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HOST FACTORS RESTRICTING HIV-1 INFECTIVITY

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ABSTRACT

The field of host-retrovirus interactions is complex and wide since multiple cellular proteins can act as positive or negative factors for viral infections or pathogenesis. In this work, we investigated the role of some cellular proteins on HIV-1 infectivity. The first examined factor is HLA-C and its potential role in AIDS progression. The analysis of the HLA-C genotype of 96 HIV-1 positive patients unveils the statistically significant association between some HLA-C variants, less stably bound to β2microglobulin/peptide complex, and a more rapid AIDS progression (p-value = 0.0143). The second considered aspect is the role of cellular proteins on HIV-associated neurocognitive disorders development (HAND). Finding new biomarkers and genetic factors linked to HIV-associated neurocognitive disorders was the aim of this second study. The analyzed factors include HLA-C, Apolipoprotein E, β2microglobulin, and Neurofilament Light Chain (NFL). A cohort of 32 patients was selected for this study and the subjects were classified according to their neurocognitive status. HLA-C and Apolipoprotein E genotypes were determined through an Allele-Specific PCR approach, while β2microglobulin and Neurofilament Light Chain plasma levels were quantified. Unfortunately, an association between the analyzed genetic factors/biomarkers and HAND development could not be determined due to the poor patient's recruitment caused by the COVID-19 pandemic. The third analyzed factor is ACOT8, a thioesterase discovered as an HIV-1 Nef protein-interacting partner. To unravel ACOT8 involvement in HIV-1 infectivity pseudotyped viruses were produced using Hek293T wild type or ACOT8 knock-out cell lines. The infection step was performed using TZM-bl wild type or TZM-bl ACOT8 knock-out cell lines. This approach revealed a probable ACOT8 role on virus production and infection that will be further analyzed.

RIASSUNTO

Il campo dello studio delle interazioni retrovirus-cellula è molto vasto data la complessità delle interazioni che si creano tra le proteine cellulari e virali. Tali interazioni possono favorire o impedire l'infettività o la patogenicità del virus. In questo lavoro di tesi sono stati analizzati alcuni fattori cellulari coinvolti nei meccanismi di infettività di HIV-1 e di progressione dell'AIDS. Per prima cosa è stato studiato il ruolo di HLA-C nella progressione dell'AIDS. Per farlo, sono stati reclutati 96 pazienti classificati in base alla diversa progressione della malattia e tramite un approccio di PCR allele specifica è stato determinato il loro genotipo HLA-C. I risultati ottenuti indicano un'associazione statisticamente significativa tra alcune varianti meno stabilmente legate al complesso β2microglobulina/peptide di HLA-C e una più rapida progressione dell'AIDS (p-value = 0.0143). Un altro aspetto preso in analisi nella presente tesi riguarda lo studio di possibili biomarcatori e fattori genetici per lo sviluppo di forme di demenza correlate ad HIV-1. In questo caso, sono stati reclutati 32 pazienti classificati in base al loro status neurocognitivo. Successivamente, tramite PCR allele specifica sono stati determinati i genotipi di HLA-C ed Apolipoproteina E. Inoltre, è stata eseguita l'analisi dei livelli plasmatici della β2microglobulina e della catena leggera del neurofilamento. Purtroppo, a causa del numero limitato di pazienti che è stato possibile reclutare, non è stato possibile identificare un'associazione tra i fattori genetici/biomarcatori analizzati e lo sviluppo di forme di demenza correlate ad HIV-1. Infine, è stato valutato il ruolo della proteina cellulare ACOT8 nell'infettività di HIV-1. In questo caso, sono stati prodotti virus pseudotipizzati in linee cellulari di Hek293T wild type o editate tramite CRISPR/Cas9 per la proteina ACOT8. Gli pseudovirus prodotti da queste due linee cellulari sono stati utilizzati per effettuare dei saggi di infezione su linee TZM-bl wild type o editate tramite CRISPR/Cas9 per la proteina ACOT8. I risultati ottenuti, anche se preliminari, indicano che ACOT8 potrebbe avere un ruolo nello step di produzione degli pseudovirus e anche nello step di infezione.

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ABBREVIATIONS

ACOT8: Acyl-coenzyme A Thioesterase 8

AIDS: Acquired Immunodeficiency Syndrome

ANI: Asymptomatic Neurocognitive Impairment

APOE: Apolipoprotein E

ASPCR: Allele-Specific Polymerase Chain Reaction

AZT: Azidothymidine

bNAbs: Broadly Neutralizing Antibodies

cART: combined Antiretroviral Therapy

Cas9: CRISPR Associated Protein 9

CCR5: CC Chemokine Receptor 5

CD4: Cluster of Differentiation 4

CNS: Central Nervous System

COL5A1: Collagen Type 5 Alpha 1 Chain

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CSF: Cerebrospinal Fluid

CXCR4: CXC Chemokine Receptor 4

Env: Envelope

Gag: Group-specific antigen

HAD: HIV-Associated Dementia

HAND: HIV-associated Neurocognitive Disorders

HBV: Hepatitis B Virus

HCV: Hepatitis C Virus

HIV-1: Human Immunodeficiency Virus type 1

HIV-2: Human Immunodeficiency Virus type 2

HLA-C: Human Leukocyte Antigen C

HPV: Human Papilloma Virus

HTLVs: Human T- Lymphotropic Viruses

KIR: Killer Ig-Like Receptors

KO: Knock-out

LTR: Long Terminal Repeat

MHC-I: Major Histocompatibility Complex Type I

MND: Mild Neurocognitive Disorder

MTAP: Methylthioadenosine Phosphorylase

Nef: Negative Regulatory Factor

NFL: Neurofilament Light Chain

NK: Natural Killer

PCR: Polymerase Chain Reaction

Pol: Polymerase

QHO: Env derived from a subject with HIV-1 subtype B infection

Rev: Regulator of Expression of Virion proteins

SIV: Simian Immunodeficiency Virus

Tat: Transactivator of transcription

TCR: T-cell receptor

TD-PCR: Touchdown Polymerase Chain reaction

UTR: Untranslated Region

Vif: Viral Infectivity Factor

Vpr: Viral Protein R

Vpu: Viral Protein U

VSV-G: Vesicular Stomatitis Virus Glycoprotein

β2m: Beta 2 microglobulin

1. **INTRODUCTION**

1.1 The *Retroviridae* family

Retroviruses are pathogens that infect a broad variety of vertebrates such as mammals, birds, reptiles, and fishes (John M Coffin et al., 1997b). Pathogens belonging to this family share some common features such as genetic material, replicative strategy, and virions structure. The retroviral genome is composed of single-stranded positive RNA molecules in which are located three essential coding domains: *gag* (encoding for virions proteins), *pol* (encoding for the reverse transcriptase, integrase, and protease), and *env* (encoding for envelope proteins) (Varmus, 1988; Weber et al., 1992). The retroviral replicative strategy is very peculiar when compared to other viruses. After the cell entry event and the viral uncoating, the reverse transcriptase initiates cDNA synthesis starting from the RNA template. Once the cDNA minus-strand synthesis is completed is exploited for plus-strand synthesis. The double-stranded DNA obtained contains at the 5' and 3' ends two identical long terminal repeats (LTRs) as a result of the reverse transcription process. These sequences are essential elements due to their binding sites for viral/cellular regulatory proteins. The dsDNA migrates into the nucleus where is integrated randomly into the cellular genome thanks to the viral integrase action. The viral genome is then transcribed exploiting cell transcription machinery and RNA polymerase II. After transcription, nuclear export and translation, the viral protease is responsible for viral proteins cleavage (J. M. Coffin, 1979; John M Coffin et al., 1997a). At the end of this process, new virions are produced and released by the infected cell. Members of the *Retroviridae* family are subdivided into seven different *genera*. Human pathogens belong to the Spumavirus *genus* (*i.e.* Human Foamy Virus: HFV), Deltaretrovirus *genus* (*i.e.* Human T- Lymphotropic Viruses: HTLVs), and Lentivirus *genus* (*i.e.* Human Immunodeficiency Viruses: HIVs) as represented in (Figure 1) (MacLachlan & Dubovi, 2017).

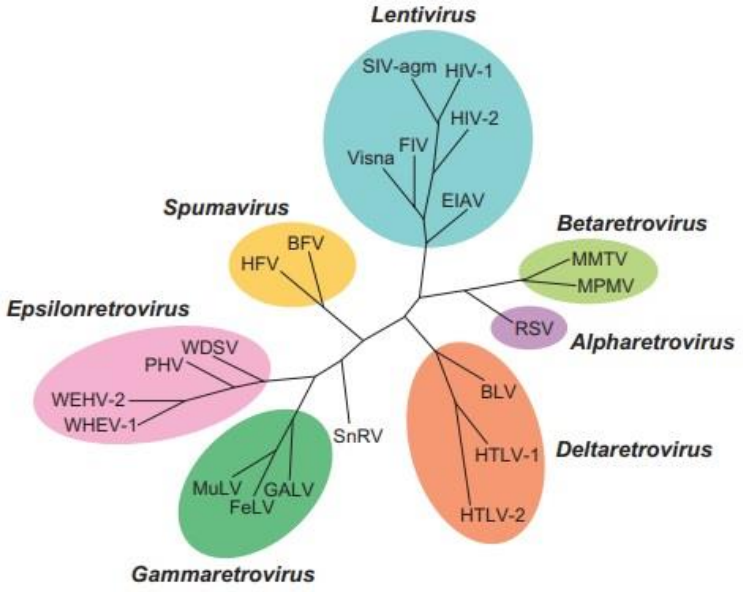

Figure 1: *Retroviridae* family classification: human pathogens belong to the Spumavirus, the Deltaretrovirus, and the Lentivirus *genera* (MacLachlan & Dubovi, 2017)

1.1.2 HIV origins and epidemiology

HIV has arisen from multiple spillover events from non-human primates lentiviruses (*i.e.,* Simian Immunodeficiency Viruses, SIVs) to humans. These zoonotic transmissions occurred in the early 1900s due to hunting, butchering, and capture activities of monkeys (Sharp & Hahn, 2011). However, this retrovirus was isolated and recognized as the causative agent of AIDS only in the 80s (Gallo et al., 1984). More than 40 species of non-human primates bear SIV and each species harbor a species-specific SIV strain. Independent cross-species transmission phenomena have generated four HIV-1 groups (known as M, N, O, and P) and eight HIV-2 groups (Nyamweya et al., 2013). SIVcpz found in chimpanzees (*i.e*. *Pan troglodytes troglodytes*) has given rise to HIV-1 M and N groups (Hemelaar, 2012; M. Peeters & Delaporte, 2012). The current worldwide HIV-1 pandemic is caused by HIV-1 group M spreading from Central Africa (the Democratic Republic of Congo is considered the epicenter), whereas the other three groups remained confined in Africa (Martine Peeters et al., 2014). The initial differences among HIV-1 groups arose from different spillover events from non-human primates; instead, HIV-1 M current differences, which gave rise to 9 subtypes, are due to expansion and diffusion in the human population (Figure 2) (Bbosa et al., 2019). HIV-2 has several similarities with HIV-1 such as genome organization, transmission mode and pathogenesis. However, HIV-2 possess reduced transmission rates, lower plasma viral load, higher CD4+ T-lymphocytes levels and lower progression to AIDS (Nyamweya et al., 2013). Global HIV statistics report that in 2020 an average of 37,7 million people were living with HIV among

them 1,5 million people became newly infected and only 28,2 million people had access to antiretroviral therapy (ART) in 2021. Moreover, another significant data is that 680000 people died in 2020 for AIDS-related diseases (*UNAIDS. Global HIV & AIDS Statistics — Fact Sheet*, n.d.).

