Long term non progressors of HIV-1 perinatally infected children: the viral, genetic and immunological profile

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I, Sarada Karicheti, hereby declare that this thesis entitled **"Long term non progressors of HIV-1 perinatally infected children: the viral, genetic and immunological profile"** submitted by me under the guidance and supervision of Prof. Anand. K. Kondapi is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled **"Long term non progressors of HIV-1 perinatally infected children: the viral, genetic and immunological profile"** is a record of bonafide work done by Ms. Sarada Karicheti a research scholar for Ph.D. Programme in the Department of Biotechnology and Bioinformatics, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

(Signature of Supervisor)

(Head of the Department)

(Dean of the School)

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Contents

Chapter I

General Introduction

Human immunodeficiency virus (HIV) as etiological agent of AIDS

Since the first of cases of AIDS reported in 1980s', scientists have made great efforts to understand the nature of human immunodeficiency virus (HIV) disease and of its causative agent, the HIV. HIV is a unique retrovirus belonged to the genus Lentivirus and family Retroviridae and causes the disease acquired immunodeficiency syndrome (AIDS) (Weiss 1993)(Douek et al. 2009). HIV mainly infect helper T cells of the immune system, such as CD4+T cells(Cunningham et al. 2010)(Dalgleish et al.)(Klatzmann et al. 1984). As the number of CD4+T cells decrease, the immune system of the host could no longer fight against opportunistic infections leading to AIDS and inevitably to death. Tudela et al. 2014, reported that over the past three decades 70 million people are infected with HIV, of the estimated 70 million HIV has claimed more than 35 million lives so far and 35 million people are living with HIV in this 3.3 million are children currently living with HIV.

HIV subtypes and global distribution

HIV is mainly two types HIV-1 and HIV-2. HIV-1 was further classified into three main groups: major (M), new (N) and outlier (O) (Thomson et al. 2002). Majority of HIV isolates belong to the group M, which has been further divided into 10 distinct clades or subgroups designated as A-J (Gaschen et al. 2002)(Stebbing & Moyle). It is also important to differentiate subtypes from recombinants, which had been represented as "circulating recombinant forms (CRFs)"(Fang et al. 2004)(Dowling et al. 2002)(McCutchan 2000). HIV-1 shows high degree of inter and intra subtype genetic diversity. Those differences in the genetic traits of HIV not only play a role in the dynamics of HIV infection but also effect the biological properties like infectivity, transmissibility, and pathogenicity (De Wolf et al. 1994)(Tscherning et al. 1998)(Kaleebu et al. 2002)(Kaleebu et al. 2007). Even though there are no reports supporting the infection of particular HIV-1 subtype with non-progressive HIV disease, individuals infected with subtype A showed to experience low risk of

progression to death compared to non-A subtype (Kiwanuka et al. 2008)(Kanki et al. 1999). In contrast, infection by HIV-1 subtype D has been shown to have a higher frequency of syncytium formation and X4 use, and increased risk of progression to death (Tscherning et al. 1998)(Kaleebu et al. 2007).

Pediatric HIV infection: epidemiology prevalence and transmission

During developmental period from fetal life to adolescence, humans expose to plethora of pathogenic organisms make them more susceptible and vulnerable to chronic viral infections (Prendergast et al. 2012). HIV-1 pathogenesis in children is characterized by rapid disease progression and shorter time to progress to AIDS and ultimately to death, compared to adults. Immune system in early life is more tolerogenic and fails to control viral replication, leading to persistent viraemia, coinfections, microbial translocation, immune dysregulation and immune exhaustion subsequently to disease progression.

It is reported that, of the estimated 35.3 million people living with HIV globally, approximately 3.2 million are children infected with HIV below 15 years of age as of 2013 and 190,000 AIDS deaths of children below 15 years of age were claimed in 2012, approximately 530 children deaths everyday despite about 770,000 children were on antiretroviral therapy (ART). An estimated 210,000 children were newly infected with in 2012 and nearly 700 children are newly infected with HIV everyday (UNAIDS, Global Report, 2014). Above two-thirds of people living with HIV/AIDS, with 91% of children residing in Sub-Saharan Africa.

HIV can be transmitted by exposure to contaminated blood and blood products, mainly through sharing of needles among intravenous drug users or by transfusion of blood. It can also be

transmitted by sexual contact (Chiasson et al. 1991). HIV transmission to the children can occur in two ways one is mother to child transmission (MTCT) or vertical transmission, 95% of the pediatric infections occur by vertical transmission. HIV transmits from HIV positive mother to her child during pregnancy, labor, delivery or breast feeding (Burger et al. 1990)(Walker et al. 2004). 5 % of the pediatric infections occur by horizontal transmission. Transmission rates range from 15-40% with the lack of any intervention. The transmission rate can be decreased to below 5% with efficacious treatment. The global community has dedicated itself to speed up progress for the prevention of MTCT through an initiative with the aim to eradicate new pediatric HIV infections by 2015 and provide better mother, newborn and child survival and health in the context of HIV (http://www.who.int/hiv/topics/mtct/en/).

HIV-1 genome and proteins

Structure of HIV is different from other retroviruses, 120nm in diameter and roughly spherical. The genome of HIV is composed of two identical, single stranded non covalently linked positive sense RNA molecules enclosed by a capsid protein p24 typical of lentiviruses. The integrated form of HIV is called as provirus. It is approximately 9749 bp and contain 5'cap, 3' tail and open reading frames (ORFs) (Wain-Hobson et al. 1985)(Lu et al. 2011). The genome of HIV has major genes, encoding structural proteins that are common to all retroviruses and non structural or accessory proteins that are unique to HIV. HIV genome has three open reading frames codes for the proteins gag, pol, and env. The *gag* encodes for the structural proteins, *pol* encodes enzymes like reverse transcriptase, integrase, protease and RNase H. And the *env* gene codes for the envelope protein gp160. In addition to this, the genome of HIV codes six accessory genes they are *vif, vpu, vpr, tat, rev and nef.* The *gag* gene product cleaved by protease into capsid and matrix proteins p24 and p17 provide the basic physical structure of the virus, pol provides the basic mechanism for virus replication, while

other proteins help HIV to enter host cell and enhance replication (Broder & Gallo 1984)(Markham et al. 1985)(Takahashi et al. 2000).

HIV pathogenesis

Soon after the identification of HIV, CD4+T cell surface molecule was recognized as a major co receptor for HIV entry (Dalgleish et al.)(Klatzmann et al. 1984)(Maddon et al. 1986). The virus entry into the host cell depends on a subsequent interaction of viral protein gp120 with CD4 molecules as a receptor and two co receptors CCR5 and CXCR4 (Yang et al. 2002)(Sanders et al. 2002). After entry into the host cells, the RNAs of HIV genome are reverse transcribed into DNA (cDNA), by reverse transcriptase eventually to virus replications. The viral cDNA get integrate into the host genome (proviral DNA) by integrase and cause a latent infection, which makes the virus very difficult to be eliminated (Greatorex 2004)(Broder & Gallo 1984). The cells that can be infected by the virus are CD4+ T lymphocytes, monocytes/macrophages, follicular dendritic cells (DC), langerhans cells and glial cells, or to some extent, the B cells. Majority of these cells express CD4 and CCR5 and/or CXCR4 on the surface (Alkhatib et al. 1996)(Feng et al. 1996)(Xiao et al. 2000). The HIV infections characteristically result in a progressive depletion of the CD4+ T lymphocytes, which had been found to correlate with development of AIDS(Denis et al. 1987).

Natural disease progression of the HIV infection

The typical course of HIV infection occurs in three phases, primary infection, latency and AIDS phase. Primary HIV infection is characterized by an initial increase in plasma viraemia and steep decline in the CD4 T cell count accompanied by acute viral illness, fever and rash (especially in combination), followed by oral ulcers and pharyngitis (Means et al. 2001)(Yerly et al. 2001). These changes can be explained by the fact that during the early phase of HIV infection, the virus can

replicated without being controlled by the immune system. This symptomatic phase of the acute HIV infection lasts from 7 to 10 days, but rarely longer than 14 days. Plasma viraemia then falls to a relatively low level, and the infected individual enters a chronic asymptomatic period known as persistent infection or clinical latency, lasting from 5 to10 years (Weiss 2003)(Wong et al. 2004)(Fiebig et al. 2003). After the primary infection phase CD4 cell count in the peripheral blood reaches to normal level but not as normal as before infection. In the end of the infection, immune destruction results characterized by a rapid increase in viral load and rapid decline in CD4 count in peripheral blood associated with opportunistic infection, malignancies and neurological disorders, which are symptoms of AIDS (Kirk et al. 2001)(Ryan et al. 2002)(Kaplan et al. 2000).

Factors attribute to the heterogeneity of the HIV infection

The natural clinical course and pathogenicity of human immunodeficiency virus type 1 (HIV-1) infection are complex and variable, and determined by many viral and host factors and their interactions and also influenced by other factors such as age, nutritional status of host, and type of HIV strain. HIV infected individuals are not equal susceptible to the infection and show differences in their viral set points, rates of decline of CD4 + T cell count, plasma viral load, emergence of cytotoxic T lymphocyte (CTL) escape mutants, and development of opportunistic infections subsequently lead to varying incubation periods of the virus (Kaur & Mehra 2009). HIV-1 infected individuals exhibit varying rates of disease progression (Xiang et al. 2004)(Mellors et al. 1997)(Schacker et al. 1998)(Butcher & Picker 1996). Based on the course of infection and disease progression individuals were classified mainly into three different groups, while most of infected individuals progress to acquired immunodeficiency syndrome (AIDS) after the infection, a small subset of HIV infected individuals about 5% -10% remain free of HIV associated symptoms, maintain stable CD4 + T cell count and low plasma viral load even in the absence of antiretroviral

therapy for over 8 to 10 years and are called as long term non progressors (LTNPs) (Barker et al. 1998)(Cecilia et al. 1999). In contrast to LTNP, rapid progressors (RP) are individuals who succumb to AIDS within four years after HIV-1 infection in the absence of ART(Fauci et al. 1996).

How these long term non progressors are able to control HIV for longer than others is still something elusive and it is further complexed because it could be due to different factors in different subjects. Several characteristics of the virus or genetic factors of the host could be partly responsible for this difference and researchers are studying LTNP to elucidate the mechanism that keep the infection under control. Besides this HIV vaccine researchers are also interested in finding the immune response that is elicited for delayed disease progression because replicating this response might be the key pushing towards a potential cure for HIV.

Virus and host factors that are associated with control of HIV infection in LTNP

Characteristics of the virus responsible for delayed disease progression probably the possible explanation for this is, these individuals are infected with inactive virus which can not infect and CD4+ T cells efficiently. The possible explanation for the host factors that control virus replication or disease progression is, at the cellular level is due to CD8+ T cells, which with the help of CD4+T cells lyse HIV infected cells. In addition, circulating soluble factors and neutralizing antibodies inhibit virus replication thus bringing down viral load. Furthermore occurrence of deletions or mutations in co receptors (CCR5/CXCR4) in CD4+ T cells (other T cells etc.) makes these cells resistant virus infection. At local level mucosal cytotoxic T lymphocytes (CTLs), mucosal IgA may inhibit virus replication. Systemically, inhibiting factors such as CTLs, antibodies, interferons and some interleukins like IL-16 are counteract with stimulating factors TNF-α, IL-1 and IL-6 (Hogan & Hammer 2001)(Poropatich & Sullivan 2011).

Earlier reports on LTNP cohorts

One of the earliest studies on non progression was identification of the Sydney Blood Bank Cohort (SBBC), where all subjects were infected via blood transfusion from a single donor who was an LTNP himself. It was found by full length HIV genome sequencing that the SBBC patients had been infected by a highly attenuated *nef* / LTR deleted mutant strain (Rhodes et al. 1999). Results of French perinatal cohort ANRS–CO1/CO11 reported that Chemokine/chemokine receptors gene mutations CCR5Δ32 (hzg), SDF-1 and HLA-b27 significantly predicts 80% LTNP vs progressors (Guérin et al. 2000). SEROCO, HEMECO, GRIV and SEROGEST cohorts had shown CCR5 delta 32 mutation (Rappaport et al. 1997).

Mechanisms for virus control in HIV controllers or long term non progressors of HIV.

HIV-1 genomic mutations in accessory genes those are associated with long term or slow progression of the disease

Accessory gene	Deletions/mutations	Functional change
1. Nef	a) deletion of 160-430 nucleotides in nef-LTR region	Reduced virus replication and viral pathogenicity
	b) 109-139 bases in nef gene and 159-204 nucleotide deletion in nef-LTR region	Loss of functional support Loss of functional support
	c) Deletion of 84 to less than 400 bases in nef – LTR region in a strain CRF01_AE.	Low level of virus replication
	d) Polymorphism at amino acid position 138	Lack of CD4 and MHC-1 down modulation
	e) substitution at amino acid positions 22-24 or 56-61	

2. Vpr	a) F72L substitution at amino acid position 72 b)amino acid substitution R77Q c) 83-89 amino acid deletion at C-terminus	Reduced nuclear localization and incorporation into newly formed virions Decreased cytopathicity Loss of ability to induce G2 cell cycle arrest
3. Vif	a) nucleotide deletion at 194, insertion at 63 and premature stop codons at 70 and 174 b) amino acid substitutions such as V13I, V55T,and L81M c) R132S substitution	Loss of functional support Loss of functional support Low level of replication capacity
4. Vpu	a) four amino acid insertion at C-terminus	Lack of functional support

Host genetic factors

Host gene	**Polymorphism/mutation**	**Functional change**
1.CCR5	32bp deletion(CCR5Δ32)	Truncated protein, low CCR5 expression
2.CCR2	Amino acid substitution at 64 position valine to isoleucine (V64I)	Reduced CXCR4 expression
3. SDF-1	Nucleotide substitution at 801 position to G to A (SDF-1 3'A)	Increased transcription

4.RANTES	Nucleotide substitution in the first intron region of RANTES T to C (RANTES In 1.1 C) RANTES 28G polymorphism promoter region	Inhibition of RANTES synthesis Increased RANTES synthesis

Innate and adaptive immune responses

Mechanism	Report
Adaptive immune responses	
Cytotoxic T cells	LTNP often have CD8+T cells that express multiple cytokines and proliferate in response to HIV infection.
T helper cells	LTNP often have CD4+T cells that express IL-2 and interferon-γ in high amounts in response to HIV-1 infection.
Neutralizing antibodies	LTNP often have high titer broadly neutralizing antibodies
Innate immune responses	
Natural killer cells	LTNP contain KIR2 receptors on NK cells receptors that enhance the function of these cells
Epap-1	Epap-1(Early pregnancy associated protein), a 90Kda anti HIV glycoprotein produced from the first trimester placental tissue. Reduces the cellular entry of HIV by competing with the gp120 of HIV.

Study design to classify children

The rate of disease progression in HIV infected pediatric population progresses more rapidly than adults and their survival pattern follows a bimodal distribution (Scott et al. 1989)(Blanche et

al. 1990). Some children, who develop early onset of disease, often associated with AIDS defining illness and died within first few years of life, have been called as rapid progressors or fast progressors. However, children who have survived beyond 8 years of age after diagnosis of HIV infection are known as long term survivors (LTS). In this group two sub populations have emerged, the long term non progressors (LTNP) who have remained asymptomatic or mildly symptomatic over a period of years in the absence of any therapy, while those who have survived despite clinical and laboratory evidence of disease progression, long term progressors (LTP) or slow progressors (SP). Long-term non progressors of HIV -1 infected children serve as ideal models to study virological, genetic and immunological characteristics to determine factors associate with slow progression to disease and death due to their natural control of HIV-1 infection.

Population characteristics

A total of 52 children were enrolled in the study. 32 children were HIV positive and 20 were healthy controls. HIV infection was diagnosed and confirmed by rapid tests, PCR and western blot. Children diagnosed positive were followed up for every six months, blood was drawn for CD4 count and to study other biochemical parameters such as serum bilirubin, albumin SGOT, SGPT etc. Clinical and immunological classification was done according to WHO classification system. Among thirty two HIV infected children 6 were LTNP, 18 were LTP or SP and 8 were RP or FP, classified based on clinical and laboratory outcome. Children who have survived more than 8 years of age, without any clinical symptoms and had a CD4 count >350 cells/µl without ant treatment were classified as LTNP (n=6), while those who have survived for 8 years, showed opportunistic infections and the CD4 count < 300 were categorized as SP or LTP (n=18, total 20 but, 2 of SP were died). Some children who had symptoms of AIDS were categorized as FP or RP (n=8) (Fig 1.1).

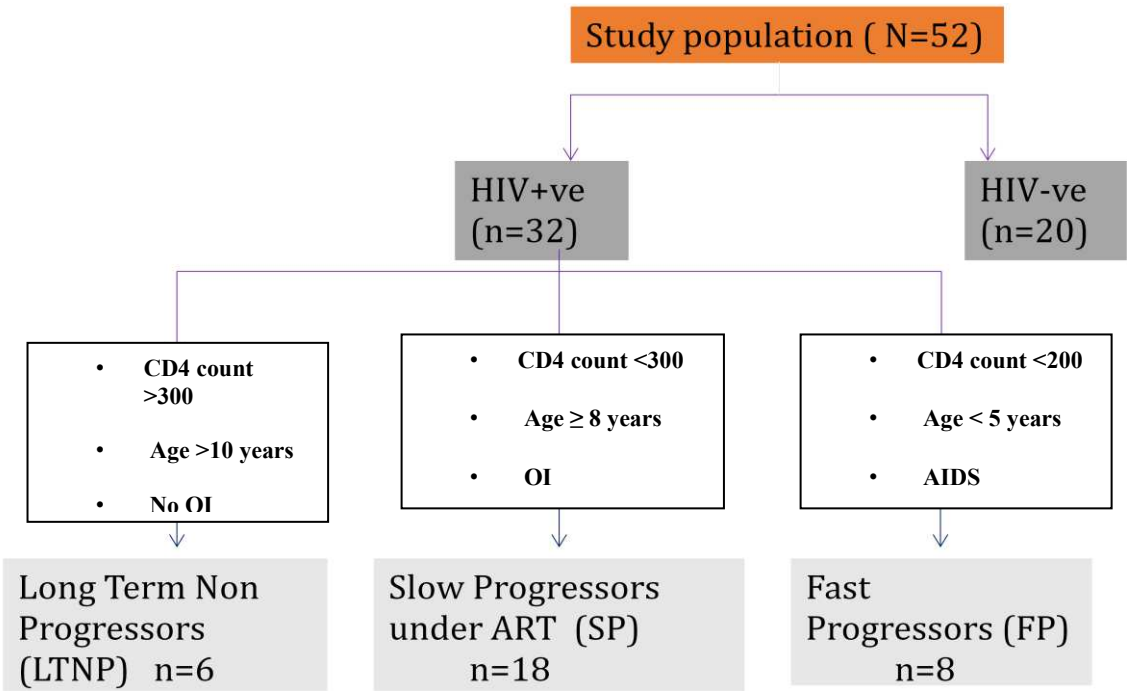


Fig1.1. Classification of HIV infected children based on disease progression and CD4+T cell count

Quantification of virus in the plasma of LTNP, SP and Progressors

The amount of virus present in the plasma of HIV infected children was determined by measuring the level of HIV-1 p24 protein. LTNP had shown a range of 8-40 pg/ml of plasma ,SP had lower level of detection to 30 pg/ml were on ART, while FP had a range of 40 to 80 ng/ml of plasma.

Subjects	p24 Antigen No .of subjects Negative	Positive	Level pg/ml
LTNP	0	6	8-40
SP(ART)	0	18	LLOD/30
FP	0	8	42-85

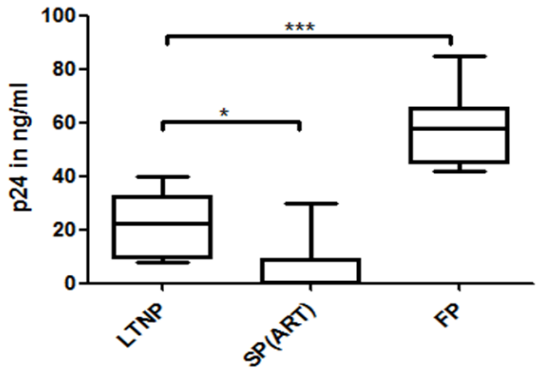


Fig 1.2. Concentration of p24 in plasma of LTNP/SP/FP HIV-1 patients. Virus p24 present in the plasma of all the groups LTNP, SP and FP were measured by ELISA kit.

Survival analysis of long term non progressors

All non progressors were alive until the last data collection point. As per the Kaplan-Meier survival analysis the incubation period in LTP patients ranged from 9 to 16 yr. The survival period in LTPs as estimated from the analysis and its dependence on CD4 in terms of median survival time (MST) in years was 10±0.66 (CI:8.69-11.30) at CD4 count <200 cells/µl; 11±0.61 yr (CI: 9.8-12.2) with CD4 count between 200 and 350 cells/µl and 12±0.18 (CI 11.63-12.37) for CD4 count >350 cells/ µl. The association between CD4 counts and survival time was found to be significant (P<0.05). The median survival time after ART was 13±0.262 yr (CI: 12.49-13.51) and the CD4 count was found to >350 cells/µl for all the patients in this group (Radhakrishna et al. 2013).

