Evaluation of potential correlation of certain host factors with HIV pathogenesis

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2022

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A thesis submitted in partial fulfillment of the requirement for the degree of

Master of Science in Healthcare Biotechnology

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2022

FORM TH-4

National University of Sciences & Technology

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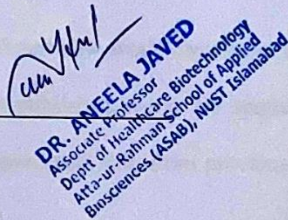

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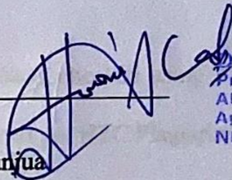

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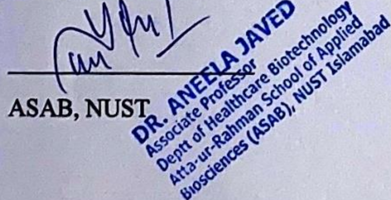

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DEDICATED TO

My Parents & Sister

For the eternal love, support and encouragement throughout this Degree

Acknowledgements

Glory be to ALLAH and praise to ALLAH and there is none worthy of worship but ALLAH, and ALLAH is the Greatest. And there is no might or power except with ALLAH, the Exalted, the Great One. All praises for Him. All respects for his Holy Prophet Hazrat Muhammad (Peace be Upon Him) who taught me the right path and enabled me to recognize the oneness of my creator.

This study comes to fruition trailing clouds of kindness of many people who deserve formal recognition for their love, help and support throughout the research.

First and foremost, I wish to thank my supervisor, **Dr. Aneela Javed**. She has been supportive since the day I began working on this project. I am grateful to her from the core of my heart for her expert guidance, skilled advice, generous criticism and sympathetic attitude without which it could not have been possible to complete this research work.

Special thanks to my guidance and Examination Committee (GEC) members, **Dr. Amjad Ali**, **Dr. Saadia Zahid** and **Dr. Ali Zohaib** for their guidance throughout the study. I would also like to thank my co-supervisor **Dr. Muhammad Faheem** for guidance throughout the project. I would also like to pay gratitude to **Dr. Sobia Manzoor** (HOD Healthcare Biotechnology) and **Dr. Hussnain A. Janjua** (Principal ASAB).

I extend my heartiest thanks to my lab fellows **Fareeha Ghori**, **Uzma Ghani**, my seniors **Hina Khan**, **Muhammad Usman** and **Maaza Sana**, and my juniors **Aaima Ayub** and **Sabahat Habib** for their utmost help and support. Special thanks to lab incharge, **Asad Ali**, who helped us throughout the experimental work.

I would also like to acknowledge my friends **Hamza Sher**, **Haiqa Khan**, **Shiza Sufiyan**, **Umar Farooq** and **Sumaiya Liaquat** for their support and help. This research has been wonderful with the help of you all.

Last but not least, I wish to thank my loving Parents and siblings, for their moral support during the entire time. Their love and support were all that kept me going.

Hafsa

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ABSTRACT

The susceptibility to HIV infection and the development of AIDS are highly influenced by host genetic factors. Single nucleotide polymorphisms in the genes that code for several cytokines, including IL-18, IL-10, and TGF-β1, have previously been associated to variable risk of HIV infection in different populations. In this research, polymorphisms in these immunomodulatory molecules have been determined through SSP PCR and related to HIV pathogenesis in Pakistani population. Our results demonstrated a significant association of TGF-β1 -509TT genotype/TGF-β1 -509C/T polymorphism with HIV susceptibility and a probable link of T allele at this position with the disease susceptibility, while C allele has shown protective role against HIV infection. Contrary to previous studies regarding IL-10 polymorphism, AG genotype has shown increased susceptibility towards HIV, while GG genotype has suggested protective role against HIV. No significant difference was found between HIV patients and controls for IL-18 polymorphisms. FAM26F is an essential regulatory protein involved in immunity, cell differentiation, infection, and anticancer activity. However, its specific role and modulatory mechanisms are yet unknown. Recently, immunofluorescence and immunoprecipitation-based techniques were employed to determine the six interaction partners of FAM26F namely, Calpain-1 catalytic subunit, Calmodulin-like protein 5, Peroxiredoxin-2, Protein S100-A7, Vinculin, and Thioredoxin. Current study has also evaluated the expression of FAM26F and its interacting partner peroxiredoxin-2 in HIV-1-infected patients and in healthy individuals to determine the association of these genes at the mRNA level with HIV pathogenesis through real-time PCR. Our results have demonstrated that FAM26F has significant association with HIV infection while peroxiredoxin-2 has no significant association with HIV infection.

Chapter 1

INTRODUCTION

Since the discovery of AIDS in the 1980s, it is estimated that 32 million individuals are HIV-positive, with 70% of infections occurring in sub-Saharan Africa. Approximately more than 25 million fatalities have been attributed to HIV/AIDS, making it one of the gravest dangers to public health in the twenty-first century. Highly active antiretroviral therapy (HAART) regimens can restrict and manage viral replication, even though there is currently no drug to eliminate the virus completely and no vaccine for AIDS in the foreseeable future(Robertson et al., 2000). The substantial genetic diversity of HIV viruses is one of their defining characteristics and a barrier in viral suppression.

Depending on environmental and genetic factors, the rate at which Human Immunodeficiency Virus-1 (HIV) advances towards immunological dysfunction varies significantly between individuals, with or without therapeutic intervention (Sobti et al., 2011b). These factors either change the availability of co-receptors or have an impact on HLA loci in hosts. (Coloccini et al., 2014). Most of these host genes are responsible for producing an array of cytokines both proinflammatory (TNFα, IL-1, IL-6, IFN-ɣ, and IL-18) and anti-inflammatory (IL-4, IL-10, IL-13, IL-1ra, and TGF-β) some of which determine the level of expression of either co-receptors or co-receptor ligands (such as chemokines) that can reduce receptor availability for HIV attachment such as (RANTES, CCR5, SDF1, and CCR2) while others are involved in immune modulation (TNFα, IL-10, MBL) (Kaur & Mehra, 2009).

HIV infection is characterized by immune activation, which also play role in the pathophysiology of the disease. It has now been shown that genetic heterogeneity in immune-related genes explains the variable susceptibility to several infectious pathogens (Keynan,

Malik, & Fowke, 2013) and provide insights on the aetiology, possible pharmacological targets, risk stratification, and treatment response against the disease (Kwok, Mentzer, & Knight, 2021).

Numerous single nucleotide polymorphisms (SNPs) in different immune related genes have been linked to variable disease progression rates and susceptibility to infection in genetic studies. Risk alleles may eliminate a binding site for a transcription factor (TF), generate a binding site for a TF, or can change the binding affinities to increase or decrease (Degtyareva, Antontseva, & Merkulova, 2021). So, it is critical to comprehend the role of host genetic factors in a disease for the development of new immune-based treatment strategies and the discovery of prognostic markers.

Association studies provide a SNP-based 'genetic profile' which can be considered as a 'fingerprint', valuable for identifying an individual's vulnerability to certain diseases, response to medications and for the 'tailoring' of therapies. In short when a gene is associated with a disease, the protein product of that gene becomes a potential therapeutic target. In the present study, we investigated the presence of correlation between polymorphic nucleotides in the IL-18, IL-10, and TGF-β gene promoters with HIV susceptibility using SSP-PCR.

A dynamic network of cellular and molecular networks makes up the mammalian immune system. Proteins regulate all these intricate networks by supporting the immune system or taking part in immunological reactions as signaling molecules, surface receptors or transcriptional regulators. Numerous new proteins have been added to this repertoire as a result of developments in molecular and structural biology.

FAM26F is one such newly discovered protein that is involved in several immune regulating responses. FAM26F's precise role and modulatory mechanisms are still unknown. Rhesus macaques infected with the simian immunodeficiency virus (SIV) showed a considerable

increase in FAM26F expression 24 and 48 hours after viral infection in two separate AIDS vaccination studies. The group of Rhesus macaques that showed increased level of FAM26F following vaccination provided superior protection against SIV, indicating a crucial function for FAM26F in infections and immunity (A. J. G. D. P. G. Javed, Leibniz-Institut für Primatenforschung, Unit of Infection Models, 2012). In order to identify the cellular process that this protein regulates, co-immunoprecipitation and immunofluorescence approaches were previously used to identify the interacting partners of FAM26F. Using immunofluorescence and confocal laser scanning microscopy, the discovered interactors, including Calmodulin-like protein 5, Calpain-1 catalytic subunit, Peroxiredoxin-2, Vinculin, Protein S100-A7, and Thioredoxin (Trx), were examined for co-localization with FAM26F. The findings showed that FAM26F strongly interacts with Trx, although some interaction with Peroxiredoxin-2 was also noted (Malik, Zafar, Younas, Zerr, & Javed, 2020).

Another protein is Peroxiredoxin 2 (Prdx2), which is an antioxidant protein that belongs to the peroxiredoxin subfamily and is primarily responsible for scavenging ROS (Duan et al., 2016). Oxidative stress has been shown to be a characteristic of several viruses from various families, including HIV (Karpenko, Valuev-Elliston, Ivanova, Smirnova, & Ivanov, 2021). Oxidative stress is defined by an enhanced generation of reactive oxygen species (ROS) and an imbalanced antioxidant response. HIV infection alters the expression of peroxiredoxins (Masutani, Ueda, Yodoi, & Differentiation, 2005), so it is interesting to analyze its expression patterns at mRNA as well.

Current study also evaluated the relative expression of FAM26F and its interacting partner Peroxiredoxin-2 in normal and diseased conditions using qPCR technique to elucidate their role in HIV pathogenesis.

Chapter 2

LITERATURE REVIEW

2.1 Origin of HIV-1

The etiologic agent of AIDS, HIV-1, was discovered in 1983. A simian origin for AIDS in humans was suspected not long after this discovery. SIVmac, the very first simian immunodeficiency virus (SIV), was found in a rhesus macaque (Macaca mulatta) with immunodeficiency and clinical indications similar to AIDS at the New England Regional Primate Research Center (NERPRC). (Daniel et al., 1985; Henrickson et al., 1983). Additionally, a survey of Senegalese citizens conducted in 1985 revealed that some people carried SIV antibodies, indicating the presence of another human retrovirus (Barin et al., 1985). This observation was confirmed in 1986, when individuals living in France who were originally from West Africa were found to have HIV-2, a novel virus related to HIV-1 (Clavel et al., 1986).Today, it is undeniably established that HIV was first spread to humans by repeated SIV transmissions from nonhuman primates (NHPs) (Sharp & Hahn, 2011).

The first SIVcpz strains, SIVcpzGab1 and SIVcpzGab2, were discovered in two captive wild-born chimpanzees in Gabon more than 20 years ago (Peeters et al., 1989). An unexpectedly high level of genetic variation among chimpanzee viruses was shown by the characterization of a third SIVcpz, known as SIVcpzANT in an animal. It is was originally from the Democratic Republic of the Congo (DRC, formerly Zaire) (HAESEVELDE et al., 1996; Peeters et al., 1992). This was connected, according to later research, to the fact that they were descended from two distinct chimpanzee subspecies (Gao et al., 1999). These early findings also demonstrated that all HIV-1 strains were more closely linked to SIVcpzPtt from Central chimpanzees (*P. t. troglodytes*) in West Central Africa than those to SIVcpzPts from Eastern chimpanzees (*P. t. schweinfurtii*) in East Central Africa.

2.2 Prevalence in Pakistan

Epidemiological data indicates that migrant workers from the Gulf States were responsible for the importation of the first HIV cases in Pakistan, which were initially documented in 1987 (Khanani, Hafeez, Rab, & Rasheed, 1988; S. Shah, Khan, Kristensen, & Vermund, 1999). Since then, non-continuous surveillance evaluations have observed significant HIV prevalence in several categories; the most current estimates for 2016-2017 were 5.6% among males who engage in sexual relations with other men and 5.6% among people who inject drugs, 7.2% among transgender people, and 38.4% among PWID (Emmanuel, Salim, Akhtar, Arshad, & Reza, 2013; Rabold et al., 2021). Estimates from the Joint United Nations Programme on HIV/AIDS (UNAIDS) for 2019 indicate that 190,000 individuals exist who are HIV positive, including 6,100 children younger than the age of 15, in the general population, or 0.1% of the population. Only 24,362 (54%) of the 44,758 PLHIV (People Living with HIV) identified with the National AIDS Control Program with a confirmed diagnosis were taking antiretroviral drugs at the end of 2020. The UNAIDS 90-90-90 HIV prevention and treatment targets (90% of HIV-positive people having aware of the symptoms; 90% taking ART; and 90% being virally controlled) are significantly missed by the majority of PLHIV (87%) in Pakistan. Public health professionals from the local and provincial levels of Pakistan's Larkana District identified a significant HIV outbreak there in April 2019 (Moher, Liberati, Tetzlaff, Altman, & Group*, 2009; Rabold et al., 2021). The provincial Sindh AIDS Control Program started a voluntary district-wide testing programme after a number of sick children with HIV-negative parents had positive HIV tests. A total of 30,192 people under the age of 15 had their HIV status tested between April 25 and June 28, 2019, and 876 (2.9%) of those were found to be positive. The World Health Organization (WHO) observed that this outbreak was the fourth HIV outbreak in Larkana since 2003 and

listed risky medical practices and inadequate infection control procedures as major risk factors for infection (Rabold et al., 2021; S. A. Shah, Altaf, Mujeeb, & Memon, 2004).

2.3 HIV subtype diversity worldwide

The main cause of the global epidemic known as AIDS is the spread of two distinct genetic lentiviruses, HIV-1 and HIV-2, from non-human primates to humans through a range of cross-species infections of simian immunodeficiency viruses. The unique HIV-1 groups M (Major), N (non-M, non-O), O (Outlier), and the most recent group P were created as a result of these distinct zoonotic viral transmissions (Sharp & Hahn, 2011). HIV-1 was first discovered in 1920s, in Kinshasa in the modern Democratic Republic of the Congo. From there, it spread through a transit network to other parts of sub-Saharan Africa, West Africa, Europe, and the rest of the world (Faria et al., 2014). A geographically confined distribution of numerous genetically diverse viruses functioned as a marker for this global distribution. Subtype B, for example, spread throughout practically all of Europe and the Americas, whereas a range of subtypes and intersubtype recombinants are found in Africa, with West Central Africa reporting the largest diversity. Since the beginning of the HIV epidemic, the majority of the world's viruses are from group M, while group N, O, and P viruses have not spread as widely. Group M viruses comprise nine subtypes (A–D, F–H, J, K) (Sharp & Hahn, 2011). Although viral incursions have been detected abroad in Europe (Portugal and France), India, and the United States, HIV-2 is still mostly limited to the western part of Africa. (Visseaux, Damond, Matheron, Descamps, & Charpentier, 2016). HIV-2 is made up of at least nine groups (formerly known as subtypes; A to I), of which groups A and D are currently circulating (Visseaux et al., 2016). It has also been shown to be less contagious than HIV-1 (Gilbert et al., 2003; Kanki et al., 1994). Until now, only a few recombinants have been identified., and there is currently a lack of information on HIV-2 subtypes (Ibe et al., 2010).