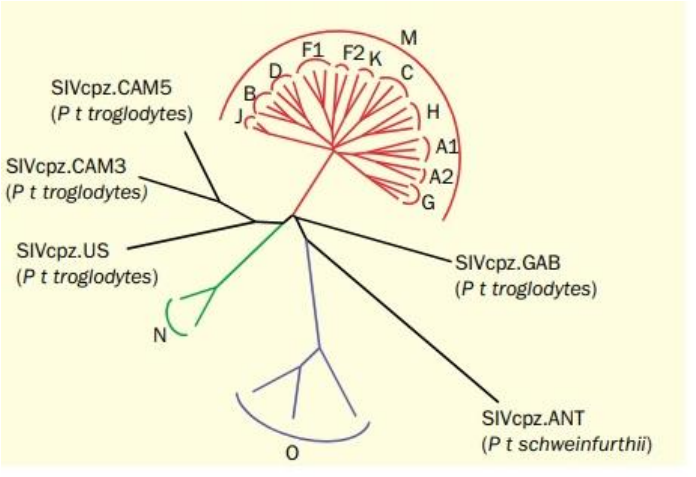

Figure 2: Phylogenetic tree generated using full-length genome sequences of HIV-1 (Thomson et al., 2002)

1.1.3 HIV-1 transmission and AIDS

HIV-1 is transmitted following the exposure of mucosal surfaces or intravenous inoculations via sexual, hematic (blood transfusions and needle sharing), and mother to child (birth and breastfeeding) transmission routes (Waymack & Sundareshan, 2021). The transmission efficacy varies depending on the transmission route, but the emergence of HIV-1 infection markers follows the same pattern (Cohen et al., 2011). Often, only a single virion is responsible for the infection, even if human infected fluids possess a mix of HIV-1 mutants called quasispecies (Yu et al., 2018). After transmission, several cell populations could be HIV-1 infection targets in the mucosa. In the first phase of infection HIV-1 replicates in the mucosa and is undetectable in plasma, this phase is called the eclipse phase and lasts between seven to twenty-one days (Cohen et al., 2011). Then HIV-1 reaches the gut-associated lymphoid tissue where CD4+ T lymphocytes become its preferential target, after this step HIV-1 spreads systematically (Hladik & McElrath, 2008). This acute phase is characterized by a peak of viral plasma levels. HIV-1 diagnosis during the acute phase relies on HIV-1 RNA, p24 protein, and antibodies detection (Cohen et al., 2011). Then, HIV-1 infection is controlled by adaptive immunity and plasma viral load values decrease. This latency period in which HIV-1 infection is partially controlled by the immune system, in absence of antiretroviral therapy, can last for months or even years. CD4+ T lymphocytes count is one of the main parameters used to monitor progression to AIDS. When CD4+ T lymphocytes level drops below 200 cells/mm^3 AIDS is diagnosed (Garcia & Guzman, 2021). The outbreak of opportunistic

infections could be a red flag indicating HIV-1 infection which helps HIV-1 diagnosis. The progression to AIDS is highly subjective and depends on numerous host and viral factors. Based on the rate of progression infected patients can be divided into three categories: Progressors (Ps), Long Term Non-Progressors (LTNPs), and Elite Controllers (ECs). Progressors have a rapid clinical progression to AIDS, instead, Long Term Non-progressors and Elite Controllers possess better control of HIV-1 infection leading to delayed AIDS development (Gebara et al., 2019). The urgent need to reduce the AIDS pandemic results in the development of new HIV-1 prevention methods such as active immunization using vaccines and passive immunization exploiting broadly neutralizing antibodies (bNAbs) (Stephenson et al., 2020).

1.1.4 AIDS treatment

Since the discovery of HIV as the causative agent of AIDS, the search for antiretroviral drugs has begun. The first approved drug for AIDS treatment was, in the late eighties, zidovudine (AZT), a nucleoside analogue that inhibits HIV reverse transcriptase enzyme.

Thereafter, new drugs were developed. Their classification is based on which HIV life-cycle step they inhibit as depicted in Figure 3 (Kemnic & Gulick, 2021; Lu et al., 2017):

- Entry inhibitors: prevent membrane fusion and HIV cell entry
- Reverse transcriptase inhibitors: nucleotide/nucleoside analogues (NRTIs) are defective nucleoside/nucleotide which acts as competitive inhibitors on reverse transcription; non-nucleotide/nucleoside inhibitors (NNRTIs) which bind an allosteric site on reverse transcriptase and act as non-competitive inhibitors
- Protease inhibitors: block HIV protein cleavage performed by its protease
- Integrase inhibitors: prevent HIV genome integration

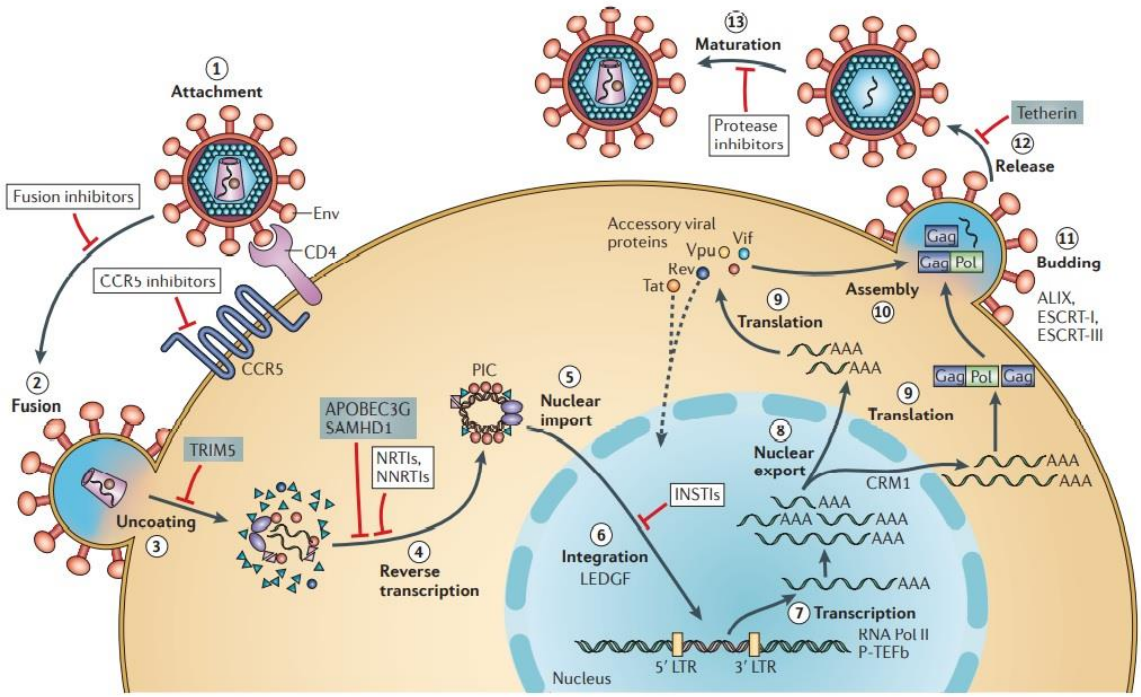

Figure 3: HIV-1 life cycle steps blocked by cellular proteins and drugs used in cART. Step 1-2: attachment and fusion inhibited by fusion and CCR5 inhibitors; Step 3-4: uncoating and reverse transcription blocked by cellular proteins (TRIM5, APOBEC3G and SAMHD1) and by reverse transcriptase inhibitors (NRTIs/NNRTIs); Step 5-6: nuclear import and integration blocked by integrase inhibitors; Step 7-11: transcription, translation, assembly and budding; Step 12-13: release and maturation inhibited by Tetherin and protease inhibitors (Engelman & Cherepanov, 2012)

Over the years, combinatorial therapy has proved to be more effective than an AIDS treatment composed of just one drug. Nowadays, the cART regimen, characterized by the use of different drugs which inhibit several HIV life-cycle steps, is the most efficient one (Becerra et al., 2016). Usually, the cART regimen is composed by two nucleotide/nucleoside reverse transcriptase inhibitors plus an integrase inhibitor or a non-nucleotide/nucleoside inhibitor or a protease inhibitor. Moreover, using cART, life expectancy is increased. However, failure to cART treatment is achievable due to HIV-1 drug resistance phenomena acquired by HIV-1 genetic mutations which impact the efficacy of the adopted cART regimen. These phenomena can be subdivided into two different categories: pre-treatment HIV-1 drug resistance and acquired drug resistance (McCluskey et al., 2019). Pre-treatment drug resistance emerges when a treatment naïve patient is infected by an HIV-drug resistant strain, these events are spreading alarmingly worldwide, reaching from 6,6 to 11% in some countries (Günthard et al., 2019; Ross et al., 2018). Instead, the acquired drug resistance arise from drugs selective pressure on HIV-1 mutated strains: HIV-1 has a high

replication and mutational rate (caused by reverse transcription mechanism which doesn't possess the proof-reading activity), thus producing HIV-1 mutated strains which can become prevalent due to the selective pressure generated by not efficacious cART (Abram et al., 2010; Günthard et al., 2019; X. Wei et al., 1995). Despite cART could control HIV-1 infection and reduce HIV-1 related morbidity and mortality, there isn't a therapy capable of eradicating HIV-1 (Bandera et al., 2019). Different cell types and tissue harbor latently HIV-1, forming the so-called viral reservoirs. The viral reservoir is mainly composed of a small subset of CD4+ lymphocytes resting memory cells bearing replication-competent proviral DNA (Fromentin & Chomont, 2021). Efforts to eradicate HIV-1 through the development of HIV-1 therapeutic vaccines are currently ongoing (Chen & Julg, 2020).

1.1.5 HIV-1 structure, genome, and proteins

HIV-1 possesses a conic core coated with a lipid envelope derived from the host cell membrane. The envelope is composed of gp120 and gp41 proteins which are responsible for the receptor (CD4) and co-receptors (CCR5 or CXCR4) binding and host cell membrane proteins such as MHC antigens. The matrix is composed of p17 protein while the capsid is formed by p24 protein. The virion contains two copies of unspliced single strand positive RNA stabilized by p7 protein, HIV-1 accessory proteins Nef, Vif, and Vpr, and enzymes that are essential for HIV-1 infection: reverse transcriptase, integrase, and protease (Arthur et al., 1992; Turner & Summers, 1999). HIV-1 genome encodes for the classical retroviral genes Gag, Pol, and Env and possesses six accessory genes: Vif, Vpr, Vpu, Tat, Rev, and Nef. RNA transcription happens after host transcription factors bind to the U3 region of the Long Terminal Repeat (LTR) integrated viral genome. Transcription exploits cellular RNA polymerase II and produces 3 major mRNA derived from different splicing events: the unspliced mRNA (approximately 9.2 Kb), analogous to the viral genome, used to produce Gag and Gag-Pol polyproteins; a single spliced mRNA (approximately 4.5 Kb) for translation of Env, Vif, Vpr, and Vpu; numerous spliced mRNA (approximately 2 Kb) coding for Tat, Rev and Nef (Figure 4) (Ferguson et al., 2002; MacLachlan & Dubovi, 2017).