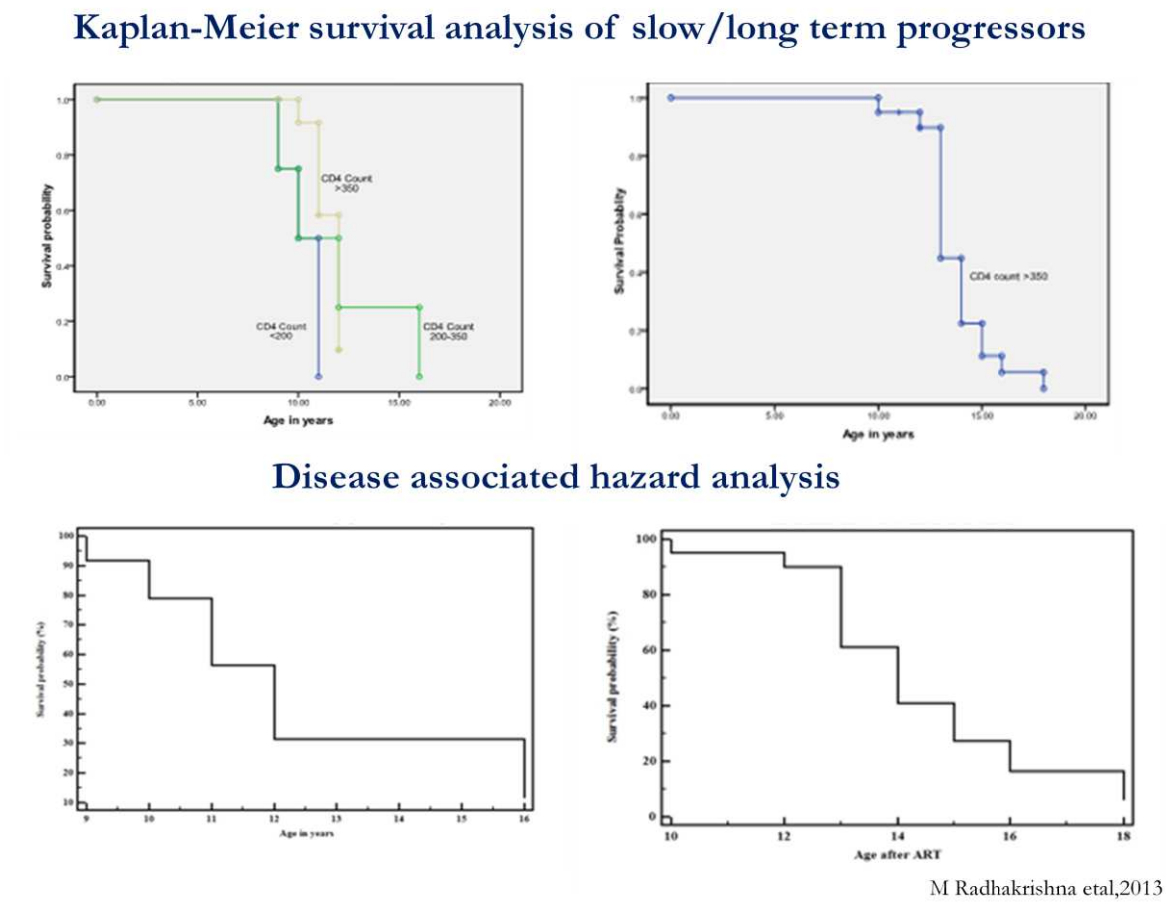


Fig1.3. Survival analysis of long term non progressors compared to SP or LTP who were under ART

Table1.1. Show the CD4 count of the study group ranged from 17 to 2214/µl with mean of 586±162 cells/µl in LTNP and SP had 661±276 cells/µl

Parameter	LTNP (n=6)	95% CI	LTP (n=20)	95% CI
Age (yr)	15.17±1.94	13.13-17.20	14.65±0.99	14.19-15.11
Height (cm)	133.17±15.04	117.38-148.95	141.40±14.42	134.65-148.15
Weight (kg)	25.67±7.66	17.63-33.71	33.55±9.71	29.00-38.10
BMI (kg/m²)	14.21±1.60	12.54-15.90	16.95±5.80	14.23-19.66
CD4⁺ T cell (Count/µl)	586.0±162	416.1-755.9	661±276.3	531.3-789.9
CD4⁺ T cell (%)	25.97±6.30	19.36-32.59	22.38±6.59	19.30-25.47
Bilirubin (mg/dl)	0.875±0.40	0.16-1.70	0.664±0.251	0.01-1.38
Serum albumin (mg/dl)	4.28±0.18	3.30-5.0	3.99±0.39*	0.9-6.50
SGOT (U/l)	58.0±12.1	27.6-100.9	52.9±19.7	19.46-48.4
SGPT (U/l)	39.4±14.8	41.64-45.77	36.3±17.7	3.5-70.2
AP (U/l)	235.8±65.0	100.9-415.5	208.1±66.8	186.57-224.9
GGT(U/l)	16.83±4.36	14.06-23.6	26.9±0.31	21.4-32.4
Serum amylase (U/l)	115.0±41.4	63.56-217.8	133.8±48.8	51.14-198.5
BUN (mg/dl)	15.87±5.40	9.98-22.6	19.02±6.15	8.77-27.2
SC (mg %)	0.908±0.21	0.86-0.99	0.92±0.25	0.29-1.61

AP, alkaline phosphates; SA, serum amylase; BUN, blood urea nitrogen; SC, serum creatinine; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase; GGT, gamma-glutamyltransferase; *$P<0.05$ compared to LTNPs

Table1.1. Characteristics of long term non progressors and slow progressors (Radhakrishna et al. 2013).

Table 1.2. shows a comparison of occurrence of opportunistic infections (OIs) in HIV infected children and the general pediatric population visiting the same hospital during the same period of time

OI/Intercurrent infection	Study children n=26*			Children visiting for primary care #N=8,500	
	With ART (LTP, N=20)		Without ART (LTNP, N=6)	Total cases (N=8,500)	
	OI (n)	%	OI (n)	OI (n)	%
Lymphaednopathy	16	80	Nil	52	0.61
Dermatitis	2	10	Nil	44	0.51
Bronchitis	6	30	Nil	442	5.2
Oral candidiasis	1	5	Nil	2	0.02
Pneumonitis	2	10	Nil	206	2.42
Granulomatous diseases of brain	2	10	Nil	53	0.62
Meningitis	1	5	Nil	20	0.24
Eardischarge (otitis media)	5	25	Nil	25	0.29
Non Hodgkins lymphoma of Burkitt type	1	5	Nil	0	0
Scabies	1	5	Nil	112	1.32
Toxoplasmosis of brain	1	5	Nil	0	0

*Total number of cases in study group -26 (LTNP 6, LTP 20, mortality 2 from LTP group);
#Data archived from medical records of the cases referred to Pediatrics department, MGM Hospital, Warangal

Table1.2. Occurrence of OIs in HIV infected patients with comparison to general pediatric population. (Radhakrishna et al. 2013)

Rationale of the study

Based on the earlier reports and characteristics above cohort, it has been shown that LTNP are distinct from other HIV infected individuals and having the characteristics such as high CD4+/CD8+ T-cell activation, absence of AIDS related symptoms for 10-15 years even in the absence of treatment and low immune activation make them as natural defense models against HIV-1 infection and disease progression. When a child survives for longer without typical AIDS-related symptoms, the host genetic and immunological framework provide various protective strategies for evading from the virus invasion and related-pathology. Further, genetic variation in virus itself would strengthen such a state. Thus, present investigation aimed at enumerating genetic, immunological and virological state in LTNPs for understanding natural defense mechanisms in this population for protection from viral pathogenesis.

Based on the above rationale the following objectives were framed.

- HIV-1 subtyping and sequence analysis of HIV-1 coded accessory genes in children with perinatal HIV infection
- Correlation of polymorphism of HIV-1 co receptors and their ligands with chemokine levels in plasma
- Elucidation of HIV-mediated immune response in HIV infected children having varied rates of disease progression

Chapter II

Materials and Methods

Materials

Cells & Virus Strains

- SupT1, Non-Hodgkin's T cell lymphoma, obtained from NIH AIDS Reference and Reagent program (Reagent contributor: Dr. James Hoxie).
- Viruses HIV-$1_{93IN101}$, HIV-$_{1\ 92BR025}$ and plasmid pNL4-3 were obtained from the NIH-AIDS Research and Reference Reagent program, USA (Reagent contributor: Dr. Robert Bollinger and the DAIDS, NIAID).

Cell Culture Materials

- RPMI-1640 and Fetal bovine serum (FBS) were purchased from Invitrogen, USA;
- Cell culture dishes and tubes were purchased from Axygen, Inc., CA, USA
- Multi-well cell culture plates and culture bottles were purchased from Corning Inc. Life Sciences, MA, and USA

Kits

- Human inflammatory cytokine CBA kit, BD Biosciences, USA
- ELISA kits, peprotech
- HIV-1 p24 Antigen Capture Assay kit, Advanced Bioscience Laboratories (ABL), Kensignton, MD, USA.
- Protein A agarose, Invitrogen, USA

Instrumentation

- Flow cytometry and cell analysis, FACS machine, CyFlow space flow cytometer, PARTEC GmBH, Germany.
- NanoDrop1000 spectrophotometer, NanoDrop Technologies, Wilmington, Delaware, USA

Methods

Study subjects

A total of fifty two children were enrolled in the study. Thirty two of them were HIV positive diagnosed by rapid tests and finally confirmed with western blot and PCR. These subjects were categorized in to three different groups based on their clinical manifestations and laboratory parameters. Namely LTNP, SP and FP. Long term non progressors (LTNP) are defined as children who maintained stable CD4 count > 350 cells/µl without any clinical symptoms for 8 years in the absence of ART, six of them were remained as LTNP. 18 were slow progressors (SP) who had been infected with HIV for 8 years and showed slow on set of clinical symptoms and declined CD4+T cells >200cells/µl. 8 patients were fast progressors (FP) who developed symptoms of AIDS within 1-4 years of infection and the CD4 count was below 200 cells/µl. Along with these 32 HIV infected children 20 healthy children were also enrolled for the study.

Inclusion criteria: Perinatally HIV infected children, who were >9 yr age (as on August 2006), had >300 CD4 cells/µl, no opportunistic infections, WHO-Stage 1 (Normal and asymptomatic) and were not receiving ART.

Exclusion criteria: Children who were not perinatally infected and were <9 yr of age (as on August 2006), had <300 CD4 cells/µl, HIV-opportunistic infections, WHO-Stages II, III, IV and under ART.

Case definitions: In the study group of 24 children (LTP and SP), baseline CD4 counts, BMI, onset of Opportunistic infections, biochemical parameters and hematology parameters complete blood picture (CBP), total lymphocyte count (TLC), Hb, erythrocyte sedimentation rate (ESR)] were monitored from 2006 to 2011. No disease progression to AIDS was monitored with the above parameters at every six month intervals over a period of five years. CD4 absolute counts and percentage were estimated at two places by flow cytometry (FACSCalibur, MultiSETV2.2, USA) at the Kakatiya Medical College, Warangal as well as at the University of Hyderabad, for the purpose of validation and to minimize bias.

Clinical evaluation: The baseline health status of the patients was reviewed every six months. Relevant investigations like chest X-ray, Mantoux test, aspiration cytology, ultrasonography, neuroimaging, were conducted and other clinical and laboratory parameters (anaemia, complete blood picture, cervical lymphadenopathy, bronchitis, otitis media, cardiomegaly, dermatitis, hepatomegaly, pneumonitis, granulomatous disease of brain, scabies, oral candidiasis, meningitis, glaucoma, non-Hodgkin's lymphoma of Burkitt type, toxoplasmosis of brain) were recorded. Patients were treated symptomatically based on their clinical presentation. Opportunistic infections were treated with a course of antibiotics.

Biochemical parameters: In addition, detailed biochemistry of bilirubin, SGOT, SGPT, serum amylase, alkaline phosphatase, GGT, blood urea nitrogen and serum creatinine were recorded with automated analyzers (photometric) in both groups for every 6 months and on onset of symptoms.

CD4 count

CD4 absolute count and percentage was estimated by flow cytometry (FACS Calibur, MultiSETV2.2) at the Medical College, Warangal and at the University of Hyderabad, Hyderabad.

PBMC isolation and plasma storage

PBMCs (Peripheral Blood Mononuclear Cells) from the blood of HIV + ve and HIV-ve samples were isolated by using Histopaque density gradient centrifugation. After drawing the blood into vacutainer tubes containing heparin, tubes were spun at 1700 rpm for 5 min at room temperature. After centrifugation, plasma in the upper layer was collected, aliquoted and stored at -70°c until use. After removing plasma, blood cells were diluted with 2 volumes of PBS (phosphate buffered saline pH7.4). Diluted blood cells were carefully loaded onto the Histopaque taken in a 50 ml falcon tube (volume ratio of Histopaque to diluted blood is 1:1.5). PBMC were separated by centrifuging the Histopaque-blood containing tubes at 1500rpm for 30 minutes without brake at room temperature. After discarding the upper layer buffer, the PBMCs in the middle layer were collected and topped up with PBS to 20 ml. The PBMCs were further washed 2 times by centrifuging the cells at 1200rpm for 10min at RT, until the supernatant changed from turbid to clear to ensure no Histopaque is left. The cell pellets of PBMCs were collected and stored at -70 °c

Isolation of genomic DNA from PBMCs

Freshly purified PBMCs or from samples frozen at -70°c were used for isolation of genomic DNA. QIAamp blood mini kit was used for isolation of genomic DNA.

Amplication viral genes envelope and accessory gene region (*nef, vif, vpu* and *vpr*)

Nested PCR was employed for both *env* and accessory genes amplification. For *env* amplification, first round PCR was conducted using primers ED3 5'-TTAGGCATCTCCTATGGCAGGAAGAAGCGG 3'; corresponding to positions 5956-5985 of the HIV-1-HXB2 genome and ED14 5'-TCTTGCCTGGAGCTGTTTGATGCCCCAGAC 3'; positions 7960-7931.These amplify a 2-kb fragment, second round primers were ED31 5'-CCTCAGCCATTACACAGGCCTGTCCAAAG-3' and ED33 5'-TTACAGTAGAAAAATTCCCCTC- 3'; amplify the 0.5-Kb C2 through C3 coding regions of envelope (Delwart et al. 1995). To amplify a 0.74-kb fragment (HXB2 positions 8696 to 9438) containing the entire *nef* gene, the following primers were used: Nef-outer 1, 5'CGCTTGAGAGACTTAATCTTGACT 3'; Nef-outer 2, 5'AGGATCTGAGGGCTCGCCACT 3'; Nef-inner 1, 5'GGGACAGATAGGATTATAGAA 3'; Nef-inner 2, 5'GTCCCTTGTAGCAAGCTCGAT 3'(Michael, Chang, L. a d'Arcy, et al. 1995). 1.4Kb fragment of the accessory gene region containing *vif, vpu* and *vpr* was amplified by using the following primers (HXB2 positions 4961-6346). ACC outer 1: 5' CGGGTTTATTACAGGGACCAACAAA3', ACC outer 2: 5' GGCATGTGTGGCCCAGATATTAT 3' , ACC inner 1: 5' GGTGAAGGGGCAGTAGTAATACAA 3'and ACC inner 2: 5' CCCATAATAGACTGTGACCCACAA 3' (Michael, Chang, L. A. d'Arcy, et al. 1995). Amplified PCR products were purified from the gel using Qiaquick gel elution kit and the purified PCR products were directly sequenced. All samples were sequenced in both directions.

Sequence and Secondary structure analysis

Env and *nef* sequences were aligned using Clustal-X version 2.1(Larkin et al. 2007) with gap-opening and extension parameters set at 10 and 0.2 respectively (default). Gonnet series weight matrix was used and each alignment step was set for iterations. Consensus sequence to understand the common features of the given sample was generated using CONSENSUS tool with the threshold set at 95%. The consensus sequence was aligned with the representative sequences of all the HIV-1 subtypes (A, B, C, D, E, F, and G). Phylogenetic tree was generated using PHYLIP version 3.69 and analyzed by neighbor joining method. Further, the aligned sequences were analyzed using RIP(Siepel et al. 1995), an online tool of HIV-LANL. The programme identifies the subtype to which the sample sequences belong by aligning the sequences with the existing database of different subtype sequences. The window size for alignment was set to 200 for subtyping of *env* sequences and 50 for *nef* sequences due to the difference in their lengths. All other parameters were set to default. The Nef and other gene sequences were also aligned in the same way (Notredame et al. 2000). The mutations were identified by comparing the sequences with the reference sequence, NL4-3. PSIpred 3.2 server was used to predict the secondary structures of the sequences that showed mutations when aligned with the reference sequence (NL4-3).

Cytometric bead array

Cytokine levels of plasma were measured by using human inflammatory cytokine CBA kit (BD Biosciences) which provide simultaneous detection or estimation of cytokines such as IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70.To estimate concentration of these cytokines in plasma, aliquots of plasma were diluted with assay diluent and assay was performed according to manufactures' instructions and all the standard safety precautions were taken throughout the assay. Six lyophilized

cytokines were reconstituted with assay diluent and allowed them to equilibrate to RT. Cytokines were serially diluted (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256) to construct a standard curve to determine the concentration of cytokines present in the plasma of unknown samples. Beads coated with the above mentioned cytokines were mixed and spun at 200g for 5 min, supernatant was removed and serum enhancement buffer wad added (equal to the volume removed) ,vortexed and incubated for 30 min at RT. After incubation 50 µl of capture bead mixture was added to 50µl of sample, incubated for 90min at RT, protected from light. Then the sample- bead mixture was washed with 1ml wash buffer at200g for 5 minutes and 50ul of phycoerythrin conjugated antibody was added and incubated for 90 min in the dark After incubation sample mixture was washed with 1ml wash buffer and resuspended the pellet in 200ul wash buffer for analysis and samples were analyzed on FACS Canto using FCAP Array software (BD Biosciences).

IgG purification from plasma

IgG was purified from the plasma by absorption with protein A- agarose. Protein A agarose was supplied as a suspension in PBS. The resin was washed for 4 times, supernatant was removed and resin was packed in a column. Protein A agarose was equilibrated with 5ml of equilibration buffer and the buffer was allowed to drain out completely. 1 ml of diluted (1:2) plasma sample with PBS was loaded onto the column and allowed to drain off completely and the column was again reloaded with the collected flow through to ensure complete removal of IgG from plasma. Column was washed with 5ml of 1x equilibration buffer. Bound IgG was eluted by using 1 ml elution buffer and the collected IgG was neutralized by neutralization buffer.

Genotyping of CCR5Δ32, CCR2 V64I, SDF 1 - 3'A, RANTES In 1.1C and MCP-1

The CCR5-Δ32, CCR2V64I, SDF1-3'A, RANTES In 1.1c and MCP-1 genotyping was done by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP).

Amplification of CCR5Δ32 with the respective primers produced amplication fragments at 177 and 149 bp respectively. Presence or absence of a 32 bp deletion in the CCR5-Δ32 gene will yield two types of amplified products and three genotypes when amplified with the CCR5-Δ32 primers. An wild type CCR5 gene will produce a177 bp fragment if the person does not have the 32 bp deletion on its two alleles, if the person is homozygous mutant for the 32 bp deletion, an amplified product of 149 bp can be seen and if the person is heterozygous (one allele is wild type and the second allele is mutant) CCR5Δ32 gene will produce two fragments of 177bp and 149bp respectively.

The amplified product of the CCR2 gene fragment is 380 bp. The PCR product was digested with the enzyme Fok I for 4 hours at 37°c. After digestion, different pattern of DNA fragments were observed at different sizes such as 380 bp, 215 bp and 165bp respectively. Mutation of valine to Isoleucine at 64 th amino acid position introduces a restriction site for the enzyme FokI. Presence of isoleucine on the two alleles that is if the person is homozygous mutant, FokI will cleave at its recognition site and yield two fragments of 215bp and 165 bp. There will be three fragments on the gel at 380 bp, 215 bp and 165 bp if the person is heterozygous for the mutation. If there is no mutation on the two alleles only one fragment will be produced at 380 bp.

The amplified product of SDF1-3'Agene is 302 bp pcr product was digested with the enzyme Msp I and the digestion pattern observed was 302, 202 and 100bp respectively. The introduction of A to G at the 801 position of SDF-1 3' UTR region removes the restriction site specific for the enzyme MspI. If the two alleles are mutated that is if the person is homozygous for the mutation, MspI will not cleave and produce only one fragment at the size of 302bp. If adenine is present on the two

alleles, in this case MspI will cleave and produce two types of fragments at the sizes of 202 and 100 bp. Three fragments of the SDF-1 products at the sizes of 302, 202 and 100 bp respectively; if the person is heterozygous.

RANTES In1.1T>C genotyping was done according to a method described by Qian et al. A 343 bp DNA region incorporating the RANTES In1.1T>C polymorphism was amplified by PCR reaction. Then the amplified PCR products were digested with MboII. The RANTES In1.1C/C will yield 225 bp and 118 bp fragments if the person is homozygous mutant, the RANTES In1.1C/T will yield 343 bp, 225 bp and 118 bp fragments if the person is heterozygous, the RANTES In1.1T/T will not be cleaved by MboII and produce a 343 bp fragment.

For MCP-1 genotyping oligonucleotide primers were utilized with PCR to amplify a 930 bp fragment of the MCP-1. The PCR products were digested with Pvu II. If the person is G/G homozygous Pvu II digested the 930 bp DNA fragment into 708 and 222 bp fragments. Only one fragment of 930bp will be seen if the person is A/A homozygous. G/A heterozygous individuals will show the fragments at 930, 708, and 222bp respectively.

ELISA

Plasma isolated from an EDTA-anticoagulated blood sample, obtained 16–24 h after extraction of venous blood, was kept at -70°c until required for ELISA determinations. ELISA was done with appropriate pairs of coating and catcher anti–antibodies, as well as with standard recombinant human proteins. The ELISA conditions for antibody coating, blocking, reagent for plasma dilution, and enzyme-developing reaction were followed as recommended by the manufacturer, all determinations were done in duplicate.

Wells were coated with a capture antibody diluted with PBS to a concentration of 0.5 to 2.0 µg/ml (0.5µg/ml for MCP-1 and RANTES 2.0µg/ml for SDF-1α) with 100 µl added to each well and plated was covered with adhesive plastic and incubated overnight at RT. After overnight incubation capture antibody was removed and plate was washed thrice with PBS. After the last wash plate was inverted on tissue paper to remove residual buffer. Then 300µl of blocking solution was added to each well and incubated for 2 hours at RT. After 2 hours of incubation blocking solution was removed and plate was washed. Mean while standards were diluted from 4ng/ml to zero in diluents and 100 µl of standard protein was added to each well and incubated for 3 hours at RT. After incubation standards were removed and plate was washed and 100µl of detection antibody was added to each well at the concentration of 0.5µg/ml and plate was incubated at RT for 2 hours. Then plate was washed and 1:2000 dilution of 100µl of avidin- peroxidise was added and plate was incubated for 30 min at RT, after 30 min plate was washed and 100µl of substrate solution was added and plate was observed for color development. Color development was monitored with an ELISA plate reader (TECAN) at 405nm with a reference wavelength at 650nm.

Preparation of cells and virus stocks

Viruses NL4-3, HIV-1 $_{93IN101}$ and HIV-1$_{92BR025}$ was propagated by infecting SupT1 cells. Sup T 1 cells were infected with undiluted virus for two hours at a cell concentration of 10^{7}/ml in 5% CO_2 incubator at 37°c. After two hours of incubation cells were washed and RPMI 1640 medium containing 10% heat-inactivated FCS was added to bring the cell concentration to 10^{6} /ml. Cells were replenished with fresh media for every two days at regular intervals. From the third day onwards after virus culture, supernatant was collected and tested for the presence of p24 antigen by ELISA. Virus was collected and stored in liquid nitrogen.