2.4 HIV structure

HIV is regarded as a "complex" retrovirus since it has six regulatory genes (tat, rev, nef, vif, vpr, and vpu). The remaining genes were given the label "accessory" because, in some cell culture settings, they are not strictly necessary for replication (Kirchhoff, 2010).HIV-1 is a member of the Retroviridae family's Lentivirus genus (lentis = slow). The HIV-1 virion structure states that the virion is spherical and has a diameter of approximately 130 nm (about 1/10,000 mm). Long terminal repeats (LTRs), including the promoter, surround the conventional retroviral genes gag, env, and pol in the HIV genome. Capsid (CA), matrix (MA), and nucleocapsid (NC) are structural proteins encoded by the gene gag. Reverse transcriptase (RT), protease (PR), and integrase (IN) are enzymes encoded by the gene pol. gp120 and gp41 are glycoproteins encoded by the gene env (van Kooyk & Geijtenbeek, 2003). The viral envelope comprises of approximately 12 trimeric complexes of the envelope (Env) protein, which is derived from the host and contains cellular proteins. External glycoprotein 120 (gp120), which facilitates viral attachment, is part of the envelope virus (env). Glycoprotein 41 (gp41), a transmembrane protein essential for viral fusion. The conical capsid of gp41 contains the viral Gag protein, p24, and is connected to the viral p17 matrix protein. Two positive polarity single stranded viral RNA with a combined length of around 10,000 nucleotides are present in the capsid. The RT and IN, as well as the nucleocapsid proteins, are connected to the RNA. The auxiliary Vif, Vpr, and Nef proteins, as well as some cellular components like tRNAlys3, which serves as a primer for reverse transcription, are also present in small quantities in the virions (Esposito & Craigie, 1999).

2.5 HIV lifecycle

There are early and late phases of replication in the complicated HIV-1 life cycle. The virion's attachment to the cell surface and integration of proviral DNA into the host genome are the first two stages of the early phase. The beginning of proviral transcription marks the

beginning of the late phase of replication, which concludes with the release of fully infectious offspring virions. The HIV life cycle only lasts one to two days in highly activated CD4+ T-lymphocyte cells and is linked to the programmed cell death of both virally infected and uninfected bystander cells.

2.5.1 Viral attachment

Infected people often only have a 20–30 minute half-life for cell-free HIV virions. Therefore, the virus must quickly locate and infect a new cell. The main co-receptors for HIV entrance are the chemokine receptors CXCR4 and CCR5, as explained below. Receptor CD4 is the primary receptor. These receptors are enough to make cells open to HIV entrance and, as a result, to decide the viral cell tropism. Env trimer and CD4 receptor concentrations, however, are usually low on virions and target cells, respectively. As a result, viral attachment is frequently ineffective and a barrier to HIV infection. These cellular receptors are enough for viral entrance and, as a result, to decide the viral cell tropism. Env trimer and CD4 receptor concentrations, however, are usually low on virions and target cells, respectively. As a result, viral attachment is frequently ineffective and a barrier to HIV infection. Numerous receptors, including poly-glycans, lectins, and others, have the ability to bind HIV virions in a less specific manner, potentially leading to significantly higher rates of viral infection (Ugolini, Mondor, & Sattentau, 1999).

2.5.2 Viral binding and viral fusion

Viral entrance which is a complicated, process with multiple steps, that provides a variety of therapeutic intervention options (Didigu & Doms, 2012). The infection process is started by the engagement of the exterior viral glycoprotein gp120 with the target cell, either directly or after unspecific binding of HIV to the infected cell. Env trimer conformational changes brought on by the cellular CD4 receptor's CD4 binding allow gp120 to engage with either the CXCR4 (X4) or CCR5 (R5) co-receptor. X4 HIV strains appear later in the course of

infection and, in the absence of antiretroviral medication, are linked to a rapid progression to AIDS (Wilen, Tilton, & Doms, 2012).

2.5.3 Viral reverse transcription

The genetic material of the virus can enter the cell once fusion is complete. The nucleocapsid shields the two positive (+ve) stranded RNAs that make up the viral genome. After fusion, a process known as RT (Reverse Transcription) converts the ss viral RNAs into linear dsDNAs. Because the sequence of activities that typically happens during transcription, the synthesis of mRNA from genomic DNA, followed by exportation into the cytoplasm and viral protein synthesis, is reversed, this process is referred to as "reverse transcription." It is carried out by an enzyme called reverse transcriptase, which is typical of retroviruses, though not exclusively, and entails a very complex chain of actions (W.-S. Hu & Hughes, 2012).

2.5.4 Viral uncoating and nuclear entry

Before importing the viral DNA into the nucleus, the viral capsid is disassembled, which is referred to as uncoating. Viral infectivity is hampered by premature uncoating brought on by point mutations in the viral capsid protein or interactions with the tripartite motif 5-alpha protein (TRIM5a). Early research revealed that viral entrance could be followed promptly by uncoating. However, imply that capsid stability—which can last for several hours—is essential for HIV-1 infection. Additionally, it appears that uncoating is closely linked to reverse transcription and occurs as the reverse transcription complex (RTCs) changes into the pre-integration complex (PIC), which is capable of integrating into the genome of the host cell (Arhel, 2010).

2.5.5 Integration

HIV must integrate its genome into the host cell's genome after successfully producing linear ds DNA and transporting it across the cellular nuclear membrane in order for gene expression

and productive infection to occur. The cell is typically infected throughout the balance of its life once the viral DNA has been incorporated into the cellular DNA by an enzyme known as integrase. The proviral DNA is reproduced alongside the host DNA as a component of the chromosome of the host cell. As a result, spreading an infection can be done by infecting new cells or by multiplying cells that already have proviral DNA. Notably, a number of substances that prevent viral integration are being utilized successfully in the clinic (Lusic & Siliciano, 2017).

2.5.6 Transcription

The integrated HIV provirus acts as a template in productively infected cells for the transcription of viral mRNAs and genomic RNA by the host cellular Pol II polymerase. The viral promoter, which can be found in the 5' LTR's U3 region and is active in a variety of cell types, starts proviral transcription. Cellular transcription factors like NFAT and NF-κB are essential for the production of viral genes. The viral transactivator protein Tat is necessary for efficient viral gene expression, therefore elongation of viral transcripts is relatively inefficient at first, resulting in poor transcriptional output (Colin & Van Lint, 2009; Karn & Stoltzfus, 2012).

2.5.7 Translation and assembly

Rev, Tat and Nef are encoded by the viral RNAs that are fully spliced. Rev facilitates the transportation of unspliced and partially spliced viral RNAs to the cytoplasm, while Tat promotes viral transcription and RNA elongation. Nef serves a variety of purposes but essentially seems to reduce the effect of immune system to detect an infected cell by downregulating a number of surface receptors like CD4 and class I MHC. Nef also affects cells in a way that makes them more efficient producers of fully infectious viral particles. The expression of the Gag-Pol and Gag precursors, which are later processed into important enzymatic and structural proteins, is made possible by the synthesis of Tat and Rev. In

parallel, single-spliced viral RNAs are used to create the Vpu, Env, and Vif proteins. The process of viral assembly is intricate and well-organized. In a nutshell, interactions between Gag proteins cause the progenitors of Gag and Gag-Pol to multimerize. The two precursors are also localized in lipid rafts in the inner leaflet of the plasma membrane where they are N-terminally myristoylated in the matrix domain (Ganser-Pornillos, Yeager, & Sundquist, 2008).

2.5.8 Budding

Budding refers to the release of virions (progeny) from infected cells. These virions pinch out of the plasma membrane and enter the host bloodstream. It involves the late p6 domain of Gag and the cellular Tsg101 protein. Notably, tetherin (BST-2) binds infectious and matured viruses to the infected cell surface and is inhibited by the HIV-1 Vpu and the Nef or Env proteins of other primate lentiviruses (Martin-Serrano & Neil, 2011).

2.5.9 Maturation

The HIV particles are discharged in an immature, infectious state that is morphologically distinguished by a thick coating of radially organized precursors of the Gag and Gag-Pol proteins. The viral protease is active during or soon after budding and splits the precursors of Gag and Gag-Pol into their mature final forms. As a result, the protein structure is altered, creating the distinctive electron-dense conical inner core and making the virus contagious (Briggs & Kräusslich, 2011).

2.6 Role of host factors in HIV pathogenesis

There are three types of variables that affect the development of diseases and infection susceptibility: 1) Viral factors impacting virus replication or immune evasion, 2) host factors affecting innate or acquired immune responses to infection, and 3) Cellular factors collaborating with viral products to govern virus replication in human cells. These factors

ultimately determine how rapidly HIV replicates /or how well it disarms the host's immune system (Lama & Planelles, 2007).

IL-18, IL-10, and TGF-β1 are important immune homeostasis regulators, and polymorphisms in these genes may cause changes in their plasma levels and impact the progression of HIV infection.

2.6.1 IL-18

IL-18, a proinflammatory cytokine, is involved in polarized natural killer and T-helper 1 cell immune responses (Chaix et al., 2008; Kato et al., 2003). Monocytes and macrophages are the main producers of interleukin (IL)-18 (K. Nakanishi, Yoshimoto, Tsutsui, Okamura, & reviews, 2001).

2.6.1.1 Effects on cells of immune system

In association with IL-12, Interleukin IL-18 has been shown to increase the production of IFN- γ by anti-CD3-stimulated Th1 cells. Alone IL-18 cannot stimulate the development of Th1 cells; however, it may stimulate mature Th1 cells to secrete IFN-γ in the presence of IL-12. As a result, IL-18 can be considered as a cytokine that promotes Type 1 responses. Additionally, IL-18 promotes type 2 responses by causing mature Th1 cells, natural killer cells, and natural killer T (NKT) cells to produce IL-3, IL-9, and IL-13 in the presence of IL-2 (K. J. F. i. i. Nakanishi, 2018).

2.6.1.2 IL-18 expression in HIV infection

Several studies indicate that patients infected with HIV type 1 (HIV-1) had much higher circulating levels of IL-18 than healthy individuals (Ahmad, Sindhu, Toma, Morisset, & Ahmad, 2002; Iannello et al., 2010; Song et al., 2006; Torre et al., 2000).

IL-18 has shown to restrict replication of HIV in macrophages due to SAMHD1 upregulation (Pauls et al., 2013). Recent study has also shown that IL-18, through the inhibition of HIV

induced caspase-3 activity, inhibits replication of HIV in Peripheral Blood Mononuclear Cells (PBMCs) and Jurkat cells (Wang, Mbondji-Wonje, Zhao, Hewlett, & communications, 2016). However, HIV infection creates an imbalance between IL-18 (upregulated) and its antagonist Interleukin-18 Binding Protein (IL-18BP) (downregulated), which may increase HIV replication in $CD4^+$ T cells (Iannello et al., 2010)

2.6.1.3 IL-18 -607C/A gene's promoter polymorphism (rs1946518)

The most prevalent single nucleotide polymorphism in the IL-18 gene, -607C/A (rs1946518), is found in the promoter region and is predicted to provide a nuclear factor binding site for the cAMP-responsive element binding protein (Takada, Suzuki, Morohashi, & Gejyo, 2002). -607C/A polymorphism has shown to be associated with HIV infection in Brazilian and North Indian population, where -607C allele and CA genotype respectively is linked with increased risk of HIV infection (Segat et al., 2006; Sobti et al., 2011a).

2.6.2 IL-10

IL-10 is a broadly active anti-inflammatory cytokine. HIV infection initiates a cascade of cytokine production usually referred to as a cytokine storm (Ribeiro et al., 2021). IL-10 is a part of this widespread "cytokine storm" in early HIV infection and reduces the functional abilities of effector CD4+ T cells (Ngcobo et al., 2022). Damage to the gastrointestinal tract barrier at an acute stage of infection results in bacterial translocation and promotes systemic inflammation and immune activation. The detection of bacterial substances by TLRs can initiate the synthesis of IL-10 (Ribeiro et al., 2021).

2.6.2.1 Effects on cells of immune system

In animal models, deletion of the IL-10 gene or inhibition of the activation pathway boosts T cell responses, leading to fast viral eradication and the development of memory T cells responses, which ultimately results in virus shedding (Brooks et al., 2006; Ejrnaes et al., 2006).

2.6.2.2 Expression in HIV infection

HIV-infected people with AIDS often have higher circulating levels of IL-10 (STYLIANOU et al., 1999). In one study, IL-10, induced by tat protein of HIV, inhibits viral replication (Masood et al., 1994). Moreover, downregulation of IL-10 results in a more rapid progression of AIDS (Kwon & Kaufmann, 2010). However, it has been shown that IL-10 expression in the early stages of infection results in suppression of HIV specific T-cell responses (Liu et al., 2014).

2.6.2.3 IL-10 -1082A/G gene’s promoter polymorphism (rs1800896)

The role of IL-10 in the pathogenesis of HIV-1 and its effects and mechanisms are highly contested. According to several studies, alleles related to low serum IL-10 levels are associated to a higher risk of HIV-1 susceptibility in the latter phases of the infection (Oleksyk et al., 2009; Shin et al., 2000). Other research suggests that the alleles (-1082A) linked to low levels of IL-10 have a protective role (Kallas et al., 2015; Naicker et al., 2009). Polymorphic site -1082A/G, lies on the proposed ETS like transcription factor binding site of IL-10 (Lazarus et al., 1997).

2.6.3 TGFβ1

TGF-β is a member of the cytokine superfamily and has three isoforms (TGF-β1, TGF-β2, and TGF-β3). Mammals produce all three isoforms with TGF-β1 being the most prominent in the immune system, where it serves as a key pleiotropic cytokine with significant immunoregulatory functions (Theron, Anderson, Rossouw, & Steel, 2017). Along with the regulation of immunological responses, TGF-βs are involved in an exceedingly variety of completely different biological processes including embryonic development, wound healing, fibrosis, angiogenesis, hematopoiesis (Prud'Homme & Piccirillo, 2000).

2.6.3.1 Effects on cells of immune system

Transforming growth factor beta-1 (TGF-β1) employs a variety of ways to mediate the inhibition of cells of the Adaptive Immune System. By inhibiting the synthesis of the transcription factors T-bet and GATA-3, respectively, this cytokine effectively prevents T-helper (Th)1 and Th2 cell differentiation and proliferation (Theron et al., 2017). Another way TGF-β1 affects humoral immune responses is by inhibiting B cell survival, proliferation, and their differentiation into antibody-producing cells (Tsuchida et al., 2017).

TGF-β1 modulates the activity of innate immune cells by decreasing the synthesis of IFN-γ by human natural killer (NK) cells, inhibiting the in-vitro activation and maturation of dendritic cells, and suppressing neutrophil degranulation (Theron et al., 2017).

2.6.3.2 Expression in HIV infection

Normally, TGF-β1 is a protective anti-inflammatory cytokine, but its excessive production can have severe pathogenic repercussions. Several investigations have found increases in the amounts of TGF-β1 in the circulation of HIV-1-infected individuals (Eaton et al., 2013) and believed to contribute towards the progression of AIDS (Theron et al., 2017). One such investigation showed a correlation between TGF-β1 levels and HIV infection. In comparison to asymptomatic patients and healthy people, an increase in plasma TGF-β1 levels was seen in HIV patients. TGF-β1 levels in plasma and CD4 or CD8 cell counts were also shown to be negatively correlated (Wiercińska-Drapalo, Flisiak, Jaroszewicz, & Prokopowicz, 2004).