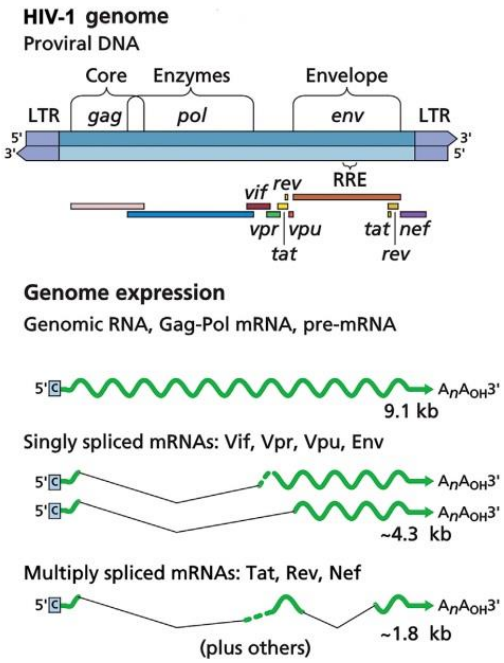

Figure 4: HIV-1 genome and mRNAs (Flint et al., 2009) The HIV-1 genome is composed by the classical retroviral genes Gag, Pol and Env and six accessory genes Vif, Vpr, Vpu, Tat, Rev, and Nef. HIV-1 genome transcription generates 3 major mRNAs: the unspliced mRNA (9.1 Kb) used to produce Gag and Gag-Pol proteins; a single spliced mRNA (4.3 Kb) to produce Vif, Vpr, Vpu and Env; multiply spliced mRNAs (1.8 Kb) to produce Tat, Rev and Nef

The assembly of the viral genome occurs in the cytoplasm through Gag and Gag-Pol precursor proteins which associate with Env glycoprotein gp160. HIV-1 protease cleaves Pol protein to form the three enzymes found in virions and Gag polyprotein to form viral structural proteins p17, p24, p7, and p9. Instead, gp160 is cleaved into gp41 and gp120 by furin (MacLachlan & Dubovi, 2017). The function of HIV-1 accessory proteins is reported in (Table 1) (Ferguson et al., 2002; MacLachlan & Dubovi, 2017).

Table 1: HIV-1 accessory proteins functions

Protein	Functions
Tat	Transactivator of transcription (Spector et al., 2019)
Rev	Implicated in un-spliced HIV-1 mRNA nuclear export (Truman et al., 2020)
Nef	Implicated in CD4 and MHC down-regulation (Geyer et al., 2001)
Vif	Inhibits APOBEC3 cellular protein action (Ooms et al., 2017)
Vpr	Contribute to cell cycle arrest in the G2 phase, enhance Env expression, and alters the expression of numerous host proteins (Lubow & Collins, 2020)
Vpu	Implicated in peroxisomes downregulation and inhibits NF-κB activation (Langer et al., 2019; Z. Xu et al., 2020)

1.1.6 Host factors involved in HIV-1 infectivity

Many host factors are involved in the protection against HIV-1 infection trying to inhibit different steps of the HIV-1 life cycle. One of the main family of cellular proteins involved in the inhibition of viral entry is the Tripartite motif 5 (TRIM5) family, in particular TRIM5α. This factor is involved in HIV-1 capsid recognition and subsequent degradation in a species-specific form. Several studies have been made on rhTRIM5α, belonging to *Rhesus Macaques*, proving its antiviral proprieties. Little is known about human TRIM5α, except that it possesses a less pronounced restriction capacity than rhTRIM5α (Cloherty et al., 2021; Grütter & Luban, 2012). Another family of proteins that are involved in the inhibition of HIV-1 entry is the Serine Incorporator (SERINC) family, in particular SERINC3 and SERINC5. Little is known about these transmembrane proteins, so far it seems that SERINC3/5 inhibit the fusion event between the viral particle and the target cell. Moreover, both of them are antagonized by HIV-1 Nef accessory protein, which promotes their internalization (Jin et al., 2020). Another protein that can play a pivotal role in HIV-1 infection is the C-C chemokine receptor type 5 (CCR5) which is one of the HIV-1 co-receptors. Several studies have analyzed the Δ32 mutation which leads to the production of a truncated protein, not exposed to the cell membrane. Homozygosity for this mutation is associated with protection against HIV-1 infection. The frequency of the CCR5Δ32 allele is low: 4-15% in the Caucasian population, mostly in the Northern Europe populations, whereas is rare in African and Asian populations (Lama & Planelles, 2007). Another evidence of CCR5 importance in HIV-1 infectivity is a case of an HIV-1 positive patient who had a hematopoietic stem cells transplantation to cure acute myeloid leukemia of a donor homozygous for the CCR5Δ32 allele. The patient maintained HIV-1 remission for 18 months after the treatment (Gupta et al., 2019). Another restriction factor

is the Mixovirus resistance 2 protein (MxB) a GTPase IFN-inducible. This protein acts as a restriction factor against various viral infections. MxB binds HIV-1 capsid preventing uncoating and nuclear import of the PIC complex (Wilbourne & Zhang, 2021; Xie et al., 2021). One of the most studied restriction factors is apolipoprotein B mRNA-editing catalytic polypeptide 3 (APOBEC3), which belongs to the cytidine deaminase family. This enzyme performs cytosines deamination to uracils in the HIV-1 ssDNA intermediates, thus inhibiting viral replication. However, HIV-1 counteracts this action through the viral accessory protein Vif, which binds APOBEC3 and targets the enzyme to proteasomal degradation (Azimi & Lee, 2020). Another important cellular factor is the Sterile Alpha Motif and Histidine Aspartate domain-containing protein 1 (SAMHD1) which weakens the HIV-1 replication step reducing the supply of deoxynucleotide triphosphates through its hydrolase activity. It's not clear if SAMHD1 possesses an RNase activity through which it can directly degrade HIV-1 viral RNA (Deutschmann & Gramberg, 2021). Tetherin/BST2 (Bone marrow stromal antigen 2) is a cellular transmembrane protein that prevents enveloped virus budding by anchoring viral particles to the cell membrane and promoting their internalization and subsequent lysosomal degradation. To bypass this cellular defense mechanism, HIV-1 Vpu promotes tetherin downregulation and degradation (Colomer-Lluch et al., 2018; le Tortorec et al., 2011).

1.2 Involvement of MHC-I complex in HIV-1 infectivity

The Major Histocompatibility Complex Class I (MHC-I) is composed of an α chain encoded by HLA-A, -B or -C, β2 microglobulin, and a short antigenic peptide. This trimeric complex is essential for the correct immune response since it presents intracellular peptides to CD8+ T lymphocytes. MHC-I complex is expressed on the cell membrane of all nucleated cells. Human Leucocyte Antigen (HLA) genes are among the most polymorphic genes in the human genome and are located in chromosome 6. HLA-A and -B possess higher expression levels and variability than HLA-C (Neefjes & Ploegh, 1988). Since its pivotal role in immune response, the relationship between MHC-I and HIV-1 infectivity is widely studied. Several studies on HIV-1 positive/AIDS cohorts have underlined how some HLA alleles seem to have a protective/deleterious effect in HIV-1 infection control and progression (Naranbhai & Carrington, 2017). For example, HLA-A*32, -A*74, -B*53, -B*57, -B*58, -B*57:03, -B*58:01, -B*27 seem to have a protective effect being associated with a slower disease progression. Instead, HLA-B*35 and -B*58:02 seem to have a negative impact on HIV-1 progression (Lazaryan et al., 2011; Lunardi et al., 2021). However, is important to underline the fact that HIV-1 infection control depends both on host and viral factors. Another important piece of evidence is that HLA homozygous phenotype is associated with worse HIV-1 control and a faster

disease progression. This phenomenon is linked to the fact that different HLA alleles have different peptide binding affinities, so having a heterozygous genotype provides a wider range of peptides that could be presented to CD8+ T lymphocytes (Carrington et al., 1999; Tang et al., 1999). It is well known that HIV-1 to escape from CD8+ T lymphocytes recognition downregulates HLA-A and -B via the accessory protein Nef (Collins et al., 1998). Other escape strategies developed by HIV-1 consist in antigen-processing escape in which a mutation on viral polypeptides prevents the proteasomal processing to constitute the peptides exposed by MHC-I; HLA-binding escape in which a mutation on viral peptides prevents their binding to HLA; TCR-escape through which the exposed viral peptide cannot be recognized by the TCR (Carlson et al., 2015).

1.2.1 HLA-C role in HIV-1 infection

HLA-C gene encodes for one α chain of the previously described MHC-I complex. Since HLA-C's pivotal role in the immune response, its correlation with HIV-1 disease progression and infectivity is widely studied. HLA-C has a reduced surface expression compared to HLA-A and -B because HLA-C binds less strongly β2microglobulin (Apps et al., 2013; Zipeto & Beretta, 2012). Moreover, it was recently discovered the occurrence of a SNP (rs67384697; "G-ins/del") in the 3' UTR region of HLA-C which regulates miR-148 binding. MiR-148 binding in turn is closely associated with HLA-C expression levels (Kulkarni et al., 2011). HLA-C alleles possess an intact binding site for miR-148 and are less expressed than HLA-C alleles with a deletion that prevents miR-148 binding (O'Huigin et al., 2011). Moreover, different HLA-C alleles show different binding stability to the β2microglobulin-peptide complex (Sibilio et al., 2008). HLA-C variants classified as unstable, are less strongly bound to the β2microglobulin-peptide complex, leading to the development of HLA-C "free chains" on the cell surface, which can be exploited by HIV-1 Env protein to enhance viral infectivity. In contrast, HLA-C alleles classified as stable, possess a stronger bound to the β2microglobulin-peptide complex thus preventing HLA-C "free chains" formation and so reducing HIV-1 infectivity (Parolini et al., 2018; Serena et al., 2017). HLA-C alleles classification as stable or unstable is reported in Table 2.

Table 2: HLA-C alleles classification as stable or unstable variants based on their binding stability to the β2microglobulin-peptide complex (Parolini et al., 2018)

HLA-C Unstable Alleles	HLA-C Stable Alleles
HLA-C*01	HLA-C*02
HLA-C*03	HLA-C*05
HLA-C*04	HLA-C*06
HLA-C*07	HLA-C*08
HLA-C*14	HLA-C*12
HLA-C*17	HLA-C*15
HLA-C*18	HLA-C*16

Moreover, HLA-C can bind KIR receptors on NK cells mediating their activation/inhibition (Biassoni et al., 1995; Körner et al., 2017). As previously described HIV-1 downmodulates HLA-A and -B exploiting its accessory protein Nef, whereas HLA-C is downregulated by the action of HIV-1 accessory protein Vpu (Apps et al., 2016; Hopfensperger et al., 2020).

1.3 HIV-associated neurocognitive disorders development and related risk factors

HIV-1 can penetrate the CNS in the early stages of infection due to high viral load levels. Monocytes CD14+ and CD16+, particularly susceptible to HIV-1 infection, can cross the blood-brain barrier due to their function thus bringing HIV-1 into CNS (Smail & Brew, 2018). The brain is considered as an HIV-1 reservoir due to the presence of the blood-brain barrier which ensures decreased immune surveillance and lack of drug penetration (Wallet et al., 2019). The presence of HIV-1 in the brain promotes an inflammatory response which leads to neuronal dysfunction and the subsequent development of HIV-1 associated neurocognitive disorders (HANDs) (Smail & Brew, 2018). The development of HANDs was particularly high in the pre-cART era; now with the advent of combined therapies, which ensure better control of HIV-1 infection, severe cases of HAND are rarer (Smail & Brew, 2018). However, HANDs are still present even if HIV-1 infection is managed through cART due to neural injuries in the first stages of the infection preceding cART initiation, neurotoxicity of the adopted therapies, and lack of drugs penetration in the CNS (Smail & Brew, 2018). The current HANDs classification was proposed in 2007 as a result of the different development of the pathology in the cART era and include HIV-associated dementia (HAD), mild neurocognitive disorder (MND), and asymptomatic neurocognitive impairment (ANI) (Antinori et al., 2007). HAD is diagnosed due to severe neurocognitive impairment which affects everyday

activities (such as inefficacy in social relations, work, and household tasks). MND is characterized by neurocognitive impairment and a moderate interference with everyday activities, whereas ANI is defined by mild neurocognitive impairment without interfering in everyday activities (Antinori et al., 2007; Eggers et al., 2017). In the pre-cART era HANDs manifestations were characterized by impairments in spoken fluency, cognitive speed, and motor abilities, instead, nowadays HANDs are dominated by learning and memory disorders (Clifford & Ances, 2013). Concerning the cART regimen, the score of CNS penetration of various agents has been assessed but is still under debate which is the best cART regimen to prevent or ameliorate HANDs (Eggers et al., 2017; Smail & Brew, 2018). Identifying HANDs plasma biomarkers is a difficult process, nowadays biomarkers correlated to the activation status of monocytes seem to be the most promising. These biomarkers include mononuclear cells carrying HIV-1 DNA and soluble CD14 and CD163 (Clifford & Ances, 2013). Risk factors linked to HANDs development comprehend aging, metabolic and genetic features, and comorbidities (Jayadev & Garden, 2009; Kompella et al., 2021). Genetic features include the Δ32 polymorphism in CCR5 co-receptor, CCL31 (CCR5 ligand) segmental duplication, and polymorphisms in TNFα (Jayadev & Garden, 2009). In another study, HLA-C alleles stability has been associated to HANDs showing that HLA-C unstable variants are more frequent in HIV-1 patients who developed HAD (Zipeto et al., 2018).