Neutralization assay with plasma

Plasma was heat inactivated (56°C/30 min), serially diluted 10-fold and incubated with virus NL4-3 for 30 min at 37°C. The virus-serum mixtures were added to the cells incubated for 4 hours at 37°C in a 5% CO2 incubator. The plasma and virus mix was removed and the cells washed, replenished with fresh media and incubated for 4 days at 37°C. On 5th day supernatants were collected to quantify p24 antigen using ELISA.

Plasma neutralization with different viral isolates

To determine the neutralization efficiency and broad neutralization of plasma of both LTNP and FP antiviral assay was performed with three different isolates NL4-3, HIV-1 $_{93IN101}$ and HIV-1 $_{92BR025}$. LTNP and FP plasma was incubated with all the viral isolates mentioned and incubated for 30min at 37°C. Then plasma and virus mix was added to cells. After 4 hours of incubation cells were washed and fresh media was added and allowed to grow for 4 days. On 5th day supernatants were collected and checked for viral replication in terms of p24 concentration.

Antiviral assay with IgG and non IgG fractions of plasma

To determine which fraction of plasma is showing antiviral activity, IgG and non IgG fraction from plasma was purified by using protein A agarose column. IgG and non IgG fractions of plasma of LTNP was treated with viral isolates and mix was added to cells. After 4 hours incubation cells were washed and grown for 4 days. 5th day supernatant collected and checked for antiviral activity of IgG and non IgG of plasma.

HIV-1 p24 antigen assay

HIV-1 p24 present in the supernatant was determined by using ELISA kit and the protocol was followed according to manufacturer's instructions. Supernatants collected were diluted and 100µl diluted supernatant was added to each well of ELISA plate containing 25µl of disruption buffer and plate was incubated at 37°c for 1 hour . After 1 hour of incubation plate was aspirated, washed with wash buffer and 100µl of conjugate solution was added and incubated the plate at 37°c for 1 hour. After incubation plate was washed and 100µl of substrate solution was added and incubated the plate at room temperature for 20 mi. After 20 minutes the reaction was stopped by adding 100µl of stop solution or 1N HCl and reading was taken at 450nm.

Statistical analysis

Statistical analysis was performed using the statistical software package SPSS Inc., Chicago, USA (Windows, version 17.0). Kaplan Meier survival analysis was used to estimate median probabilities and cumulative probabilities with 95% confidence intervals. The non parametric Chi-square test and t-test were also applied in comparative analysis results between different groups. Graph pad prism 5 was used for ELISA determinations.

Primers used for PCR-RFLP

Name	Sequence
CCR5Δ32-F	5- TGTTTGCGTCTCTCCCAGGAA -3
CCR5Δ32-R	5-GAGTAGCAGATGACCATGACAA-3
CCR2V64I-F	5-GGATTGAACAAGGACGCATTTCCCC-3
CCR2V64I-R	5-TTGCACATTGCATTCCCAAAGACCC-3
SDF 1-3'A-F	5-CAGTCAACCTGGGCAAAGCC-3

SDF 1-3'A-R	5- CCTGAGAGTCCTTTTGCGGG-3
RANTES In 1.1 T/C	5-CCTGGTCTTGACCACCACA-3
RANTES In 1.1T/C-R	5-GCTGACAGGCATGAGTCAGA-3
MCP-1-F	5-CCGAGATGTTCCCAGCACAG-3
MCP-1-R	5-CTGCTTTGCTTGTGCCTCTT-3

Restriction enzymes and digestion pattern of genetic variants

Mutation	Enzyme	Digestion pattern
CCR5Δ32	None	177(wt), 145(mut) bp
CCR2V64I	Fok 1	G – 380 bp, A – 215+165 bp
SDF 1-3'A	Msp1	G – 193+100 bp A - 293 bp
RANTES In 1.1 T/C	MboII	T – 343bp C – 225+118bp
MCP-1 A/G	PvuII	A – 930 bp G - 708+222 bp

Chapter III

HIV -1 subtyping and sequence analysis of HIV -1 coded accessory genes in children with perinatal HIV infection

Introduction

Long term non progression in HIV infected individuals is a complex interplay between viral and host factors (Mikhail et al.)(Rodés et al. 2004)(Anastassopoulou & Kostrikis 2003)(Mangano et al. 2000)(den Uyl et al.). HIV genome and its proteins has become the subject of interest ever since it is discovered (Broder & Gallo 1984). Among all the nine genes coded by HIV, the six accessory or regulatory genes (*nef, vif, tat, rev, vpr* and *vpu*) are unique to HIV and play an important role in viral replication and pathogenesis. Intensive research on HIV genome, mainly in accessory/regulatory genes has been reported that certain genetic abnormalities may provide protection to the host.

Progression rate of the disease may also get affected by the subtype of HIV circulated in the infected individual. HIV-1 exhibits high intra and inter subtype genetic diversity. Such differences in the genetic features of HIV can influence biological properties like infection, transmission and pathogenesis (De Wolf et al. 1994)(Tscherning et al. 1998). Although there are no reports supporting infection of particular HIV-1 subtype with non-progressive HIV disease, individuals infected by subtype A appear to undergo less level of risk of progression to AIDS compared to non-A subtype (Kiwanuka et al. 2008)(Kanki et al. 1999). Thus studying viral genome characteristics provide great opportunities in understanding HIV gene functions and their contribution to viral pathogenesis.

The largest cohort of LTNP infected with defective strain of HIV-1 has been reported in the Sydney blood bank cohort (SBBC) (n=8) whose members were infected with an HIV-1 strain having defective *nef* gene after being transfused with blood from a common donor (Deacon et al. 1995)(Estable et al. 1996)(Learmont et al. 1992)(Rhodes et al. 1999)(Michael, Chang, L. a d'Arcy, et al. 1995). In spite of the fact that the accessory genes of HIV initially thought to be not essential for

infection, While present reports show their importance in establishment of viral replication and pathogenesis in vivo (Wang 2013).

One key determinant of viral pathogenicity is the HIV-1 accessory protein Nef (Weiss 1993). HIV-1 Nef, a 27-35Kda myristoylated protein, emerged as one of the viral protein that plays an important role in viral life cycle and pathogenesis (Corró et al. 2012). Although initially considered non essential for HIV replication, it can enhance viral infectivity by 5 fold in vitro (Spina et al. 1994). This protein exhibit spectrum of biological activities including down modulation of CD4 and major histocompatability complex MHC-1 on cell surface *etc* (Greenway et al. 2003; Mariani & Skowronski 1993; Aiken et al. 1994; Anderson et al. 1994; Miller et al. 1994; Stoddart et al. 2003). Furthermore, Nef binds to host cell proteins such as Lck,Hck,Akt,Raf,Alix-1 and others modulating many T cell signaling pathways (Geyer et al. 2001a). The activity of Nef is related to some key motifs, such as $MGxxxS_{1}$, WL_{57}, $EEEE_{62}$, $PxxPxR_{72}$, FPD_{121}, EE_{154}, $D/ExxxLL_{160}$, and DD_{174}. Most of the Nef functions are achieved by its flexible regions by binding to different cellular components. The flexible regions include membrane anchoring region contain a core domain designed for signalling and retention, and a second flexible loop for trafficking and internalization (Geyer et al. 2001b).

In view of the great versatility of Nef protein, one may hypothesize that besides large deletions in Nef, single amino acid substitution via point mutation can lead to premature termination of protein synthesis that in turn leads to reduced viral fitness and replication (Casartelli et al. 2003). Vpr (Viral protein R) is one the highly conserved small basic protein. The functions of Vpr include G2 cell cycle arrest, apoptosis and nuclear localization of HIV-1 preintegration complex. Vpr also plays an important role in long term disease progression causing infection in non dividing cells such as macrophages (Sherman et al. 2002)(Brenner & Kroemer 2003)(Chui et al. 2006). Vif (virion

infectivity factor) promotes viral infectivity by enhancing HIV replication and inducing degradation of antiviral factor APOBEC3G (apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3G)(Sheehy et al. 2002)(Zhang et al. 2008). Only few polymorphisms or mutations have been identified in the vif gene in association with delayed disease progression. Vpu (Viral protein U) is a transmembrane protein play a key role in degrading newly synthesized CD4 molecule by proteasome pathway. Additionally, Vpu enhances virus release from virus-producer cells and down modulation of CD4 and MHC-11(Nomaguchi et al. 2008) (Chen et al. 1993)(Bour et al. 1995)(Kerkau 1997)(Neil et al. 2008).

Many studies have reported an association between defective *nef* gene and slow or non disease progression of HIV infection, mostly in adult cohorts (Salvi et al. 1998; Brambilla et al. 1999; Deacon et al. 1995; Michael, Chang, L. A. d'Arcy, et al. 1995; Kirchhoff et al. 1999). However, few have been concentrated on *nef* gene defects in vertically infected children (Casartelli et al. 2003; Geffin et al. 2000) and it has not been reported that amino acid variations in different stages of AIDS development.

So, our aim was to study mutations and deletions in the accessory gene region to understand their role in slow or non progression of disease in pediatric population choosing unique group of patients with different clinical outcomes. We found that point mutations or deletions in c terminal region or total absence of nef and other accessory genes could affect their function and diminish HIV-1 pathogenicity in vivo as indicated by low viral loads and stable CD4+T cell counts in LTNP children.

Results

In the present study we have analyzed the *nef and* other accessory genes *vif, vpu* and *vpr* sequences from the study population to understand the subtype, amino acid variability and presence of deletions and mutations. Envelope, *nef* and other accessory genes were amplified by nested PCR using genomic DNA derived from PBMC of the HIV-1 infected children of category LTNP (n=6), SP(n=18) and FP (n=8). Sequences were aligned with the reference sequence (NL4-3) and were studied for mutations. Mutational analysis was performed. *Env* and *nef* sequences were aligned using Clustal-X version 2.1. For subtyping the consensus sequence of *env* and *nef* aligned with the representative sequences of all the HIV-1 subtypes (A, B, C, D, E, F, and G). Phylogenetic tree was generated to understand the grouping of consensus sequence with the subtypes. The analysis was performed using PHYLIP version 3.69 by neighbor joining method. Further the aligned sequences were analyzed using RIP (Siepel et al. 1995), an online tool of HIV-LANL. The programme identifies the subtype to which the sample sequences belong by aligning the sequences with the existing database of different subtype sequences. Further *nef , vif, vpu* and *vpr* sequences were aligned using Clustal-X version 2.1 and also T-COFFEE (Notredame et al. 2000) online server with the reference NL4-3 sequence. The mutations were identified by comparing the sequences with the reference sequence. PSIpred 3.2 server was used to predict the secondary structures of the nef sequences that showed mutations when aligned with the reference sequence (NL4-3).

Virus subtyping using *env* and *nef sequences*

Envelope sequences contain information on the different subtypes circulating in any given geographic area and most of the Indian samples were subtyped based on this structural gene, thus this region is usually exploited for subtyping of the sample sequences during clinical studies (Larkin et al. 2007) and much data is not available concerning the variability of *nef* gene for subtyping

analysis. Considering the role of *nef* in genetic diversity we also have subtyped the samples based on *nef* gene. From the analyses (Fig 3.1A, B, C & D) of both *env* and *nef* it was observed that the samples belonged to subtype B.

Sequence analysis of the samples

As discussed above studies have identified Nef and other accessory proteins to be crucial in the maintenance of optimal viral replication and in the progression of infection to acquired immune deficiency syndrome (AIDS). Some important putative motifs have been reported in the Nef sequence that is responsible for its modification, interaction with cell surface receptors, signalling, trafficking, *etc.* We have analyzed the samples to understand the variations in the sequences specifically in these functionally important motifs. It was observed that most of the motifs were well conserved in all sequences, while mutations were observed in few important regions. The details of the analysis are discussed in the next section.

Conserved motifs

$MGxxxS_{1}$ motif responsible for Nef myristoylation by acting as a substrate for N-myristoyl transferase protein and $PxxPxR_{72}$ motif that mediates interaction between Nef and signalling molecules essential to induce cellular activation and aids in down-regulation of MHC- I and MHC-II were well conserved in all sequences. Similarly FPD_{121} responsible for interaction of Nef with human thioesterase reported to influence Nef-mediated endocytosis, M_{20} and $EEEE_{62}$ responsible for MHC-I down-regulation were also observed to be conserved throughout the sequences. TA_{48} motif observed to affect Nef's binding to CD4 cytoplasmic domain was also well conserved. Other motifs in the accessory proteins, Vpr, Vpu and Vif were motif R77 Q and F72L, play a role in

cytopathicity, nuclear accumulation and localization of virions in host cell and also were well conserved in all the samples (Table 3.2).

Mutations Observed

In the EE_{154} motif responsible for CD_4 down regulation, E155 (Glutamic Acid) is substituted by aspartic acid (D) in the sample LTNP_3. The motif D/ExxxLL$_{160}$, responsible for the viral pathogenicity, was observed with mutations at E_{160} (Glutamic Acid) to D (Aspartic Acid), S_{163} (Serine) and L_{164} (Leucine) to N (Asparagine) and H (Histidine) respectively in the sample LTNP_3.Substitution of, S_{163}(Serine) with R (Ariginine) was observed in sample SP_21. In the binding motif DDPXXE$_{174}$ for c-Raf1 kinase reported to be involved in the mitogen-activated protein kinase pathway, E_{177} (Glutamic Acid) is substituted by D (Aspartic Acid) and E_{179} (Glutamic Acid) by K (Lysine) in sample SP_18. This entire motif was found to be mutated in sample LTNP_3. The C-terminal sequence of LTNP_3 was observed to be mutated to a large extent. SP_18 showed some more mutations reported in the Fig 3.2 & Fig 3.3. Mutation at T_{15} supposed to be frequent in LTNPs was reported in all sequences. However this mutation was also seen in the wild type reference strain NL4-3. At the same time H_{102} was seen in all sequences including the wild type reference strain NL4-3 (Foster et al. 2011).

Some samples were reported with premature stop codons resulting in shortening of the sequences. Some other novel mutations were observed in our study such as N_{157} (Asparagine) substituted by I (Isoleucin) in LTNP_3. V_{180} (Valine) was seen substituted by C (Cysteine) and M (Methionine) with in LTNP_3 and SP_18 respectively. Two additional amino acid residues F,V (Phenylalanine, Valine) respectively were seen at positions 174,175 in LTNP_3. The Nef CTL epitopes were conserved among all the groups. Table 3.1 depicts mutations observed in all the groups.

Fig 3.6 depicts nested PCR amplication of viral accessory genes *vif, vpu* and *vpr* from this amplification pattern it was observed that LTNP 4 had a truncated accessory gene region and after sequence analysis it was confirmed that deletion of *vpu* gene in LTNP 4. Among the accessory proteins it was also noticed that the protein product *Vpu* was totally absent in one of the sample. It is clear that the sample LTNP_10 has the maximum mutations in all of the accessory proteins which can be attributed to the non-progression of the disease. Other samples noticed with mutations are LTNP_7 and LTNP_3. (Fig 3.7, 3.8 & 3.9).

Secondary structure

Solving the full length structure of Nef is difficult due to the presence of flexible loops that are difficult to crystallize owing to their flexibility. The complete structure can be understood by studying the component fragment and by modeling the flexible loop. HIV-1 Nef possesses a genetically diverse and structurally flexible N-terminal membrane anchor region of about 60 residues (2-57), followed by a well-conserved and folded C-terminal core domain of about 130 residues (56-206).

The mutations in the sequences reflect in the secondary structure resulting in changes in the regions responsible for interactions. To understand the implication of the mutations in this study, secondary structures of sequences with mutations were predicted using PSIpred. The generated structures were compared with that of the reference sequence (NL4-3). Fig 3.4. depicts the structures of reference NL4-3sequence, LTNP_3, and SP_16. As observed in Fig 3.4B and 4C, structures of samples LTNP_3 and SP_16 show a helix break in region 81-94. Helix at position 190 to 197 is absent in sample LTNP_3 as observed from the secondary structure generated.

Discussion

Motif	***Function***	***Observation***	***Occurance***
$MGxxxS_{1}$	Nef myristoylation	*Conserved*	32 sequences
T_{15}	Observed commonly among LTNP	*Conserved*	32 sequences
M_{20}	MHC-I down-regulation	*Conserved*	32 sequences
TA_{48}	CD4 cytoplasmic domain binding	*Conserved*	32 sequences
$CAWLEAxEEEE_{55}$	MHC-I down-regulation	*Conserved*	32 sequences
$PxxPxR_{72}$	Nef and signaling molecules interaction, MHC I and II Downregulation	*Conserved*	32 sequences
H_{102}	Observed commonly among LTNP	*Conserved*	32 sequences
FPD_{121}	Responsible to influence Nef-mediated endocytosis	*Conserved*	32 sequences
EE_{154}	CD_4 downregulation	$E_{155}D$	1 sequence
$D/ExxxLL_{160}$	Viral pathogenicity	$S_{163}N$ $S_{163}R$ $L_{164}H$	1 sequence.

$DDPxxE_{174}$	Binding motif for c-Raf1 kinase involved in the mitogen-activated protein kinase pathway	$E_{177}D$ $R_{178}K$ $E_{179}R$ $E_{179}K$	2 sequences 1 sequence

Table 3.1. Summary of functional motifs of Nef and the observations in the study population

Gene/Change in motif	Function	Observation	Occurrence
Vpr/ F72L	Hinders vpr localization into nucleus	Conserved	32 sequences
Vpr/ R77Q	Reduced cytopathicity	Conserved	32 sequences
Vpr/ C-terminal a.a deletion 83-89	Defective nuclear localization	Amino acid substitution	5 LTNP,1 SP and 2 FP And conserved in 24 sequences
Vif/ V13I,V55T,L81M	No functional transport	Conserved	32 sequences
Vif/R132 S	Reduced replication capacity	Conserved	32 sequences
Vif/a.a54-117	No functional support	Amino acid substitutions	LTNP 7 and 10

Vpu/4 a.a insertion in the N-terminus and total deletion of vpu	No functional support	Conserved deleted	32 sequences 1 LTNP

Table 3.2: Summary of functional motifs mutations in Vif, Vpr and Vpu associate with LTNP

From the analysis it was observed that most of the functional motifs were conserved in all the study subjects suggesting that Nef alone might not be responsible for the delayed progression of the disease. However some mutations were observed in motifs responsible for the overall maintenance of the virus. The mutations with their occurrences are given in Table 3.1. Furthermore some mutations that have never been reported earlier were observed in this study.

To understand the effect of all the mutations the sequences and observed substitutions were submitted to the PROVEAN (Protein Variation Effect Analyzer) v1.1 server, a tool to analyze whether a protein sequence variation affects protein function (Choi et al. 2012). The score cut-off was set -2.5 and all other parameters were set to default. PROVEAN calculates a score based on the reference and variant versions of a protein query sequence with respect to sequence homologs collected from the NCBI NR protein database through BLAST. As observed from the result Fig 3.5, it can be noted that few of the substitutions are above the mentioned threshold value implying that the substitutions were not common in the aligned homologs. It can be assumed that they could be detrimental to the function of the protein.

The implication of these substitutions with respect to their position in the secondary structure was analyzed by the ConSurf server (Celniker et al. 2013; Ashkenazy et al. 2010; Berezin et al. 2004). The

novel mutations identified are of the residues in the exposed region of the protein. The mutations thus might have a role to play in important interactions and in turn in the proper functioning of the protein. These mutations also seem to be of residues that are well conserved throughout the alignment thus studying the secondary structures of the proteins might reveal important information. In the secondary structures generated a break in the helix at 81-94 region was observed that was shown to be highly conserved in all *nef* alleles, implying the importance in its function. It has also been reported that the helices present in the region of 70-120 form a cavity that probably are responsible for interaction with Src family kinases (Geyer et al. 2001b). Another difference observed is the absence of helix at position 190 to 197 which is a part of the core domain, reported to be the only region of Nef that has a stable tertiary fold. This also can be attributed to the loss of function of Nef. Thus these changes might be responsible for the loss of function of Nef and in turn contribute to the late or non- progression of HIV.

Conclusion

Above study was aimed at understanding the role of Nef and other accessory gene sequence mutations in rendering Long tern non-progressor status to a specific population. The samples were observed to be clustered with subtype B reference sequence, while subtype C is commonly observed in the Indian population. From the study it was observed that sequence from LTNP-3 sample showed mutations in three important motifs EE,ExxxLL and DDPxxE. Interestingly, changes in its secondary structure were also observed in comparison with the reference sequence. A break in the helix (region 81-94) and absence of a helix (Region 190-197) in the C-terminal region could be attributed to the loss of function of Nef. Similar mutations in some motifs were observed in Slow Progressor samples SP_18 and SP_21. Long Term Non-Progressor 10(LTNP_10) and slow progressor 2(SP_2) have premature stop codon because of which the Nef protein is shorter in

length resulting in loss of a part of C-Terminal responsible for interactions with Src family kinases. Some mutations like N_{157} (Gutamine) substituted by I(Isoleucin), V_{182} (Valine) with C (Cysteine) and M (Methionine) and L_{183} (Leucine) substituted by M (Methionine) were observed. Among the accessory proteins it was also noticed that the protein product *Vpu* was totally absent in one of the sample. It is clear that the sample LTNP_10 has the maximum mutations in all of the accessory proteins which can be attributed to the non-progression of the disease. Other samples noticed with mutations are LTNP_7 and LTNP_3.