HIV-1 proteins may also have a role in TGF-β1 synthesis. It has been demonstrated that the HIV-1 Trans activator of transcription (Tat) protein induces the production of TGF-β1 by human leukocytes (Reinhold, Wrenger, Kähne, & Ansorge, 1999). HIV-1 glycoprotein gp160 also promotes considerable mRNA expression and TGF-β1 secretion in PBMC from HIV-seronegative, healthy donors, according to a previous research (R. Hu et al., 1996).

2.6.3.3 TGF-β1 gene's promoter polymorphism -509C/T (rs1800469)

Polymorphisms in cytokine genes especially in promoter regions can change gene transcription and result in disparities in cytokine production across individuals.

TGF-β1-509C > T allele has been linked to higher plasma levels of TGF-β1, decreased T cell proliferation and it is positioned inside a YY1 consensus binding site (Rathod & Tripathy, 2015). This polymorphism is a C-to-T base exchange that results in the creation of YY1 consensus sequence and is found in a promoter region linked to negative transcriptional regulation (Hobbs et al., 1998). In Thailand population, it was shown that TT and CT frequency at -509C/T promotor site of TGF-β1 was remarkably higher in people living with HIV (PLWH) and suffering from non-AIDS illnesses, hence, making it a good candidate for association studies with respect to AIDS (Akekawatchai, Phuegsilp, Changsri, Soimanee, & Sretapunya, 2022).

In this context, the study's aim was to investigate how the TGF-β1 gene's promoter polymorphism -509C/T (rs1800469) influenced Pakistani patients' susceptibility to infection with HIV.

2.6.4 SSP-PCR

For the discriminating of alleles resulting from single base substitutions, the polymerase chain reaction using sequence specific primers (PCR-SSP) offers a potent method (Kirschbaum, Foster, & haemostasis, 1995).

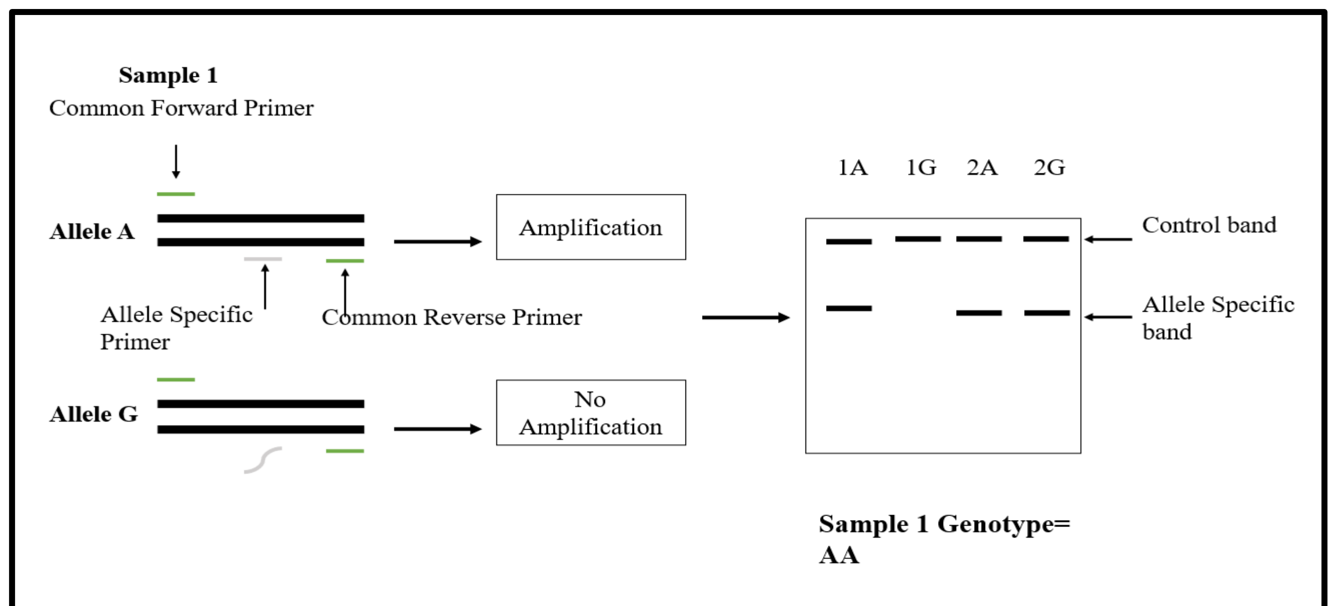

Figure 1: Principle of SSP-PCR.

2.7 FAM26F

FAM26F is a protein that is highly expressed in several immune cell types, indicating its significance in modulating a variety of immunological responses. FAM26F (family with sequence similarity 26, member F) is a relatively new term that has gained interest as an immunological regulator. The 315-amino-acid protein FAM26F, commonly known as CALHM6, is stable and weighs 34.258 kDa. It possesses 3-5 transmembrane helices and an immunoglobin-like shape, indicating its immunological significance(Malik et al., 2020). Investigating the role of FAM26F in SIV infection revealed that pre-infection levels of FAM26F are inversely correlated with overall viral load of plasma. As a result, FAM26F can be regarded as one of the earliest prognostic markers that, can provide us with information about severity and progression of antiviral immune response in the early stages of the infection (A. Javed et al., 2016).

Previously, a little was known about functional characterization and subcellular localization of FAM26F. Recently, it was demonstrated that FAM26F is mostly localized in the cell's Golgi apparatus, albeit a little amount of it is also found in the ER (Malik et al., 2020).

To discover the biological process that FAM26F regulates, one study used co-immunoprecipitation and immunofluorescence-based methods to identify the interaction partners of FAM26F. Proteins were co-immunoprecipitated using Dyna beads after cells had been transfected with the FAM26F plasmid and lysed. To find the interacting proteins, the eluates underwent MS analysis. Majority of the proteins were found to be involved in the innate immune system and the calcium homeostasis mechanism of the cell. Later, proteins were immunostained and their co-localization with FAM26F was viewed using confocal laser scanning microscopy to further assess the degree of functional relatedness between each of these proteins and FAM26F. The protein Thioredoxin and the protein Peroxiredoxin-2 were shown to be co-localizing with FAM26F primarily (Malik et al., 2020).

To ascertain whether these genes are associated with HIV pathogenesis, it is essential to evaluate the expression of FAM26F and its interacting partners in HIV-1-infected patients and in healthy individuals at the mRNA level as well.

2.8 Peroxiredoxin-2 (PRDX2)

Peroxiredoxins (Prdxs), family of cysteine-dependent peroxidase enzymes are widely distributed and perform important functions in maintaining the levels of peroxide in cells (Perkins, Nelson, Parsonage, Poole, & Karplus, 2015). Prdxs are H2O2 metabolizing thioredoxin-specific antioxidants that were initially discovered in yeast (Zhao, Wang, & bioscience, 2012).

A member of the peroxiredoxin family, peroxiredoxin 2 (Prdx2) is an antioxidant protein whose primary function is to combat ROS (Duan et al., 2016). Prdx2 is present in the nucleus and cytoplasm. It has also been shown to bind to the erythrocyte plasma membrane in oxidative stress (Karpenko et al., 2021).

Oxidative stress, which is characterized by increased formation of reactive oxygen species (ROS) and an unbalanced antioxidant response, has been shown to be a common feature of numerous viruses from different families. They include herpesviruses, several respiratory viruses, the hepatitis B and C viruses, the Human Immunodeficiency Virus (HIV), etc (Karpenko et al., 2021). Cellular protective factors including Bcl-2, TRX, and peroxiredoxins exhibit altered expression because of HIV infection (Masutani et al., 2005).

Thus, evaluation of these host factors can help to find novel Host-based therapeutic targets whose overexpression or silencing could prove to be potential anti-HIV therapy.

So, the Objectives of present study were:

1. To evaluate the role of SNPs in important immunomodulatory cytokines such as IL-10, IL-18, and TGF-β1 in HIV infected Pakistani population.

2. To analyse the expression of FAM26F and interacting partner Peroxiredoxin at the mRNA level in healthy individuals and HIV-infected patients using quantitative polymerase chain reaction (qPCR) to elucidate their role in HIV pathogenesis.

Chapter 3

MATERIALS AND METHODS

3.1 SNP analysis

3.1.1 Primer designing

Manually designed Sequence Specific primers were used to genotype SNPs using Sequence Specific Primer PCR (SSP-PCR).

The sequences of human genomic regions containing our target SNPs were obtained from the NCBI SNP database or UCSC Genome Browser. Primer3 software (Untergasser et al., 2012) was used to design these SNP-specific primers. Primer blast (Ye et al., 2012) software was used to test the specificity of binding to our target template; or else, modifications were made to prevent non-specific amplifications. OligoCalc, Multiple Primer Analyser, and idtDNA tools were used to analyze hairpin formation, heterodimers formation, and the possibility of primer dimer formation between forward and reverse primers, as well as among allele specific primers. Salt solution melting temperature were measured. Candidates with a higher potential for cross-hybridization were removed, and new primers were designed.

To facilitate the determination of common annealing temperatures, the melting temperatures of all four primers were maintained within a 2 °C range. GC content was kept between 30-70% and melting temperature ranges were established between 50-70 °C. The allele was kept at the 3' end of allele-specific primers. To prevent the formation of potential dimers, the primer length of allele-specific primers was altered according to the sequence and kept between 16 and 30 bp.

Table 1: List of primers designed for reported HIV SNPs.

Sr. no.	Labels	Sequence (5’-3’)	Product	Ta (°C)

			size (bp)	
1.	IL-18F	CTTTGCTATCATTCCAGGAA	301	55
	IL-18R	TAACCTCATTCAGGACTTCC		
	1L-18F(C)	GTTGCAGAAAGTGTAAAAATTATTAC	196	
	1L-18F(A)	GTTGCAGAAAGTGTAAAAATTATTAA		
2.	IL-10F	TATCTGAAGAAGTCCTGATGTC	318	55
	IL-10R	TTCTTTTAGTTGTAAGCTTCTGTG		
	IL-10F(A)	CTACTAAGGCTTCTTTGGGAA	168	
	IL-10F(G)	CTACTAAGGCTTCTTTGGGAG		
3.	TGF-βF	CTGACCCCAGCTAAGGCATG	384	66
	TGF-βR	AGAGGACCAGGCGGAGAAG		
	TGF-βF(C)	CCTCCTGACCCTTCCATCCC	197	
	TGF-βF(T)	CCTCCTGACCCTTCCATCCT		

In accordance with the manufacturer's instructions, stock solutions for primers were prepared by adding the required quantity of water to each primer. Using 10 µL of the stock solution and 90 µL of nuclease-free water, primer dilutions were made. These procedures were carried out inside a biosafety cabinet to prevent contamination of the stock solutions and dilutions.

3.1.2 Sampling

As experimental group, inclusion criteria were HIV-positive patients with a confirmed positive status. As a control group, 50 HIV-negative samples were collected.

3.1.2.1 Study group selection

With the patients' informed consent, blood samples were obtained from 103 HIV-positive patients at the Pakistan Institute of Medical Sciences (PIMS) in Islamabad and the Punjab AIDS Control Program (PACP) in Lahore for this study.

3.1.2.2 Ethical statement

Ethical Review Board of Shaheed Zulfiqar Ali Bhutto Medical University (SZABMU), Islamabad, Pakistan, and Institutional Review Board (IRB) of ASAB, NUST, Islamabad, Pakistan, granted approval for this research. It was completed in conjunction with the NACP (National AIDS Control Program), its referral laboratory at the Pakistan Institute of Medical Sciences (PIMS), Islamabad, and its provisional counterpart PACP (Punjab AIDS Control Program) located in Lahore.

3.1.2.3 Blood sample collection

Blood samples were required for the genotyping of SNPs under study. Our study comprised of 103 HIV-positive patients and 50 samples of healthy individuals from control population.

Blood samples were taken from HIV-infected/AIDS patients with explicit informed consent. Patients' blood was taken in sterile EDTA tubes and kept at 4 degrees Celsius until further processing. Prior to the study, patients' clinical data including age, gender, plasma viral load, and CD4 cell count were obtained.

Control blood samples were obtained from students at the Atta-Ur-Rahman School of Applied Biosciences (ASAB), National Institute of Sciences and Technology (NUST), Islamabad, randomly. For each subject, information such as gender and HIV history in the

family was collected to ensure they did not violate inclusion criteria. To avoid coagulation prior to DNA extraction, all blood samples were collected in EDTA tubes and stored at 4 °C in a refrigerator.

3.1.3 DNA extraction

DNA extraction from control and patient samples was performed utilizing Phenol-Chloroform method. All the procedures were carried out at the PACP laboratory in Lahore.

Table 2: Chemical composition of DNA extraction solutions.

<table>
<tr><th>Solution A</th><th>Solution B</th><th>Solution C</th><th>Solution D</th></tr>
<tr><td>0.32M Sucrose</td><td>400mM NaCl</td><td rowspan="4">400 µL Phenol</td><td rowspan="2">Chloroform (24 vol)</td></tr>
<tr><td>10mM Tris (pH 7.5)</td><td>10mM tris (pH 7.5)</td></tr>
<tr><td>5mM MgCl2</td><td>2mM EDTA (pH 8)</td><td rowspan="2">Isoamyl alcohol (1 vol)</td></tr>
<tr><td>Add 1% (v/v) triton X-100 after autoclaving the above solution</td><td></td></tr>
</table>

For each sample, 750 µL of Solution A and 750 µL of blood were added to a 2 mL Eppendorf tube, mixed by inversion, and left at room temperature for 5–10 minutes. The tubes were centrifuged for 1 minute at 13000 rpm. The pellet was resuspended in 400 µL of Solution A after removing the supernatant. After 1 minute of centrifugation at 13000 rpm, the supernatant was discarded and the nuclear pellet was dissolved in 400 µL of Solution B. 12 µL of 20% SDS, and 5 µL of Proteinase K was added to the solution prior to overnight incubation at 35 °C. SDS was added for denaturation of the proteins, and Proteinase K was added to inactivate the nucleases.

Next day, 500 µL of a fresh mixture of Solution C (250 µL phenol) and Solution D (250 µL (24 Chloroform: 1 Isoamyl alcohol)) were added to each tube, well mixed through inversion, and then centrifuged at 13000 rpm for 10 minutes. Calculations for the preparation of solution C and D were done according to the number of samples being processed.

Three layers were obtained. In fresh 1.5 mL Eppendorf tubes, the aqueous (upper) layer was collected, measured, and equal volumes (ranging from 300 to 500 µL) of Solution D were added. The centrifugation step was then repeated for 10 minutes. After that, the aqueous layer was collected in fresh tubes, and DNA was precipitated by adding 55 µL of 3M Sodium Acetate (pH 6), along with an equivalent volume of chilled iso-propanol (equal to aqueous layer). The tubes were inverted many times to guarantee DNA precipitation, centrifuged for a further ten minutes, and the supernatant was removed completely. The DNA pellet was washed by adding 200 µL of 70% ethanol, centrifugation was done at 13000 rpm for seven minutes, and ethanol was discarded. The tubes were dried on absorbent paper, and the DNA pellet was dried by incubating it at 37 °C for 20 minutes. The DNA pellet was later dissolved in 75 µL of TE buffer (Tris-EDTA). Using the Thermo Scientific™ NanoDrop, the DNA yield was determined prior to storing the samples at -20 °C.

