocytose and kill mycobacteria and stimulates apoptosis of macrophages, depriving bacilli of host cells and leading to death and presentation by dendritic cells of mycobacterial antigens (253).

M. tuberculosis evolved and has developed different mechanisms which they interact and modulate the host immune response. Mycobacterium expresses surface antigens which can induce the production of IL-10 and IL-4, which typically have anti-inflammatory effects (466, 467). Higher the expression of IL- 4, it has been implicated as a virulence factor, both for the anti-inflammatory ability and also for its apparent capacity to promote tissue damage in association with TNF- α (255) These studies suggest that IL-4 (alone or jointly with TNF- α) may play a role in tissue destruction and/or cell death during infection by *M. tuberculosis*. TNF- α is one of the most powerful controlling factors for the recruitment of monocytes and is a potent inducer of cell death by apoptosis (255). Necrosis, on the other hand, is associated with the lysis of the infected cell, the release of feasible *M. tuberculosis*, and damage to the surrounding tissues (468).

One of the study showing that TNF- α may be associated with immunopathological response in tuberculosis leading as main mediator of the destruction to an excessive inflammation with necrosis and cachexia (252, 469). Different experimental models have shown that TNF- α plays important role not only in host responses against MTB but also in the immunopathology of tuberculosis.

One study finding of increase in TNF mRNA expression levels are compatible with previous studies Lee *et al.* have highlighted that these pro-inflammatory cytokines are likely to accumulate in infected areas to deal with intracellular bacteria. However, the cytokine levels are likely to be lower in the MDR-TB population. Cytokine levels appear to be an indicator of disease activity. Many investigators have documented that TNF- α levels are higher in newly diagnosed TB and lower in chronic infection (470, 471) and are likely to be an indicator of local disease activity. In MDR-TB where the cytokines levels are low, it is reasonable to assume that this reflects a low immune response to the infection. These cytokines are important in the response to advanced pulmonary TB and the levels decrease as the disease progresses (472, 473) Furthermore, in the progressive form of TB, IL-4 secretion increases and

the expression of TNF- α and IL-1 α is inhibited. As the cytokine levels decrease, the disease worsens and the bacteria proliferate.

Patients with tuberculosis (TB) often have profound wasting, and this is regarded as one of the cardinal features of the disease. Wasting associated with TB is likely caused by a combination of decreased appetite, leading to a decrease in energy intake, interacting with increased losses and altered metabolism as part of the inflammatory and immune responses (474, 475). Wasting leads to impaired physical function (476) and increased mortality in patients with TB (477, 478). Although body weight usually increases during TB treatment, recovery may be slow and significant wasting may persist for months after the start of effective TB therapy (479).

There is an uncertainty surrounding cause as well as effect of a poor nutritional status in tuberculosis patients. The cause or pathogenesis of tuberculosis-associated wasting is incompletely understood, although it is likely that inflammatory mediators such as tumor necrosis factor- (TNF) do play a role. Similarly, a poor nutritional status is known to suppress cellular immunity, which is essential against *Mycobacterium tuberculosis*, but the precise mechanism remains uncertain (452, 480). Leptin is one of the causative factors for the wasting syndrome in the TB patients. Body fat mass is the most important determinant of plasma leptin concentrations, but starvation, hormones (including insulin and cortisol), as well as inflammatory mediators are able to modulate leptin production (481). Animal studies have shown that LPS, TNF, and IL-1 raise leptin concentrations in serum and leptin mRNA in adipose tissue (482). Similarly, in cancer patients, recombinant TNF (481) and IL-1 (483) increased plasma leptin. In sepsis patients, leptin levels were found to be elevated (484, 485). Attenuation of the acute phase response and proinflammatory cytokine production during antituberculosis treatment was accompanied by an impressive increase of plasma leptin concentrations. Of course, the acute inflammatory response in the animal and patient studies described above is different from the more chronic response in tuberculosis patients. As discussed earlier about the role of TNF- α HIV and TB diseased condition, it behaves very differently when it comes with the HIV+TB dual infection. The incidence and mortality rates for the new AIDS –defining opportunistic infections are higher when the individuals with HIV are co-infected with TB. Various epidemiological data have shown that MTB infection has a negative impact on the immune response to HIV and on progression of AIDS.

TNF production in response to *M. tuberculosis* infection is required for control of bacterial growth; TNF is known to activate HIV replication in macrophages, demonstrating that the host immune response initiated against one pathogen may promote the replication of another. Thus, both HIV and *M. tuberculosis* stimulate TNF release from infected cells, and TNF hampers bacterial growth while enhancing HIV replication.

While the, *M. tuberculosis* survives in DCs and actively down-regulates their pro-inflammatory activity and antigen-presenting function, with concurrent induction of anti-inflammatory cytokines (486). Similarly, HIV does infect and manipulate DCs and the ensuing T-cell functions (487). In HIV infection, not only is DC-mediated activation of T cells impaired, but the migration of infected DCs can also contribute to pathogen dissemination.

Also various reports had suggested that TB patients have a microenvironment which facilitates HIV infection by various factors such as i) increasing the expression of co-receptors CXCR4 and CCR5 regulated by *M. tuberculosis* products; ii) increasing pro-inflammatory cytokines, especially TNF; and iii) down-regulation of CCL5. It has also been shown that *M. tuberculosis* and its cell wall component, lipoarabinomannan (LAM) (349), may activate replication of HIV in provirus-carrying cells by inducing TNF and IL-6 production through the NF- κ B pathway, which in turn triggers transcriptional activation of the long terminal repeat (LTR) promoter and supports replication of HIV.