Figures

Phylogenetic analysis of *nef* and *env*

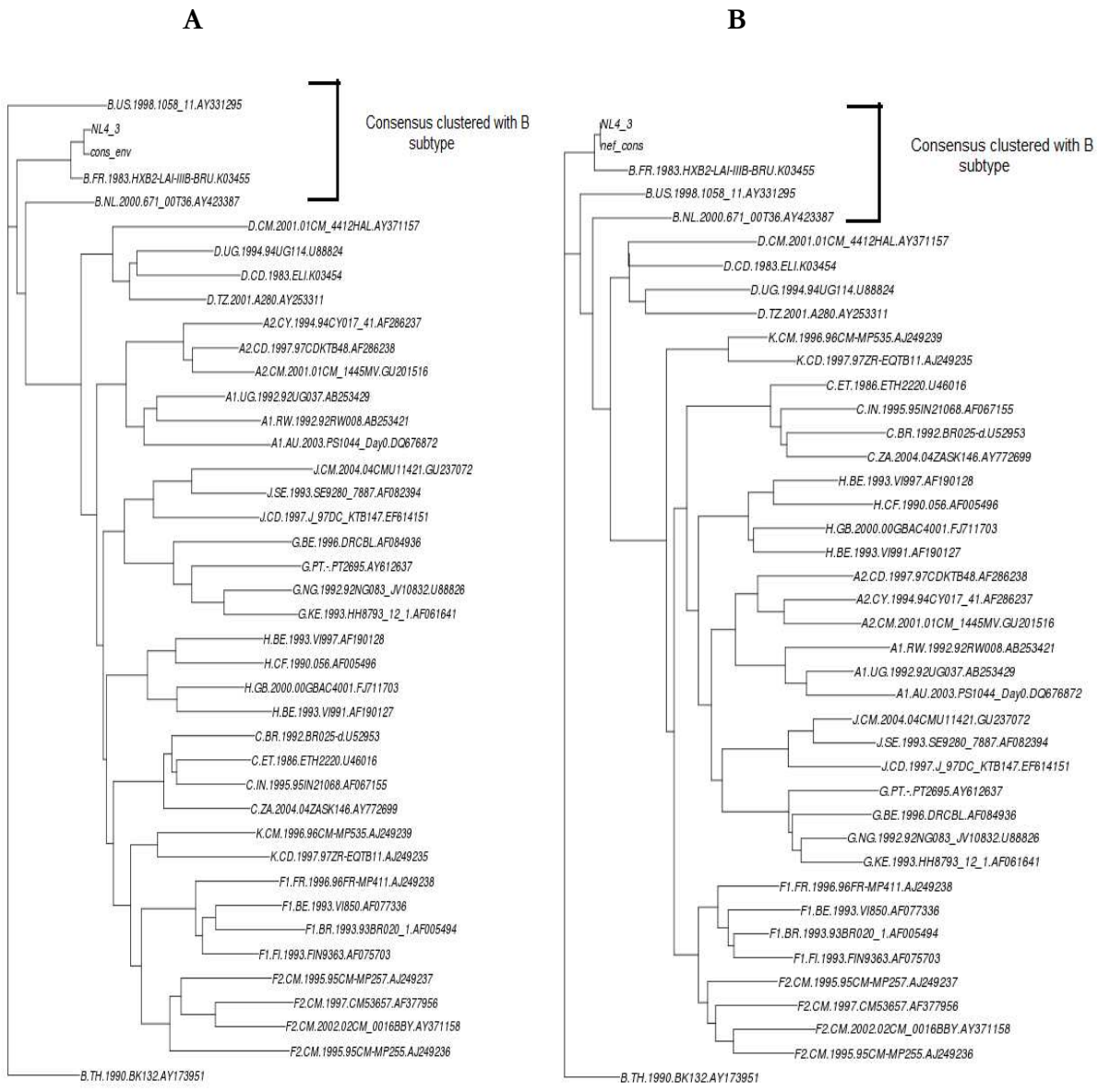
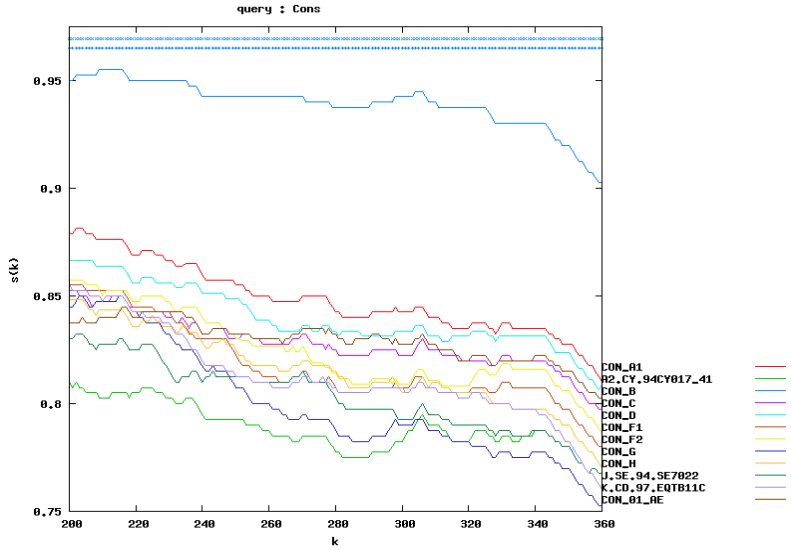


Fig.3.1(A & B): The phylogenetic tree was constructed by aligning the consensus sequences of the sample *nef* and *env* using the HIV Align tool of HIV-LANL where all the subtype sequences from the existing database are used for the alignment

C



D

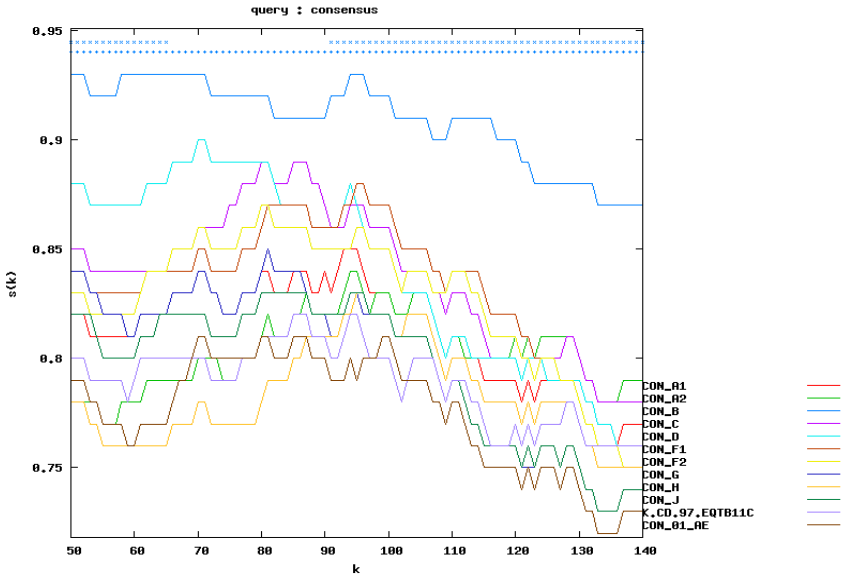


Fig 3.1(C): Plot generated by RIP depicting the similarity of the env consensus with different subtype sequences. **Fig1(**D**)**sequences. In the RIP plot,the x-axis (k) represents the query sequence position at the center of the moving window. The y-axis, s(k), shows the similarity between that window of sequence and each of the background sequences. In the plot **1(c)**, consensus B (dark blue) is the sequence with the highest similarity to the background sequences; it begins with a similarity of 0.95 and falls to a similarity of about 0.90 towards the C-terminal. In the plot **1(d)**, consensus B (dark blue) is the sequence with the highest similarity to the background sequences; it begins with a similarity of 0.925 and falls to a similarity of about 0.85 towards the C-terminal.

Aminoacid alignment of Nef

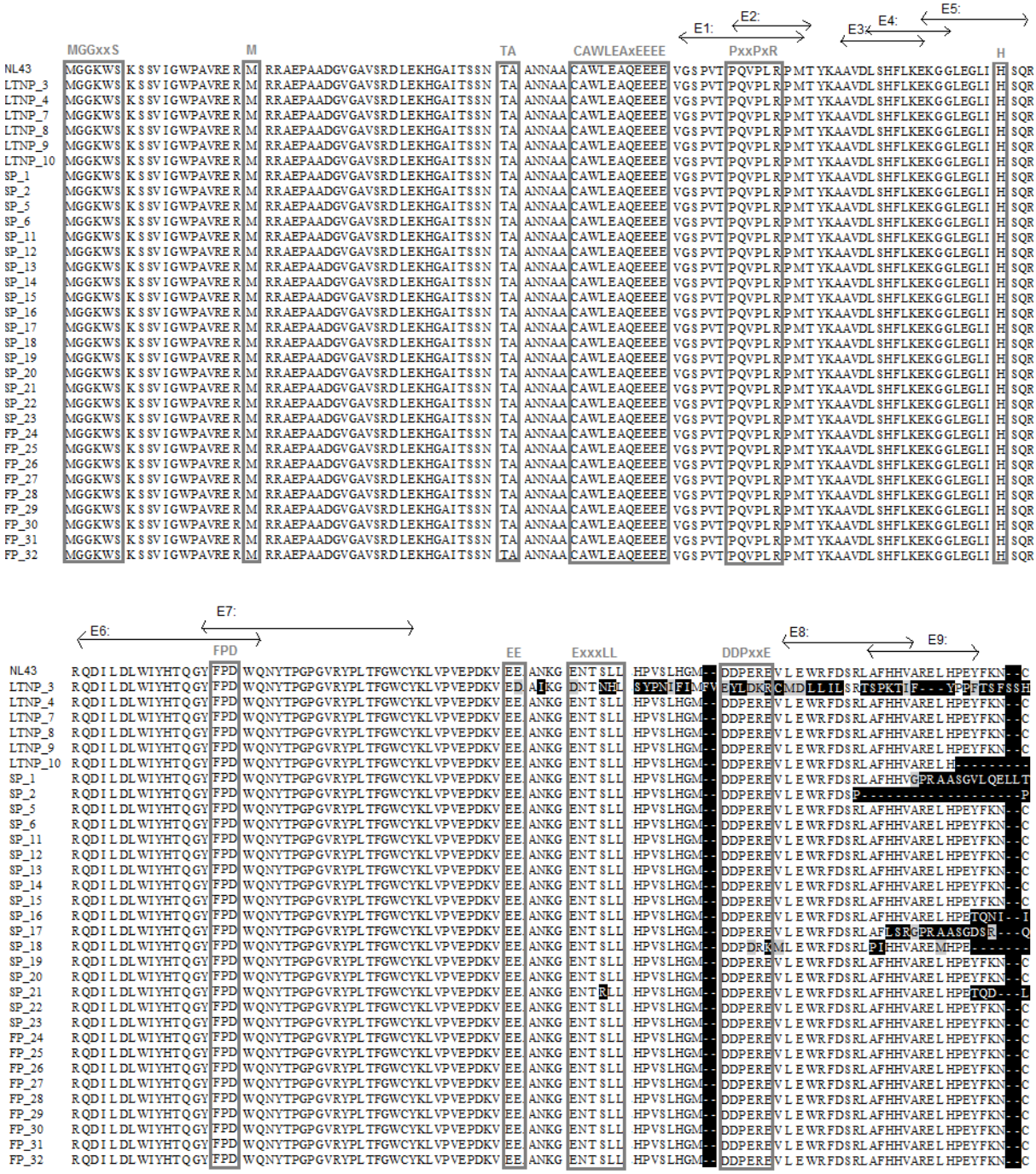


Fig 3.2: Aminoacid alignment of Nef. Comparision of the deduced aminoacid sequences with the reference sequence NL4-3 from the different groups. From top to bottom the sequences are from LTNPs,SPsand FPs.The position was numbered by the reference sequence NL4-3.The locations of functionally important regions are noted in boxes and all were in frame. CTL epitopes are represented as arrows from E1 to E9.

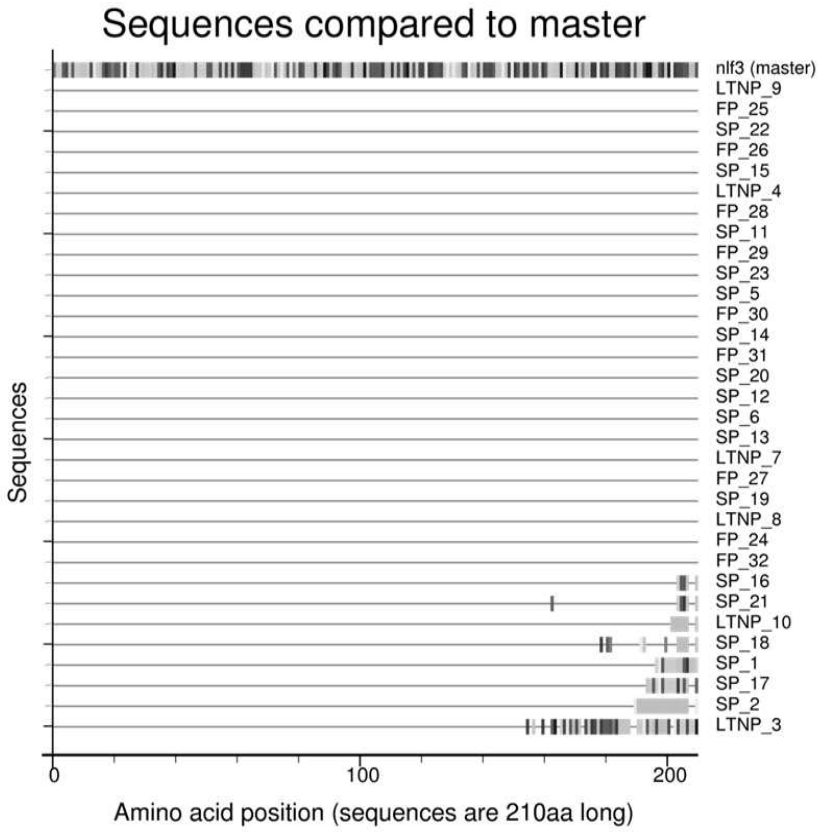
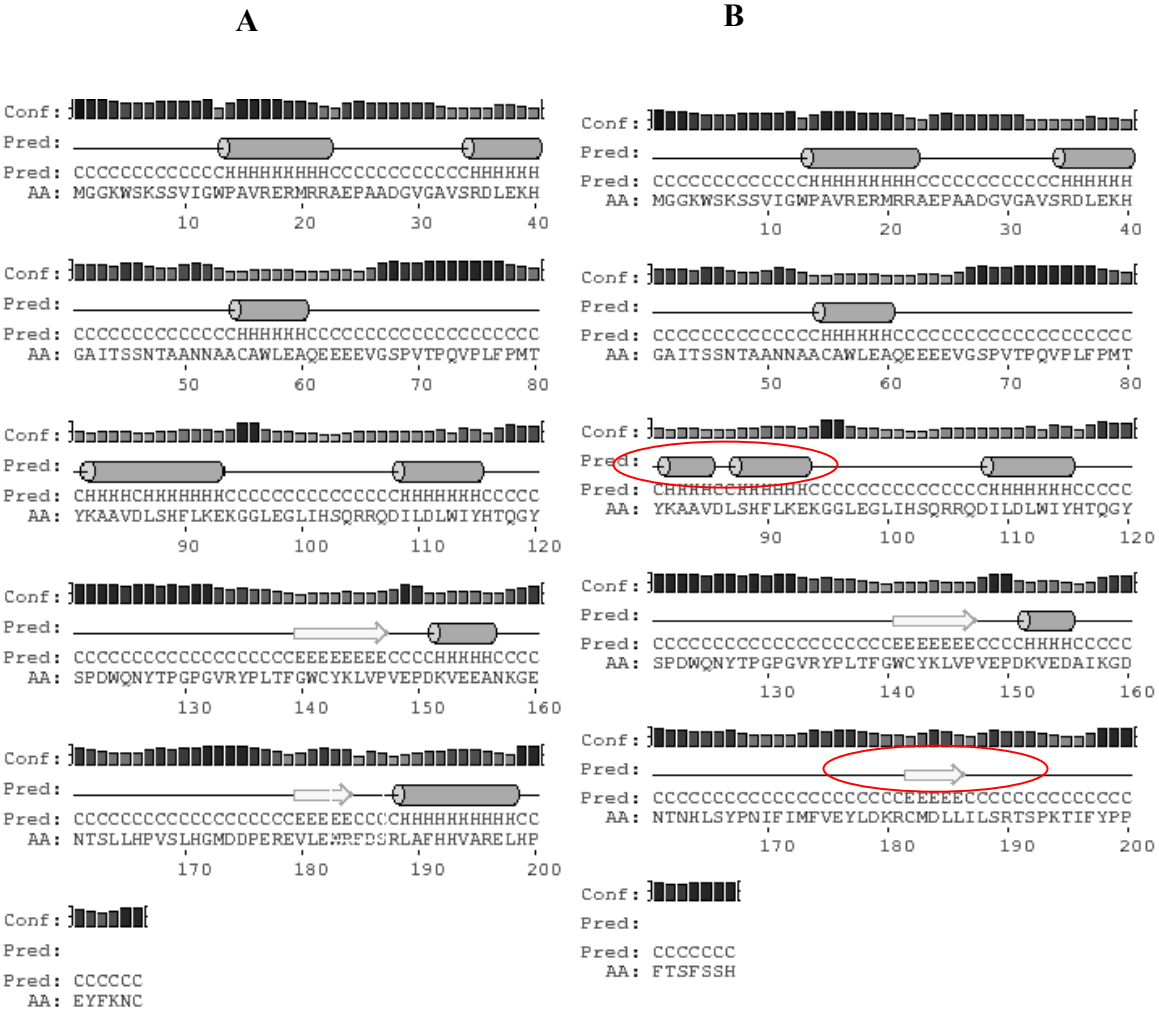


Fig 3.3: Plot showing the difference of the sequences with respect to the reference (NL4-3) sequence.

Secondary structure of Nef



C

Fig 3.4:Secondary Structre predicted using PSIpred **a)**Structure predicted with the reference sequence NL4-3 **b)**Structure with sample LTNP_3 **c)**Structure with sample SP_18

Amino acid substitution effect: generated by PROVEAN Server

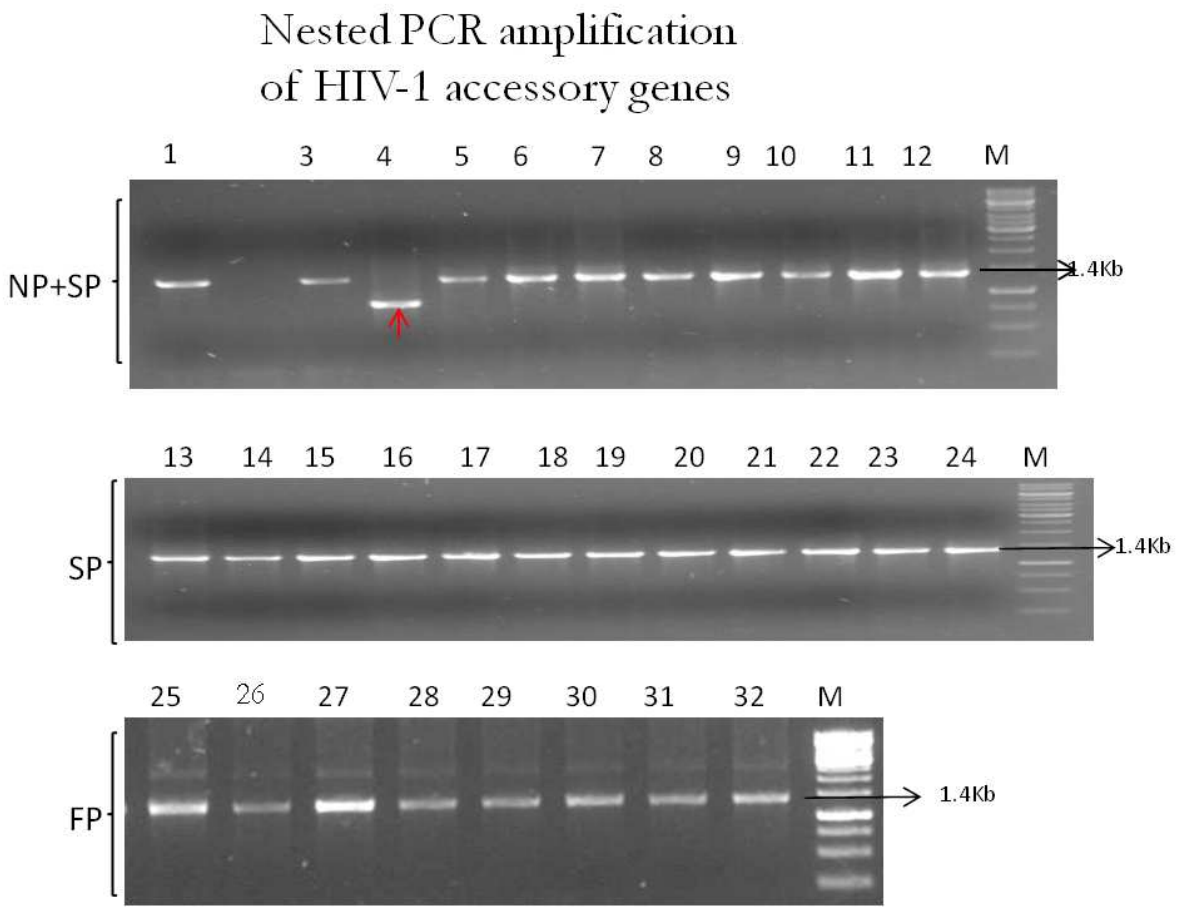
Variant	PROVEAN score	Prediction (cutoff= -2.5)
E155D	-2.050	Neutral
S163N	-1.792	Neutral
S163R	-2.546	Deleterious
L164H	-5.420	Deleterious
E177D	-1.399	Neutral
R178K	-0.499	Neutral
E179R	-3.848	Deleterious
E179K	-3.081	Deleterious
N157I	-4.508	Deleterious
V180C	-3.555	Deleterious
V180M	-2.139	Neutral

Table 3.3: Result generated by PROVEAN server. Substitutions S163R, L164H, E179R, E179K, N157I, V180C have scores above the threshold score -2.5, implying the rare occurrence of the substitutions.

Overall conserved and functional residues in Nef

Fig 3.5: The predicted conserved sites and functional residues in the Nef sequence using the alignment of the LTNP sample sequences.