3.1.4 DNA quantification

DNA Quantification was done using Nanodrop spectrophotometer. A spectrophotometer can precisely determine the purity and concentration of DNA in as little as 1µl of sample.

On the pedestal, 1 µL of each sample was dispensed, and absorbance was measured. The NanoDrop software computes the DNA concentration based on the obtained absorbance values. The absorbance of DNA is maximum near 260 nm. The A260/A280 ratio is an indicator of the purity of the DNA samples, and values of 1.8 or higher indicate pure DNA samples.

As a secondary measure of nucleic acid purity, 260/230 ratio is used. Generally, the 260/230 values for pure nucleic acid are greater than the corresponding 260/280 values. Expected 260/230 values typically range between 2.0 and 2.2. When the ratio of absorbance 260/230 is less than 1.8, it indicates contamination by organic or chaotropic compounds that absorb at 230 nm. Whereas, high A260/A230 ratio may be the result of a blank measurement on an uncleaned pedestal or the use of the incorrect Blank measurement solution. The pH and ionic strength of the blank solution should be similar to the sample solution.

3.1.5 Validation of DNA extraction by gel electrophoresis

The DNA extraction was validated by analyzing it on 1% agarose gel made in a 1X TAE buffer solution. 7 µL of sample and 3 µL of loading dye was used for loading into wells and gel was run in gel tank containing a similar concentration of 1X TAE buffer.

To make a 50X TAE buffer stock solution, 242 g of Tris base was mixed with 700 mL of distilled water. Then 100 mL of 0.5 M EDTA, 57.1 mL of Glacial acetic acid (GAA) was added, the pH was adjusted to 8, and the volume was increased to 1 litre at the end with distilled water.

2 mL of 50X buffer and 98 mL of distilled water were mixed to prepare 100 mL of 1X TAE working solution.

3.1.6 Genotyping through SSP PCR

The genotyping of each sample was performed through SSP-PCR. Two 25 µL reaction mixtures were prepared for each SNP using 12.5 µL Zokeyo 2xHLingene PCR Master Mix (serial number HPR001-01), 1 µL (10 pmol) of either allele specific primer, 1 µL (10 pmol) of control forward primer and 1.5 µL (15 pmol) of common reverse primer; 2 µL (0.1 to 2.5 µg) of DNA and 9 µL of nuclease free water. The following cycling conditions were established: initial denaturation at 95 °C for 15 minutes, followed by 35 cycles of 95 °C for

45 seconds, Ta °C for 45 seconds, and 72 °C for 45 seconds, and a final extension at 72 °C for 10 minutes. TGF-β1 was optimized at Ta of 64 °C, whereas IL-10 and IL-18 had a Ta of 55 °C.

3.1.7 Gel electrophoresis

2% agarose gel prepared in 1X TAE buffer was used for the analysis of the SSP PCR products. For the preparation of 100 mL agarose gel, 2 g of agarose was dissolved in 100 mL of TAE buffer and heated in a microwave until it became clear. After it cooled down to room temperature, 10 µL of ethidium bromide was added for staining the gel and then swirled for mixing. 3 µL of loading dye was mixed with 7 µL of PCR product for loading purpose. Total 10 µL was loaded in each well. At 90 V and 500 mA current, the gel was run for 50 minutes. The Dolphin ™ Gel Documentation System was used to analyze and obtain pictures of the gel.

3.1.8 Statistical analysis

In both infected and control samples, direct counting was done to estimate the allelic and genotypic frequencies for each polymorphic site, then these frequencies were compared using the Chi-square test in Graph Pad Prism version 8.0.1. P values under 0.05 were considered significant. Using the free statistical program MedCalc, odds ratios (OR) were calculated with a 95% confidence interval (CI) (Waqar, Altaf, Nazir, Javed, & Evolution, 2020).

3.2 Gene expression analysis

3.2.1 Primer designing

Primers for the housekeeping genes (glyceraldehyde phosphate 3-dehydrogenase (gapdh), actin beta (*actb*), glucuronidase beta (*gusb*), hypoxanthine phosphoribosyltransferase 1 (*hprt1*), phosphoglycerate kinase 1 (*pgk1*), peptidylprolyle isomerase A (*ppia*), beta-2-microglobulin (*b2m*), TATA-box binding protein (*tbp*), transferrin receptor (*tfrc*), tyrosine 3-

monooxygenase (*ywhaz*), ATP synthase peripheral stalk-membrane subunit b (*atp5bp*)), *calhm6* and *prdx2* were designed using NCBI primer BLAST (Ye et al., 2012) employing following conditions:

- Size of PCR products: 70–200 bp
- Tm range: 60–63 °C
- An exon-exon junction must be covered by primer.
- Refseq mRNA database was chosen.
- Only *Homo sapiens* were the focus of the primer's specificity.

The ability of these primers to create secondary structures was further examined using the Oligo Calc: Oligonucleotide Properties Calculator (Kibbe, 2007). Finally, using UCSC Genome Browser's In-Silico PCR (Kent et al., 2002), the melting temperature and specificity of these primers were once more examined.

3.2.2 Sample collection

Patients who had confirmed HIV infection met the requirements for participation in the experimental group. As a control group, 30 samples that tested HIV-negative were gathered.

With their informed agreement, 50 HIV-positive patients at the Punjab AIDS Control Program (PACP) in Lahore supplied blood samples for this study.

The Ethical Review Board of Shaheed Zulfiqar Ali Bhutto Medical University (SZABMU), Islamabad, Pakistan, and the Institutional Review Board (IRB) of ASAB, NUST, Islamabad, Pakistan, both approved this work. With the collaboration of Punjab AIDS Control Program (PACP) in Lahore and its interim equivalent, the NACP (National AIDS Control Program), this project was completed.

Before blood samples were taken, patients with HIV infection or AIDS gave their explicit, informed consent. Blood from the patients was taken in sterile EDTA tubes and processed immediately. Prior to the trial, clinical data on the patients, such as their age, gender, plasma viral load, and CD4 cell count, were collected.

Control blood samples were chosen at random from students at the Atta-Ur-Rahman School of Applied Biosciences (ASAB), National Institute of Sciences and Technology (NUST), Islamabad. Data on each subject's gender and family history of HIV infection were gathered to make sure that participants did not go against the inclusion criteria. Each blood sample was taken in sterile EDTA tube and immediately processed.

3.2.3 RNA extraction

All HIV-positive samples had their RNA extracted at PACP. RBC lysis buffer pH 7.3 (17.98 g NH4Cl, 2 g KHCO3, 400 µL 0.5 M EDTA in 200 mL total volume) and Solarbio Triquick Reagent R1100 were used to extract the RNA.

To do this, 25 mL of RBC lysis buffer were combined with 5 mL of blood and let to sit at room temperature for 10 minutes. Supernatant from the mixture's 10-minute centrifugation at 600 rcf was discarded. Reconstituted pellet was incubated for five minutes at room temperature with 1 mL of lysis buffer. After that, a pellet of white blood cells (WBCs) was resuspended in 1 mL of PBS after being centrifuged at 3000 rpm for 2 minutes. The final pellet was resuspended in 1 mL of Solarbio Triquick Reagent R1100 after being centrifuged once more at 3000 rpm for 2 minutes. At -80 °C, this can be kept in storage indefinitely.

Following the use of Solarbio Triquick Reagent R1100 and effective WBC extraction, 200 µL of chloroform was added, and then vortexed for 30 seconds. This mixture was then centrifuged at 12000 rcf for 10 minutes at 4 °C after being incubated at -20 °C for 3 minutes. 500 µL of isopropanol was added after the aqueous phase had been properly transferred to a

new microcentrifuge tube. This mixture was inverted numerous times, followed by a 10-minute incubation period at -20 °C. This mixture was then centrifuged for 10 minutes at 4 °C at 12000 rcf. After removing the supernatant, the pellet was washed with 1 mL of 75% ethanol. At 4 °C, a centrifuge was run for 2 minutes at 12000 rcf. The supernatant was discarded, and before being suspended in nuclease-free water, the final RNA pellet was air dried at 4 °C for 10 minutes. Denaturing gel electrophoresis was used to confirm the presence of RNA and was carried out at 90 V, 90 mA, and for 50 minutes.

3.2.4 cDNA synthesis

Nanodrop was used to measure the amount of extracted RNA, and Thermo Scientific's RevertAid First Strand cDNA Synthesis Kit K1622 was used to create cDNA from 1 µg of RNA. In a total capacity of 12 µL, 1 µg of RNA and 1 µL of random hexamer are combined for this purpose. For five minutes, this mixture was incubated at 65 °C. Then, to this mixture, were added 4 µL of 5X buffer, 1 µL of ribolock, 2 µL of 10 mM dNTPs, and 1 µL of RT enzyme. For this reaction, the temperature range was 25 °C for 5 minutes, 42 °C for 60 minutes, and then 70 °C for 5 minutes. Then, a 1:3 dilution of the synthesized cDNA was prepared.

3.2.5 Primer optimization

Using 2% agarose gel electrophoresis and gradient PCR, the *calhm6* and *prdx2* primers were optimized. Using Zokeyo 2X HLiHRPene PCR Master Mix HPR001 in a final concentration of 1X HLiHRPene PCR Master Mix, conventional PCR was carried out using 44.4 ng of cDNA, 10 pmol of forward primer, and 10 pmol of reverse primer. The reaction volume was up to 25 µL. Gradient PCR was run at 95 °C for 5 minutes of initial denaturation, 35 cycles of 95 °C for 45 seconds of denaturation, 57 °C to 63 °C for annealing, 72 °C for 45 seconds of extension, and 72 °C for 7 minutes of final extension. Finally, 50 minutes of 90 V, 500 mA 2% agarose gel electrophoresis were performed.

3.2.6 Real-time PCR and analysis

Real-time PCR was used to initially finalize the house-keeping gene before additional gene expression research on infected and uninfected samples. Then, each sample was subjected to a series of triplicate real-time PCR procedures to determine the Ct values of the house-keeping gene, capn1, and vcl. In a reaction volume of 20 µL, real-time PCR was carried out using Solis BioDyne 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) at a final concentration of 1X, 5 pmol of forward and reverse primers, and 44.44 ng of cDNA. Real-time PCR was carried out at 95 °C for 12 minutes of initial activation, then 40 cycles of 95 °C denaturation for 15 seconds, 60 °C annealing for 20 seconds, and 72 °C extension for 20 seconds. EvaGreen's fluorescence was photographed during the extension stage.

Following real-time PCR, the gene copy number and fold change for *calhm6* and *prdx2* were determined for each sample. The following formulas were used for copy number:

$$\Delta C_t = C_{t_{(target)}} - C_{t_{(house\ keeping)}}$$

$$Copy\ number = 100 \times 2^{-\Delta C_t}$$

These formulas provide us a relative measurement of how many copies of the target gene (*calhm6* or *prdx2*) will be present for every 100 copies of house-keeping genes.

The average of the Ct values for the infected and uninfected groups were determined separately for both genes to determine the fold change. Then, the formulas below were used:

$$\Delta\Delta C_t = \Delta C_{t_{(infected)}} - \Delta C_{t_{(uninfected)}}$$

$$Fold\ change = 2^{-\Delta\Delta C_t}$$

3.2.7 Statistical analysis

GraphPad Prism 8.0.1 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com, was used to conduct the statistical analysis. Using an unpaired t-test, the

calhm6 copy number of 30 uninfected and 46 infected samples (4 samples did not show expression) was compared. In order to create column graphs, mean with SEM was used. Unpaired t-test was also used to compare the *prdx2* copy number in 50 infected samples and 30 uninfected samples. In order to create column graphs, mean with SEM was used. 30 uninfected samples of *calhm6* and *prdx2* underwent linear regression analysis, and an XY graph was also produced. 46 infected samples of *calhm6* and *prdx2* underwent linear regression analysis as well, and a corresponding XY graph was produced. As 14 samples exhibited undetectable viral loads, 32 samples and 36 samples underwent linear regression analysis of *calhm6* and *prdx2*, respectively, with log10 viral load, and XY graphs were produced, respectively. With 39 samples (11 samples' CD4 counts were unavailable), a linear regression analysis of *calhm6* and *prdx2* with CD4 count was also carried out, and XY graphs were produced, respectively. Unpaired t-tests were used to determine the gender distribution of uninfected *calhm6* (12 males and 18 females) and *prdx2* (12 males and 18 females), and column graphs (mean with SEM) were produced, respectively. Unpaired t-tests were used to determine the gender distribution of the infected *calhm6* (30 males, 16 females) and *prdx2* (33 males, 17 females), and column graphs (mean with SEM) were produced, accordingly.

Chapter 4

RESULTS

4.1 SNP analysis

4.1.1 Validation of DNA extraction by gel electrophoresis

DNA extraction was validated using agarose gel electrophoresis to check the DNA genomic quality. Unsheared genomic DNA usually gives a thick band at around 23 kb. Fragmentation of genomic DNA was observed in patient samples. Gel results are listed in the figure and figure.

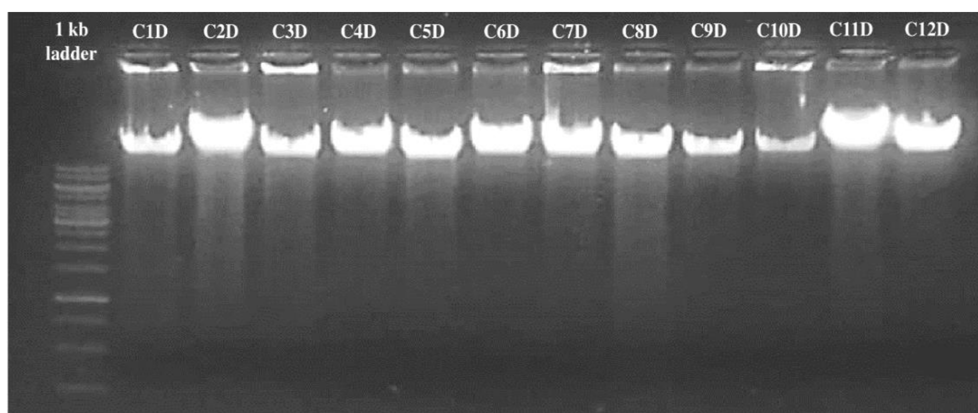

Figure 2: Confirmation of extraction from control samples on 1% agarose gel electrophoresis. M: 1 kb ladder, control samples C1D-C12D.