IL-6 gene expression levels in study cohorts.

In present study the IL-6 mRNA levels were significantly increased in HIV study cohort when compared with control cohort showing to 43 –fold increase. In our study we found that the IL-6 mRNA levels in TB study cohorts were increased when compared with control cohort showing there was 758 –fold increase.

In our study the mRNA expression levels in the HIV+TB co-infection study cohorts were significantly increased when compared with the healthy control cohorts. There was a 784 fold increase found which was found to highly significant. The family of IL-6-type cytokines comprises IL-6, IL-11, LIF (leukaemia inhibitory factor), OSM (oncostatin M), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1) and CLC (cardiotrophin-like cytokine) (Heinrich et al; 2003). IL-6 is a pleiotropic cytokine that is commonly produced at local tissue sites and released

into circulation in almost all situations of homeostatic perturbation typically including endotoxemia, endotoxic lung, trauma, and acute infections.

Human immunodeficiency virus (HIV) infection has long been shown to induce expression and secretion of IL-6 (86) by monocytes and macrophages (488). Even when virologically suppressed, treated HIV-infected persons have significantly higher plasma levels of IL-6 than well-matched uninfected controls.

Activated inflammation, as demonstrated by persistently higher IL-6 levels, may have profound and far-reaching clinical implications. Reports involving both HIV-infected and HIV-uninfected persons have linked increased plasma IL-6 levels to a variety of adverse clinical outcomes, including anaemia (489), cancer (490, 491), cardiovascular disease (492), and death.

The reasons HIV infection is associated with a chronic inflammatory state are not entirely understood. In of the study a random sample of 499 individuals participating in the Strategies for Management of Anti-Retroviral Therapy (SMART) trial(493), older age and higher body mass index (BMI) were independently associated with elevated IL-6 levels. Among SMART participants co-infected with hepatitis C virus (HCV), IL-6 levels were particularly higher in those with pre-existing hepatic fibrosis, as defined by abnormally elevated levels of the liver fibrosis marker hyaluronic acid [18]. Identifying additional factors associated with higher IL-6 levels could elucidate the mechanisms driving inflammation during HIV infection.

Our results matches with some of the studies where there is been increase in the levels of IL-6 in the Tb patients where they have estimated the serum IL-6 levels from the patients with active tuberculosis. IL-6 secreted by macrophages infected with *M. tb* is able to inhibit the response to INF- γ by non-infected macrophages adjacent to infected ones. These results reveal that IL-6 maybe involved in the inability of the cellular immune response to eradicate the infection. This cytokine can also be harmful to mycobacterial infections, insofar as it is able to inhibit the production of TNF- α and interleukin-1 beta (IL-1 β) (494). It is possible that high concentrations of IL-6, as observed in active pulmonary TB, may contribute to the disease progression, whereas intermediate concentrations found in contacts could contribute to the maintenance of the latent state.

IL-6 is a multifunctional cytokine secreted by lymphoid and non-lymphoid cells regulating B- and T-cell function and is a potent inducer of the acute-phase protein response. In active tuberculosis, the role of IL-6 may be predominantly

negative. This is supported by two facts : (1) IL-6 promotes the growth of mycobacteria in peripheral blood monocytes and (2) IL-6 inhibits the production of TNF- α and IL-1 β , which may enhance the intracellular killing of microorganisms and the development of granuloma (495).

IL-6, together with tumor necrosis factor and IL-1, belongs to the group of proinflammatory cytokines which initiate early inflammatory responses. More attempts to establish the role of IL-6 as in the anti-infective immune response have shown its proinflammatory potential during acute viral or bacterial infections (496). Also IL-6 has been regarded to have both pro-inflammatory and anti-inflammatory properties. In the setting of chronic disease, it may be a key player in the elicitation of cellular immune responses against affected cells and of mucosal humoral responses against infection (497).

All the TB patients included were having pulmonary tuberculosis. Macrophage activation by cytokines is the main mechanism underlying acquired resistance to pathogen; however, even activated macrophages fail to fully eradicate the pathogen []. It has been shown that IL-6 is required for the rapid expression of an initial protective IFN- γ response during *M. tuberculosis* infection. This early response occurs in the lung and is which is important in the initial control of mycobacterial growth in this organ. Sahashi et al. (498) suggest a similar action for IL-6 studying the lung in sarcoidosis; in which they showed that it defends its participation in the alveolitis that precedes granuloma formation – the characteristic feature of sarcoidosis.

Studies about the cytokine net and tuberculosis are important because it is known that drugs used for treatment do not kill mycobacteria, they only block the bacterial cell cycle. Moreover, efficacy of the immune response against tuberculosis might be increased. Simbirtsev et al. have demonstrated the preliminary action of SCV-07 (gamma-glutamyl-tryptophan), a synthetic dipeptide that stimulates T-lymphocyte differentiation and specific response and enhances IL-2 and IFN- γ in mice. They observed an increased anti-tuberculosis efficacy when it was used in combination with the standard chemotherapy. Thus, modern therapy against tuberculosis should be based on stimulation of anti-tuberculosis immune response associated with a standard chemotherapy, especially in multi-drug resistant cases. The IL-6 levels were increased in the HIV+TB co-infection groups. The dual infection increases the expression levels.