Nested PCR amplification of HIV-1 accessory genes

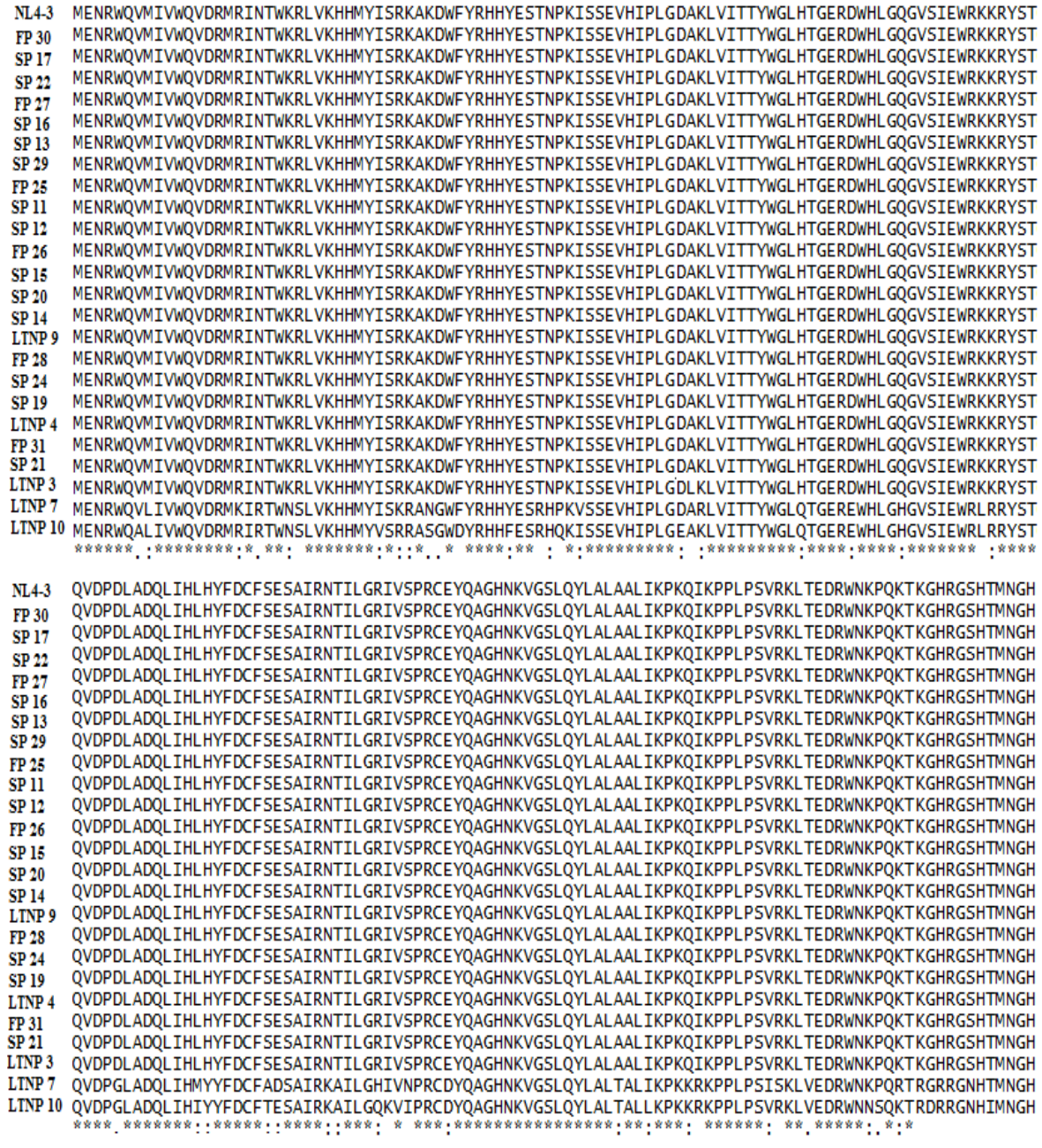


LTNP: Non progressor (3, 4, 7, 8, 9, 10)
SP: Slow progressor (1, 2, 5, 7, 11, 12, 13-24)
FP: Fast progressor (25-32)

Fig 3.6. Nested PCR amplication of accessory gene *(vif, vpr* and *vpu)* region. PCR was performed using genomic DNA of patients LTNP, SP and FP.

Amino acid alignment of Vif, Vpr and Vpu

A



B

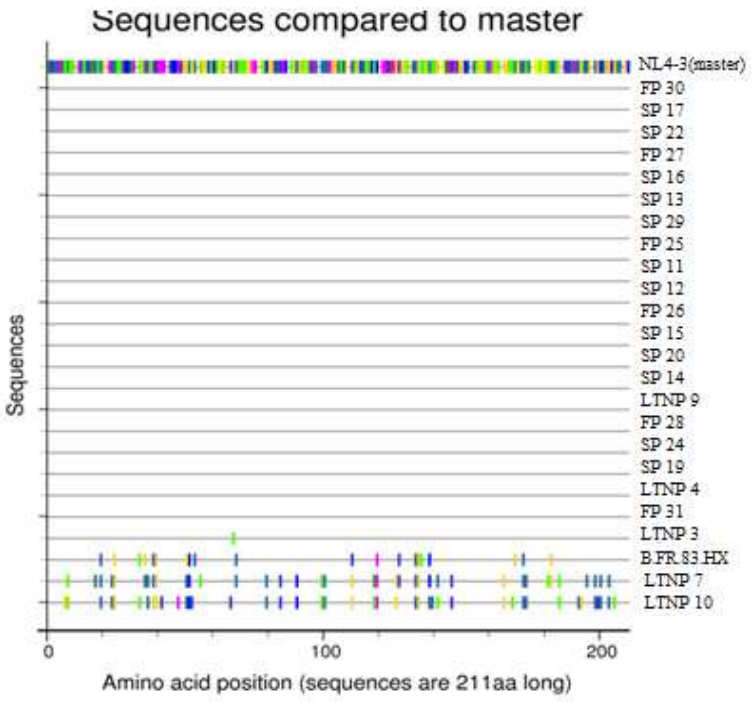


Fig3.7.A) Aminoacid alignment of Vif. From top to bottom the sequences are from LTNPs,SPsand FPs.The position was numbered by the reference sequence NL4-3. B) Plot showing the difference of the sequences with respect to the reference (NL4-3) sequence. Comparision of the deduced aminoacid sequences with the reference sequence NL4-3 from the different groups.

A

```
NL4-3   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 22   MLQK--SNRPQREPYNEWTLESLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 18   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 13   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
FP 29   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
FP 25   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 12   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 11   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 13   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 15   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 14   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 21   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 24   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 19   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 23   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
FP 31   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
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FP 26   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLRQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
SP 20   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLRQHIYETYGDTWAGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGVTRQR---RARNGASRS---------
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FP 27   MEQAPEDQGPQREPYNELTLEILEELKQEAVRHFPPPWLHSLGQYIYETYGDTWTGVETIIR-ILQQLLFIHFR-IG--CQHSRIGILQQR---RARNGASRP---------
LTNP 7  MEQLPEDQGPQREPYNEWALEILEDLKQEAVRHFPRPWLHSLGQYIYNTYGDTWTGVEAIIR-ILQQLLFTHFR-IG--CHHSRIGILRQR---RARNGASRS---------
LTNP 9  MEQPPEDQGPQREPYNEWTLELLEELKQEAVRHFPRPWLHSLGQYIYETYGDTWTGVEAIIR-ILQQLLFVHFR-IG--CQHSRIGILRQR---RARNGANRS---------
LTNP 10 MEQPPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHSLGQHIYETYGDTWTGVEAIIR-ILQQLLFIHFR-IG--CRHSRIGISRQR---RARNGASRS---------
FP 29   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWGRSGSHNKEFCNNCCLSIFR-IG--CRHSRIGVTRQR---RARNGASRS---------
FP 30   MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWGRSGSHNKEFCNNCCLSIFR-IG--CRHSRIGVTRQR---RARNGASRS---------
LTNP 3  MEQPPEDQGPQREPYNEWTLELLEELKQEAVRHFPGPWLHGLGQYVYETY-DTWDRSCRSNKECYNNCCLLDLQNLGAIVRPRRAGNGSCRSYPGASEASGKSA---------
SP 17   MEQAPEDQGPQREPYNEWTLQLL----------------------------------------------------------------------------------------------
LTNP 4  MEQPPEDQGPQREPYNEWTLELLEELKQEAVRHFPRPWLHGLGQYVYETYGDNWTGVRIRER--AEDSGNESEGDADELCNMVDLGIVDAF---RIIDNVIESLVITVPINA
        * *   .: ******** :*: *
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B

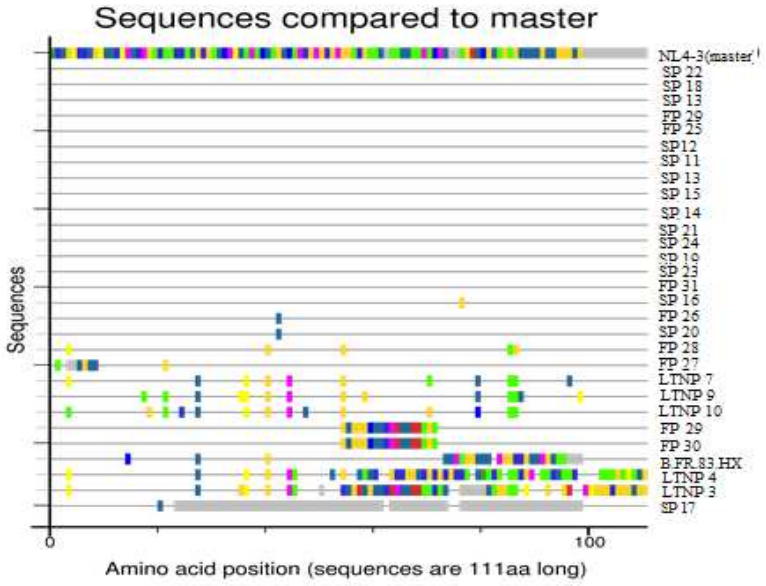

Fig 3.8.A) Aminoacid alignment of Vpr. From top to bottom the sequences are from LTNPs,SPsand FPs.The position was numbered by the reference sequence NL4-3. B)Plot showing the difference of the sequences with respect to the reference (NL4-3) sequence. Comparision of the deduced aminoacid sequences with the reference sequence NL4-3 from the different groups.

A

```
NL4-3    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
FP 30    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 17    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 22    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
LTNP 3   ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
FP 27    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 13    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 16    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
FP 29    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
FP 25    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 11    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 12    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
FP 26    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 15    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
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SP 14    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
LTNP 7   ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
FP 28    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 24    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
SP 19    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
LTNP 9   ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
FP 31    ----MQPI--IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL-
LTNP 10  MVDLLERIDYRVGVGALLVALIIAIVVWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELSTM-VDMGNLRLWMVMTCL
             :: *   *.: **:**:*******:** *****::****** **.*: ************:.. *  * *:**:   * :
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B

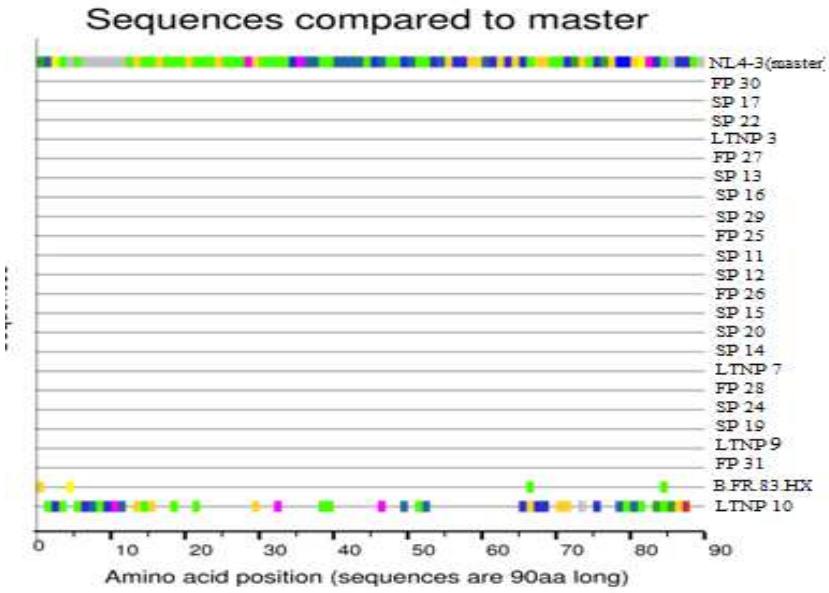


Fig 3.9.A) Aminoacid alignment of Vpr. From top to bottom the sequences are from LTNPs,SPsand FPs.The position was numbered by the reference sequence NL4-3. B) Plot showing the difference of the sequences with respect to the reference (NL4-3) sequence. Comparision of the deduced aminoacid sequences with the reference sequence NL4-3 from the different groups.

Chapter IV

Correlation of polymorphism of HIV-1 coreceptors and their ligands with chemokine levels in plasma

Introduction

Susceptibility to HIV shows a steady level of individual heterogeneity, much of which can be granted by genetic variation of the host, some to the virus and some to the immune system. In an attempt to find host factors necessary for HIV replication, to recognize important pathogenic pathways, and to find out the full armament of host defense mechanisms, there has been a shift from candidate-gene studies to impartial genome-wide genetic and functional studies. Nevertheless, the number of securely determined host factors involved in HIV disease remains less, describing only approximately 15-20% of the observed heterogeneity (An & Winkler 2010). Humans are persistently in contact with infectious pathogens. Selective pressure may lead to genetic changes that may be crucial to escape from infections or to defense against infection (Barreiro & Quintana-Murci 2010). Studies on interaction of HIV and chemokine receptors has dramatically improved our understanding of the molecular processes involved in HIV infection (Lusso 2006).

Genetic variations of the host may be one of the factors responsible for the susceptibility to infection and disease progression. Although, no single gene or polymorphism is likely to be responsible for the outcome. HIV-1 requires at least 250 host-derived relying factors for gaining entry into host cells and accomplishing its life cycle (Brass et al. 2008). Therefore, multiple genetic factors are happened to be involved in susceptibility, pathogenesis, and disease progression following HIV-1 infection. Some of them, that have been existed with HIV infection conclusively are, genes affecting viral entry by changing the expression on cell surface levels of chemokine receptors and their natural ligands chemokines as well (Reiche et al. 2007)(Arenzana-Seisdedos & Parmentier 2006). In addition to CD4, HIV requires co receptors to enter into host cell; important common co receptors are CCR5 and CXCR4. CCR5 is used by macrophage tropic viruses or non syncitium inducing viruses and T- tropic viruses or syncitium inducing viruses use CXCR4 as well

(Boring et al. 1997)(Gonzalez et al. 2002). Genomic studies have shown that variants of the genes for co receptors and chemokines can influence transmission and rate of disease progression (M Dean et al. 1996)(Winkler et al. 1998) (Martin, Dean, et al. 1998).

In particular, allelic polymorphisms of the genes for CCR5, CCR2, RANTES, SDF-1 and MCP-1 have been found to be associated with susceptibility or resistance to HIV infection and on the clinical course of the disease (Reiche et al. 2007). Although, the true affect of these gene variants on host susceptibility to HIV infection and progression to AIDS remains controversial. Mutations in genes of chemokines or their receptors contribute to the inter-individual heterogeneity in risk of HIV infection and pathogenesis (Boven et al. 1999)(O'Brien & Moore 2000)(Martin, Carrington, et al. 1998)(Paxton et al. 1998)(An et al. 2002)(Blanpain et al. 2002). Hence, identification of the functionally active polymorphisms in the gene of HIV co-receptors and chemokines will be significant in assessing the transmission and pathogenesis of HIV.

.

CC chemokine receptor 5 (CCR5) is a member of rhodopsin-like family of seven-transmembrane G protein-coupled receptors (GPCR) contained 352 amino acid (Mummidi et al. 1998) The protein has 3 extra-cytoplasmic loops (ECL), 7 transmembrane (TM) domains and 4 intra-cytoplasmic loops (ICL)(Farber & Berger 2002).CCR5 gene encodes CCR5 protein located on chromosome 3p amongst a cluster of genes that encode chemokine receptors (Mummidi et al. 1997). It had been identified that the CCR5 works as a prime co-receptor for the HIV virus of M-tropic or R5 strains. The in vivo expression of the CCR5 had been identified on the activated/memory T lymphocytes, monocytes/macrophages, dendritic cells and microglial cells (Bleul et al. 1997)(Wu et al. 1997). Moreover studies of the polymorphism in the CCR5 gene had demonstrated that a number of mutations in CCR5 ORF were capable of affecting the susceptibility to HIV infection or the natural

course of disease progression. One notable example is the mutant allele of CCR5 bearing 32-bp deletion (Δ32) within the ORF, results in the formation of a truncated protein and deficiency of CCR5 membrane presentation (Deng et al. 1996). Homozygous CCR5 Δ 32 carriers are protected from HIV infection while heterozygous carriers display slow disease progression after HIV infection. This had been attributed to the lower level or null CCR5 expression on the cell surface (Carrington et al. 1999) (Libert et al. 1998)(Nguyên et al. 1999)

Homozygous carriers of CCR5 Δ32 had been protected from the HIV infection in an AIDS Cohort Study (MACS) in multiple centers in US. The study had described the mutation in homozygous status in two homosexual men who had remained uninfected with the HIV in spite of the repeated high-risk exposure(M Dean et al. 1996). Subsequently, the homozygous CCR5 Δ32 carriers had been found significantly enriched in several highly-exposure-while-uninfected in (EU) cohort studies; conversely, the homozygous CCR5 Δ32 carriers had not been found among several thousands of HIV infected individuals (Rana et al. 1997)(Samson et al. 1996). CCR5 Δ32mutant had been proved to be a common allele in Caucasians, while it rarely happened in the Middle Easterners, Africans or native Asians (Carrington et al. 1999) . The allele frequency of CCR5 Δ32 mutant in the Caucasians resided in North American is around 1%, with heterozygotes representing 2% and homozygotes representing 1% respectively (Martinson et al. 1997).

Despite the critical role played by CCR5 as the major co-receptor for HIV-1, polymorphisms in other chemokine receptors appear to also show a certain level of protection against HIV-1 infection and disease progression. The most convincing evidence comes from the CCR2V64I variant, an allelic variant in which valine 64 is replaced by isoleucine (Smith et al. 1997). Heterozygous individuals for CCR2V64I delay progression to AIDS; however no clear effect in protecting against

HIV-1 infection has been reported. Not all studies have proved this association (Michael et al. 1997)(Schinkel et al. 1999), and the influence of the CCR2V64I remains controversial. Interestingly, the CCR2 receptor is used only by a few strains in vivo, so the mechanism of action of the CCR2V64I variant is unclear. CCR2 located 17.5 kb upstream of the CCR5 promoter, and it has been recommended that the 64I variant may be in linkage disequilibrium with genetic variations in the CCR5 gene (Lee et al. 1998). To date, most studies have investigated no changes in the expression levels of CCR2 or CCR5 in CCR2V64I individuals (Lee et al. 1998)(Mariani et al. 1999). One study, even though, reported lower levels of surface CCR5 and suggested, yet it did not prove, that CCR2V64I may bind with increased affinity to CCR5 intracellularly and thus abrogates the expression of CCR5 at the cell surface. Another report suggested interference with CXCR4 as an alternative explanation, demonstrating that the 64I gene product dimerizes with CXCR4 more effectively than the wild-type CCR2 (Mellado et al. 1999). One mutation associated with the CC-chemokine receptor, CCR2V64Iis shown mostly in Africa compared to others. However, regardless of high prevalence of HIV in Africa, the CCR2V64I mutation is one of the possible factors in HIV/AIDS progress. Overall frequency of this mutation worldwide follows is :Africa (17.2%), Gambia (4.3%), and Central Africa (20.2%)(Martinson et al. 2000). In some Spanish populations the prevalence is also high (14% - 30%) (Acosta et al. 2003).

SDF-1 (Stromal Derived Factor 1) is also named as pre-B-cell growth stimulating factor precursor (PBSF), had been initially isolated from murine bone marrow stromal cell line. SDF-1 had been thought to play an important role in the B-cell development (Nagasawa et al. 1994)(Nagasawa et al. 1996)(Nomura et al. 1996)(Ma et al. 1998). The knockout of SDF-1 gene had been lethal in mouse embryos (Aiuti et al. 1997). The mRNAs of both CXCR4 and SDF-1 had been found in various adult tissues and are constantly expressed during the embryogenesis, specifying their functional

significance to the organogenesis and maintenance of the immune, hematopoietic, and central nervous systems (Ward et al. 1998)(Tachibana et al. 1998).

A common G/A transition at position of 801 bp relative to the non coding region of SDF-1 gene, in the 3'UTR had been characterized in the SDF-1α transcript and named as SDF-1-3'A (Winkler et al. 1998) . The polymorphism had been found in all ethnic groups , with allelic frequencies of 21.1% in Caucasians, of 16% in Hispanics, of 5.7% in AA, and of 25.7% in Asians (Winkler et al. 1998). The mutant locates in a conserved fragment of the 3'UTR of the SDF-1α transcript, which could potentially serve as a target for cis-acting factors that influence production or transport of the product. HIV infected individuals who are homozygous or heterozygous carriers of the SDF-1-3'A had exhibited a delayed progression to AIDS, which had been suggested that the up-regulated SDF-1 expression. The effect had been firstly reported in Caucasians; subsequently it was reported in French genetics resistance to infection by HIV-1 (GRIV) cohort (Hendel et al. 1998)(Lathey et al. 2001). However, other epidemiological studies did not support these observations. A study into the European EU subjects showed that SDF-1-3'A homozygotes were not associated with the LTNP (Soriano et al. 2002). It shows that the inter-population variations existed in determining the effects of the SDF-1-3'A mutation on the HIV pathogenesis.

The human CCL5/RANTES (Regulated on activation normal T cell expressed and secreted) is an 8 Kda protein and the gene position is on chromosome 17. RANTES is normally produced by T lymphocytes, monocytes and platelets. Higher levels of RANTES could control HIV infection of R5 tropic strains possibly by competing with the virus for receptor binding In HIV-infected individuals, those with increased level of RANTES was found to delay the onset of AIDS-defining illness (Furci et al. 1997)(Mikawa et al. 2002)(Paxton et al. 1996). RANTES exhibit different types of

polymorphisms in both the promoter region as well as in regulatory region.SNP in the promoter region of RANTES such as -28G and -403A has been associated with delayed disease progression (Liu et al. 1999). Conversely, the SNP in the gene regulatory region RANTES In 1.1C, demonstrated to be associated with progression to AIDS by suppressing protein synthesis. Individuals having the mutation In1.1c progressed to AIDS faster than persons carrying -28G polymorphism, along with those subjects who did not carry any mutation (Wichukchinda et al. 2006).

MCP-1 (Monocyte Chemotactic Protein-1) is a chemokine produced by variety of cells such as monocytes, fibroblasts and endothelial cells. MCP-1 plays an important role in recruiting and activating macrophages to the site of inflammation and granuloma formation. In HIV infected individuals with tuberculosis elevated levels of MCP-1 has been observed. MCP-1 A/G polymorphism has been associated with elevated levels of MCP-1 in HIV -1 infection.