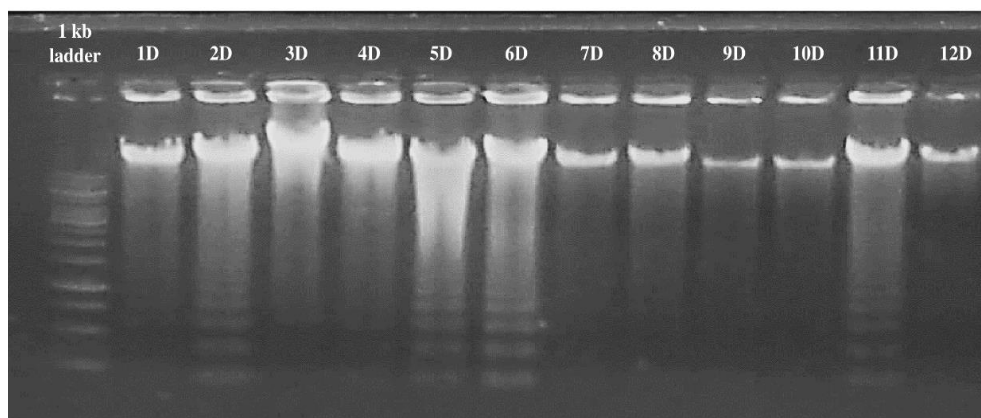

Figure 3: Confirmation of extraction from patient samples on 1% agarose gel electrophoresis. M: 1 kb ladder, HIV patient samples 1D-12D.

4.1.2 IL-18 -607C/A, IL-10 -1082A/G, and TGF-β1 -509C/T primer optimization for Ta °C determination

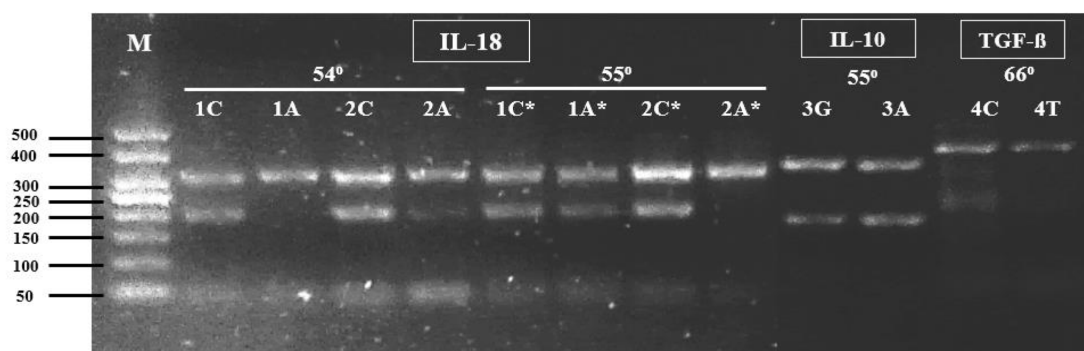

Figure 4: Primer optimization of IL-18 -607C/A, IL-10 -1082A/G, and TGF-β1 -509C/T on control samples. 2 control samples and 2 different predicted temperatures used for IL-18 SNP primer set. IL-10 and TGF-β run on 55 °C and 66 °C respectively as predicted temperatures. Product size for control fragment and allele specific fragment of IL-18: 301 bp and 197 bp, IL-10: 318 bp and 168 bp, TGF-β: 384 bp and 197 bp respectively. M: 50 bp ladder.

4.1.3 Genotyping using SSP PCR and gel electrophoresis

The participants in the study were divided into two groups. HIV-infected group consisted of 73 males and 30 females, had a mean age of 31.21±12.78 years, a median CD4+ count of 372.5 cells/L, and a median viral load of 137,206 IU/ml. The control group had a mean age of 25.18±8.83 years and consisted of 16 males and 34 females.

IL-18 -607C/A, IL-10 -1082A/G, and TGF-β1 -509C/T genotyping was done using SSP PCR and agarose gel electrophoresis on extracted DNA samples.

4.1.3.1 Genotyping of IL-18 -607C/A on control and HIV-infected samples using SSP PCR

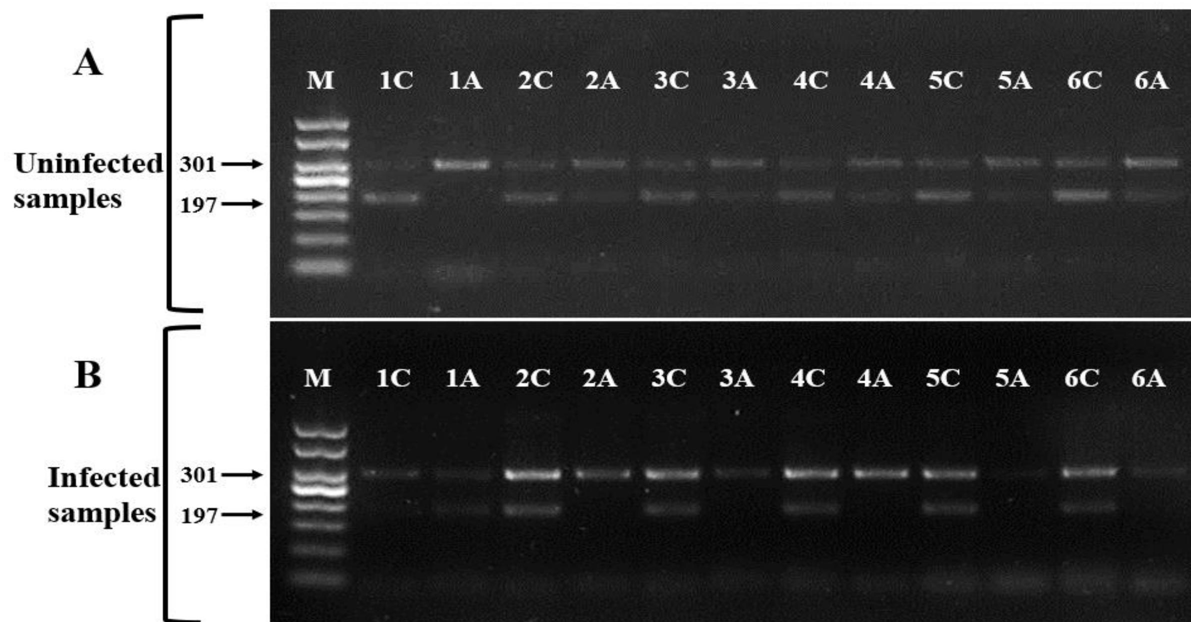

Figure 5: IL-18 (rs1946518) polymorphism screening in control and infected samples via SSP-PCR. (**A**) represents control samples. (**B**) represents infected samples. C represent PCR with C allele specific primer and A represents PCR with A allele specific primer. Size of control fragment:301 bp and allele specific fragment: 197 bp. M: 50 bp ladder.

4.1.3.2 Genotyping of IL-10 -1082A/G on control and HIV-infected samples using SSP PCR

In our study, 50 samples of healthy persons and 103 samples of HIV-infected patients were analysed for IL-10 -1082A/G polymorphism.

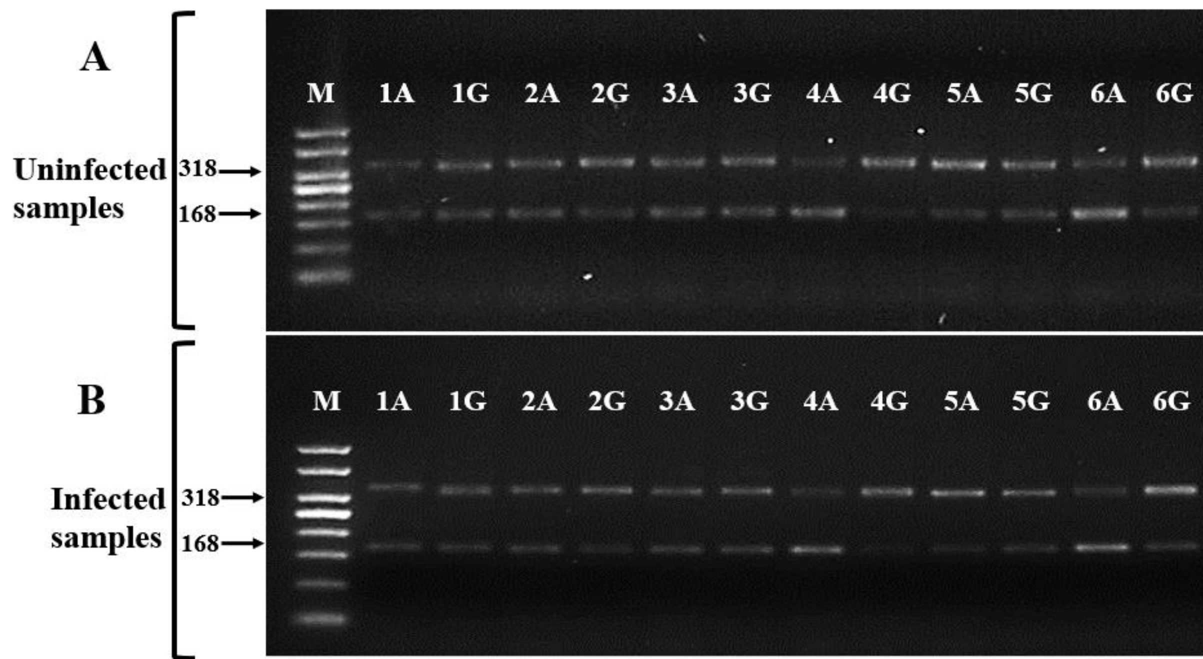

Figure 6: IL-10 (rs1800896) polymorphism screening in control and infected samples via SSP-PCR. (**A**) represents control samples. (**B**) represents infected samples. A represent PCR with A allele specific primer and G represents PCR with G allele specific primer. Size of control fragment:318 bp and allele specific fragment: 168 bp. M: 50 bp ladder.

4.1.3.3 Genotyping of TGF-β1 -509C/T on control and HIV-infected samples using SSP PCR

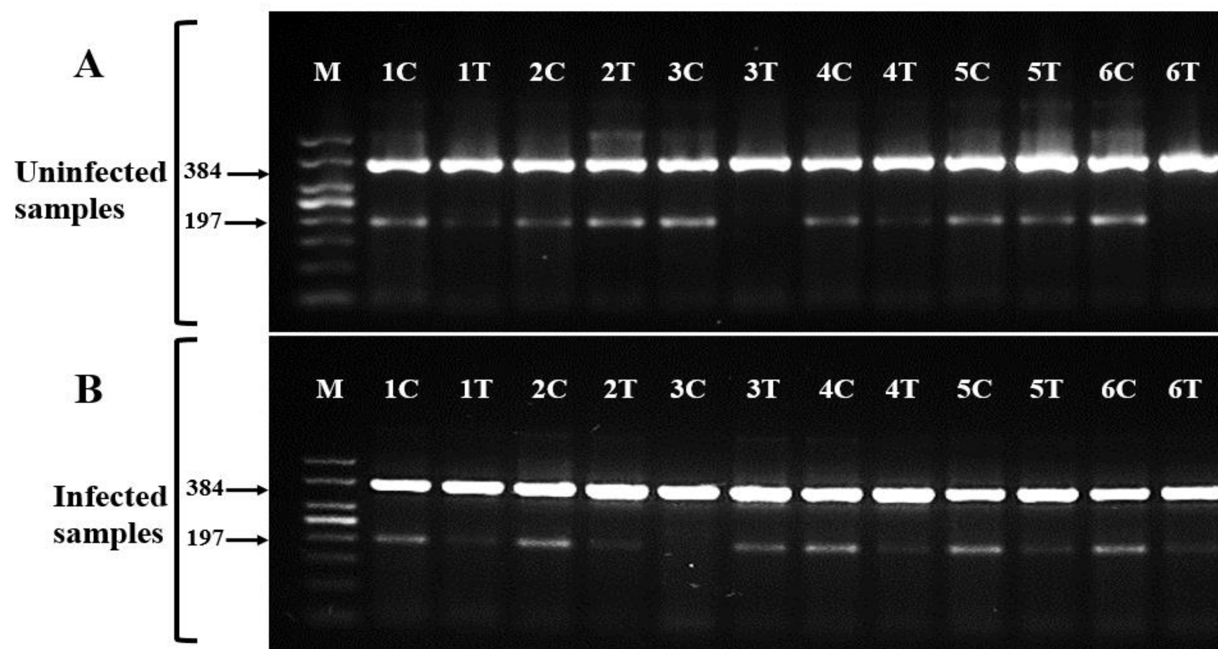

Figure 7: TGF-β1 (rs1800469) polymorphism screening in control and infected samples via SSP-PCR. (**A**) represents control samples. (**B**) represents infected samples. A represent PCR with A allele specific primer and G represents PCR with G allele specific primer. Size of control fragment:318 bp and allele specific fragment: 168 bp. M: 50 bp ladder.

4.1.4 Statistical analysis

4.1.4.1 IL-18 -607C/A statistical analysis

Table 3: Genotypic and allelic frequencies of IL-18 -607C/A polymorphisms.

Genotypic and Allelic frequencies of IL-18 -607C/A polymorphisms				
Polymorphism			**Patient frequency**	**Control frequency**
IL-18 -607C/A	**Genotype**	**CC**	36% (37/103)	34% (17/50)
		AA	2% (2/103)	6% (3/50)
		CA	62% (64/103	60% (30/50)
	Allele	**C**	67% (138/206)	64% (64/100)
		A	33% (68/206)	36% (36/100)

Table 4: Statistical analysis and genotypic and allelic significance of IL-18 -607C/A.

Polymorphism			HIV+	HIV-	Odds Ratio (OR)	95% CI	p-value	Significance P<0.05
IL-18 -607C/A	Genotype	CC	0.359	0.34	1.0882	0.5349 to 2.2140	0.8155	
		AA	0.019	0.06	0.3102	0.0501 to 1.9193	0.2081	
		CA	0.62	0.6	1.0940	0.5477 to 2.1852	0.7991	
	Allele	C	0.66	0.64	1.1415	0.6916 to 1.8841	0.6046	
		A	0.33	0.36	0.8760	0.5308 to 1.4458	0.6046	

Figure 8 depicts the genotypic distribution of the IL-18 -607C/A polymorphism, whereas **Figure 9** demonstrates the allelic frequency of the C and A alleles.

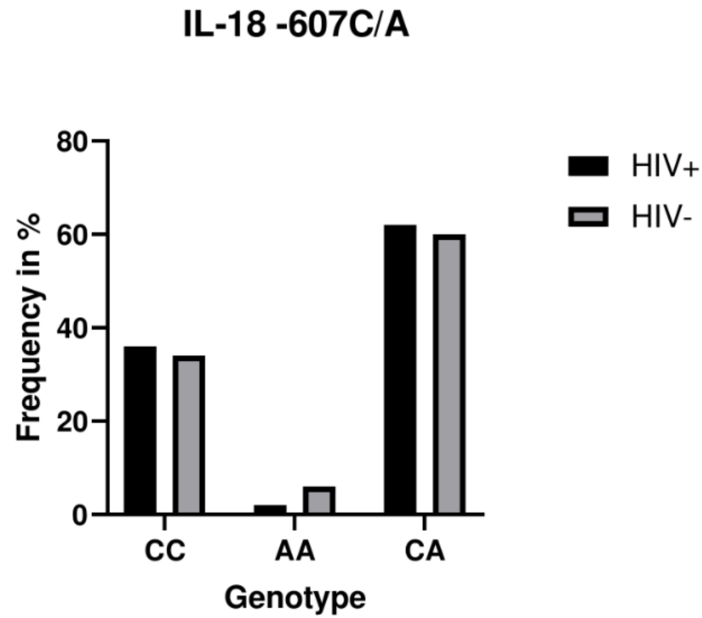

Figure 8: Genotypic distribution of IL-18 -607C/A in control and HIV groups.