IFN- γ levels in study cohorts

In our study the mRNA levels of IFN- γ were increased in HIV study cohorts when compared to control study cohorts. Though there was no significant increase seen, the levels were elevated showing a 6 –fold upregulation was seen in case of HIV cohorts. In our study the mRNA expression levels in the TB study cohorts were significantly increased when compared with the healthy control cohorts. There was 11 fold increase found which was found to be highly significant.

In our study the mRNA expression levels of IFN- γ in the HIV+TB co-infection study cohorts were significantly increased when compared with the healthy control cohorts. There was a 20 fold increase found which was found to be highly significant. Interferon- γ (IFN- γ) plays various roles in the pathogenesis of HIV/AIDS. In an HIV-1 infected individual, the production of IFN- γ is detected as early as the acute phase and continually detected throughout the course of infection. Initially produced to clear the primary infection, IFN- γ together with other inflammatory cytokines are involved in establishing a chronic immune activation that exacerbates clinical diseases associated with AIDS. Unlike Type 1 IFNs, IFN- γ has no direct antiviral activity against HIV-1 in primary cultures, as supported by the *in vivo* findings of IFN- γ therapy in infected subjects. Results from both *in vitro* and *ex vivo* studies show that IFN- γ can instead enhance HIV-1 replication and its associated diseases, and therapies aimed at decreasing its production are under consideration. On the other hand, IFN- γ has been shown to enhance cytotoxic T lymphocytes and NK cell activities against HIV-1 infected cells.

Throughout the acute stage of HIV-1 infection, IFN- γ levels in infected adults steadily increase, with a peak approximately 20–24 days post-infection (499). In chronic, stable disease, IFN- γ levels decline to a steady state that is often equivalent to healthy controls (499). Although there is a predictable elevated trend in overall IFN- γ expression during HIV-1 clinical disease, no significant difference has been reported in HIV-1 specific IFN- γ response of the CTLs in both progressor and long-term nonprogressor patients with chronic disease. Although overall expression of IFN- γ by CD8 CTLs does not correlate to stage or chronicity of clinical disease, significantly larger numbers of HIV-1 specific CTLs are maintained in long-term non-progressors (500, 501). There is a significant trend of steady increase of IFN- γ levels in chronic, progressive disease however, there is marked patient to patient variability in overall expression of IFN- γ with no demonstrable correlation between IFN- γ

expression and viral load, viral set point, viral clearance, or chronicity (502, 503). In a cross-sectional study performed by Wantanabe et al. (504), proinflammatory cytokines TNF- α , IL-6, IL-10, IL-18, and IL-7 levels had a significant correlation with CD4 count in HIV- patients, but IFN- γ levels were often continuously elevated and variable between patients with no significant correlation to progressors and long-term non-progressors in chronically infected patients (504). Several theories exist as to why IFN- γ response does not correlate with disease progression and largely center on the polyfunctional and proinflammatory effects of IFN- γ (503). It has been suggested that cytokine expression and immunologic profiles in HIVC patients are more proinflammatory than immunoregulatory when compared to uninfected but exposed controls (505). It is also likely that HIV-1 infection results in modification of antigen presentation in macrophages and dendritic cell lines, resulting in anergy of HIV-1 specific CD4C and CD8C T cells (506). Another theory suggests diminished response to IFN- γ in target populations may alter immunomodulation of Th1/Th2 response through the production of synergistic (IL-2, TNF- α) or inhibitory (TGF- β , IL- 10, IL-13) cytokines. A recent study evaluated a combined proinflammatory and immunomodulatory cytokine panel including IFN- γ to predict viral load set point 12 months after infection (507). In the combined panel, IFN- γ , IL-12p40/70, IL-7, and IL-15 levels predicted 66% of viral set point variation in acute phase patients (507). In addition to the polyfunctional effects of IFN- γ , and due to the marked complexity of the antiviral response, it is likely that monitoring of HIV-1 patients by a proinflammatory cytokine panel rather than relying on a single cytokine will better predict viral load set point and progression of clinical disease.

Clinical assessment of IFN- γ levels in the serum of HIV positive adult subjects at different clinical stages has been used to determine the importance of IFN- γ in the pathogenesis of HIV-1. Clinical studies have focused on the changes in cytokine levels upon introduction of highly active anti-retroviral therapy (HAART), or ART. The elevation of multifunctional cytokines such as IFN- γ and TNF- α , can either enhance or control HIV-1 infection depending on the clinical stage of HIV-1 infection.

Protective role of IFN- γ in HIV

In the HIV infection it is generally accepted that early innate immune responses are crucial factors against HIV-1 in determining the course of infection.

This following the mucosal transmission of HIV, viral RNA remains undetectable in the circulation for a period of 10 days. This is referred as the “eclipsed period “or period from the viral entry into the cell which leads to production of new virions. Later during time the viral level increases exponentially after the eclipse period to reach at its maximum peak around 21-28 days post infection. One of major response of immune system to HIV infection there is a rush in levels of expression of inflammatory cytokines such as IFN- γ and IFN- α . The levels coincide with a gradual rise in HIV-1 viral load and contribute to symptoms of acute retroviral syndrome. This increase in viremia was shown to be associated with rapid and transient increase in IFN- α as well as other proinflammatory cytokines.