1.3.1 Apolipoprotein E and neurocognitive impairment onset

Apolipoprotein E (APOE) is a protein involved in lipid metabolism mediating its transport and uptake. This 34KDa secreted protein is produced mainly by the kidney, liver, macrophages, and brain. APOE gene is located in chromosome 19 and to date, three different variants APOE2, E3, and E4 have been characterized. The difference between these three haplotypes lies on two SNPs (rs429358 and rs7412) which result in the presence of cysteine or arginine at positions 112 and 158. APOE2 possesses two cysteine residues, APOE3 one cysteine, and one arginine and APOE4 two arginine residues (Jayadev & Garden, 2009; Siddiqui et al., 2018; Zhong et al., 2016). It's widely recognized APOE4 haplotype involvement in Alzheimer's disease onset (Corder et al., 1993; Saunders et al., 1993). APOE4 role in HANDs onset is still a matter of debate due to controversial results emerging from numerous studies. It is difficult to determine the APOE effect on HAND development due to other factors such as aging, comorbidities, and cART which can impact neurocognitive impairment. However, the association between APOE4 and HANDs development has been demonstrated in some previously published papers: two studies demonstrated that HIV-1 positive patients bearing APOE4 had worse cognitive performance and memory impairments

(Hoare et al., 2013; Wendelken et al., 2016). In another study, the homozygosity for APOE4 haplotype resulted in a more rapid HIV progression, but an association with HAD development was not detected (Burt et al., 2008). A fourth study didn't find an association between APOE4 and HANDs development even taking into consideration an APOE4 dose-dependent correlation (Morgan et al., 2013).

1.3.2 Beta2microglobulin and NFL as biomarkers linked to the development of HAND

Since HANDs diagnosis is difficult and complex, the attention is focused on finding biomarkers to detect HIV-related neurocognitive impairment to help HANDs diagnosis process. Two of the examined biomarkers are Beta2microglobulin and neurofilament light chain. Beta2microglobulin (β2m) is the invariant chain of the MHC class I complex. The release of β2m in serum occurs as the result of MHC-I turn-over. In patients suffering from kidney failure, β2m is not appropriately degraded thus increasing β2m concentration and consequent aggregates formation. So, it is well known its ability to form amyloid aggregates primarily found in joints of patients subjected to hemodialysis (Eakin & Miranker, 2005). Another study demonstrates the association between plasma β2m levels and advanced Alzheimer's disease compared to the levels of patients suffering from mild cognitive impairment (Dominici et al., 2018). In other studies, the role of β2m in the development of HANDs was examined. In fact, in a previously published work, the β2m levels in cerebrospinal fluid were strongly associated with the severity of the AIDS dementia complex (Brew et al., 1992). In another paper, CSF β2microglobulin levels were associated with reduced CD4+ T lymphocytes and its levels in CSF of HANDs patients were elevated regardless of serum levels (McArthur et al., 1992). Therefore, βeta2microglobulin could be exploited as a biomarker of HANDs development (Price et al., 2007). Another marker of CNS injury is the neurofilament light chain protein (NFL). Neurofilament proteins constitute about 85% of the cytoskeleton and are composed of three chains: light, medium, and heavy. NF proteins are mainly constituted by the light chain which is also the most soluble one. Due to these proprieties, NFL can be used as a marker of axonal degeneration and can be found in CSF and plasma (Yilmaz et al., 2017). NFL plasma levels, despite being 50-fold lower, strongly correlate with NFL levels in CSF. Moreover, NFL concentration seems to be higher with aging, in HAD patients, and in HIV-1 positive patients ART-naïve with low CD4+ T lymphocytes (Gisslén et al., 2015). In another study, NFL concentration in CSF positively correlates with plasma viral load and aging. Moreover, NFL levels were higher in patients with neurocognitive impairment (Guha et al., 2019). In a previously published work, NFL was used as a marker of CNS injury in HIV infected patients finding that NFL concentration in CSF

is higher in HAD patients and in patients with low CD4+ T lymphocytes and during the early stages of HIV-1 infection (Peluso et al., 2013; Peterson et al., 2014). NFL can be exploited as a biomarker to follow HIV-1 associated neurodegeneration (Abdulle et al., 2007; Gisslén et al., 2007).

1.4 ACOT8: a host factor participating in HIV-1 infectivity

Acyl-CoA Thioesterases (ACOTs) are enzymes involved in lipid metabolism which can hydrolyze the thioester bond releasing free fatty acids and Coenzyme A. ACOT enzymes are mainly present in peroxisomes and are subdivided into two subfamilies Type I and Type II (Hunt et al., 2012). ACOT8 is a member of Type II thioesterases and was initially described as a Nef interacting protein (Liu et al., 1997; Watanabe et al., 1997). ACOT8 gene is located in the long arm of chromosome 20, possesses 6 exons and in the upstream region, there is a putative peroxisome proliferator responsive element (PPRE) which can be exploited for ACOT8 expression regulation (Hunt et al., 2012). ACOT8 is a highly conserved protein being expressed from bacteria to man; in man and mouse possesses a wide tissue expression. Moreover, mouse ACOT8 has a wide substrate specificity hydrolising medium to large fatty acids (Hunt et al., 2012). As previously described, ACOT8 was discovered as a Nef interacting partner. It is known that Nef is responsible for CD4 downregulation. Previously published reviews suggested how ACOT8 could be involved in Nef-mediated CD4 downregulation. Nef-ACOT8 complexes could have multiple cellular localizations, among which is the plasma membrane. In the plasma membrane, ACOT8 can deacetylate the lymphocyte-specific protein tyrosine kinase $p56^{lck}$ (Lck) promoting its removal. Lck binds CD4 cytoplasmatic tail, thus Lck removal could support CD4 internalization (Lazarow, 2011; Palmeira et al., 2019). Mutations that impair Nef and ACOT8 interactions have been previously characterized (Liu et al., 2000; Serena et al., 2016). The role of ACOT8-Nef interaction needs to be further investigated to assess its involvement in HIV-1 infectivity. Moreover, ACOT8 involvement in cancer development has been studied in clear cell renal cell carcinoma, hepatocellular carcinoma, and lung cancer (Hung et al., 2014; Jung et al., 2013; C. L. Xu et al., 2020).

2. AIM OF THE RESEARCH

This thesis aims to investigate the role of host factors in HIV-1 infectivity and pathogenicity. The impact of some host factors (*i.e.,* HLA-C, APOE, beta2microglobulin, and Neurofilament light chain) in AIDS progression, HIV-1-associated neurocognitive disorder development, and HIV-1 infectivity has been analyzed. The thesis is organized into three sections dedicated to the following main aims:

1- Analyse the possible correlation between HLA-C stability and AIDS progression. To this aim, HLA-C genotyping of 96 HIV-1 infected patients was performed. Patients were distributed into three categories based on their AIDS progression status. Statistical analysis has been employed to examine the potential association between HLA-C unstable variants and a more rapid AIDS progression.

2- Evaluate genetic factors and biomarkers linked to the development of HIV-1-associated neurocognitive disorders development. To this aim, 32 HIV-1 positive patients were enrolled, submitted to the neurocognitive test, and subdivided based on their neurocognitive impairment status. Recruited subjects had different stages of progression of HIV-1 infection (between A1 and C3 status of the CDC classification system). HLA-C and APOE genotype were analyzed to assess a potential correlation between HLA-C unstable alleles/APOE4 and HANDs development. The evaluated biomarkers comprehend beta2microgloubulin and neurofilament light chain to determine if there is a link between their plasma level and HANDs development.

3- Study the role of ACOT8 in HIV-1 infectivity. To this aim ACOT8, knock-out cell lines were produced exploiting the CRISPR/Cas9 technique and subsequently used to perform production and infection assays using pseudotyped viruses.

3. MATERIALS AND METHODS

3.1 HIV-1 infected patients cohort progression status

A cohort of 96 HIV-1 infected subjects was enrolled in this study. Patients, based on their HIV-1 progression status (defined by CD4+ T lymphocytes cell count trend and plasma HIV-1 RNA level), were divided into three categories: Progressors (Ps), Long Term Non-Progressors (LTNPs), and Elite Controllers (ECs). Subjects gave informed consent, and the research protocols were approved by the corresponding institutional review boards Research Ethics Committees: Núcleo de Medicina Tropical (# 275.456/2013), from Federal University of Pará Brazil; Ethics Committee of the Health Department of the Federal District (#066/07) from hospitals of the public network of the Federal District. Brazil; Ethics Committee for Analysis of Research Projects (CAPPesq) of Hospital das Clínicas HCFMUSP (#CAPPesq #0306/10, online registration #5867) Faculdade de Medicina da Universidade de Sao Paulo, Brazil.

3.1.1 HIV-1 positive patient cohort

A total of 32 patients was enrolled in this study following the subsequent admission criteria: HIV-1 positive subjects with different stages of disease progression (between A1 and C3 status of the CDC classification system), observing a cART regimen, aged 18-65 and with suppressed plasma viral levels. Exclusion criteria from the study include the patient's history of recurrent drug abuse, current drug addiction, the presence of neurocognitive or psychological disorders. All the enrolled subjects gave informed consent, and this study was approved by the Ethics Committee (2459CESC). Patients were recruited by Dr. Lanzafame, Dr. Lattuada, and Dr. Malena from the Operative unit of infectious diseases, AOUI Verona, and from the Operative unit of infectious diseases, ULSS9, Verona. The neurocognitive status of the patients was established by Dr. Federico and Dr. Mantovani, following a battery of neurocognitive tests (Antinori et al., 2007; Gisslén et al., 2011). The enrolled patients based on the test results were divided into two main categories (HIV-1 positive subjects without neurocognitive impairment and HAND patients).

3.2 DNA extraction from blood

The salting-out method was employed to extract DNA from blood samples (Longmire et al., 1988). Plasma was divided through centrifugation and stored at -80°C before proceeding with DNA extraction. After cell lysis, samples were digested overnight followed by protein dehydration and precipitation. DNA was extracted through precipitation using ethanol, resuspended with distilled H_2O, and quantified using NanoDrop (ThermoFisher Scientific).