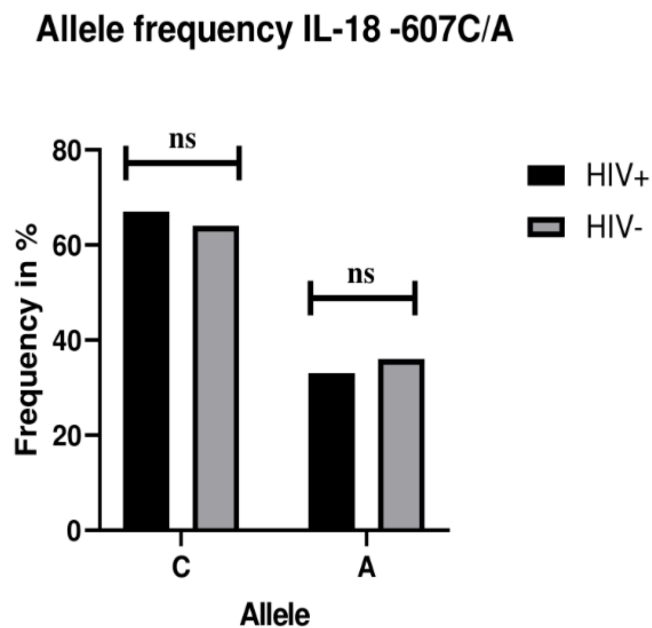

Figure 9: Allelic frequency in % of IL-18 -607C/A in control and HIV group.

Gender-based distribution data was available for only 62 infected samples. Statistical analysis was performed using chi-square test.

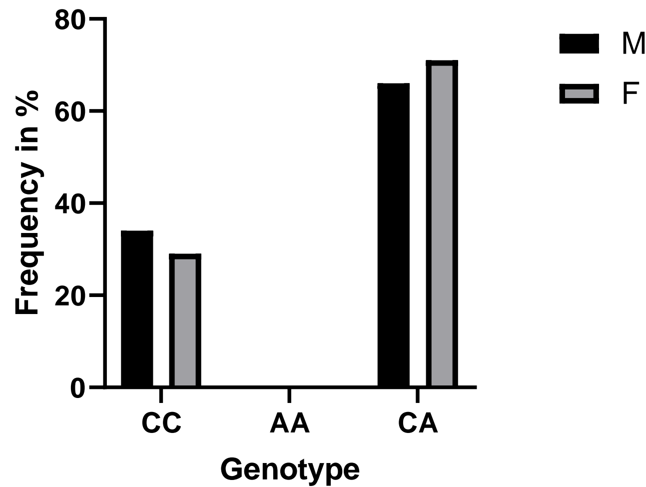

Figure 10: Genotypic distribution of IL-18 -607C/A for HIV patients in male and female groups.

Due to unavailability of viral load data of all the infected samples, only 59 samples were analysed for viral load associations. Statistical analysis was performed using unpaired t-test for IL-18 -607C/A CA and CC genotype. No significant results were produced.

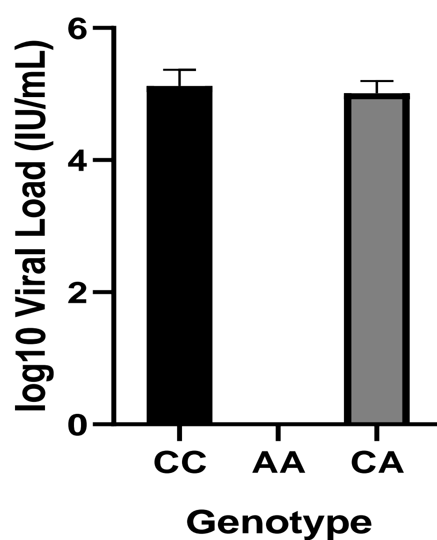

Figure 11: Viral load in different genotypes of IL-18 -607C/A.

61 samples were analysed for CD4 count association with genotypes. Statistical analysis was performed using unpaired t-test for IL-18 -607C/A CA and CC genotype.

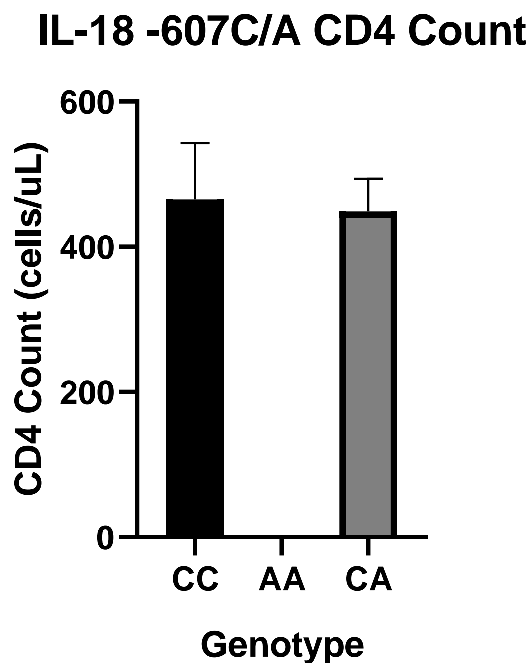

Figure 12: CD4 count in different genotypes of IL-18 -607C/A.

4.1.4.2 IL-10 -1082A/G statistical analysis

Table 5: Genotypic and allelic frequencies of IL-10 -1082A/G polymorphisms.

Genotypic and Allelic frequencies of IL-10 -1082A/G polymorphisms				
Polymorphism			**Patient frequency**	**Control frequency**
IL-10 -1082A/G	**Genotype**	**AA**	5% (5/103)	8% (4/50)
		GG	0% (0/103)	8% (4/50)
		AG	95% (98/103)	84% (42/50)
	Allele	**A**	52% (108/206)	50% (50/100)
		G	48% (98/206)	50% (50/100)

Table 6: Statistical analysis and genotypic and allelic significance of IL-10 -1082A/G.

Polymorphism			**HIV+**	**HIV-**	**Odds Ratio (OR)**	**95% CI**	**p-value**	**Significance P<0.05**
IL-10 -1082A/G	**Genotype**	**AA**	0.048	0.08	0.5867	0.1505 to 2.2876	0.4425	
		GG	$^{\Delta}$0	0.08	0.0499	0.0026 to 0.9464	**0.0459**	*
		AG	0.95	0.84	3.7333	1.1536 to 12.0817	**0.0279**	*
	Allele	**A**	0.52	0.5	1.1020	0.6833 to 1.7773	0.6903	
		G	0.47	0.5	0.9074	0.5626 to 1.4634	0.6903	

Figure 13 depicts the genotypic distribution of the IL-10 -1082A/G polymorphism, whereas **Figure 14** demonstrates the allelic frequency of the A and G alleles.

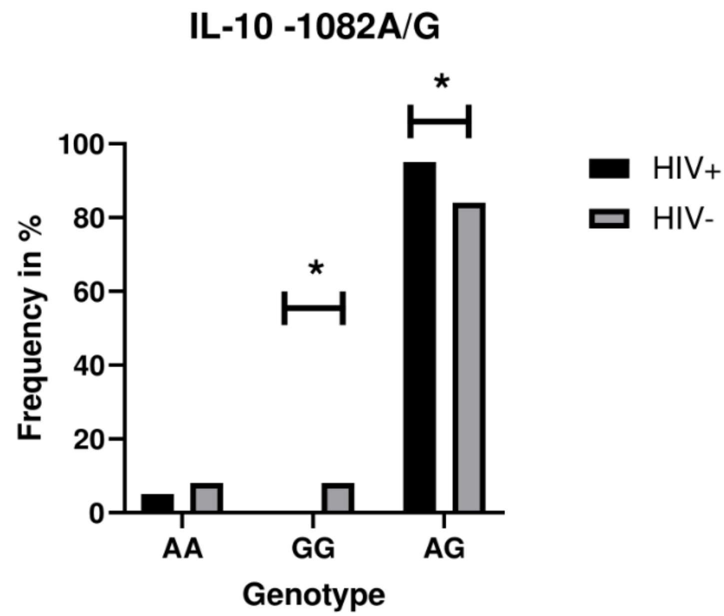

Figure 13: Genotypic distribution of IL-10 -1082A/G in control and HIV groups.

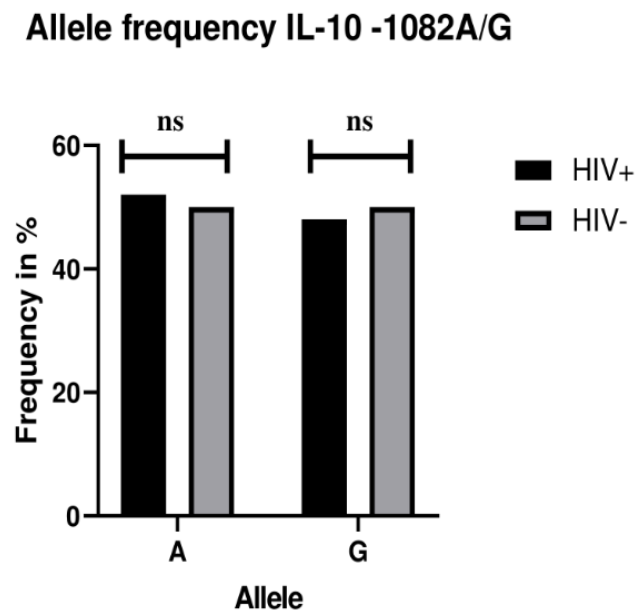

Figure 14: Allelic frequency in % of IL-10 -1082A/G in control and HIV group.

IL-10 -1082A/G analysis with respect to gender distribution, viral load and CD4 count was not performed because these details were available for only 62, 59, and 61 samples,

respectively, and all these samples had only one genotype dominant in infected samples so statistical tests were not applied.

4.1.4.3 TGF-β1 -509C/T statistical analysis

Table 7: Genotypic and allelic frequencies of TGF-β1 -509C/T polymorphisms.

Genotypic and Allelic frequencies of TGF-β1 -509C/T polymorphisms				
Polymorphism			**Patient frequency**	**Control frequency**
TGF-β1 -509C/T	**Genotype**	**CC**	23% (24/103)	40% (20/50)
		TT	33% (34/103)	2% (1/50)
		CT	44% (45/103)	58% (29/50)
	Allele	**C**	45% (93/206)	69% (69/100)
		T	55% (113/206)	31% (31/100)

Table 8: Statistical analysis and genotypic and allelic significance of TGF-β1 -509C/T.

Polymorphism			**HIV+**	**HIV-**	**Odds Ratio (OR)**	**95% CI**	**p-value**	**Significance P<0.05**
TGF-β1 -509C/T	**Genotype**	**CC**	0.23	0.4	0.4557	0.2202 to 0.9430	**0.034**	*
		TT	0.33	0.02	24.1449	3.1964 to 182.3848	**0.002**	**
		CT	0.43	0.58	0.5618	0.2837 to	0.098	

						1.1127		
	Allele	**C**	0.45	0.69	0.3698	0.2232 to 0.6126	**0.0001**	***
		T	0.54	0.31	2.7045	1.6324 to 4.4807	**0.0001**	***

Figure 15 depicts the genotypic distribution of the TGF-β1 -509C/T polymorphism, whereas **Figure 16** demonstrates the allelic frequency of the C and T alleles.

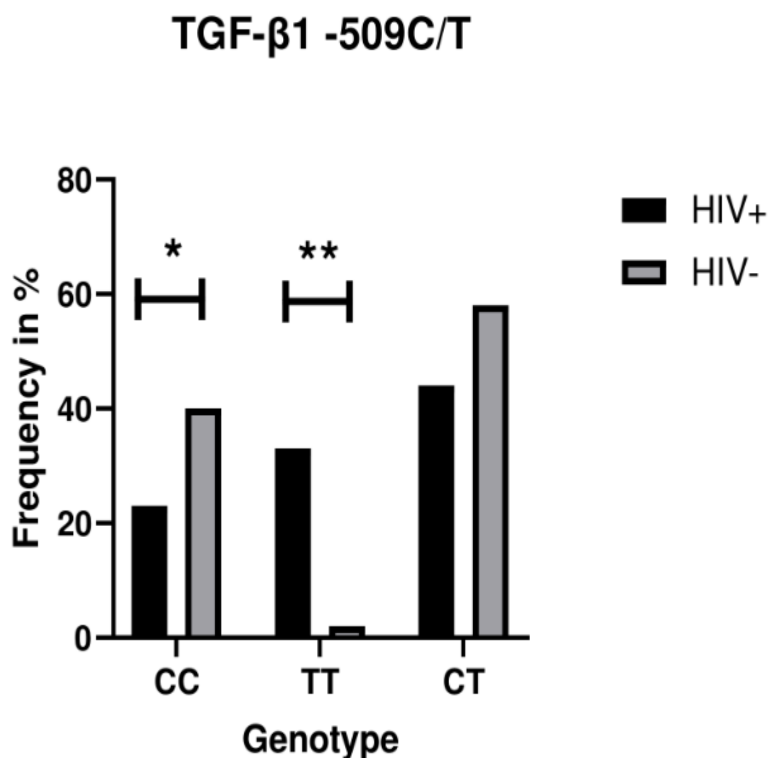

Figure 15: Genotypic distribution of TGF-β1 -509C/T in control and HIV groups.

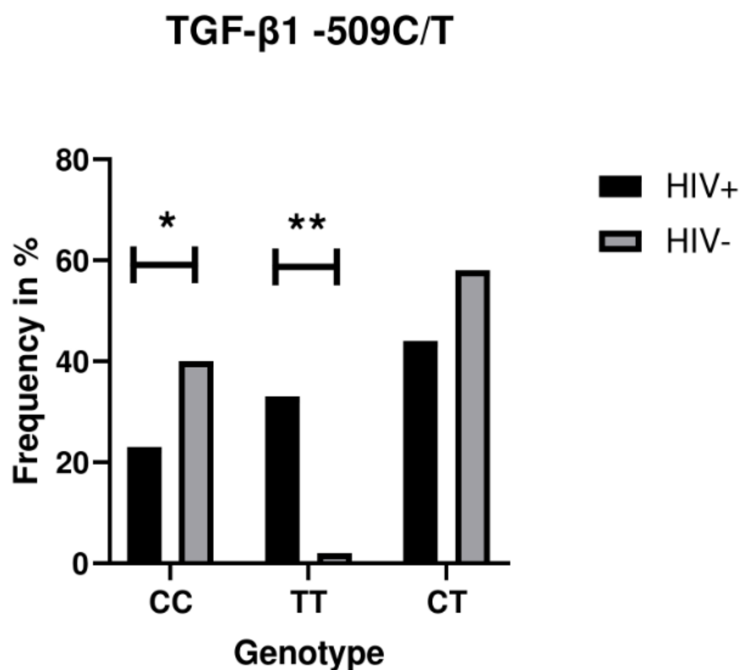

Figure 16: Allelic Frequency in % of TGF-β1 -509C/T in control and HIV groups.