The progression of HIV disease is marked by the loss of CD 4 + cells which ultimately leads to AIDS condition and then ultimately it leads to death. Many mechanisms had been proposed to lead to this rapidly loss of CD4+ cells including the chronic immune activation and cytopathic effects of HIV on CD4 + T cells. Elevated levels of IFN- ω , (508), IFN- β (509), IFN- γ (510) and IFN- α in patients with AIDS had been reported as earlier studies. IFN- α has been identified as one of the major cytokines markers of HIV pathogenesis.

It is also found that effect of IFN- α is majority affecting the HIV disease progression and pathogenesis but there is a very little known about the potential effects of other IFN subtypes which has a role in course of infection. Interferon- γ – induced NO has been showed to be highly increased in the serum of patients infected with HIV, especially in patients with low CD4 counts. Nitric oxide induced by HIV-tat has been implicated in neurotoxicity and the pathogenesis of HIV associated dementia (511).This shows the IFN- γ and perhaps other IFN-mediated host antiviral response such e.g. NO production contributes to HIV pathogenesis.

In-vitro studies and some data from HESN cohorts had indicated the IFN responses are crucial in preventing the initial HIV replication and establishment of infection. Interferons act as a double edge sword in protection against and also the exacerbation of HIV infection. These Interferons restricts HIV infection at the exposure point and control viral spread during acute infection. These IFNs contributes to persistent immune activation and CD4+ cells depletion during the chronic infection. Overproduction of IFNs at the later stages of the disease eventually leads to active viral replication and thus worsens the disease outcome. Modulation of the IFN responses prior to HIV infection also during the later stage of infection has a great

potential to prevent HIV transmission, and then helps to clear the transmission and also helps to inhibit chronic immune activation.

At the site of MTB infection, IFN- γ is the major cytokine of Th-1 cells, therefore, as a pro-inflammatory immune response, it protects against MTB. IFN- γ activates macrophages to kill intracellular mycobacteria. IFN- γ is the chief cytokine involved in the protective immune response against mycobacterial infection. It is produced primarily by CD4 and CD8 T lymphocytes and NK cells. It is also known that natural killer T cells (NKT) and $\gamma\delta$ T lymphocytes, cells with a narrow repertoire of antigen recognition, can also produce IFN- γ in response to mycobacterial stimulation, displaying protection against *M. tuberculosis* infection both in vitro and in vivo (512).

The inflammatory cytokine response is linked to development of clinical manifestations of TB. For instance, higher levels of IFN- and IL-10 are associated with lower body weights and wasting in newly infected TB patients. Whilst a higher IFN- γ to IL-10 ratio is associated with protection and TB cure (513), a lower IFN- γ to IL-10 ratio is linked to TB relapse. Furthermore, increased plasma adiponectin levels are associated with severe TB characterized by extensive pulmonary lesions. Although adiponectin promotes IL-10 release and impairs IFN- γ secretion in human macrophages (514), (515) the magnitude of IFN- γ and IL-10 to adiponectin production as an indicator of inflammatory response and clinical manifestations in TB is largely unknown.

Mycobacterium tuberculosis infections evolve through an early stage and subsequently a chronic course of disease. Proinflammatory cytokines such as IFN- γ are upregulated in the early phase followed by counteractive increase in anti-inflammatory cytokines such as IL-10 and adiponectin in the chronic phase of disease (516). Since the magnitude and timing of the cytokine response in TB are influenced by underlying host factors (517), one study showed relationship between these factors and clinical manifestation of TB disease.

The elevated circulating IFN- γ levels in the TB cases suggest upregulation in the pro-inflammatory response. These results are consistent with previous studies showing elevated plasma levels of IFN- γ in newly infected TB patients (518, 519). Hyperactivation of macrophages and dendritic cells leads to overproduction of proinflammatory mediators that mediate development of clinical manifestations in

early stage disease. It is, therefore, possible that the upregulation of the proinflammatory response in early phase of *M. tuberculosis* infection is capable of controlling TB infection.

The indirect link of IFN- γ levels and body weight, body mass index, and hip girth in the TB cases may indicate that IFN- γ promotes weight loss in newly infected TB patients. These findings corroborate previous studies showing higher levels of IFN- γ in TB cases presenting with lower body weight (520) it is possible that IFN- γ contributes to weight loss in newly infected TB patients. For instance, increased IFN- γ levels induces anorexia and upregulates IL-12 promoting weight loss through epithelial cell damage (521, 522).

In our study the mRNA expression levels of IFN- γ in the HIV+TB co-infection study cohorts were significantly increased when compared with the healthy control cohorts. There was a 20 fold increase found which was found to highly significant.

NF- κ B levels in study cohorts

In our present study we found that NF- κ B levels were significantly increased in all three study cohort when compared with the healthy cohorts.

The NF-kappa B (NF- κ B)/Rel family of transcription factors serves an important regulatory role in the production of immune responses and inflammation. The NF- κ B family member RelB displays unusual properties in comparison to the other family members, as it exudes inhibitory effects on the pathway along with its traditional function of transcription initiation

The activation of NF- κ B is hallmark of all viral infection. Due to its different abilities to promote the expression of different protein which are involved in the innate and adaptive immunity, NF- κ B coordinates different aspects of immune function which is required for resistance to infection. The activation of NF- κ B during viral infection has interpreted as a protective response of the host to the viral pathogen. This hypothesis had been supported by various studies in mice deficient members of the NF- κ B family which are more susceptible to the infection (523) . In addition to this different virus had evolved strategies to interfere in the NF- κ B activation such as vaccinia virus (524), African swine fever virus (ASFV) where there is a protein involved which helps to form a dimer to prevent its nuclear translocation.