3.3 Genotyping through ASPCR

The Allele Specific Polymerase Chain Reaction (ASPCR) is a peculiar type of PCR that allows the discrimination of very similar sequences that differ even for a single nucleotide. This specific amplification is enabled thanks to particular primer pairs and very strict PCR conditions. Primer pairs are designed to display a mismatch at the 3´ side which doesn't allow the amplification of sequences very similar to the desired template. This PCR approach has been used to perform both HLA-C and APOE genotyping since both these genes possess extremely similar alleles. In some cases, the Touchdown PCR (TD-PCR) procedure has been used to further increase the PCR specificity. All the ASPCR reactions were carried out using Life Touch Thermal Cycler, BIOER Technology, and using positive and negative controls (*i.e.*, samples with known HLA-C/APOE genotype used as positive/negative controls for each ASPCR reaction). Moreover, in all the ASPCR reactions an internal control gene was co-amplified along with the tested HLA-C/APOE allele to avoid false-negative results. For this purpose previously published primer pair for the amplification of Collagen Type 5 Alpha 1 Chain (COL5A1) (Greenspan & Pasquinelli, 1994) was used. The COL5A1 amplified fragment (667 base pair) can be easily distinguished from the HLA-C/APOE alleles PCR products and can be amplified in a wide range of annealing temperatures. In the case of HLA-C *05 and HLA-C *07:04 a specific primer pair to amplify Methylthioadenosine Phosphorylase (MTAP) (Sangalli et al., 2017) was used as an internal control gene to better discriminate HLA-C amplified products. PCR products were analyzed through 2% agarose gel. To visualize the amplified products the EuroSafe Nucleic Acid Stain Solution (Euroclone) was added to the gel. The samples were subjected to agarose gel electrophoresis with Loading dye 1X (5% glycerol, 0,04% bromophenol blue/xylene cyanol) along with a molecular weight marker (100 bp DNA Ladder, Promega). PCR products were visualized using UVIDoc H2D (Uvitec Cambridge).

3.3.1 HLA-C genotyping, sequencing, and bioinformatic analysis

To perform HLA-C genotyping an ASPCR approach has been employed exploiting previously published primer pairs (M. Bunce et al., 1995, 1996; Mike Bunce et al., 1994; Tonks et al., 1999). Primer pairs sequences are reported in Tables 3 and 4.

Table 3: Forward primer pairs used to perform HLA-C genotyping through ASPCR

Antigens	**Alleles**	**Forward primers**	**Sequence**
HLA-C*01	C*01:01-02	368	CACAGACTGACCGAGTGAG
HLA-C*02/*17	C*02:01-02; *17:01	366	CCGAGTGAACCTGCGGAAA
HLA-C*03	C*03:02-04	368	CACAGACTGACCGAGTGAG
HLA-C*04	C*04:01-03; *18:01	366	CCGAGTGAACCTGCGGAAA
HLA-C*05	C*05:01	366	CCGAGTGAACCTGCGGAAA
HLA-C*06	C*06:02	367	TACTACAACCAGAGCGAGGA
HLA-C*07	C*07:01-03	130	CCGCGGGTATGACCAGTC
HLA-C*07:04	C*07:04	367	TACTACAACCAGAGCGAGGA
HLA-C*08	C*08:01-03	165	ACGACACGCAGTTCGTGCA
HLA-C*12:01-02	C*12:01-02	368	CACAGACTGACCGAGTGAG
HLA-C*12:03	C*12:03	368	CACAGACTGACCGAGTGAG
HLA-C*14	C*14:02-03	371	CCACTCCATGAGGTATTTCTC
HLA-C*15	C*15:01-05	366	CCGAGTGAACCTGCGGAAA
HLA-C*16:01	C*16:01	368	CACAGACTGACCGAGTGAG
HLA-C*16:02	C*16:02	366	CCGAGTGAACCTGCGGAAA
HLA-C*17	C*17:01	366	CCGAGTGAACCTGCGGAAA
HLA-C*18	C*18:01; B*82:01	271	GGGAGCCCCGCTTCATCT

Table 4: Reverse primer pairs used to perform HLA-C genotyping through ASPCR

Antigens	Alleles	Reverse primers	Sequence
HLA-C*01	C*01:01-02	315	CCCCAGGTCGCAGCCAC
HLA-C*02/*17	C*02:01-02; *17:01	145	GAGCCACTCCACGCACTC
HLA-C*03	C*03:02-04	389	AGCGTCTCCTTCCCATTCTT
HLA-C*04	C*04:01-03; *18:01	143	GCCCCAGGTCGCAGCCAA
HLA-C*05	C*05:01	379	CGCGCGCTGCAGCGTCTT
HLA-C*06	C*06:02	127	GGTCGCAGCCATACATCCA
HLA-C*07	C*07:01-03	378	CAGCCCCTCGTGCTGCAT
HLA-C*07:04	C*07:04	379	CGCGCGCTGCAGCGTCTT
HLA-C*08	C*08:01-03	166	GCGCAGGTTCCGCAGGC
HLA-C*12:01-02	C*12:01-02	126	TGAGCCGCCGTGTCCGCA
HLA-C*12:03	C*12:03	157	CCGCCGTGTCCGCGGCA
HLA-C*14	C*14:02-03	388	GGTCGCAGCCAAACATCCA
HLA-C*15	C*15:01-05	223	GCCATACATCCTCTGGATGA
HLA-C*16:01	C*16:01	146	CCCTCCAGGTAGGCTCTCT
HLA-C*16:02	C*16:02	146	CCCTCCAGGTAGGCTCTCT
HLA-C*17	C*17:01	319	CTCACGGGCCGCCTCCA
HLA-C*18	C*18:01; B*82:01	143	GCCCCAGGTCGCAGCCAA

The ASPCR reaction components are reported in Table 5.

Table 5: Composition of ASPCR reactions performed to genotype HLA-C alleles

Reagents	Reaction 1X quantity
Wonder Taq Buffer 5X (Euroclone)	1X (1mM dNTPs, 1.5mM $MgCl_2$)
Primer Forward HLA-C	10-15 pmol
Primer Reverse HLA-C	10-15 pmol
Primer Forward COL5A1/MTAP	5-10 pmol
Primer Reverse COL5A1/MATAP	5-10 pmol
Wonder Taq polymerase (Euroclone)	1,25 U
DNA	20 ng
H_2O	Up to 25 µl

All the ASPCR Thermal profiles used to genotype HLA-C are reported in Tables 6-20.

Table 6: ASPCR thermal profile employed to genotype HLA-C*01; *02:01-02; *17:01

HLA-C*01 (F368; R315) and HLA-C*02:01-02; *17:01 (F366; R145)

Initial Denaturation	95°C	2’	
Denaturation	95°C	20’’	40 cycles
Annealing	65°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 7: ASPCR thermal profile employed to genotype HLA-C*03:02-04

HLA-C*03:02-04 (F368; R389)

Initial Denaturation	95°C	2’	
Denaturation	95°C	20’’	40 cycles
Annealing	55°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 8: ASPCR thermal profile employed to genotype HLA-C*04:01-03; *18:01

HLA-C*04:01-03 and HLA-C *18:01 (F366; R143)

Initial Denaturation	95°C	2’	
Denaturation	95°C	20’’	40 cycles
Annealing	59°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 9: ASPCR thermal profile employed to genotype HLA-C*05:01

HLA-C*05:01 (F366; R379)

Initial Denaturation	95°C	3cx’	
Denaturation	95°C	20’’	40 cycles
Annealing	59°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 10: ASPCR thermal profile employed to genotype HLA-C*06:02

HLA-C*06:02 (F367; R127)

Initial Denaturation	95°C	3’	
Denaturation	95°C	20’’	40 cycles
Annealing	55°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 11: ASPCR thermal profile employed to genotype HLA-C*07:01-03

HLA-C*07:01-03 (F130; R378)

Initial Denaturation	95°C	3’	
Denaturation	95°C	20’’	40 cycles
Annealing	61°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 12: ASPCR thermal profile employed to genotype HLA-C*07:04

HLA-C*07:04 (F367; R379)

Initial Denaturation	95°C	3’	
Denaturation	95°C	20’’	35 cycles
Annealing	58°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 13: ASPCR thermal profile employed to genotype HLA-C*08:01-03

HLA-C*08:01-03 (F165; R166)

Initial Denaturation	95°C	3’	
Denaturation	95°C	20’’	35 cycles
Annealing	62°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 14: ASPCR thermal profile employed to genotype HLA-C*14:02-03 and HLA-C*15:01-05

HLA-C*14:02-03 (F371; R388) and HLA-C*15:01-05 (F366; R223)

Initial Denaturation	95°C	3’	
Denaturation	95°C	20’’	35 cycles
Annealing	55°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 15: ASPCR thermal profile employed to genotype HLA-C*12:01-02

HLA-C*12:01-02 (F368; R126)

Initial Denaturation	95°C	2’	
Denaturation	95°C	20’’	40 cycles
Annealing	58°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 16: ASPCR thermal profile employed to genotype HLA-C*12:03

HLA-C*12:03 (F368; R157)

Initial Denaturation	94°C	2’	
Denaturation	94°C	20’’	40 cycles
Annealing	55°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 17: ASPCR thermal profile employed to genotype HLA-C*16:01

HLA-C*16:01 (F368; R146)

Initial Denaturation	94°C	3’	
Denaturation	94°C	20’’	16 cycles (-0,5°C each cycle)
Annealing	72°C	20’’	
Extension	72°C	20’’	
Denaturation	94°C	20’’	30 cycles
Annealing	57°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 18: ASPCR thermal profile employed to genotype HLA-C*16:01

HLA-C*16:02 (F366; R146)

Initial Denaturation	94°C	3’	
Denaturation	94°C	20’’	16 cycles (-0,5°C each cycle)
Annealing	74°C	20’’	
Extension	72°C	20’’	
Denaturation	94°C	20’’	35 cycles
Annealing	64°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Table 19: ASPCR thermal profile employed to genotype HLA-C*17:01

HLA-C*17:01 (F366; R319)

Initial Denaturation	95°C	1’	
Denaturation	95°C	15’’	35 cycles
Annealing	57°C	15’’	
Extension	72°C	15’’	
Final Extension	72°C	2’	

Table 20: ASPCR thermal profile employed to genotype HLA-C*18:01

HLA-C*18:01 (F271; R143)

Initial Denaturation	94°C	2’	
Denaturation	94°C	20’’	16 cycles (-0,5°C each cycle)
Annealing	75°C	20’’	
Extension	72°C	20’’	
Denaturation	94°C	20’’	25 cycles
Annealing	62°C	20’’	
Extension	72°C	20’’	
Final Extension	72°C	2’	

Sanger sequencing was applied when the HLA-C genotype was not determined by ASPCR. To amplify HLA-C exon 2 and exon 3, which are among the HLA-C most variable regions, a previously published primer pair was used (Lazaro et al., 2013). Primer pair sequences used to amplify and sequence HLA-C exon 2 and 3 are reported in Table 21.

Table 21: Primers used to amplify (5CIn1-61 and 3BCIn3-12) and sequence (Cex2F) HLA-C

Primer	**Sequence**
5CIn1-61	AGCGAGGKGCCCGCCCGGCGA
3BCIn3-12	GGAGATGGGGAAGGCTCCCCACT
CEx2F	GGGTCGGGCGGGTCTCAGCC

The reaction components are reported in Table 22.

Table 22: Composition of PCR reactions performed to amplify HLA-C exon 2 and 3

Reagents	**Reaction 1X quantity**
Wonder Taq Buffer 5X (Euroclone)	1X (1mM dNTPs, 1.5mM $MgCl_2$)
5CIn1-61	5 pmol
3BCIn3-12	5 pmol
Wonder Taq polymerase (Euroclone)	1,25 U
DNA	20 ng
H_2O	Up to 25 µl

The PCR thermal profile is reported in Table 23.

Table 23: PCR thermal profile employed to amplify a region covering HLA-C exon 2 and 3

HLA-C exon 2 and 3 (5CIn1-61; 3BCIn3-12)

Initial Denaturation	98°C	3’	
Denaturation	98°C	10’’	40 cycles
Annealing	68°C	15’’	
Extension	72°C	1’	
Final Extension	72°C	2’	

All the PCR reaction products were visualized through 2% agarose gel electrophoresis as previously described. To purify and concentrate the PCR products Micorcon 30K (Millipore) was employed following the manufacturer’s procedure. The purified samples were visualized and quantified through 2% agarose gel electrophoresis using Mass Ruler DNA Ladder Mix (ThermoFisher Scientific). Samples were then prepared for Sanger sequencing adding 36 ng of PCR product, 6,4 pmol of CEx2F, or 3BCIn3-12 primer and submitted to evaporation at 65°C for approximately 30 minutes. Sequencing was performed by BMR Genomics (https://www.bmr-genomics.it/) company.