Total number of samples for gender-based distribution was 62. Statistical analysis was performed using chi-square test.

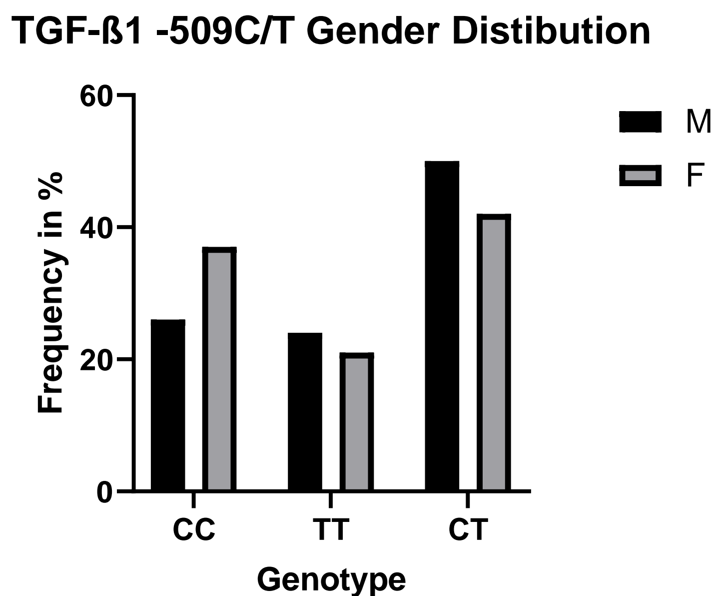

Figure 17: Genotypic distribution of TGF-β1 -509C/T for HIV patients in male and female groups.

TGF-β1 -509C/T analysis was performed using one-way ANOVA. 59 samples were analysed for viral load associations. Graphs were generated using mean with SEM.

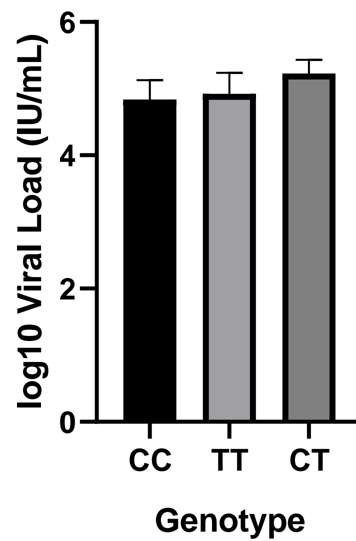

Figure 18: Viral load in different genotypes of TGF-β1 -509C/T.

61 infected samples were analysed for CD4 count association with genotypes. Analysis was performed using one-way ANOVA. Graphs were generated using mean with SEM.

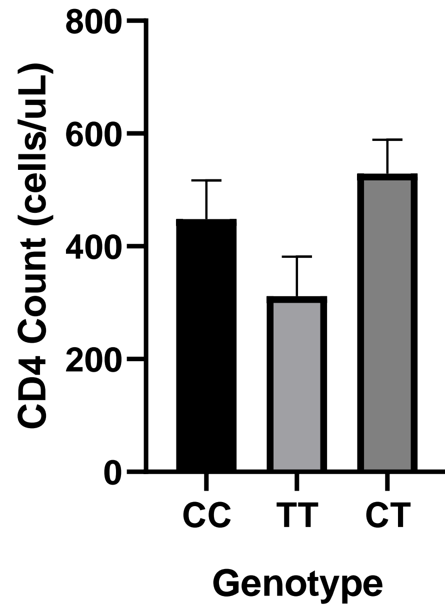

Figure 19: Viral load in different genotypes of TGF-β1 -509C/T.

58 samples were analysed for viral load vs CD4 count correlation. Linear regression analysis was performed.

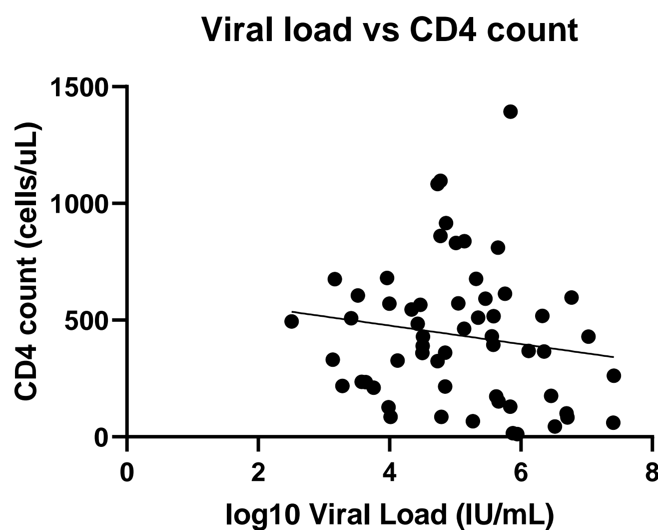

Figure 20: Viral load vs CD4 count correlation.

4.2 Gene expression analysis

4.2.1 Primer designing and optimization

Table 9 contains information about specified primers and their optimum annealing temperature. Additionally, **Figure 21** shows the gradient PCR results for *calhm6* and *prdx2* after electrophoresis on agarose gel.

Table 9: Designed primer sequences, GC content, and annealing temperature information.

	Sequence (5'-3')	GC (%)	Ta (°C)
calhm6			
Forward primer	CACCCGATGCCTATCTCCAG	60	60
Reverse primer	TTTGCTGCCACTCTTTCATGC	48	
prdx2			
Forward primer	AGACGAGCATGGGGAAGTTTG	52	60
Reverse primer	GGGCACAAGCTCACTATCCG	60	
gusb			
Forward primer	TGGAGCAAGACAGTGGGCT	57.89	60
Reverse primer	ACCTTAAGTTGGCCCTGGGT	55	
hprt1			
Forward primer	CCCTGGCGTCGTGATTAGTG	60	60
Reverse primer	CGAGCAAGACGTTCAGTCCT	55	
pgk1			
Forward primer	CCCACAGCTCCATGGTAGGA	60	60
Reverse primer	TCTGCAACTTTAGCTCCGCC	55	
ppia			

Forward primer	GCCGAGGAAAACCGTGTACTAT	50	60
Reverse primer	CTTGTCTGCAAACAGCTCAAAGG	48	
rps18			
Forward primer	GGATGGAAAATACAGCCAGGTCC	52	60
Reverse primer	GAAGTGACGCAGCCCTCTATG	57	
b2m			
Forward primer	TTGTCTTTCAGCAAGGACTGGT	45	60
Reverse primer	GCTTACATGTCTCGATCCCACTT	48	
tbp			
Forward primer	AGTGAGGTCGGGCAGGTTC	63	60
Reverse primer	CCAAGAAACAGTGATGCTGGGT	50	
tfrc			
Forward primer	CTCGTGTCCTCCCTTCATCCT	57	60
Reverse primer	CTGCCACACAGAAGAACCTGC	57	
ywhaz			
Forward primer	GACTGGGTCTGGCCCTTAAC	60	60
Reverse primer	ATCCGATGTCCACAATGTCAAGT	43	
atp5pb			
Forward primer	TCACAGGGACGCTAAGATTGC	52	60
Reverse primer	TTGCCTGCAATACCCCTGGAC	57	
gapdh			
Forward primer	CCCACTCCTCCACCTTTGAC	60	60
Reverse primer	TCCTCTTGTGCTCTTGCTGG	55	

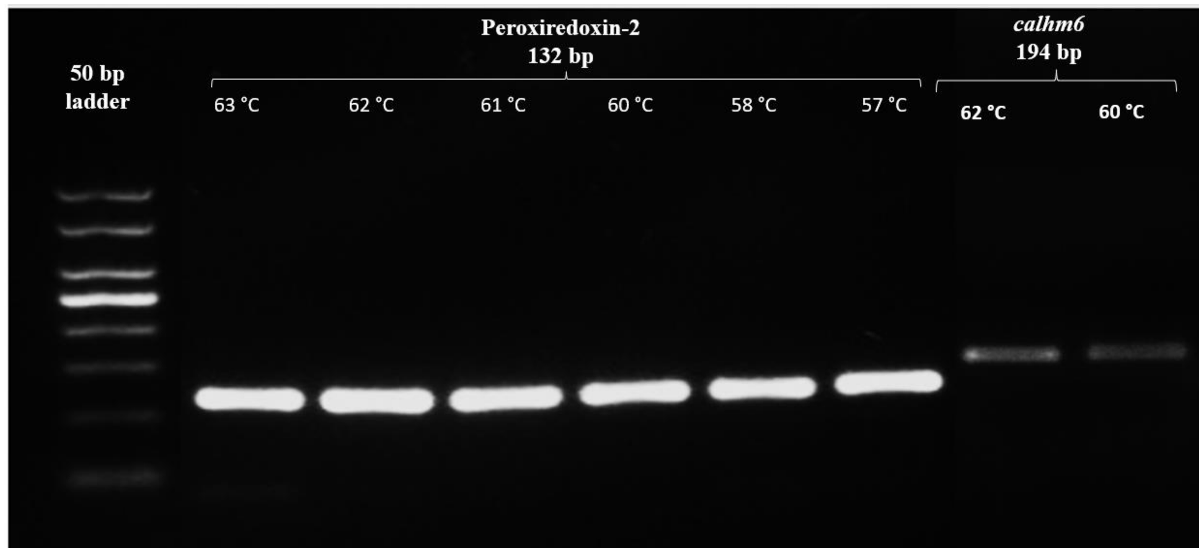

Figure 21: Results of gradient PCR for calhm6 and prdx2 on gel electrophoresis. Results of the *calhm6* gradient PCR at 60 °C, and 62 °C, and results of the *prdx2* gradient PCR at 57 °C, 58 °C, and 60 °C to 63 °C.

Our findings showed that *clhm6* and *prdx2* could be run over a wide range of Ta, whereas the Ta of other house-keeping genes was confirmed using real-time PCR.

4.2.2 RNA extraction

50 uninfected samples and 30 uninfected samples were processed for RNA extraction. **Figure 22** displays representative RNA denaturing gels for uninfected and infected samples.

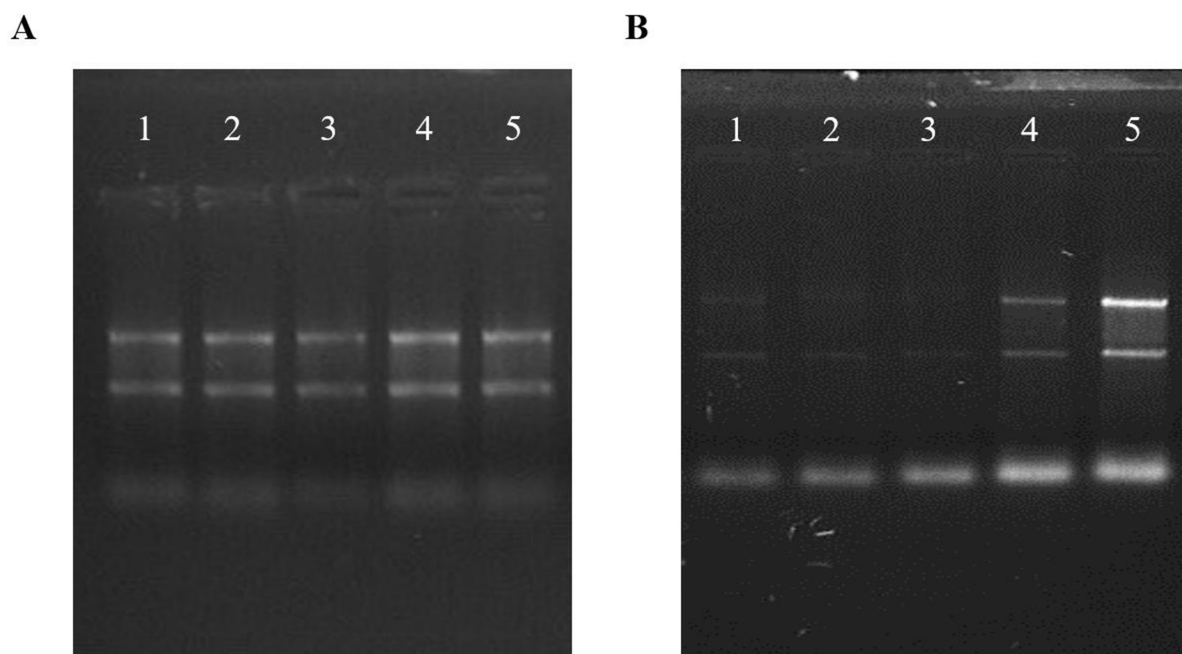

Figure 22: Results of RNA denaturing of infected and uninfected samples. (**A**) RNA of infected samples 1 – 5 and (**B**) uninfected samples 1 – 5.

4.2.3 Real-time results and statistical analysis

Real-time results and additional calculations showed that there was significant difference in the expression levels of the *calhm6*, while no significant difference was seen in *prdx2* gene between the uninfected and infected groups. The fold changes for *calhm6* and *prdx2* in infected samples were found to be -3.61 and -1.61, respectively, compared to uninfected samples.

Statistical analysis further revealed that gender had no significant impact on *calhm6* and *prdx2* copy number. **Figure 23** and **Figure 24** showed that there was no association among viral load, CD4 count, *calhm6*, and *prdx2*. While **Figure 25** shows that the expression of the *calhm6* and *prdx2* genes are significantly correlated in the case of uninfected individuals, while in case of infection, no correlation was observed.

It was observed that the p-value for uninfected individuals was 0.0002, indicating that this link was significant. In contrast, the p-value for infected people is 0.9751, showing that there is no correlation between *calhm6* and *prdx2* between infected individuals.