Also viruses such as cowpox, raccoon pox viruses were found to inhibit NF- κ B – activation by interfering the I κ BA degradation.

Other studies have found that HIV accessory protein Vpu inhibits the NF- κ B activation by interfering with β -trcp mediated degradation of I κ B α and to promote the apoptosis of the infected cell by suppressing NF- κ B dependent expression of the anti-apoptotic factors (83).

Various studies had made clear point focusing on the viruses which can directly activate NF- κ B and utilize it in different ways. NF- κ B activation could be a strategy evolved by the different viruses in order to block the apoptosis pathway and thus helps for prolong survival of the host cells in order to gain back more time for replication process and increase the viral progeny production. Various viruses such as HIV, HSV, HCV, EMCV had shown such function (104).

The role of NF- κ B in activating HIV transcription has been extensively studied. In normal human CD4⁺ T lymphocytes, NF- κ B binding activity is low and consists predominantly of p50, but not p65, DNA binding. T-cell activation results in the formation of p50-p65 NF- κ B complexes and enhancer-dependent HIV LTR transactivation, indicating that unstimulated CD4⁺ T lymphocytes offer a cellular environment of low permissiveness to HIV LTR function. In HIV-infected T cells, NF- κ B–dependent transactivation is essential for HIV-LTR induction; interestingly, even the function of HIV Tat in resting CD4 T lymphocytes depends on κ B responsive elements in the LTR. Furthermore, CD4⁺ T lymphocytes carrying an infectious HIV provirus with point mutations in these elements fail to transcribe viral RNA upon cell activation (108).

Increasing the intracellular levels of NF- κ B may permit high levels of HIV-1 gene expression and could thus provide a favourable environment for HIV replication. Upstream of NF- κ B activity, the IKK complex is constitutively activated in chronically HIV-1–infected myeloid cells (525, 526).

HIV infection of immune system cells is itself proven as a potent and persistent cause of immune activation. Several endogenous cytokines which are released by the cells affects the HIV disease progression. The expression of such cytokines is dysregulated by the HIV infected individuals. Cytokines such as IL-1 β , IL-2, -3, -4, -6, -7, -15, -16, TNF- α , growth factors M-CSF, GM-CSF induces HIV expression. Some studies carried in TGF- β and IFN- γ , this activates the NF- κ B via I κ B. This activation leads to enhance the cytokine and LTR- directed gene expression.

In human macrophages, inhibition of NF- κ B activation reduced the viability of intracellular MTB through increased induction of apoptosis and autophagy (527). This showed that inhibition of NF- κ B enhanced macrophage response to MTB, known that fact that NF- κ B is a ubiquitous transcription factor which is involved in many cellular processes associated with inflammation and infections. For example, suboptimal binding of NF- κ B to a cisregulatory site on the 5'-flanking region of the IFN- γ gene promoter leads to decreased production of IFN- γ (528), a pro-inflammatory cytokine which is critically important in host-defence against mycobacterial infections. While some other studies has explained that NF- κ B activation in mouse macrophages resulted in increased killing of mycobacteria, those studies primarily used non-pathogenic *Mycobacterium smegmatis* (529). These studies suggest that NF- κ B plays roles in both host-defence and host-susceptibility, depending on the microbial pathogen and the host species.

TNF- α is also shown to be having role in inducing the apoptosis in context of MTB infection. But there is little information on TNF- α whether it shown any induction in THP-1 cells or MDM infected with MTB. Dhiman and co-workers previously showed that THP-1 cells, in which NF- κ B was suppressed via transfection with a mutated, dominant-negative form of I κ B α , were less burdened by intracellular MTB (530).

The increased vulnerability of AIDS patients to TB is most likely related to impaired effector T cell function (531, 532). However, increased NF- κ B activation seen in HIV positive individuals could also impair the ability of their MTB-infected macrophages to undergo apoptosis (339), providing another mechanism for their predisposition to TB. So we conclude that NF- κ B expression does help in killing of the MT but it also leads to apoptosis and also leads to the deleterious effects on the host and subsequently increases the disease progression.

As described earlier there is in increased in the levels on NF- κ B in HIV and TB study cohorts individually when they were studied. The HIV+TB dual infection shows the both viral and bacterial factors play important role in the disease progression and thus leading to ultimate death of the individual.

Multiple studies shown had shown to determine whether M. tuberculosis influences HIV replication. The data on the replication of HIV within M. tuberculosis-coinfected macrophages is bit controversial, which is supporting both increase (533-536) and decrease in viral replication. Some of the In vitro studies have demonstrated

the importance of the macrophage activation state and the presence of proinflammatory cytokines in inducing HIV replication. THP-1 macrophages in contact with lymphocytes or neutrophils induce viral replication(534). These coinfecting macrophages which in turn increase HIV replication when the CCAAT enhancer binding protein beta (C/EBP β) this transcription factor is inhibited and an increase in NF- κ B production is induced, and NF- κ B binds to the HIV long terminal repeat and initiates viral transcription. HIV replication decreased in the presence of neutralizing antibodies to tumor necrosis factor (TNF) and interleukin-6 (IL-6) and increased in the presence of antibodies to IL-10 and transforming growth factor (537), which further supports the idea that coinfecting activated macrophages increase HIV replication.