The electropherograms were analyzed and the sequences were compared with the HLA-C alleles aligned sequences from the IPD database (https://www.ebi.ac.uk/ipd/imgt/hla/). A bioinformatic approach was exploited to identify the putative individuals' HLA-C genotype.

3.3.2 APOE genotyping

To perform APOE genotyping an ASPCR approach was employed using a Touch-down PCR (TD-PCR) procedure. Previously published primer pairs mapping two different SNPs (rs429358 and rs7412) were employed to discriminate the three different APOE haplotypes (Zhong et al., 2016). Primer sequences are reported in Table 24.

Table 24: Primer sequences employed to perform APOE genotyping

Primer	**Sequence**
E2-E3 Forward	CGGACATGGAGGACGTGT
E2 Reverse	CTGGTACACTGCCAGGCA
E4 Forward	CGGACATGGAGGACGTGC
E3-E4 Reverse	CTGGTACACTGCCAGGCG

Even in this case, an internal control gene (COL5A1) was co-amplified to avoid false-negative results as previously described. The reaction components are reported in Table 25.

Table 25: ASPCR reaction components to genotype APOE

Reagents	**Reaction 1X quantity**
Wonder Taq Buffer 5X (Euroclone)	1X (1mM dNTPs, 1.5mM $MgCl_2$)
Primer Forward APOE	15 pmol
Primer Reverse APOE	15 pmol
Primer Forward COL5A1	10 pmol
Primer Reverse COL5A1	10 pmol
Wonder Taq polymerase (Euroclone)	1,25 U
DNA	20 ng
H_2O	Up to 25 µl

The TD-PCR thermal profiles are reported in Tables 26 and 27.

Table 26: ASPCR thermal profile employed to genotype APOE2

APOE2 (E2-E3 Forward; E2 Reverse)

Initial Denaturation	95°C	2’	
Denaturation	95°C	15’’	10 cycles (-0,5°C each cycle)
Annealing	72°C	10’’	
Extension	72°C	10’’	
Denaturation	95°C	15’’	35 cycles
Annealing	55°C	10’’	
Extension	72°C	10’’	

Table 27: ASPCR thermal profile employed to genotype APOE3 and APOE4

APOE3 (E2-E3 Forward; E3-E4 Reverse) and APOE4 (E4 Forward; E3-E4 Reverse)

Initial Denaturation	95°C	2’	
Denaturation	95°C	15’’	10 cycles (-0,5°C each cycle)
Annealing	72°C	10’’	
Extension	72°C	10’’	
Denaturation	95°C	15’’	35 cycles
Annealing	65°C	10’’	
Extension	72°C	10’’	

The PCR products were visualized using a 2% agarose gel electrophoresis as previously described.

3.4 Statistical analysis

The Mann-Whitney T-test, Chi-Square test, and the descriptive statistics were performed using GraphPad Prism version 7.03 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3.5 B2microglobulin and Neurofilament light chain plasma quantification

The quantification of the β2m plasma levels was conducted through nephelometry using (N Latex β2–Microglobulina; Siemens) following the manufacturer’s procedure. Samples were diluted and then subjected to the quantification process. The methodology is based on the formation of aggregates between β2m contained in the plasma samples and polystyrene particles coated with anti-β2-microglobulin antibodies. These aggregates cause the dispersion of the incident light which is related to β2m plasma levels. The NFL plasma levels were quantified using the digital immunoassay SIMOA (Nf-light kit, SR-X immunoassay analyzer, Quanterix Corporation) following the manufacturer’s procedure.

3.6 Cell lines

Cell lines (Hek293T, Hek293T ACOT8 KO, TZM-bl, and TZM-bl ACOT8 KO) were maintained in Dulbecco's Modified Eagle Medium (DMEM) complemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, and 1% Penicillin/Streptomycin. All the cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2. For pseudotyped virus production co-transfecting, two vectors $3X10^5$ Hek293T and Hek293T ACOT8 KO cells were seeded in 6-well plates; for pseudotyped viruses production co-transfecting, three vectors $2X10^5$ Hek293T and Hek293T ACOT8 KO cells were seeded in 6-well plates. For the infection test, $1X10^4$ TZM-bl and TZM-bl ACOT8 KO cells were seeded in each well of 96-well plates.

3.7 Knock-out cell lines produced through CRISPR/Cas9 system

Three gRNAs were selected to target ACOT8 first exon (5´-CGGTCGTGACCAAGACGCTACGG-3´; 5´-GAACTAGATGTCGTCCCCGCAGG-3´ and 5´-GCGACCGCGGCGATCCCCCTGGG-3´). Each guide RNA was cloned independently into pSpCas9(BB)-2A-Puro (PX459) V2.0 vector (#62988, Addgene) using BbSI restriction site as previously described by (Ran et al., 2013). The transfection of the three vectors containing the three different gRNAs was executed using $6X10^5$ Hek293T and TZM-bl seeded in a 60 mm dish using Trans-IT®-LT1 transfection reagent (MIR2300, Mirus Bio). To select the transfected cells, seven hours after the transfection 0.5 µg/ml of Puromycin was added to the culture medium. After 5 days of selection, cells were subjected to clonal selection through limiting dilution to obtain clonal cell lines. ACOT8 KO was verified by Western Blot. Total proteins were extracted from the selected clones using RIPA buffer and quantified through Bradford assay. The extracted proteins were resolved using a 10% polyacrylamide gel through SDS-PAGE electrophoresis and transferred to a PVDF membrane (ThermoFisher Scientific). The membrane was saturated using a solution composed of 5% non-fat milk dissolved into TBS 0,1% Tween20 and then incubated with an anti-ACOT8 primary antibody (Santa Cruz) diluted 1:100 in milk 5% TBS-Tween 0,05% and with an anti- α/β tubulin (Cell Signaling) diluted 1:2000 in milk 5% TBS-Tween 0,05% as control. After the incubation with secondary antibodies, the signal was detected using an ECL detection reagent following the manufacturer's procedure.

3.8 Pseudotyped virus preparation and p24 quantification

Two different methods were applied to produce pseudotyped viruses: the first one implies a co-transfection using two vectors (backbone and envelope) whereas the second one implies a co-transfection using three vectors (backbone, envelope, and packaging) as reported in Figure 5. In the first method, the transfection was performed using Trans-IT®-LT1 transfection reagent (MIR2300, Mirus Bio) following the manufacturer's protocol, whereas in the second method the transfection was performed using Calcium phosphate. The p24 quantification was executed through One-Wash™ Lentivirus Titer Kit, p24 ELISA (TR30038, Origene) following the manufacturer's protocol. This step is needed to standardize the infection assay using the same quantity of pseudotyped viruses.

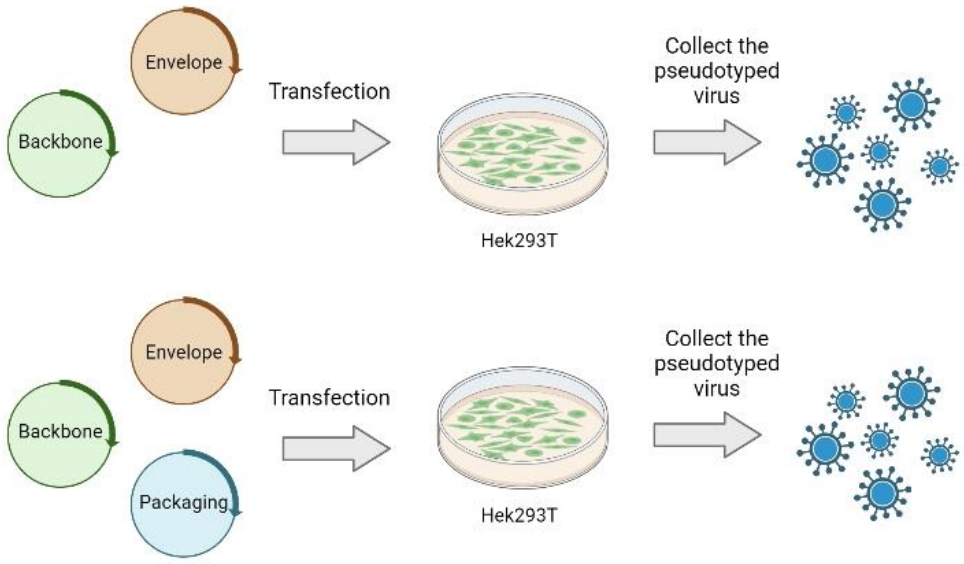

Figure 5: Distinctive steps of pseudotyped virus production. This figure was created with BioRender.com.

3.8.1 Pseudotyped virus preparation co-transfecting two vectors

To perform pseudotyped virus preparation seed $3X10^5$ Hek293T/Hek293T ACOT8 KO and after 24 hours perform the transfection using two vectors (backbone and envelope) in ratio 2:1. In this case, $PSG3^{\Delta Env}$ backbone vector and QHO/VSV-G envelope plasmids were used. The transfection was performed using TransIT-LT1 transfection reagent (MIR2300, Mirus Bio) following the manufacturer's procedure. After 48 hours form transfection collects the supernatants, filter and freeze the aliquots at -80°C. Perform the p24 quantification using One-Wash™ Lentivirus Titer Kit, p24 ELISA diluting all the supernatants 1:1000 using DMEM.

3.8.2 Pseudotyped virus preparation co-transfecting three vectors

To produce pseudotyped virus seed $2X10^5$ Hek293T/Hek293T ACOT8 KO, after 24 hours perform the transfection using three vectors (backbone, envelope, and packaging). In this case, p.8.91 backbone vector, pCSFLW packaging vector, and QHO/VSV-G envelope vectors were employed. The transfection was performed using Calcium phosphate (Graham & van der Eb, 1973). After 48 hours collect, filter, and freeze at -80°C the supernatant. Perform the p24 quantification using One-Wash™ Lentivirus Titer Kit, p24 ELISA diluting all the supernatants 1:1000 using DMEM.

3.9 Infectivity-Luciferase assay and statistical analysis

To perform the infectivity-luciferase assay use 300 pg of quantified supernatants (for the protocol with the two vectors system) and 625 pg (for the protocol with the three vectors system) and then execute 1:5 dilutions. Prepare the TZM-bl/TZM-bl ACOT8 KO cell suspension ($1x10^4$ cells/100 µl of DMEM for each well of 96-well plates) and add 40 µg/ml of DEAE-Dextran. Incubate for 48h and read the luciferase signal using Britelite™ Plus (PerkinElmer) reagent following the manufacturer's procedure (Figure 6). The Mann-Whitney T-test was performed on the highest pseudotyped virus concentration *i.e.* 300pg or 625 pg using GraphPad Prism version 7.03 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

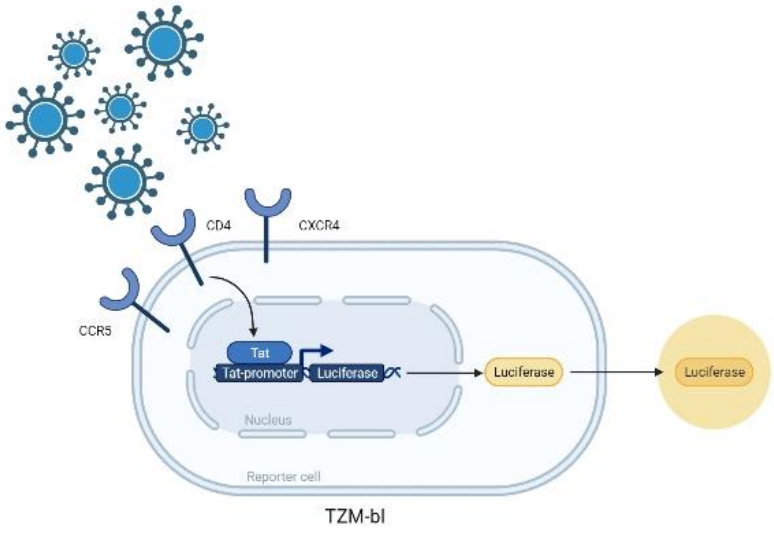

Figure 6: Distinctive steps of the infection assay. This figure was created with BioRender.com.