The possible effect of genetic heterogeneity in HIV chemokines and their receptors on the HIV infection and pathogenesis have been perceived. Our current knowledge is majorly based on naturally occurring mutations mainly isolated from Caucasians, Africans and Japanese. The study aimed to study genetic polymorphisms in pediatric population from South India. It was planned to investigate the polymorphism in chemokines and receptors to find out the association between the host genetic polymorphisms and the HIV pathogenesis.

Results

Genetic factors of host can affect susceptibility, pathogenesis and disease progression in HIV-1 infected person. In this chapter, we evaluated the host gene polymorphisms, allelic distribution and protein expression levels of some of the host genes for chemokines and their receptors that play

an important role in HIV-1 disease progression, comparing different groups of HIV-infected individuals named as LTNP, SP and FPs as well as healthy subjects (HC). The goal of this study was to investigate the role of these host genetic factors in susceptibility or resistance to HIV

To investigate the distribution of antiretroviral gene variants CCR5Δ32, CCR2 64I, SDF-1, RANTES and MCP-1 we have enrolled 52 subjects for study including healthy subjects. They were both HIV positive and HIV negative. We classified them as different groups based on their clinical outcome. LTNP group (n=6) were all above 9 years with the absolute CD4 count >300 cells/µl, did not show any AIDS related illness, asymptomatic and showed normal activity according to WHO classification system I and they were not on ART. While 18 subjects in the SP group have CD4 counts below 300 cells/µl, showed opportunistic infections and on ART. FP group had 8 subjects who showed symptoms of AIDS and were on ART, the CD4 count is below 200cells/µl. We also collected the blood samples from 20 healthy HIV negative children. All the children among all the groups were both age and sex matched. Transmission was mother to child and none of the patients had any other risk factors like intravenous drug use *etc* (Radhakrishna et al. 2013).

Genotyping: Allelic distribution of CCR5-Δ32, CCR2V641, RANTES In1.1C, SDF-1-3'A and MCP-1 in LTNP, SP, FP and HC subjects.

The allelic frequencies of CCR5-Δ32, CCR2V641, RANTES In1.1C, SDF-1-3'A and MCP-1 in all the cohorts was reported by genotyping and PCR-RFLP using primers specific for each of these allele. No homozygous CCR5-Δ32 mutation was identified in our study group except in one patient from SP group was identified as heterozygous (Fig 4.1).Two of the LTNP patients and one of the SP patient shown heterozygous mutant allele CCR2V64I (Fig 4.2). No difference in the allelic distribution of RANTES In 1.1c was observed among all the groups (Fig 4.4A), however a

significantly higher allelic distribution of SDF-1-3'A and MCP-1 was observed in LTNP group compared to SP and FP cohorts (Fig 4.3 A) (Fig 4.5 A).

Expression levels of chemokines SDF-1α, RANTES and MCP-1.

SDF-1α, RANTES and MCP-1 protein expression in the plasma of LTNP, SP, FP and HC patients was quantitated using commercially available ELISA kits. Data in Fig. 4.3D, 4.4C, 4.5D show the SDF-1α, RANTES and MCP-1 levels in the LTNP, SP, FP and healthy (HIV-1 negative age and sex matched) control samples. Statistical comparisons were made between levels of SDF-1α, RANTES and MCP-1 in the LTNP and FP cohorts. Our data show a significantly increased SDF-1alevels in LTNP ($p< 0.05$) as compared to the FP patient cohort. On the other hand, a significant decrease was observed in the levels of MCP-1 in LTNP compared to FP respectively. . The RANTES protein expression levels in the LTNP and FP groups were not statistically significant (P=NS). These data report that gene significant changes in the levels of the respective chemokines can affect HIV-1 disease progression.

Discussion

Host genetic factors may be one of the factors that play an important role in susceptibility or resistant to HIV infection and the rate of disease progression in different patients of HIV cohorts that represent varied spectrums in HIV-1 disease. In the present study our aim was to investigate the allelic distribution and protein expression levels of some of the important polymorphic alleles such as CCR5-Δ32, CCR2V641, RANTES In1.1C, SDF-1-3' A, and MCP-1 that are critical to progression of HIV-1 infection. In view of this, we compared the allelic distribution and the expression of these important genetic polymorphisms in different groups of HIV cohorts though the comparison was made mainly between LTNPs and FPs cohorts who represent totally opposite

end of the HIV-1 disease progression scale. Therefore genetic markers may be useful in predicting disease status and may establish unique targets for therapy.

Studies have reported that polymorphisms in CCR5 and CCR2 are protective against HIV-1 infections and their distribution varies according to ethnicity (M. Dean et al. 1996)]. A deletion of 32bp in the coding region of the CCR5 gene named as CCR5Δ32 is protective in both heterozygous and homozygous individuals against HIV-1 infections. In the CCR2-64I polymorphism substitution of valine to isoleucine at 64 position of CCR2 is protective in heterozygous individuals against disease progression. It was revealed that, Caucasians have higher frequency of CCR5Δ32 and lower in Asians and Africans (Sabeti et al. 2005). The CCR2V64 I distribution was 13% in Africans and in Cameroons 0 to 7% (Anzala et al. 1998). Since no CCR5Δ32 allele was observed in LTNP cohort and even in other cohorts proved that CCR5Δ32 frequency was almost very low in this region and it could not account for slow disease progression in LTNP. The distribution of CCR2V 64I was 33.3 % in LTNP and 0% in FP. CCR2V64I in the LTNP cohort as compared to the RP cohort suggesting that the expression of this allele may partly contribute to the mechanism underlying the delayed disease progression. CCR2V64I has no effect on the occurrence of HIV-1 infection, but homozygotes/heterozygotes for this allele have a slower rate of HIV-1 disease progression; also the effect of CCR2V64I polymorphism on disease progression may vary according to the disease status and interaction with other genetic variants (Choi et al. 2007).

The total protein levels of SDF-1α, RANTES, and MCP-1 were quantified using commercially available ELISA kits. Statistical significance was determined using ANOVA based on comparisons between the LTNP and FP groups. The allelic distribution of the SDF-1-3'A allele also varies among different populations. In the US, the SDF-1-3'A has an allele frequency of 21% in Caucasians and

6% in African American populations. Studies have reported that the protective effect of SDF-1- 3' A, was twice, as effective as the effects contributed by CCR5 and CCR2 allelic variants. Our results showed a significantly higher allelic distribution of the SDF-1- 3'A allele in the LTNP cohort as compared to the FP cohort. Additionally significantly higher levels of SDF-1α were also observed in the plasma of LTNP patients as compared to the FP group.

Expression levels of RANTES can be influenced by the SNPs that have been reported in the RANTES. So far three SNPs have been reported two in the promoter region 28C/G and 403A/G and one in the first intron region In1.1/C. The SNPs 28C/G and 403A/G variants increase RANTES synthesis, while the In1.1 T/C variant inhibits RANTES gene expression (Liu et al. 1999). The distribution of RANTES In1.1C allele is 13.8% US Caucasians and is lower in African American populations [12]. In the current study, we did not observe any difference in the allelic distribution of RANTES In 1.1/C among all the groups and no significant in the protein expression level indicating may not confer to the slower disease progression seen in our LTNP subjects.

In the current study two different HIV-1 cohorts, LTNPs and FPs, along with SP and HC were investigated for the expression of polymorphic alleles that could provide details of the progression or lack thereof of HIV-1 infections. Here we studied the polymorphic alleles, CCR5Δ32, CCR2V641, RANTES In1.1C, SDF-1-3'A, and MCP-1 can be used to predict disease outcomes of HIV-1 infections. While the present study is novel in that these two dinstinct HIV-1 cohorts, LTNPs and FPs who were therapy naive were investigated, additional multi-center, studies with an increased number of patients from similar cohorts are necessary to determine the mechanisms underlying the functional effects of these genetic polymorphisms.

Monitoring the HIV-1 individuals for these genetic markers based on ethnicity and studying the response of these subjects with polymorphisms in chemokine and chemokine receptor genes will provide us to facilitate rational, probable decisions on treatment regimens and to determine important mechanisms across viral phenotype, host genotype, and HIV disease progression

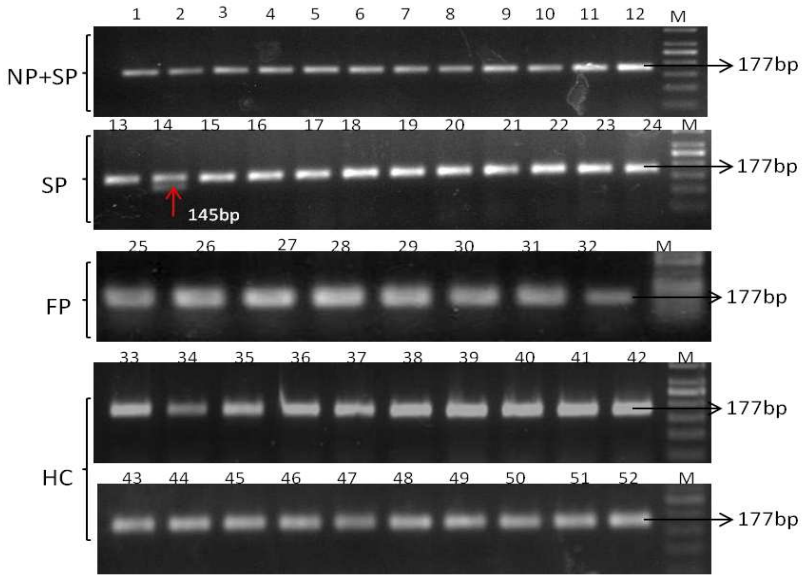
Conclusion

In conclusion, we investigated the distribution of different antiretroviral gene variants in HIV infected children and in seronegative population as well. Not surprisingly, we found no CCR5Δ32 homozygous allele. The distribution of CCR2V64I, RANTES was almost similar among all the groups, probably did not have any impact in disease progression. Elevated levels of SDF-1-3'A and decreased levels of MCP-1 correlating to allelic distribution was observed in the LTNP group could be attributed to delayed progression. From the study done on the ARG variants their expression levels and in light of the limited size of the population, further cohorts with an increased number of patients are to be studied to determine more conclusive results and to understand the effect of these polymorphisms.

Figures

Genotyping of CCR5Δ32 in LTNP, SP, FP and HC groups

A



LTNP: Non progressor (3, 4, 7, 8, 9, 10)
SP: Slow progressor (1, 2, 5, 7, 11, 12, 13-24)
FP: Fast progressor (25-32)

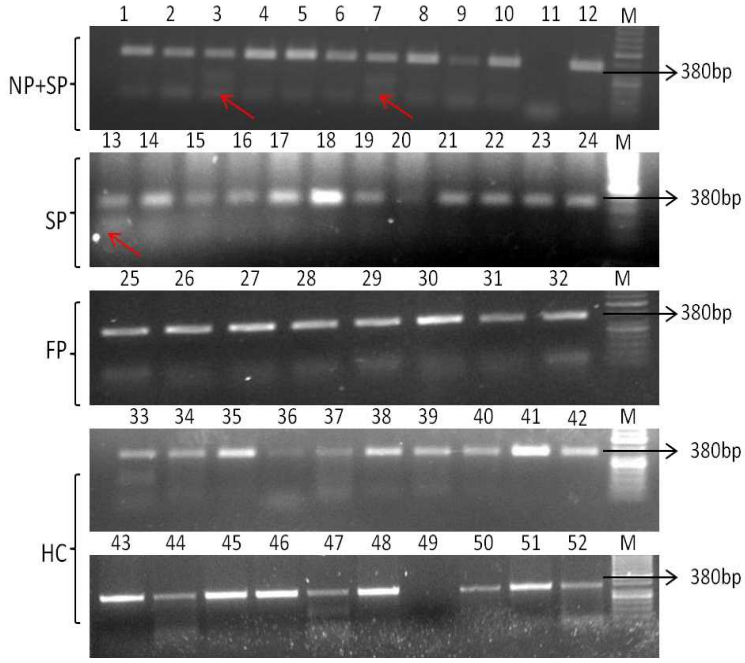
B

Gene Variant		LTNP(%)	LTP(%)	TP(%)	HC(%)
CCR5	WT/WT	6(100)	17(94.4)	8(100)	20(100)
	WT/Δ32	0(0.0)	1(5.6)	0(0.0)	0(0.0)
	Δ32/Δ32	0(0.0)	0(0.0)	0(0.0)	0(0.0)

Fig 4.1: A) Genotyping of CCR5Δ32 in LTNP, SP, FP and HC groups. Genomic DNA was isolated from the patients and was used to determine the genotype for CCR5Δ32 mutation in all the cohorts. Heterozygous allele was indicated by arrow. B) Allelic distribution of CCR5Δ32 in different groups, no significant difference in the heterozygous frequency of CCR5 allele was observed. The stastical significance was determined P=NS

PCR-RFLP Analysis of CCR2V64I

A



LTNP: Non progressor (3, 4, 7, 8, 9, 10)
SP: Slow progressor (1, 2, 5, 7, 11, 12, 13-24)
FP: Fast progressor (25-32)

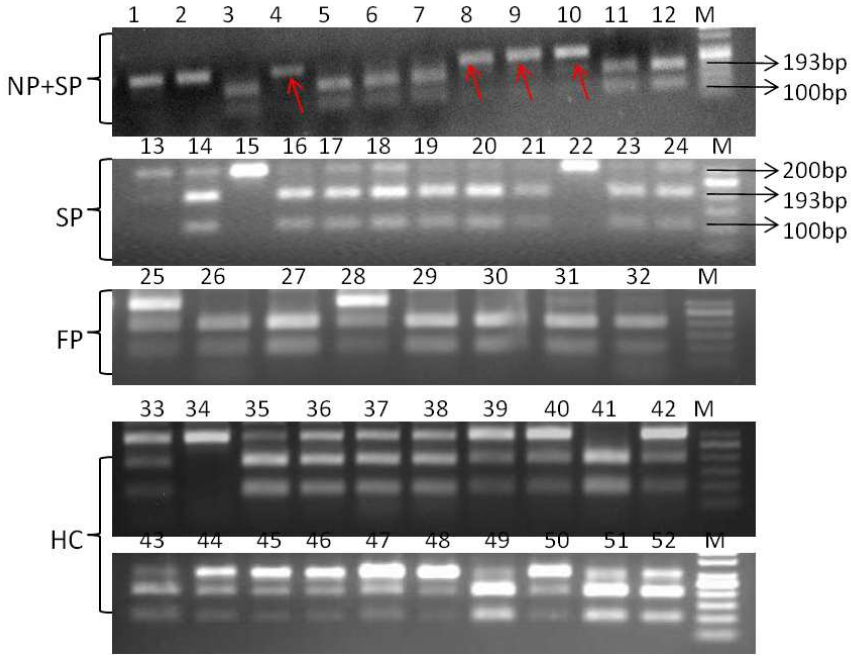
B

Gene Variant		LTNP(%)	SP(%)	FP(%)	HC(%)
CCR264I	V/V	4(66.6)	13(72.2)	8(100)	17(89.4)
	V/I	2(33.3)	4(22.2)	0(0.00)	2(10.6)
	I/I	0(0.00)	0(0.00)	0(0.00)	0(0.00)

Fig.4.2. A) PCR-RFLP analysis of CCR2V64I in LTNP, SP, FP and HC groups. Genomic DNA was isolated from the patients, pcr amplified and pcr product was digested with the restriction enzyme Fok 1 for 3 hours at 37°c,digested products were ran on agarose gel (2%) to determine the genotype for CCR2V64I mutation in all the cohorts. Arrows represent patients carrying protective allele heterozygous CCR2 64 I. B) Allelic distribution of CCR264I in all the groups.

PCR-RFLP analysis of stromal cell derived factor -1 3'A (SDF-1 3'A)

A



LTNP: Non progressor (3, 4, 7, 8, 9, 10)
SP: Slow progressor (1, 2, 5, 7, 11, 12, 13-24)
FP: Fast progressor (25-32)

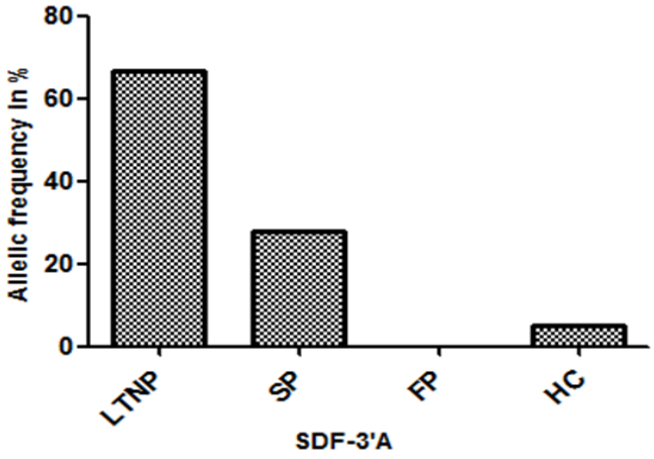
B

Gene Variant		LTNP(%)	SP(%)	FP(%)	HC(%)
SDF 3'A	+/+	2(33.3)	0 (0.00)	6(75)	1(5)
	+/3'A	0 (0)	13(72.2)	2(25)	18(90)
	3'A/3'A	4(66.6)	5(27.7)	0(0)	1(5)

Fig .4.3.A) Genotyping Of SDF-1 3'A by PCR –RFLP. Genomic DNA was isolated,sdf-1 was pcr amplified and pcr product digested with the restriction enzyme Msp1 for 3 hours at 37°c to determine the genotype for SDF-1 3'A allele in all the cohorts. Protective alleles were represented by arrows B) Allelic distribution of SDF-1 3'Ain different groups, mainly significant difference was observed between LTNP and FP group. $P<0.05$

Allelic distribution of SDF1 -3'A and SDF-1 α ELISA

C



D

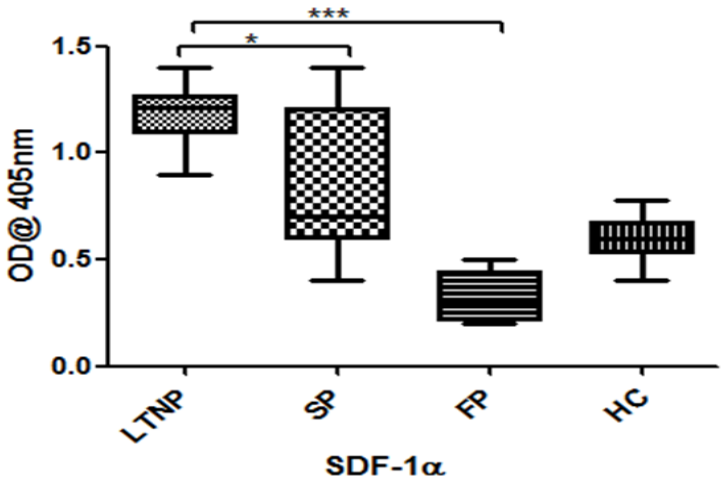
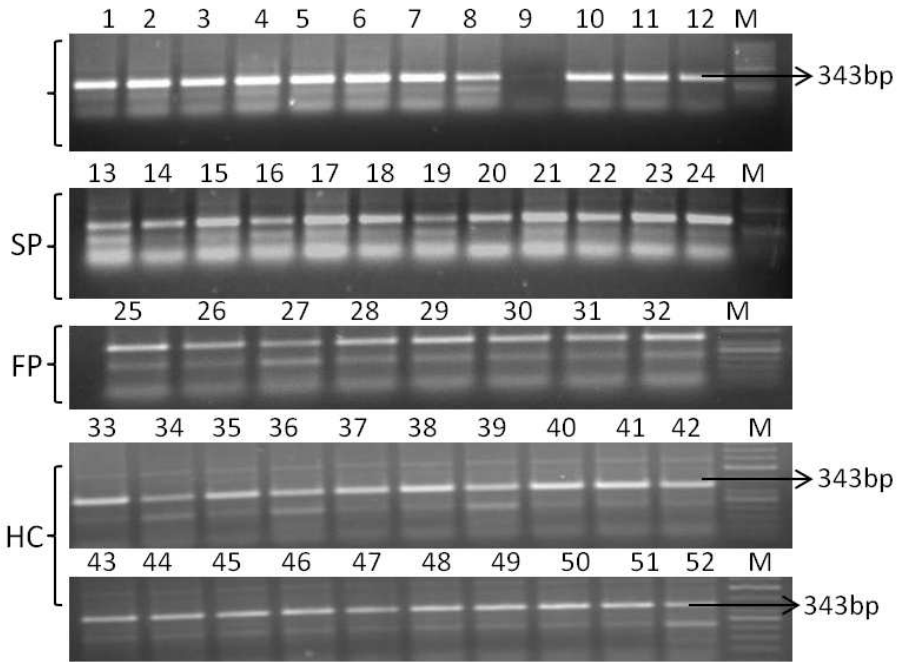


Fig 4.3. C) : A) Histogram for showing allelic distribution of SDF-3'A protective allele in LTNP,SP,FP and HC groups. D) Protein expression level of SDF-1α in all the groups, estimated using commercially available ELISA kit. Statistical significance was determined using ANOVA based on comparisons between the LTNP, SP, FP, and HC groups P value is <0.05

PCR-RFLP analysis of RANTES In 1.1C

A



LTNP: Non progressor (3, 4, 7, 8, 9, 10)
SP: Slow progressor (1, 2, 5, 7, 11, 12, 13-24)
FP: Fast progressor (25-32)

B

Gene Variant		LTNP(%)	LTP(%)	TP(%)	HC(%)
RANTES 1.1C	+/+	0(0)	0(0)	0(0)	0(0)
	+/C	6(100)	18(100)	8(100)	20(100)
	C/C	0(0.0)	0(0.0)	0(0.0)	0(0.0)

Fig 4.4.A) PCR-RFLP analysis of RANTES In 1.1C in LTNP, SP, FP and HC groups. Genomic DNA was isolated, pcr product digested with MboII was used to determine the genotype for RANTES In 1.1c B) Allelic distribution of RANTES In 1.1c in all groups. No stastical significance among all the groups was observed.