The immune environment created by *M. tuberculosis* also promotes viral replication *ex vivo*. *M. tuberculosis* causes an increase in inflammatory cytokines *in vivo*, which can lead to activation of T cells and macrophages, which then induces viral replication of HIV. Garrait et al. showed from the study that incubation of HIV-infected PBMC with pleural fluid from individuals with TB induces more replication than incubation with pleural fluid from individuals without TB (538). Thus the increase in replication was dependent on IL-6 and TNF- α , which supports the perception that activated cells are induced by proinflammatory environments may help increase HIV replication. This supports work that confirmed that HIV replication increases in activated CD4⁺ T cells (539) and CD14⁺ macrophages (540), which are prevalent at sites of *M. tuberculosis* infection.

ROS

Evidence suggested that HIV-1 infected patients are under chronic oxidative stress. This effect is subsequent to depletion of endogenous antioxidant moieties and to an increased production of ROS. Observation of the multiple pathogenic interactions between ROS and the HIV-1 has drawn attention to the possibility that these types of the interaction may play a role in the pathogenesis of many other viruses as well. ROS has been suggested to be involved in many aspects of HIV-1 disease pathogenesis, including increase viral replication, inflammatory response, and decrease of immune cell proliferation, loss of immune function, chronic weight loss, and increase sensitivity to drug toxicity. In addition, antiretroviral combination

therapy increases protein oxidation as well as the level of oxidative stress already present in HIV-1 infection (Ngondi et al., 2006).

One aspect of the role of ROS in HIV-1 pathogenesis is the positive modulatory effect on the immune activation, important both in eradication of viral infection but also in immune induced cellular injury (Schwarz, 1996). HIV-1 infections cause a chronic inflammation as shown by high plasma levels of pro and inflammatory cytokines, chemokines and ROS in seropositive individuals (Israel & Gougerot-Pocidalo, 1997). Increased production of ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide may be related to an increased activation of polymorphonuclear leukocytes during HIV-1 infection or influenced by the pro-oxidant effect of pro-inflammatory cytokines produced by activated macrophages during the course of HIV-1 infection (Das et al., 1990).

8 SUMMARY AND CONCLUSION

The results presented in the present study marks out the oxidative stress in HIV, TB and HIV+TB co-infection patients. 50 patients in each study cohort were included each study cohort with healthy controls. The free radicals help in accentuating the disease progression in these patients. The pro-oxidant markers, anti-oxidant markers, cytokine gene expression, NF- κ B expression was studied in this study.

- 1) The serum nitric oxide levels in HIV study cohort was increased significantly $17.40 \pm 3.18 \mu\text{M}$ when compared with controls.
- 2) The Serum TBARS levels were found to be significantly increased in HIV+TB $14.9 \pm 1.44 \mu\text{M}$ study cohort when compared with controls/
- 3) The serum CRP levels where found to be significantly increase in HIV+TB $4.47 \pm 0.63 \text{ mg/dL}$ study cohort and TB $3.67 \pm 0.41 \text{ mg/dL}$ study cohort when compared with controls $0.14 \pm 0.06 \text{ mg/dL}$.
- 4) The intracellular SOA levels were significantly increased in HIV study cohort $0.69 \pm 0.14 \text{ O.D units}$; there was increased found in HIV+TB $4.47 \pm 0.63 \text{ O.D units}$ study cohort and TB $3.67 \pm 0.41 \text{ O.D units}$ but not statistically significant when compared with controls.
- 5) The GSH levels showed different results the Total thiols showed significant increase in HIV study cohorts $582.8 \pm 45.42 \mu\text{M}$, while in TB $362.9 \pm 26.92 \mu\text{M}$, and HIV+TB 294.0 ± 24.63 when compared with controls $715.2 \pm 21.37 \mu\text{M}$. While the free thiols showed significantly decreased in the TB study cohorts $11.95 \pm 1.53 \mu\text{M}$, in HIV $29.10 \pm 3.25 \mu\text{M}$, HIV+TB $27.50 \pm 4.51 \mu\text{M}$, when compared with control $397.7 \pm 6.52 \mu\text{M}$. The protein thiols have significantly decreased in HIV study cohorts $553.7 \pm 43.76 \mu\text{M}$, while in TB $350.9 \pm 27.3 \mu\text{M}$, while in HIV+TB $266.5 \pm 24.5 \mu\text{M}$, also in other groups but not statistically significant decrease was seen when compared with controls.
- 6) The enzymatic antioxidant enzymes such SOD, CAT, G-ST, G-PX, GR, were studied.
- 7) The serum Catalase activity was significantly reduced in TB $1.40 \pm 0.20 \text{ U/mg of protein}$ and HIV $1.74 \pm 0.27 \text{ U/mg of protein}$ study cohort while there was no difference in the HIV+TB $2.96 \pm 0.40 \text{ U/mg of protein}$ when compared with control study cohort.