4. RESULTS

4.1 Association between HLA-C alleles and AIDS progression

This study aims to determine if HLA-C unstable alleles are correlated with a more rapid progression to AIDS stage. For this purpose, HLA-C genotyping of 96 HIV-1 infected subjects was performed. These patients were divided, based on their HIV-1 progression status, into three categories (as previously defined, section 3.1): Progressors (Ps), Long Term Non-Progressors (LTNPs), and Elite Controllers (ECs).

4.1.1 Description of the patient cohort

The 96 patients enrolled in this study were divided into the previously described three categories:

-48 Progressors (Ps)

-37 Long Term Non-Progressors (LTNPs)

-11 Elite Controllers (ECs)

The characteristics of the studied population and the analysis of the parameters which lead to the subject's classification (such as the evaluation of the CD4+ T lymphocytes, the viral load levels, and the estimated follow-up) are listed in Table 28.

Table 28: Description of HIV-1 infected patients characteristics (Ps indicates Progressors, LTNPs indicates Long Term Non-Progressors, and ECs indicates Elite Controllers). The age is reported as median and Interquartile distance (IQR); male, ethnicity, and origin are reported as numbers and percentage. The CD4+ T lymphocytes, Viral load and follow up are reported as median and interquartile distance (IQR). Not all the data were available for the recruited subjects.

		Ps (n=48)	**LTNPs (n=37)**	**ECs (n=11)**
Age, median (IQR), years		40.5 (30-52.5)	43 (33.5-52.5)	33 (27-41)
Male, n (%)		37 (77)	27 (72.9)	7 (63.6)
Ethnicity, n (%)	Caucasian	44 (91.6)	31 (83.8)	7 (63.6)
	Black	4 (8.4)	6 (16.2)	4 (36.4)
Origin, n (%)	Brazil	28 (58.4)	19 (51.4)	-
	Canada	14 (37.8)	14 (37.8)	11 (100)
	USA	6 (12.5)	4 (10.8)	-

	Ps (n=44)	LTNPs (n=37)	ECs (n=11)
CD4, median (IQR), lymphocytes/mm³	324 (219.5-431)	500 (451-627)	500 (400-500)
	Ps (n=39)	**LTNPs (n=29)**	**ECs (n=11)**
Plasma HIV RNA level, median (IQR), copies/mm³	17698 (3688-100000)	4119 (2691- 20000)	50 (50-50)
	Ps (n=48)	**LTNPs (n=35)**	**ECs (n=10)**
Follow up, median (IQR), years	6 (4-8.9)	9.6 (6-13)	7.14 (4.4-12.5)

The analysis of the studied group median age applying the Mann-Whitney test resulted in a statistically significant difference between LTNPs and ECs (p-value = 0.0143) as reported in Figure 7. Applying the Chi-square statistical analysis there aren't statistically significant differences between the distribution of male and female and the ethnicity in the three categories. On the contrary, there is a statistically significant difference regarding the sample origin (p-value = 0.0009).

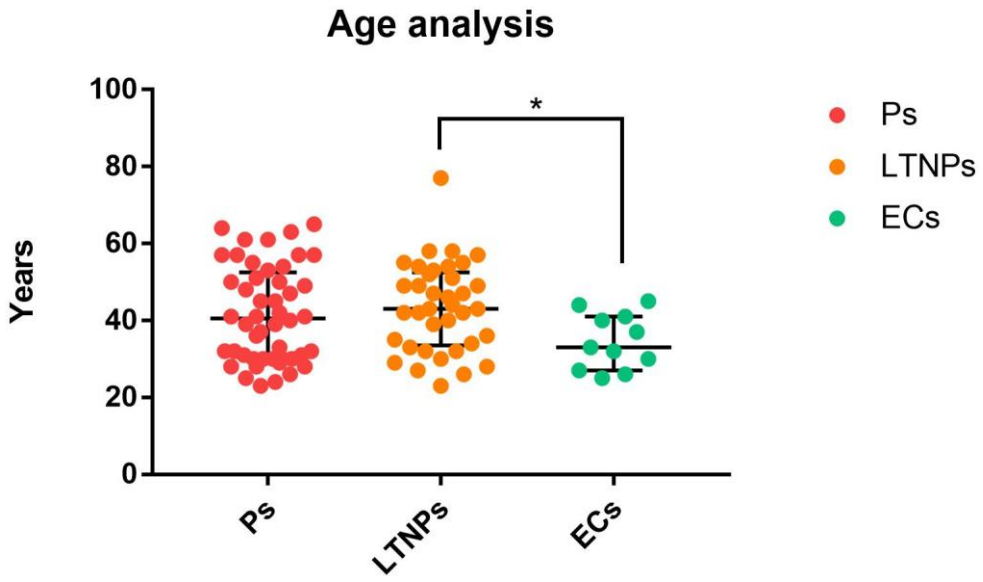

Figure 7: Age analysis of HIV-1 infected patients (Ps indicates Progressors, LTNPs indicates Long Term Non-Progressors, and ECs indicates Elite Controllers). The p-value is indicated as follows: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001; **** = p-value ≤ 0.0001.

The Mann-Whitney test was applied to examine the differences between CD4+ T lymphocytes levels, viral load levels, and follow-up time between the three categories. The analysis on the CD4+

T lymphocytes levels underlined a statistically significant difference between LTNPs and Ps (p-value < 0.0001) and between ECs and Ps (p-value = 0.0017) as represented in Figure 8.

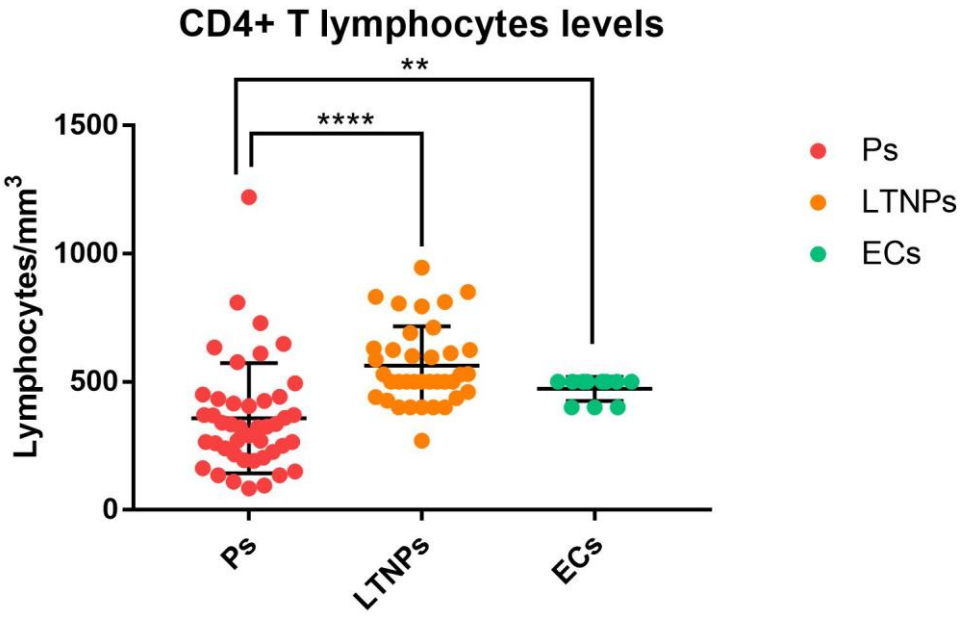

Figure 8: CD4+ T lymphocytes levels analysis of HIV-1 infected patients (Ps indicates Progressors, LTNPs indicates Long Term Non-progressors and ECs indicates Elite Controllers). The p-value is indicated as follows: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001; **** = p-value ≤ 0.0001.

Regarding the viral load, statistically, significant differences have been found between ECs and Ps (p-value < 0.0001) and between LTNPs and ECs (p-value < 0.0001) as depicted in Figure 9.

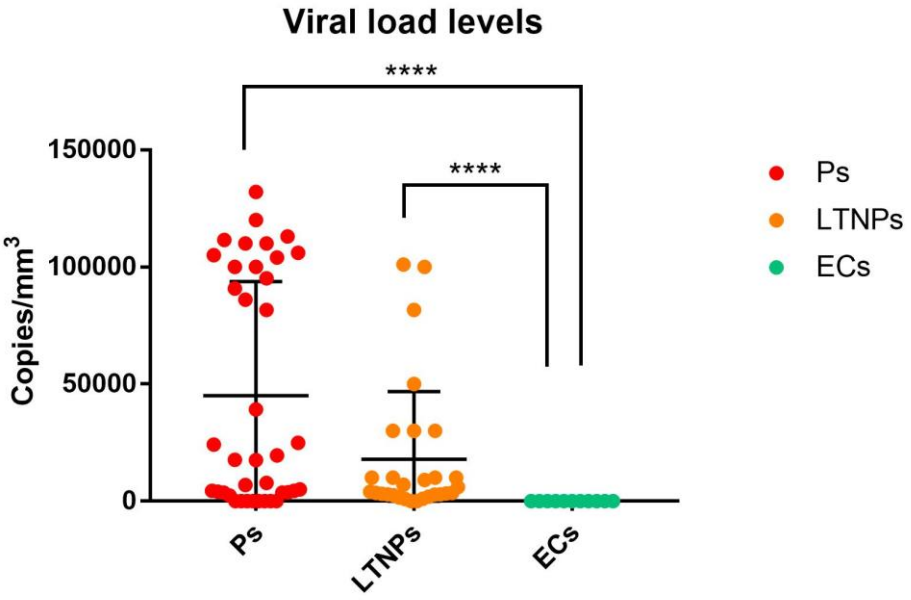

Figure 9: Viral load levels analysis of HIV-1 infected patients (Ps indicates Progressors, LTNPs indicates Long Term Non-progressors and ECs indicates Elite Controllers). The p-value is indicated as follows: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001; **** = p-value ≤ 0.0001.

Concerning the follow-up time, a statistically significant difference has been found between LTNPs and Ps (p-value = 0.0235) as represented in Figure 10.

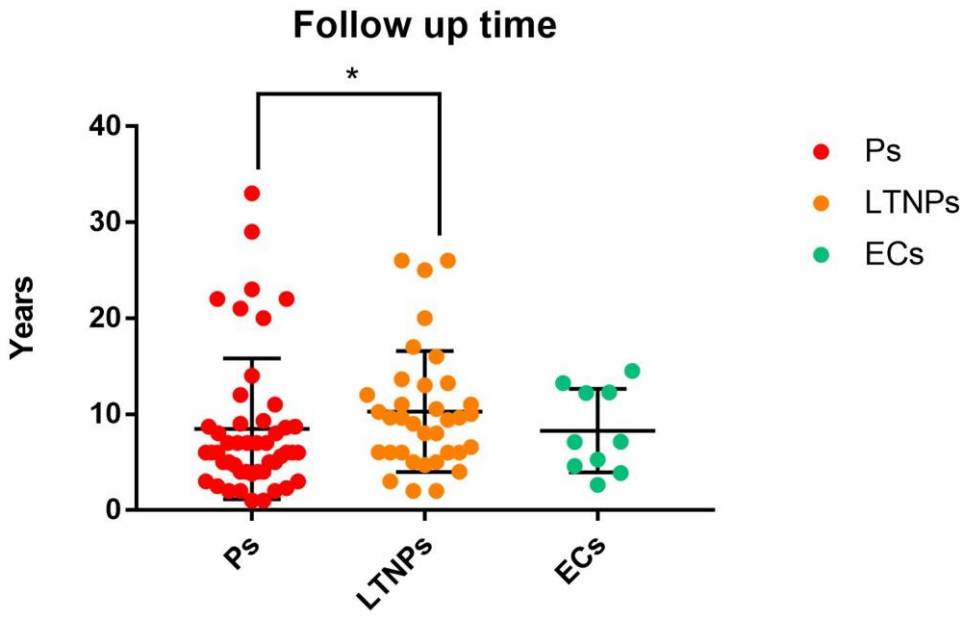

Figure 10: Follow up time analysis of HIV-1 infected patients (Ps indicates progressors, LTNPs indicates Long Term Non-Progressors, and ECs indicates Elite Controllers). The p-value is indicated as follows: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001; **** = p-value ≤ 0.0001.