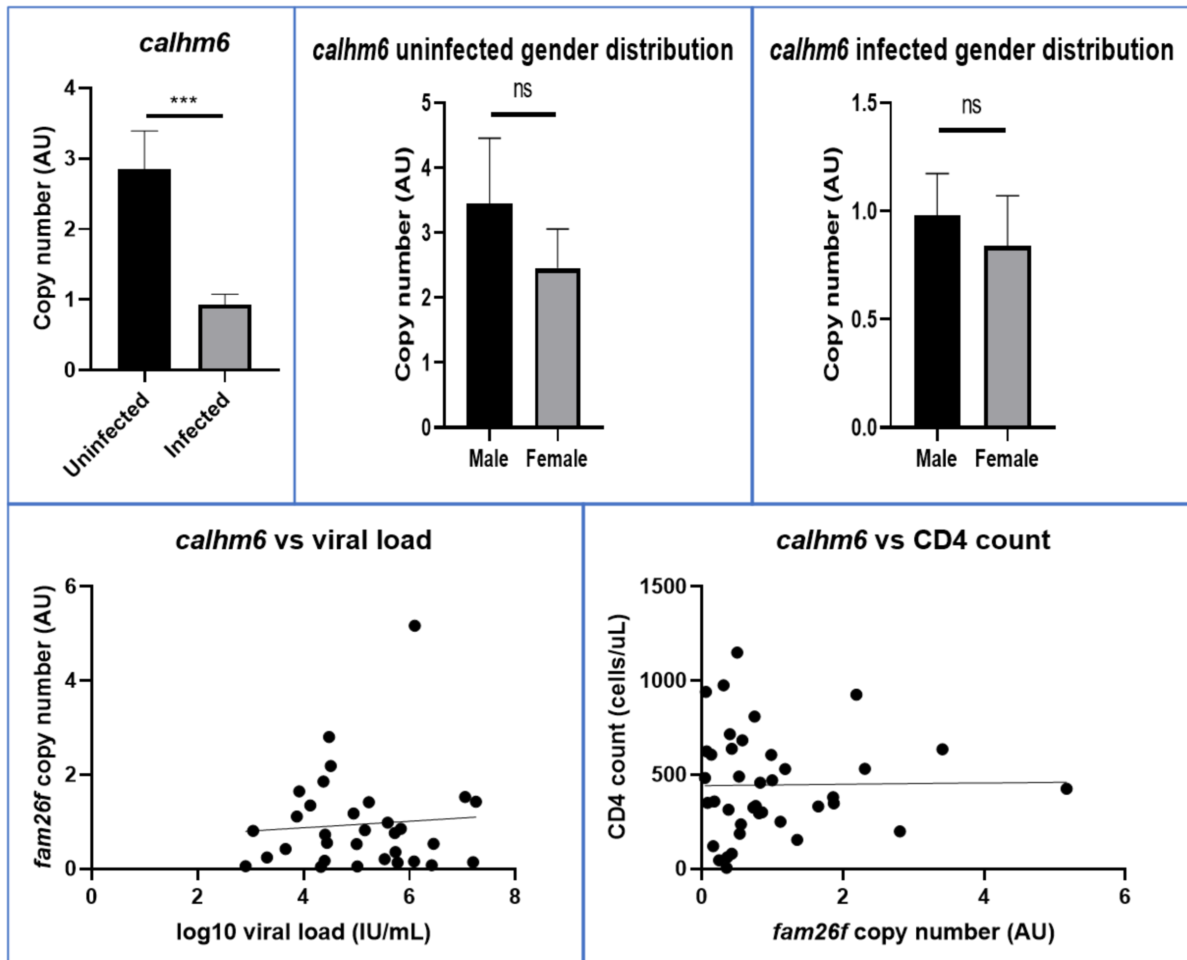

Figure 23: *calhm6* gene expression analysis. *calhm6* expression based on gender distribution, infected and uninfected groups, and its linear regression analysis with viral load and CD4 count.

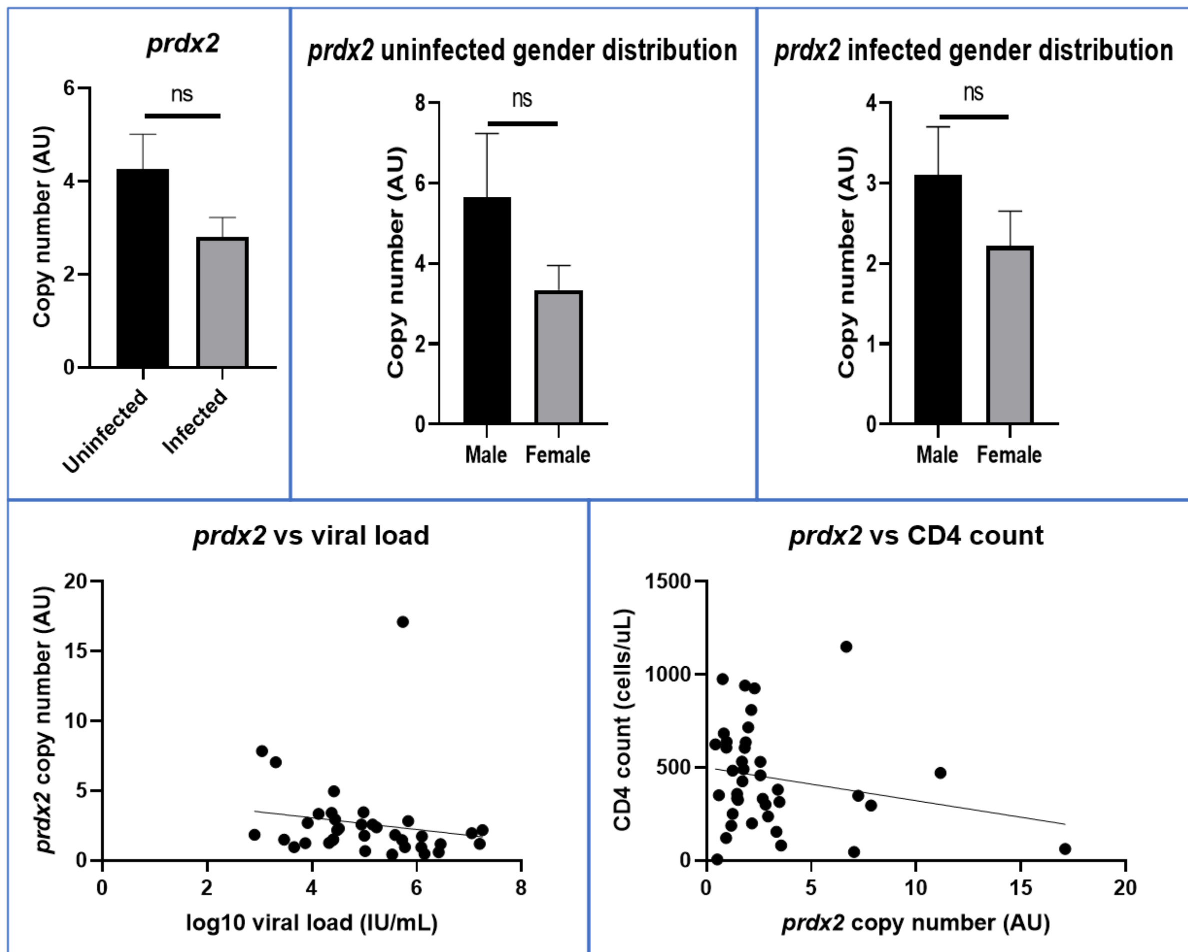

Figure 24: *prdx2* gene expression analysis. *prdx2* expression based on gender distribution, infected and uninfected groups, and its linear regression analysis with viral load and CD4 count.

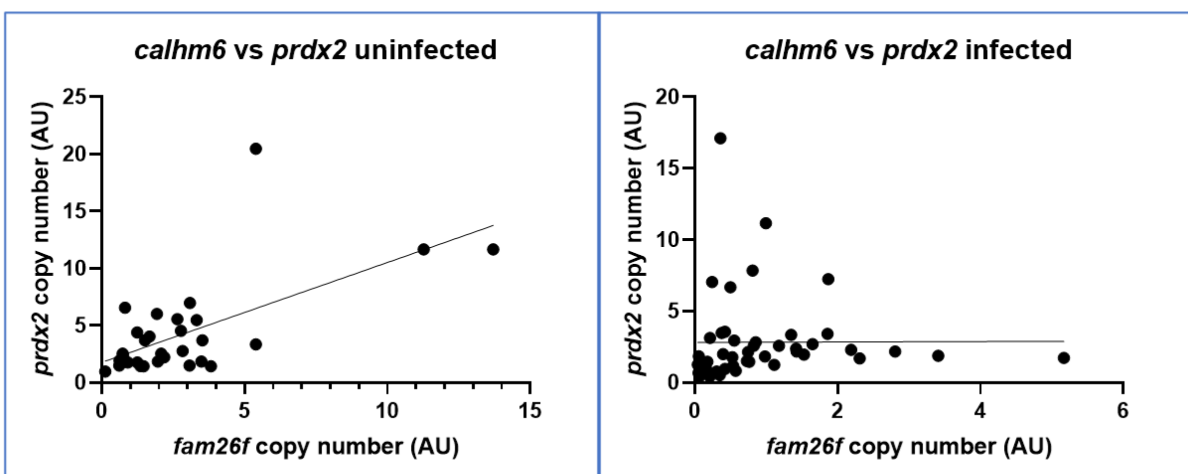

Figure 25: Analysis of linear regression between *calhm6* and *prdx2* in samples that were both infected and uninfected.

Chapter 5

DISCUSSION

The global aim of eradicating HIV/AIDS by 2030 necessitates underpinning of multifaceted mechanisms and a variety of factors that play a crucial role in this disease. It has been observed that most of the polymorphisms are typically located within the introns and promoter regions, thereby regulating the expression of certain cytokine genes and affecting the binding ability of transcriptional factors. The host factors included in this research were chosen due to their possible influence on pathogenicity of HIV infection, their reaction to treatment and infection control. Upon evaluating the genetic and allelic distribution of SNPs within the study, IL-18 exhibited non-significant results, however IL-10 and TGF-β1 demonstrated significant results.

IL-18 is a proinflammatory cytokine that increases HIV replication. SNPs in the promoter region of the IL-18 gene alter both the quantitative expression as well as transcriptional activity of this cytokine. Sobti et al. found that IL-18 -607CA and CC genotypes were associated with a higher level of IL-18 and rapid progression to AIDS in a study including 500 HIV/AIDS-infected individuals from North India. 15 The AA genotypes corresponds to a lower level of IL-18. The C to A transition has been shown to disrupt the binding of CREB protein (Giedraitis, He, Huang, & Hillert, 2001). An investigation carried out by Segat et al. in Brazilian children infected with HIV also related this polymorphism to differential HIV susceptibility (Segat et al., 2006). In current study, the CC and CA genotypes did not differ significantly between the case and control population group (P= 0.8155 and 0.7991, respectively) neither did the C and A allele frequency (P=0.6046 each). The CC and CA genotypes were found in higher number in both the control as well as case group which previously have been associated with a rapid progression to AIDS. A larger sample size is

needed to justify this claim if whether these genotypes prevail amongst the general Pakistani population too.

The second polymorphism under study was the IL-10 -1082A/G polymorphism. Freitas et al. had studied the associations of 5 SNPs with HIV infection and pathogenicity out of which 3 exhibited significant results (Bonfim Freitas et al., 2015). One of these polymorphisms is -1082A/G in IL-10 which in theory lowers $CD8^+$ T cells count. IL-10 is a macrophage growth inhibiting cytokine also constraining HIV replication in these cells (Kollmann et al., 1996). Different IL-10 genetic variants have exhibited various association to AIDS progression such as lower IL-10 serum levels have been linked to a higher risk of rapid progression to AIDS while at the same time conferring protection in other groups such as a study carried out by Winkler et al. showed that a transition from A>C at position -592 within the IL-10 promoter was linked to a slower progression to AIDS (Shin et al., 2000). Naicker et al. found that the AA and AG genotypes at position -1082 conferred protection by enhancing the immune response of HIV specific $CD8^+$ T-cells (Naicker et al., 2012). In the present study, an equal number of AA genotype indicate that its frequency did not differ significantly between the case and control population (P=0.4425) as well as the allele frequency of A and G was non-significant occurring at almost equal frequencies (P=0.6903). However, AG genotype at -1082 position has shown to increase susceptibility towards HIV infection with P=0.0279. Surprisingly, the GG genotypes which is associated with a rapid progression to AIDS was found within the control population with P=0.0459.

TGF-β1 is recognized as a CTLA-4 stimulant and its expression is correlated to progression markers, and IL-2 inhibition (Blobe, Schiemann, & Lodish, 2000). The 509C > T variant is located within a YY1 consensus binding site, and the 509T allele has been associated with elevated plasma levels of TGF-β1*.In HIV infected people, CTLA-4 positive T-regulatory cells are amplified triggering virus related immune dysfunction (Andersson et al., 2005). The

study suggested T allele's likely association with a greater HIV susceptibility which can partially be described by TGF-β1's linkage to increased expression of the HIV coreceptors CCR5 and CXCR4, vital for viral entry (Riley et al., 2000). In this study, the TT genotype differed significantly between the control and case group (P=0.002) with a higher TT genotype in HIV patients being linked to a higher level of TGF-β1. Similarly, T allele at this position also showed significant results with P=0.0001 suggesting a strong linkage to HIV susceptibility. However, CC genotype and C allele also showed significant difference between control and case group (P=0.034 and 0.0001, respectively) with a higher CC genotype and C allele in the control group hence suggesting their protective role against HIV susceptibility.

The genetic variability of cytokines in HIV-1-infected individuals in Pakistan and their impact on disease development are poorly understood. Therefore, this research will help to assess the prevalence of these polymorphisms and their impact on people's vulnerability to HIV infection.

The biological modification of the immune system has emerged during the past several years as a therapeutic option in medicine. We examined putative immune mediators in HIV patients while emphasising their significance as host factors that may help us better understand the biological complexity of HIV pathogenesis.

FAM26F is a newly identified immune-regulating protein. Unknown are FAM26F's role and modulatory mechanisms. Co-immunoprecipitation and immunofluorescence were employed to discover FAM26F's interaction partners and cellular process. Calmodulin-like protein 5, Calpain-1 catalytic subunit, Peroxiredoxin-2, Vinculin, Protein S100-A7, and Thioredoxin (Trx) were tested for co-localization with FAM26F. FAM26F interacts substantially with Trx and Peroxiredoxin-2 (Malik et al., 2020).

FAM26F in SIV infections has revealed that its pre-infection levels are inversely correlated to viral load (A. Javed et al., 2016). It was also seen in HBV infections, that FAM26F expression was significantly downregulated (Jabeen et al., 2021). Our results further endorse this previous literature, as it is demonstrated in our study that *calhm6* is significantly downregulated in case of HIV expression.

PRDX2 has previously shown overexpression in case of HIV exposed-uninfected individuals (Peretz, Cameron, Sékaly, & AIDS, 2012). In our study, it was also demonstrated that *prdx2* expression was decreased non-significantly in case of HIV infections.

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ABSTRACT

The susceptibility to HIV infection and the development of AIDS are highly influenced by host genetic factors. Single nucleotide polymorphisms in the genes that code for several cytokines, including IL-18, IL-10, and TGF-β1, have previously been associated to variable risk of HIV infection in different populations. In this research, polymorphisms in these immunomodulatory molecules have been determined through SSP PCR and related to HIV pathogenesis in Pakistani population. Our results demonstrated a significant association of TGF-β1 -509TT genotype/TGF-β1 -509C/T polymorphism with HIV susceptibility and a probable link of T allele at this position with the disease susceptibility, while C allele has shown protective role against HIV infection. Contrary to previous studies regarding IL-10 polymorphism, AG genotype has shown increased susceptibility towards HIV, while GG genotype has suggested protective role against HIV. No significant difference was found between HIV patients and controls for IL-18 polymorphisms. FAM26F is an essential regulatory protein involved in immunity, cell differentiation, infection, and anticancer activity. However, its specific role and modulatory mechanisms are yet unknown. Recently, immunoprecipitation and immunofluorescence-based techniques were employed to determine the six interaction partners of FAM26F namely, Calpain-1 catalytic subunit, Calmodulin-like protein 5, Peroxiredoxin-2, Protein S100-A7, Vinculin, and Thioredoxin. Current study has also evaluated the expression of FAM26F and its interacting partner peroxiredoxin-2 in HIV-1-infected patients and in healthy individuals to determine the association of these genes at the mRNA level with HIV pathogenesis through real-time PCR. Our results have demonstrated that FAM26F has significant association with HIV infection while peroxiredoxin-2 has no significant association with HIV infection.

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