RANTES quantification by ELISA

C

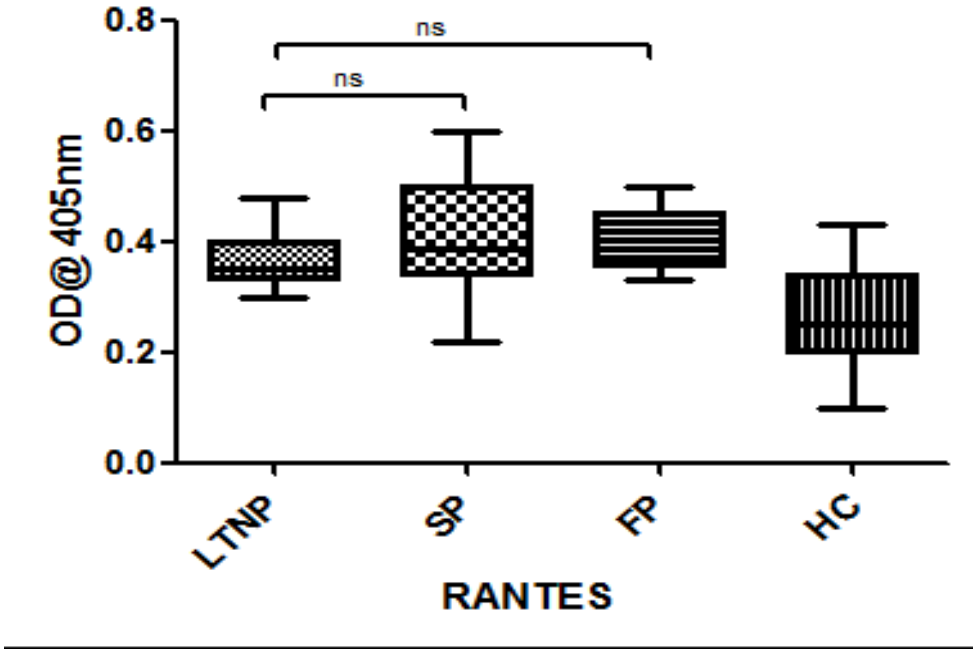
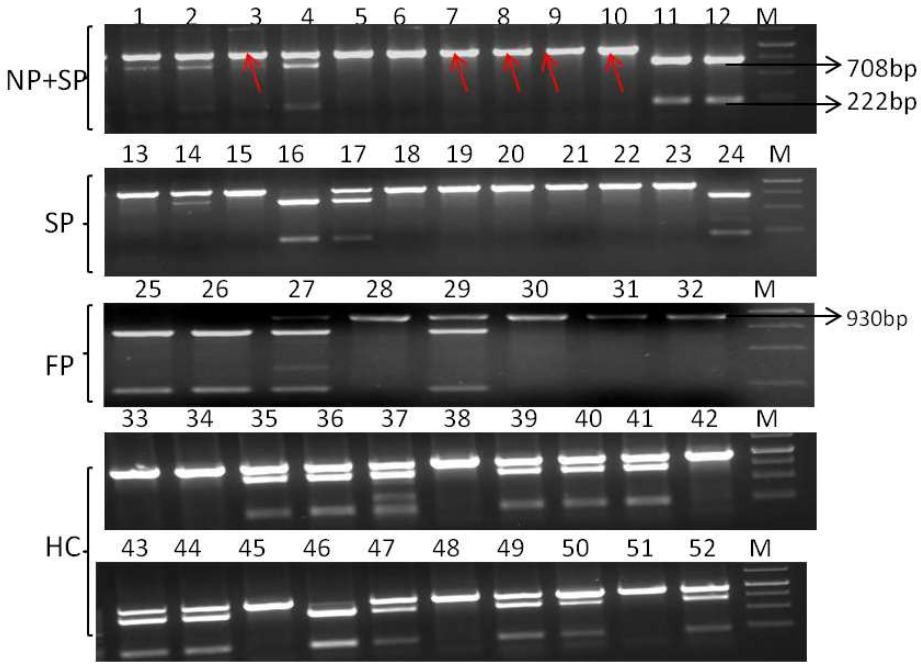


Fig4.4.C) Protein expression level of RANTES In1.1c in all the groups, estimated using commercially available ELISA kit. Statistical significance was determined using ANOVA based on comparisons between the LTNP, SP, FP, and HC groups. P value is NS (not significant)

PCR-RFLP analysis of MCP-1

A



LTNP: Non progressor (3, 4, 7, 8, 9, 10)
SP: Slow progressor (1, 2, 5, 7, 11, 12, 13-24)
FP: Fast progressor (25-32)

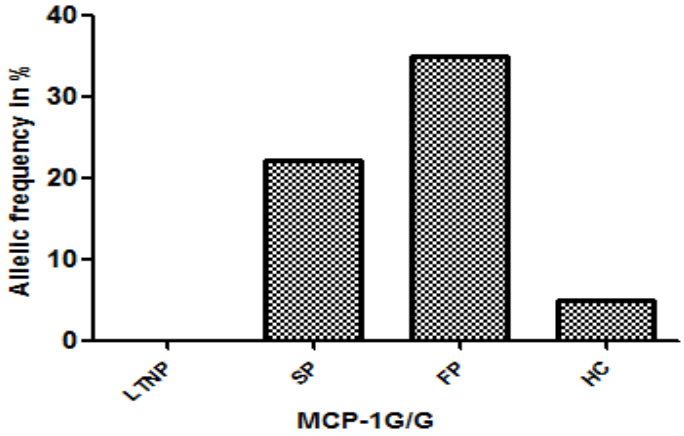
B

Gene Variant		LTNP(%)	SP(%)	FP(%)	HC(%)
MCP-1A/G	A/A	5(83.3)	10(55.6)	4(50)	7(35)
	A/G	1 (16.6)	4(22.2)	1(12.5)	12(60)
	G/G	0(0.00)	4(22.2)	3 (37.5)	1(5)

Fig 4.5. A) PCR-RFLP analysis of MCP-1 in LTNP, SP, FP and HC group.MCP-1 was pcr amplified, digested pcr product with restriction enzyme Pvu II to determine the genotype for MCP-1 in all the cohorts. Arrow represents protective allele B) Allelic distribution of MCP-1 in different groups.

Allelic distribution of MCP-1 and quantification by ELISA

C



D

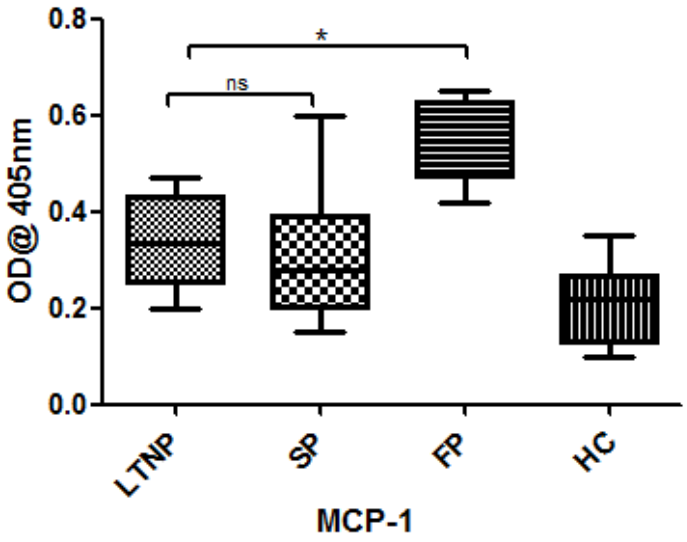


Fig 4.5. C): A) Histogram for showing allelic distribution of MCP-1 allele in LTNP, SP, FP and HC groups. D) Protein expression level of MP-1 in all the groups, estimated using commercially available ELISA kit. Statistical significance was determined using ANOVA based on comparisons between the LTNP, SP, FP, and HC groups P value is <0.05

Overall distribution of different genes

Gene polymorphism	LTNP (no=6)	SP(no=18)	FP(no=8)
CCR5Δ32	-	+(1),-(17)	-
CCR2V64I	+(2), -(4)	+(1), -(17)	-
RANTES In1.1c	-	-	-
SDF-1α	++ (4)/6	+(4)/18	-
MCP-1	+++(5)/6	++(9)/18	+(3)/8

Table 4.3. Overall distribution of Chemokine receptors and chemokines

CCR5, CCR2 and RANTES distribution was similar in all the groups but increased SDF-1 MCP-1 Polymorphism has been observed in LTNP group suggest that these two alleles may delay the progression towards AIDS

Chapter V

Elucidation of HIV-1 mediated immune response in HIV-1 infected children having varied rates of disease progression

Introduction

The Human Immunodeficiency Virus (HIV) primarily targets the immune system and weakens its surveillance and defense mechanism, subsequently HIV infected individuals becoming more susceptible to a broad range of opportunistic infections normally cleared by the immune system of a healthy individual. Both cell mediated and humoral immunity are considered to be play an important role in HIV infection despite the nature of the protective immune response in HIV infection is unknown (Fauci 1993). HIV infection is associated with depletion in the number of CD4 +T cells, defective immunological memory and dysregulation of cytokine production *in vivo* (Diamond et al. 1988)(Biggs et al. 1995)(Kedzierska et al. 2000). HIV associated immunodeficiency could be due to decreased production of immunological factors associated virus defense (Eg. Cytokines *etc.*) and increased production of factors promoting virus replication (cell cycle arrest *etc.*) (Clerici & Shearer 1993). Cytokines play a crucial role in maintaining the homeostasis of the immune system and nature of the immune response is demonstrated by the level of cytokines in the plasma of an infected individual.

Cytokines play a role in modulating the susceptibility of cells to productive HIV infection and replication by promoting the activation of T lymphocytes and differentiation as well as monocytes/macrophage differentiation (Zack et al. 1990)(Sonza et al. 1996). So, the effect of cytokines on HIV-1 replication can be stimulatory, inhibitory and bifunctional indicating that, infection and replication of HIV *in vitro* and *in vivo* is under constant regulation of complex network of cytokines produced by different cells of the immune system.

When the cells of the immune system identify an intracellular pathogen, the immune response that results is particularly defined and targeted to the clearance of the pathogen (Dinarello 2000). CD4

+T cells of the immune system play a critical role and are necessary in accomplishing this task. T helper cells, also named CD4+ T helper cells (Th), are immune cells with an important role in directing the response necessary to accomplish this task. Depending on the nature of the immune response to pathogens, the response is of two types , Th1 or cell mediated immune response and Th2 or antibody mediated immune response When an intracellular pathogen is detected a Th1 response is induced, conversely, Th2 response is induced when the clearance of an extracellular pathogen is needed (Dinarello 2000).

As integral components of the immune system and based on their role in infection, generally cytokines can be categorized into 2 major categories, the pro-inflammatory cytokines, which activates the immune system, or the anti-inflammatory cytokines suppress the immune system (Dinarello 2000)(Clerici et al. 1993)(Esser et al. 1991). Besides playing a regulatory role CD4+ T cells also play a role in inflammatory disease progression this is accomplished by cytokine produced by cells of the innate immune system (Elenkov & Chrousos 1999). For instance IL-12 is produced by antigen presenting cells (APC), induces naive T-cells into the Th1 (CD4+) subset. IL-2 and interferon gamma (IFN-γ) produced by Th1 cells, activate macrophages essential for eliminating intracellular pathogens (Park et al. 2005)(Fan et al. 1993).

Reports from the studies carried out have shown that HIV-infected individuals have a weaker immune system due to the inability of CD4+ T cells to proliferate and differentiate due to decreased levels of IL-12 (Noble et al. 2001), which in turn leads to decrease in IL-2 and IFN-γ, leading to immunosuppression and allowing room for opportunistic infections, a hallmark of HIV progression. HIV dampens the immune system towards intracellular pathogens by decreasing pro- inflammatory cytokines and increasing anti-inflammatory cytokines. For example levels of anti-

inflammatory cytokines interleukin 10 (IL-10), should be relatively decreased in healthy individuals with intracellular infections, allowing the infection to be cleared by the appropriate Th1 response.

The role of the humoral immune response or antibody mediated immune response in delaying disease progression or neutralizing HIV infection. This phenomenon is not clearly understood while, the available reports suggest that this type of the immune system is not fully protective against chronic infection while a combination of cellular and humoral immunity is crucial. In the quest of developing a prophylactic vaccine studying HIV specific neutralizing antibodies has become the subject of intense research and it is further prompted by the results of the RV144 trail which showed a good efficacy (Rerks-Ngarm et al. 2009). An important function of antibodies is the ability to neutralize the virus by binding to the viral surface and inhibit cellular infection. Hence, immune response elicited by an effective protective vaccine would need to be broadly neutralizing across different viral subtypes (Walker & Burton 2008). These broadly neutralizing antibodies (bNABs) have been produced naturally during a process of intense somatic hypermutation (Wu et al. 2010) in a minority (15%) of HIV-infected subjects over the course of several years of post-infection (Sather et al. 2009)(Walker et al. 2011)(Gray et al. 2011). Some studies have demonstrated that frequency of neutralizing antibodies is higher in LTNPs than progressors (Cecilia et al. 1999)(Pilgrim et al. 1997), while others are not shown (Doria-Rose et al. 2010)(Pereyra et al. 2008). From the existing reports it is shown that LTNPs have a stronger virus-specific neutralizing antibody (Nab) response against both autologous and heterologous viral variants (Braibant et al. 2008)(Mahalanabis et al. 2009)(Sreepian et al. 2004), and level of neutralizing antibodies rise at the later stage of infection due to decreased viral replication and presence of more heterogeneous virus populations, While in progressors due to the decrease in number of CD4 T cells, they lost their ability to

produce NAb response over the course of infection(Lopalco 2004). A recent study reported that several young children with HIV-specific antibodies with high neutralizing breadth (Goo et al. 2014).

The measurement of cytokines and neutralizing activity of antibodies in patients with varied rates of disease progression may provide extra information to understand disease progression. So the study was aimed to determine the plasma levels of IL-1β, IL-6, IL-8, IL-10, 1L-12 and TNF-α and also neutralization breadth of the antibodies present in the plasma was performed by purifying IgG to verify neutralization activity is antibody mediated.

Results

The group studied was composed of 32 HIV-1perinatally infected children and 20 healthy subjects HIV infected children were classified as different groups based on the clinical characteristics and rate of disease progression. 6 were non progressors without any symptoms, 18 children were slow progressors with symptoms of opportunistic infections and were on ART and 8 children were fast progressors with symptoms of AIDS.

Plasma cytokines

Cytometric bead assay (CBA) was used to measure the plasma concentration of TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 in plasma samples from different groups of HIV-1 infected children and healthy individuals. HIV infected children had elevated levels of TNF-α, IL-1β, IL-6, IL-8 and IL-10 specifically in fast progressor group compared to healthy controls and IL-12 levels were higher in LTNP and healthy individuals. Stastical comparisons were mainly made between LTNP and FP, the two groups that had different rates of disease progression. Cytokines that stimulate HIV- 1 infection and replication like proinflammatory cytokines tumor necrosis factor –α (TNF-α), IL-1β, IL-8, IL-

10 and IL-6 (Fig 5.1 A&B, 5.2 A&B and 5.3 A) were significantly increased in fast progressor group with the P values $p<0.05$ and $P<0.001$. On the other hand cytokines that enhances immune response to HIV-1 infection such as IL-12p70 was increased in LTNP and below detection level was observed in fast progressor group. Overall imbalance in the cytokine levels were observed and uncontrolled HIV replication in fast progressor group is associated with increased proinflammatory cytokines and perturbation in the levels of cytokines.

Neutralization activity of whole plasma

HIV neutralization of plasma was performed with different dilutions of (serial 10 fold dilutions) plasma. Plasma was heat inactivated at 56°c for 30 min and incubated with virus NL4-3 for 30 min at 37°c. The plasma and virus mixture was added to PBMCs after 2 hours of incubation plasma – virus mixture was removed and fresh media was added. Cells were allowed to grow until 5th day supernatants were collected to p24 concentration was determined by ELISA. When the neutralization activity of plasma was examined by different HIV cohorts, plasma from all the groups neutralized or reduced HIV replication above 50% inhibition at low dilutions (10^{-1}). The neutralization was stastically significant ($P < 0.01$) between LTNP and FP cohort at higher dilutions (10^{-2} to 10^{-4}) where LTNP plasma showed higher neutralization activity than FP plasma (Fig 5.4.).

Neutralization activity of plasma against primary isolates

Plasma samples obtained from LTNP and FP were assessed for neutralization breadth with three HIV strains (NL4-3, HIV-$1_{92BR025}$ and HIV-$1_{93IN101}$). 6 non progressors and 6 fast progressors samples were used for evaluation. From Fig.5.5 it showed that plasma from fast progressors could neutralize only one strain NL4-3. No neutralization of other two isolates HIV-$1_{92BR025}$ and HIV-$1_{93IN101}$ was not observed with the plasmas from fast progressors, while minimum 3 samples (50%) of

LTNP could neutralize all the 3 isolates and 4 (66.6%) of LTNP neutralized minimum 2 isolates. The neutralization breadth of plasmas from LTNP was greater than FP.

Neutralization of plasma is antibody mediated

To determine whether antibodies or other factors present in the plasmas were responsible for neutralization activity we fractionated plasma as IgG fraction and non IgG fraction. IgG of plasma was removed by using protein-A agarose column chromatography and flow through was used as non IgG fraction, separation of IgG was determined by SDS-PAGE (Fig. 5.6). Plasmas were again re-evaluated for virus neutralization with both IgG and non IgG fractions. Flow through fractions showed only 10 to 20% neutralizing activity, where as antibody fraction displayed 60 to 80% neutralizing activity (Fig 5.7.A&B) indicating that neutralization of plasma was attributed to antibodies IgG.

Discussion

The survival of small fraction (5-15%) of individuals without immunologic deterioration after being infected with HIV for more than 8-10 years without any therapy is still elusive. Understanding the factors that contribute to this non progression phenomenon may provide information to develop anti HIV regimens and vaccines against HIV-1. HIV-1 infection results in chronic immune activation and dysregulation in cytokine production *in vivo*. During the course of HIV-1 infection, a shift from Th1 (IL-2, IFN-γ and IL-12) to Th2 cytokine profile (IL-1, L-6, IL-8) has been proposed to play a role in disease progression. HIV-1 infection is associated with decreased production of Th1 cytokines and increased production of Th2 cytokines IL-10, proinflammatory cytokines IL-1β, TNF-α and other cytokines IL-6 and IL-10 are thought to enhance HIV replication, activation and expression. Such imbalance in the cytokine profile may play a role in the immunopathogenesis of the

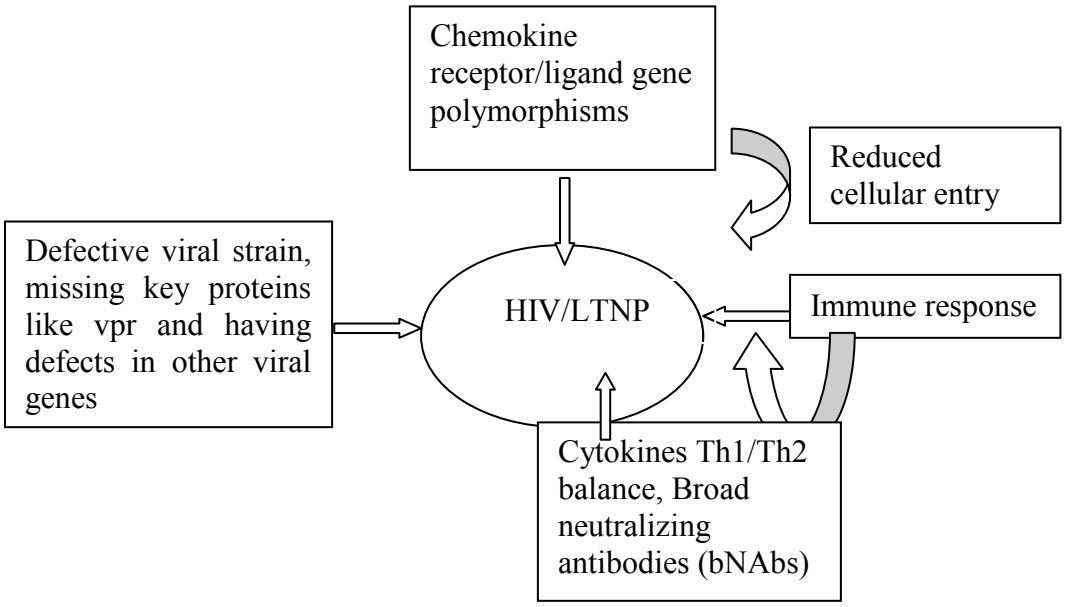
disease. It is also important to note that imbalance of cytokine profile in HIV-1 infection and as potential biomarkers to predict the rate of disease progression. With the aim to understand the differences in cytokine profile role in disease progression, we measured cytokine levels in patients with different rates of disease progression and their cytokine levels were compared. In our study, we measured six cytokines by CBA kit. Based on the data, we could see a clear stastical difference between LTNP and FP. Our results showed increased levels of IL-12 in LTNP which in turn activate Th1 subset to maintain cellular immunity in LTNP. In contrast to this other cytokines such as, TNF-α, IL-1β, IL-6, IL-8 and IL-10 decreased in LTNP and increased in FP group.

Several reports identified the importance of neutralizing antibodies (NAbs) in long term non progression of HIV infection. Most of the studies were cross sectional where they compared the NAb levels in AIDS patients with compromised immune function with LTNP who had intact immune system. It has been reported that presence of higher level of neutralizing antibodies in LTNP with comparison to fast progressors at later stages of infection is attributed to two phenomena. The first phenomenon is the low level of NAbs in the plasma of the FPs. This cannot be due to a lack of immunogen, since progressors higher viral load which is associated with disease progression to AIDS (Tetali et al. 1996). The decreased antibody levels in rapid or fast progressors are certainly caused by the deterioration of the immune function, which has been reported not only in terms of decreased antibody titers, lower immunoglobulin levels, and declined CD4+T cell counts (Farzadegan et al. 1996), but also increased apoptosis of CD4+ T and B cells (Samuelsson et al. 1997), decreased antibody-dependent cellular cytotoxicity (ADCC) (Baum et al. 1996), and decreased proliferative responses(Dyer et al. 1997). A second phenomenon contributing to the presence of higher level of NAbs in LTNP (Zhang et al. 1997), which is attributed to low level of virus replication, maturation of humoral immune response exhibiting increase in the number of B

cells to produce neutralizing antibodies and presence of more heterogeneous immune cell populations in LTNPs (Delwart et al. 1997), which in turn may provide stronger and more diverse antigenic stimulation. Similar to these reports, our results showed that LTNP have higher level of neutralizing antibodies, titers and increased breadth of neutralization. From our results it was also evident that neutralization was antibody mediated came to know that when we performed neutralization assay with both IgG and non IgG fraction, lack of neutralization was observed in non IgG fraction of plasma. Thus plasma neutralization is likely antibody mediated.