- 8) The serum GST activity was decreased significantly in all three study cohorts; HIV 1.73 ± 0.12 U/mg of Protein, in TB 1.33 ± 0.13 U/mg of Protein, in HIV+TB 2.09 ± 0.23 U/mg of Protein when compared with controls 3.67 ± 0.13 U/mg of Protein
- 9) The serum SOD activity was seen significantly increased in TB study cohort 0.51 ± 0.16 U/mg of Protein while there was increased in HIV 0.06 ± 0.005 U/mg of Protein and HIV +TB 0.1 ± 0.01 U/mg of Protein but increased was not statistically significant when compared with control cohorts 0.06 ± 0.004 U/mg of Protein.
- 10) The serum GPX activity was increased in all study cohorts but the increase was not statistically significant. 0.068 ± 0.09 U/mg of Protein activity in controls while in HIV group 0.17 ± 0.04 U/mg of Protein, while in TB 0.18 ± 0.07 U/mg of Protein while in HIV+TB 0.14 ± 0.03 U/mg of Protein.
- 11) The serum GR activity was significantly increased in HIV study groups 1.15 ± 0.28 U/mg of Protein while though there was increase in activity in TB 0.48 ± 0.09 U/mg of Protein and HIV+TB cohort 0.51 ± 0.12 U/mg of Protein it was not significant when compared with control cohort 0.38 ± 0.06 U/mg of Protein
- 12) The mean total serum proteins were found to be elevated in all study cohorts, in HIV 12.23 ± 0.53 , TB study cohort 16.02 ± 0.47 , And HIV+TB 11.07 ± 0.54 mg/dl when compared with control cohorts showing the hyperproteinaemia.
- 13) The mean serum albumin levels were found to be increased in TB study cohort 6.2 ± 0.17 mg/dL while there was decrease in HIV 4.55 ± 0.19 mg/dL and HIV+TB study cohorts 4.64 ± 0.18 mg/dL
- 14) The mean serum globulin levels were found significantly elevated in all three study cohorts; in HIV 7.67 ± 0.37 mg/dL, in TB 9.63 ± 0.34 mg/dL, and HIV+TB 6.42 ± 0.42 mg/dL when compared with controls 3.12 ± 0.12 mg/dL
- 15) The serum A/G ratio we levels were found significantly decreased in all three study cohorts ; in HIV study group 0.63 ± 0.02 , in TB study group 0.67 ± 0.02 in HIV+TB 0.85 ± 0.05 when compared with controls 1.89 ± 0.18 .
- 16) The cytokine gene expression was studied to see the expression of proinflammatory cytokines such as TNF- α , IL-6, IFN- γ .
- 17) The TNF- α gene expression showed significantly increase in all study cohorts with 3309 –fold change in HIV+TB, 994 –fold in HIV and 930 in TB study cohort when compared with controls.

- 18) The IFN- γ gene expression was found to be significantly elevated in all study cohorts showing 20-fold change in HIV+TB, 11.20 in TB and 6.70-fold change in HIV study cohorts.
- 19) The Il-6 gene expression was found to be significantly elevated in all study cohorts showing 784-fold change in HIV+TB, 735 –fold change in TB and 43-fold change in HIV study cohorts.
- 20) The NF- κ B levels were found to be significantly increased in all three study cohorts 0.33 in TB, 0.31 in HIV+TB, and 0.26 in HIV+TB study cohorts when compared with controls.

9 CONCLUSION

The pathology of both HIV and TB are attributed to immune dysfunction. Multiple parameters contribute in maintaining immune homeostasis. Critical amongst these are the inflammatory pathways and pathways regulating redox homeostasis, which in turn are intricately linked to each other. Co-infection of tuberculosis in HIV patients is known to worsen the prognosis of the former disease however very little information is available regarding the clinical parameters concerning the prognosis of HIV, TB and HIV+TB co-infection. The present study exhibits the modulation of pivotal inflammatory and redox markers in HIV, TB and HIV+TB co-infection. Co-infection TB in HIV patients worsens the parameters that maintain immune and redox homeostasis. Since these two pathways were found to be critically affected by these diseases specific interventions concerning these pathways i.e. use of anti-inflammatory and anti-oxidant agents may be incorporated along with conventional ART and AKT regimes.

10 LIMITATIONS OF THE STUDY

The study was carried out in patients at single time points. Following Up the patients over the course of disease would have given better inputs in to the exact dynamics of these parameters.

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12 APPENDIX

A) NITRIC OXIDE ASSAY

- i. 0.1 M Sodium Nitrate (# 617545305001730- Merck)
0.069 g in 10 ml of D/W diluted to 1:1000 to make the working stock and use this for standard preparation.
- ii. 0.1 % NED (# 14495 -SRL chemicals)
0.01g in NED in 10 ml of D/W
- iii. 1 % sulphanilamide reagent
0.1 g in 10 ml of 5 % O- Phosphoric acid

B) TBARS ASSAY

- i. TRIS HCL BUFFER (20 mM)
0.315 g of TRIS (#SRL CHEMICALS MOL BIO GRADE) in 100 ml of D/W.
Adjust the pH-7.2 with 0.1 N HCL.
- ii. Thiobarbituric Acid (# Sigma Aldrich)
Add 0.033 g of TBA to 5 mL of Glacial Acetic Acid and 5 ml of D/W. Prepare freshly and keep in Amber Colour glass bottle.
- iii. Ortho Phosphoric Acid (1.22 M # Qualigens / Fishers scientific)
1.67 mL of OPA added into 5 ml of distilled water and make volume up to 20 ml of distilled water.
- iv. Tetramethoxy Propane (#Sigma Aldrich Chemicals)
Add 1.75 uL of TMP in 10 ml of TRIS -HCL buffer.
- v. 0.1 N NaOH
0.4 g in 100 ml of D/WS

C) SUPEROXIDE ANION

- i. Nitro Blue Tetrazolium (NBT) - 1mg/mL

Final volume: 10 ml made up with distilled water NBT-10 mg

The solution should be freshly prepared, sterile filtered using 0.22 u syringe filter and kept in amber glass vials

- ii. 2N KOH

Final volume : 10 mL, made up with the distilled water KOH-1.12 g

D) TOTAL SULFHYDRYL GROUPS (TOTAL-SH), PROTEIN BOUND SULFHYDRYL GROUPS (PROTEIN-SH) AND FREE SULFHYDRYL GROUPS (FREE-SH)

- i. PBS; pH 7.0 - Total volume: 250 mL

Add following reagents in half volume of D/W.