Conclusion

In conclusion, our results from this study demonstrated that level of cytokines among HIV infected individuals with the highest levels were seen in fast progressor individuals than long term non progressors. So progression of disease is associated with increased levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) in turn lead to impaired cellular immunity. Also our study about neutralizing activity of plasma revealed that LTNP had higher level of neutralizing antibodies and broad neutralization activity against primary isolates and the neutralization activity of plasma is attributed to antibody IgG fraction. Our results provide some insight between levels of cytokines, antibody titer and breadth of neutralization about disease progression.

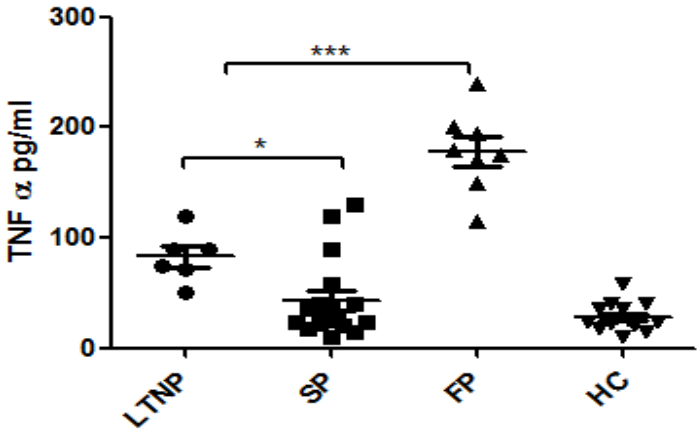


Possible factors that are associated with delayed progression of the disease in HIV -1 infected long term non progressors of children studied.

Figures

ELISA: TNF-α and IL-1β

A



B

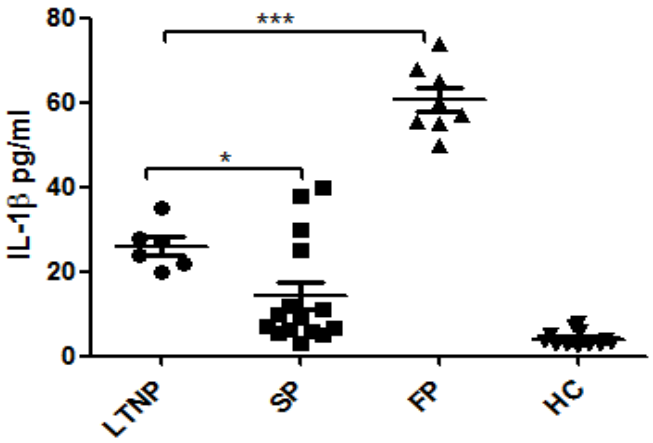
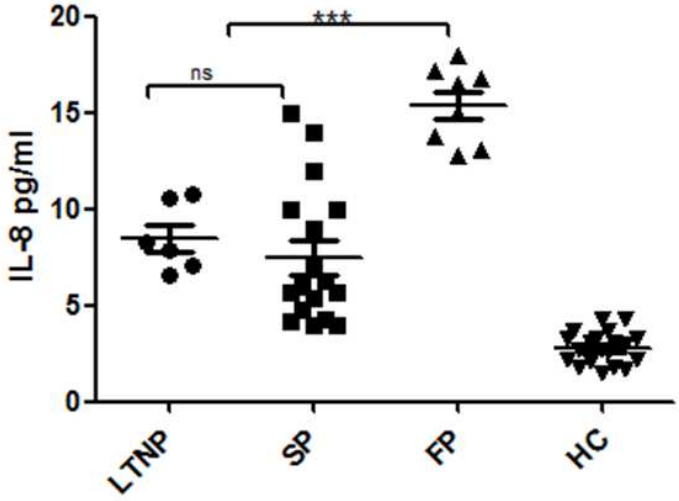


Fig 5.1.A &B: Mean plasma concentration of cytokines TNF-α and IL-1β. Measured from all the HIV positive children and HIV negative children. Plasma cytokine levels were measured by cytometric bead array (CBA) using human inflammatory cytokine CBA assay kit (BD Bioseciencs) followed according to the protocol given. P value is significant between LTNP and FP. ***P < 0.001

ELISA: IL-8 and IL-10

A



B

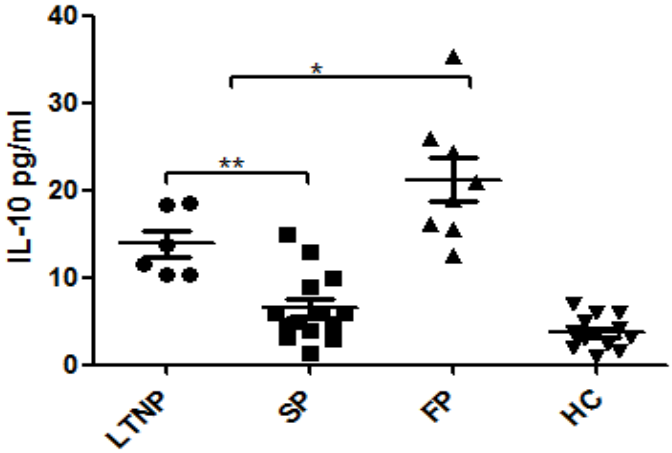
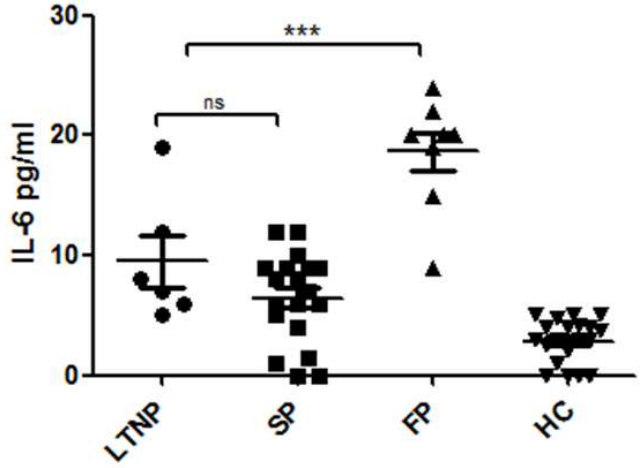


Fig 5.2. A &B: Plasma concentration of cytokines IL-8 and IL-10 measured using CBA kit (BD Biosciences) in all the cohorts both in HIV +ve and HIV – ve children. Stastical analysis was done using ANOVA. P value is significant between LTNP and FP. P value for IL-8 is ***P < 0.001 and for IL-10 is *P < 0.05.

ELISA : IL-6 and IL-12p70

A



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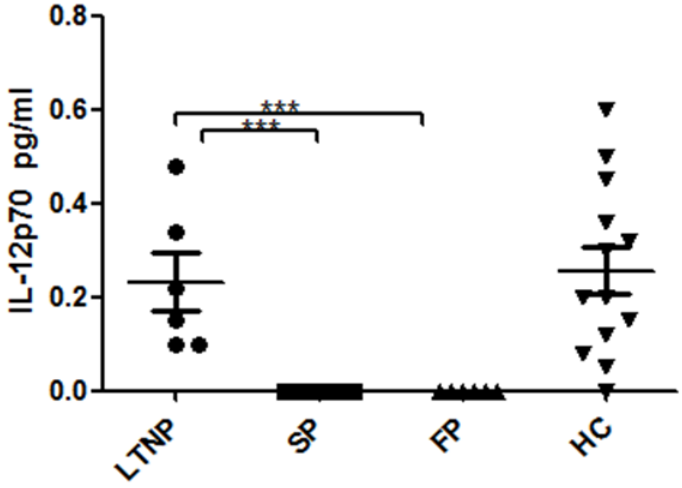


Fig 5.3. A &B: Mean plasma concentration of cytokines IL-6 and IL-12p70 measured using CBA kit (BD Biosciences) in all the cohorts both in HIV +ve and HIV – ve children. Stastical analysis was done using ANOVA. P value is significant between LTNP and FP. P value for IL-6 and IL-12 is ***P < 0.001.

Neutralization activity of plasma with different dilutions

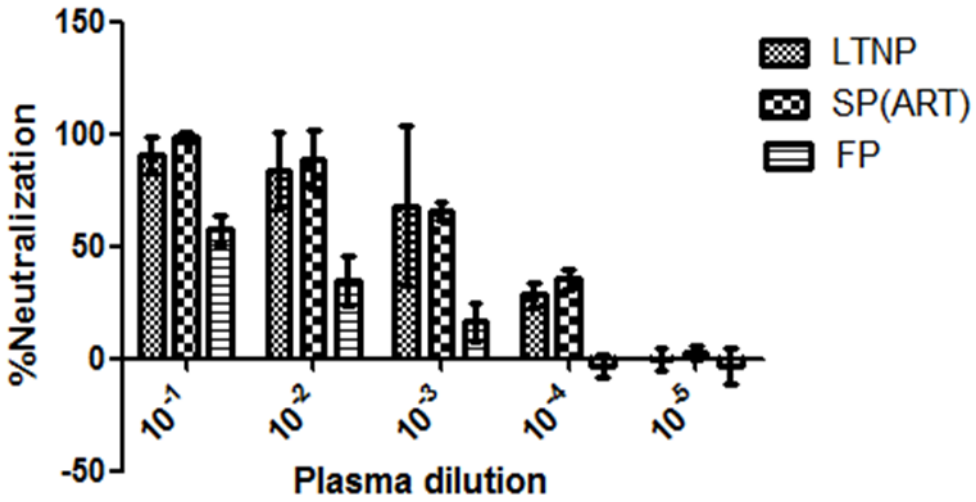


Fig. 5.4. Neutralization of HIV-$1_{93IN101}$ replication by plasma taken in different dilutions (10 fold) from nonprogressor, slow and fast progressors. Virus replication was determined by p24 antigen (pg/ml) in culture fluids collected at 96 hours. Plasma from long term non progressors could neutralize the virus even at higher dilution (10^{-4}).

Plasma neutralization with different viral isolates

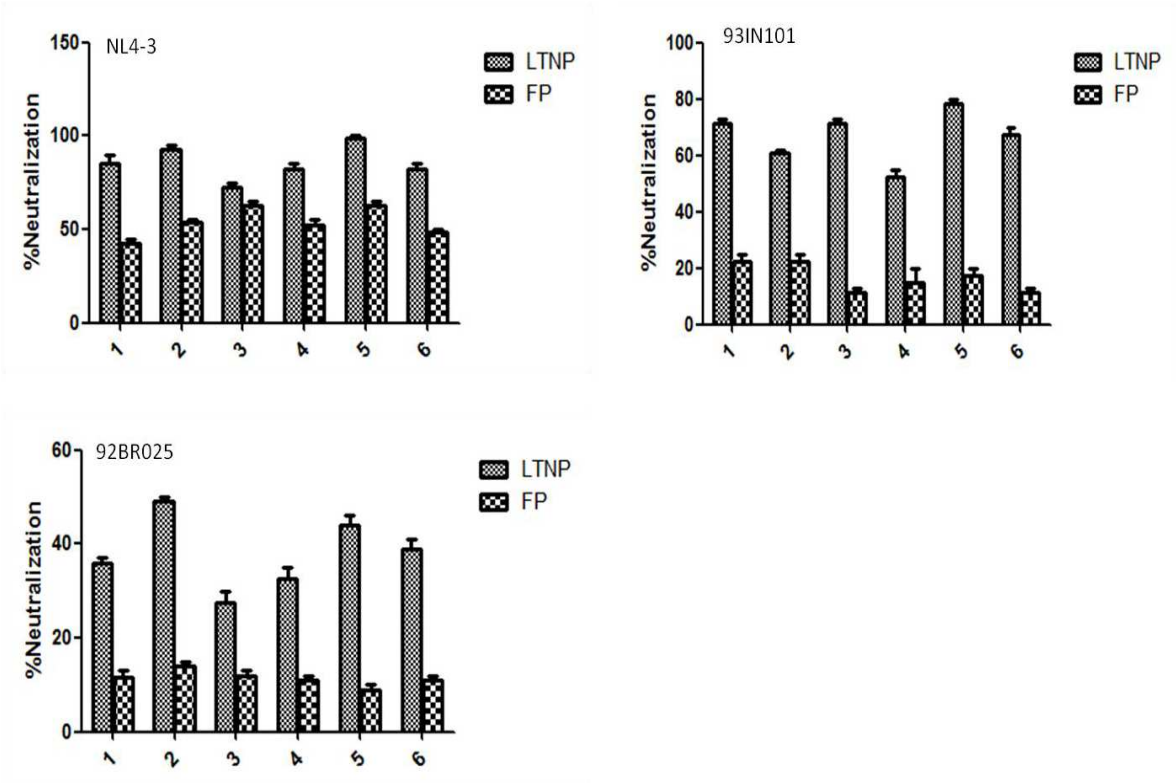


Fig.5.5: Neutralization activity of plasma against different isolates. LTNP and FP plasma was treated with three different HIV-1 strains NL4-3, HIV-$1_{93IN101}$ and HIV-1 $_{92BR025}$. Plasma and virus mix was added to PBMCs after 2 hours of incubation cells were washed and replenished with fresh media and allowed to grow until day 5, Supernatants were collected and checked for concentration of p24 by ELISA.

IgG purification

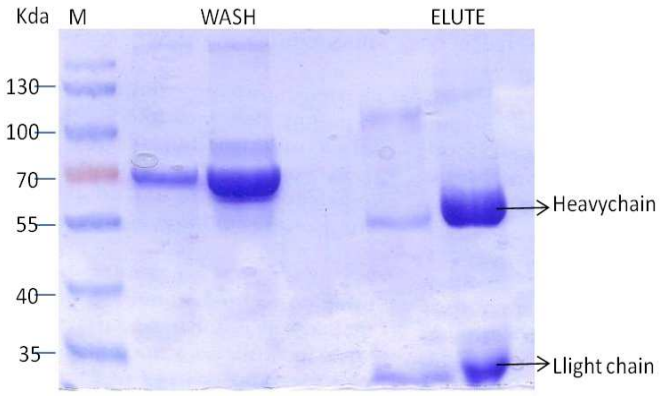


Fig.5.6. IgG purification from plasma. IgG was purified from plasma using protein A agarose.

Neutralization activity of IgG and non IgG fractions of plasma

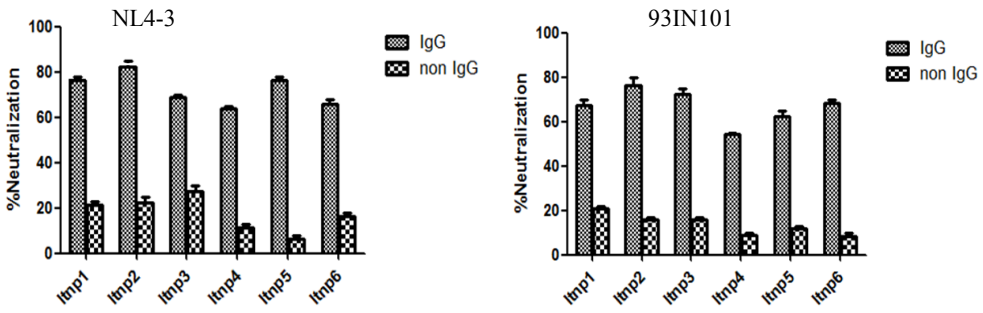


Fig.5.7: Neutralization activity of IgG and non IgG fractions of plasma. Neutralization of virus isolates NL4-3 and HIV-$1_{93IN101}$ was evaluated with both IgG and flow through (non IgG) was done and the antiviral activity was mediated by antibody fraction of plasma.

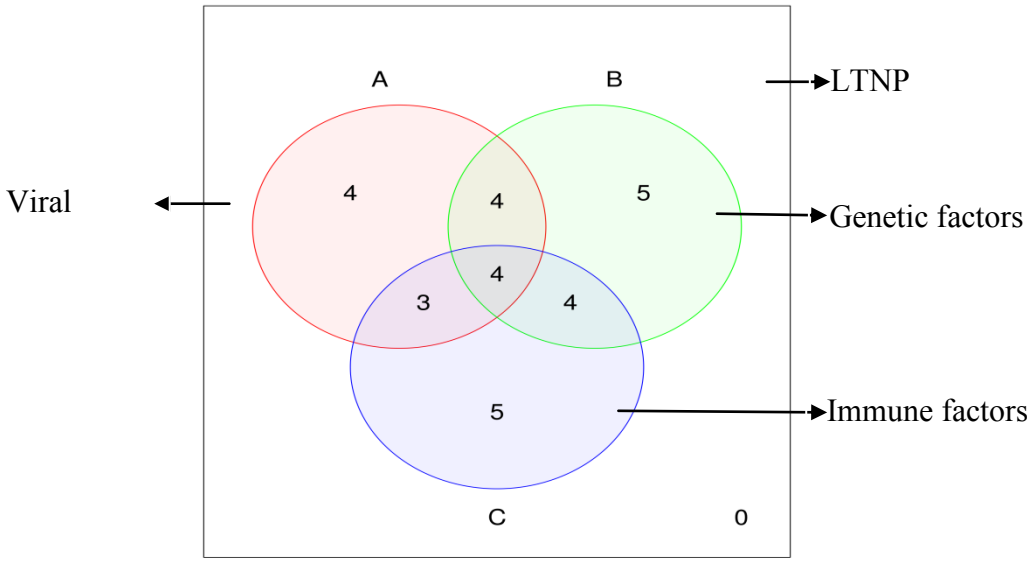
Consolidated Summary

- ✓ Long term non progressors (LTNP) are a small subset (5-15%) of HIV-1 infected individuals who are asymptomatic, maintain favorable course of infection after being infected with HIV -1 for more than 8-10 years in the absence of antiretroviral therapy (ART). What makes these individuals to control infection is a piece of the puzzle and prompted researchers to understand their ways of natural control of infection.
- ✓ The possible explanation for this is LTNP may harbor attenuated viral strain along with host genetics and immune function. Here in this thesis we studied the viral, genetic and immunological profile in long term non progressors of pediatric population with comparison to progressors.
- ✓ With respect to virological features, phylogenetic and subtyping analysis of structural gene *env* and accessory gene *nef* showed that population enrolled in this study were subtype B. The amino acid analysis of Nef and other accessory proteins (Vif, Vpu and Vpr) in LTNP revealed no common profile of deletions, while it was found that this group of LTNP possess certain point mutations and deletions, amino acid replacement in the functional motifs of Nef and other accessory proteins were observed.
- ✓ Most motifs crucial for the Nef function were well conserved except motifs EE, ExxxLL and DDPxxE in LTNP sample 3. LTNP-10 had premature stop codon in the Nef region. Similarly slow progressor SP-2 had premature termination. Some point mutations such as N_{157} (Glutamine) substituted by I (Isoleucine), V_{182} (Valine) with C (Cysteine) and M (Methionine) and L_{183} (Leucine) substituted by M (Methionine) were observed both in LTNP and SP group, which may affect Nef function.
- ✓ A comparison of Nef secondary structure of LTNP-3, SP-18 with the reference sequence NL4-3 showed that loss of one helix and a break in the helix was observed. Other accessory proteins (Vif, Vpu and Vpr) total deletion of Vpu was observed in the LTNP- 4. Amino acid sequences of sample LTNP-10 has the maximum mutations in all of the accessory proteins which can be

attributed to the non-progression of the disease. Other samples noticed with mutations are LTNP_7 and LTNP_3, where as absolutely no changes were seen in fast progressor group.

- ✓ This study demonstrated few changes in the accessory region of viral strains circulating infected LTNP along with this subjects genetic back ground may likely to be a one o f the factor contribute to delayed disease progression.
- ✓ Host genetic factors play an important role in slow progression of the disease to AIDS. HIV uses many host factors for gaining entry in to the host cell and to complete its life cycle. Hence multi genetic factors are thought to play a role in susceptibility, pathogenesis and disease progression. Some of these genetic factors are the genes that could be involved during entry undergoes modification in expression levels of surface receptors and their ligands. Hence, distribution of some of the gene variants such as CCR5Δ32, CCR2V64I, RANTES, SDF-1α and MCP-1, along with protein levels of (SDF-1α, RANTES and MCP-1) was studied.
- ✓ The results demonstrated that CCR5Δ32 allele was not observed, CCR2 V 64I heterozygous alleles were observed in 3 of the LTNP children and none of the other groups had the polymorphism. RANTES distribution was similar in all the groups correlating ,which was correlated with levels of RANTES was similar in all the groups, indicated that RANTES had no role in delaying of disease progression in this group of LTNP children. While we observed increased frequency of SDF-1 3'A and higher levels of SDF-1α in LTNP group and frequency of MCP-1 allele G/G and its protein levels were decreased in LTNP and increased in FP group, decreased levels of MCP-1 and increased levels of SDF-1α in this group of LTNP may impact disease progression.
- ✓ The rate of disease progression not only get affected by viral genetic variability and host genetic factors but also influenced by host immune function as well. HIV infection is associated with declined CD4+ T cell counts which in turn affect both the cell mediated as well as humoral immunity.

- ✓ We observed cytokine levels and antibody neutralization in HIV infected children. A switch in the cytokine pattern was observed in fast progressor group where they have increased levels of Th2 cytokines IL-10, proinflammatory cytokines IL-1β, IL-6, IL-18 and TNF-α and decreased levels of IL-12p70, and opposite was seen in LTNP. No significant difference was identified between LTNP and healthy controls, also LTNP have higher levels of neutralizing antibodies and increased neutralization breadth than FP and the neutralization activity was mainly due to IgG present in the plasma. However, larger number of population need to include in the study to get more conclusive results and the virus genetic variability observed need to be further studied.
- ✓ In conclusion, present study demonstrated following changes in LTNP children virus variability and host genetic and immune background, such as point mutations, deletions , loss of one helix in the secondary structure of Nef, total deletion of one of the viral key protein Vpu, higher levels of chemokine SDF-1α,decreased pro inflammatory cytokines and increased neutralizing antibody response was seen. All these factors compositely may associate with long term non progression in this group of population.



Venn diagram of 6 LTNP of pediatric group studied: A, B and C represent viral, host genetic and immune factors. It showed that 4 LTNP out of 6 children had defective viral genes, host genetic polymorphisms and low level proinflammatory cytokines along with broad neutralizing antibodies.

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