Reagents	Amount	CHEMICAL DETAILS
NaCl	2 g	SRL CHEMICALS #1940103 MOL BIO GRADE
KCl	0.05g	QUALIGENS A.R. GRADE
Na ₂ HPO ₄	0.36 g	SRL CHEMICALS #194413 MOL BIO GRADE
KH ₂ PO ₄	0.06 g	HIMEDIA # RM 249

I. Make up the volume to 240 mL

II. Adjust the pH

III. Make the volume to 250 ml

1. TEA; 20mM (#T58300- #SIGMA ALDRICH) Dissolve 0.186 g of TEA in 50 ml of distilled water. Store at 4⁰C.

2. DTNB; 5mM (#D8130- # SIGMA ALDRICH)

Dissolve 0.00792 g of DTNB in 10ml of PBS

3. Glutathione stock; 30mM (# 074013 SRL CHEMICAL)

Dissolve 31.1 mg of reduced glutathione in 5 mL of 20 mM TEA.

Prepare fresh daily and keep on ice until used.

E) CATALASE ACTIVITY

- i. Phosphate buffer; 50mM pH 7.0
 Solution A: Dissolve 0.69 g of KH_2PO_4 in 100ml d/w
 Solution B: Dissolve 1.33 g of Na_2HPO_4 in 150ml d/w
 Mix solutions (A) and (B) in ratio of 1:1.55
- ii. H_2O_2 ; 30mM (SRL CHEMICAL 30 % W/V)
 Diluted 0.34 ml 30% H_2O_2 with phosphate buffer to 100 ml.
 Prepare just before use.

F) GLUTATHIONE S-TRANSFERASE (GST) ACTIVITY

- i. Phosphate buffer; 0.3 M pH 6.0
 Total volume: 100 ml
 Solution A: Dissolve 5.23 g of K_2HPO_4 in 100ml D/W.
 Solution B: Dissolve 4.08 g of KH_2PO_4 in 100ml D/W.
 Mix solution A and B of 31.5 ml and 68.5 ml respectively.
 Adjust the pH to 6.0
- ii. GSH; 30 mM (# 074013 SRL CHEMICALS)
 Dissolve 0.92 g of reduced GSH in 100ml D/W
- iii. CDNB; 30mM (#GRM 507 # HIMEDIA)
 Dissolve 0.61 g of CDNB in 100 ml of 95% ethanol.

G) SUPEROXIDE DISMUTASE (SOD) ACTIVITY

- i. Hydrochloric acid; 10mM (FISHER SCIENTIFIC)
 Add 84 μl of HCL in 25 ml d/w. Make up the volume to 100ml
- ii. Pyrogallol; 30 mM (#16040 SIGMA ALDRICH)
 Dissolve 37.8 mg of pyrogallol in 10 mL of 10 mM HCL
- iii. TCB; pH 8.2 Total volume: 500 ml
 Dissolve 6.69 gm of sodium cacodylate (# C0250 -10G SIGMA ALDRICH)
 and 245.9 mg of DTPA (# 32318 FLUKA /SIGMA ADLRICH) in d/w. Make
 up the volume 500 mL with D/W Adjust the pH to 8.2 with 1M TRIS (#
 204892 -SRL CHEMCALS)

H) GLUTATHIONE PEROXIDASE ACTIVITY

- i. 4 mM EDTA (#054448 SRL MOL BIO GRADE)
Weigh 30 .0 mg in 20 ml of D/W
- ii. Reduced Glutathione 4 mM (# 074013 SRL CHEMICALS) 24 g in 20 ml of D/W
- iii. 0.2 mM NADPH (#14495 SRL CHEMICALS) 8.33 g in 50 ml D/W
- iv. H₂O₂ 0.25 mM (SD fine chemicals)
Add 0.078 ml of H₂O₂ in 50 ml of D/W
- v. Sodium Azide -4 mM (# Himedia GRM 1038) – 10 mg in 20 ml D/W
- vi. Glutathione Reductase (# G3664 sigma Aldrich)
50 µL from stock and to 450 µl of D/W and mix properly. Then add this mixture to 4500 µl of D/W to make the total volume of 5 ml of 1 I.U of enzyme stock.

I) GLUTATHIONE REDUCTASE ACTIVITY

- i. 0.5mM EDTA (#054448 SRL MOL BIO GRADE)
9.30g in 50 ml D/W
- ii. 1 mM Oxidized Glutathione (074017 SRL extra pure grade)
12.25 g in 20 ml D/W
- iii. NADPH 0.1 mM (#14495 SRL CHEMICALS)
3.98 mg in 50 ml D/W
- iv. 0.1M phosphate buffer
 - 1) Na₂HPO₄: 0.1 mM - 1.41gm in 100 ml D/W
 - 2) NaH₂PO₄: 0.1 mM – 1.19 GM in 100 ml D/Wmix 1) 86.6 ml and 2) 13.4 ml and adjust pH 7.6