All the 96 subjects enrolled in this study were not taking antiretroviral therapies at the time of sampling. The HIV-1 clade more represented in this cohort of samples is clade B.

4.1.2 HLA-C genotyping and sequencing analysis

To genotype HLA-C, 17 reactions of ASPCR have been performed for each sample. An example of the ASPCR obtained results is represented in Figure 11 in which is reported HLA-C*06 genotyping of ten samples, two of them possess the HLA-C*06 allele (sample 76 and 85).

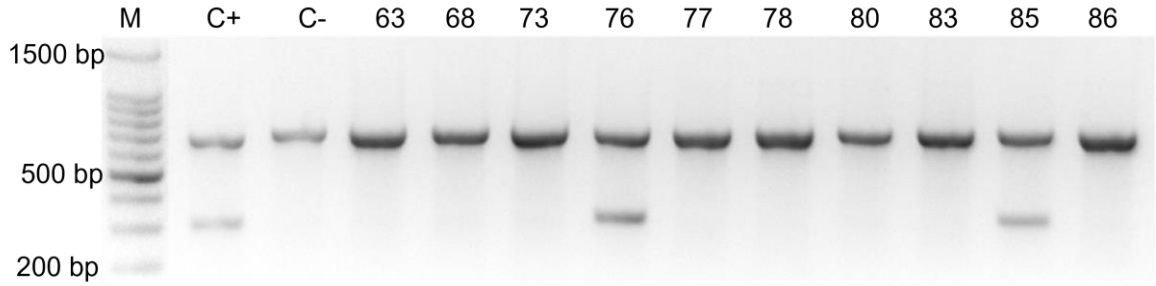

Figure 11: HLA-C*06 genotyping through ASPCR. M indicates Molecular weight marker (100 bp DNA Ladder Promega); C+ and C- indicate positive and negative controls. PCR bands represent HLA-C*06 (297 bp) and internal control gene COL5A1 (667 bp).

In some cases, the HLA-C genotype couldn't be determined through the ASPCR approach (some samples tested positive for more than two alleles or only 1 allele), in these cases samples were prepared and sent to BMR genomics to perform Sanger sequencing on exon 2 and 3. An example of an obtained electropherogram is represented in Figure 12 in which are represented two variation points (a SNP in the left box and an in/del the right box). The in/del "TGGAT" in this position is a peculiarity of HLA-C*07 alleles in which is present and the electropherogram varies its phase if the subject has a heterozygous genotype.

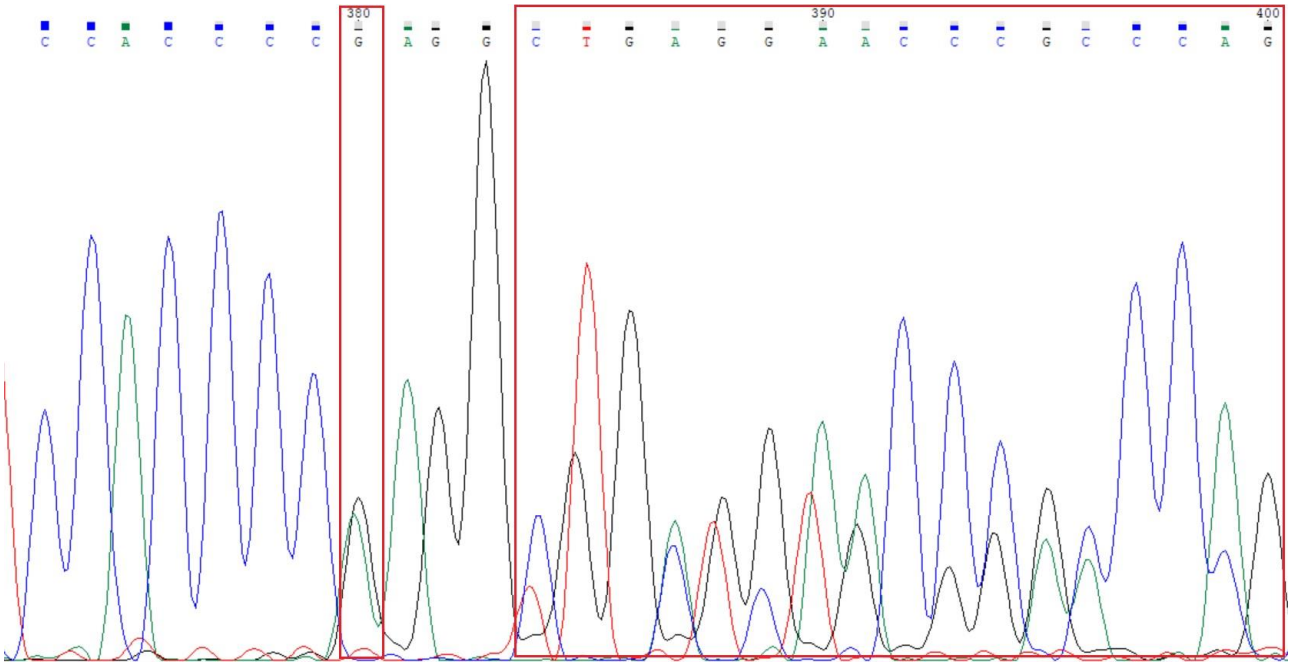

Figure 12: HLA-C electropherogram: SNP on the left red box, in/del on the right red box

After the annotation of all the variation points present on the electropherograms, the putative individuals' genotype was examined with a bioinformatic approach based on sequence alignments

using the entire database of all known HLA-C alleles. The HLA-C alleles frequencies results are indicated in Table 29.

Table 29: HLA-C alleles frequencies results reported as number of alleles found and percentage of alleles of the total in each category. Ps indicates progressors, LTNPs indicates Long Term Non-Progressors, and ECs indicates Elite Controllers

	Ps alleles (n=96)	**LTNPs alleles (n=74)**	**ECs alleles (n=22)**
HLA-C*01, n (%)	1 (1)	4 (5.4)	1 (4.5)
HLA-C*02, n (%)	4 (4.2)	5 (6.75)	3 (13.6)
HLA-C*03, n (%)	10 (10.4)	5 (6.75)	1 (4.5)
HLA-C*04, n (%)	21 (21.8)	10 (13.5)	2 (9)
HLA-C*05, n (%)	4 (4.2)	9 (12.2)	1 (4.5)
HLA-C*06, n (%)	8 (8.3)	9 (12.2)	0 (0)
HLA-C*07, n (%)	29 (30.2)	11 (14.9)	6 (27.3)
HLA-C*08, n (%)	3 (3.1)	7 (9.4)	3 (13.6)
HLA-C*12, n (%)	6 (6.25)	9 (12.2)	2 (9)
HLA-C*14, n (%)	0 (0)	2 (2.7)	0 (0)
HLA-C*15, n (%)	4 (4.2)	1 (1.35)	0 (0)
HLA-C*16, n (%)	4 (4.42)	2 (2.7)	1 (4.5)
HLA-C*17, n (%)	1 (1)	0 (0)	1 (4.5)
HLA-C*18, n (%)	1 (1)	0 (0)	1 (4.5)

4.1.3 HLA-C unstable alleles are associated with AIDS progression

To assess the correlation between HLA-C stability and a more rapid AIDS progression, HLA-C genotyping was performed on the 96 enrolled subjects. The result of the Chi-square test indicates that there is a statistically significant correlation between HLA-C unstable alleles and a more rapid AIDS progression (p-value = 0.0143) as represented in Figure 13.

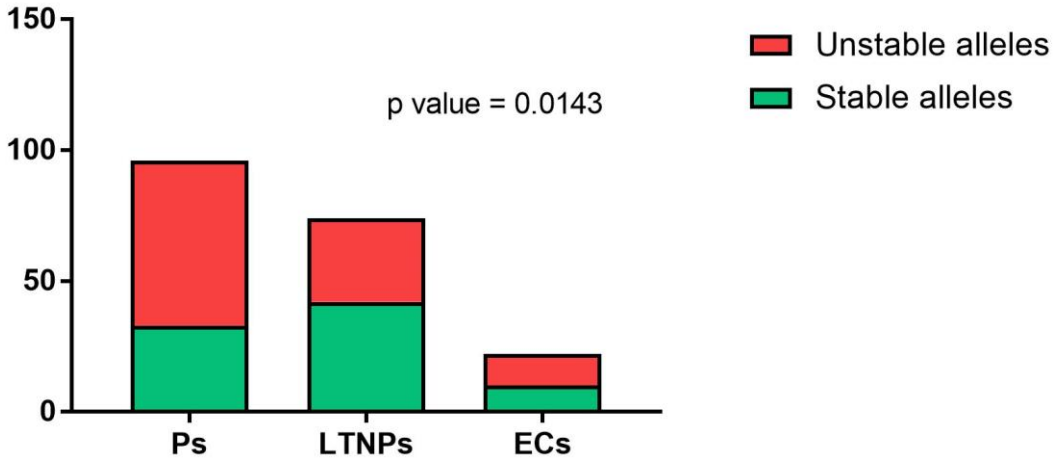

Figure 13: Representation of HLA-C stable and unstable alleles for each category of AIDS patients (Ps indicates Progressors, LTNPs indicates Long Term Non-progressors and ECs indicates Elite Controllers)

4.2 Host factors influence on HIV-associated neurocognitive disorders

The study aims to find clinical biomarkers and genetic factors linked to the development of HIV-1 associated neurocognitive disorders. A cohort of 32 patients was enrolled and submitted to a battery of neurocognitive tests. Based on the test results, patients were divided into two main categories: patients with HIV-associated neurocognitive disorders (HANDs) which include ANI (Asymptomatic Neurocognitive Impairment), MND (Mild Neurocognitive Disorder), and HAD (HIV-Associated Dementia), and a control group constituted by HIV-1 positive subjects without neurocognitive impairment. The genetic factors and clinical biomarkers evaluated in this study include HLA-C and APOE genotype and β2microglobulin (β2m) and Neurofilament Light Chain (NFL) plasma levels.

4.2.1 Description of the patient cohort

Patients enrolled in the study were divided into two groups:

- 23 HIV-1 Controls: HIV-1 positive patients without neurological impairments

-9 HAND patients: HIV-1 positive patients with neurological impairments (5 ANI and 4 HAD patients)

The characteristics of the studied patient's cohort are listed in Table 30.

Table 30: Characteristics of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive Disorders). The age is reported as median and Interquartile distance (IQR); the number of males is reported as number and percentage. Not all the data were available for the recruited subjects.

	HIV-1 Controls (n=22)	**HAND patients (n=9)**
Age, median (IQR), years	56 (50-60)	53 (49-59.5)
Male, n (%)	16 (72.7)	6 (66.7)

Applying the Mann-Whitney statistical analysis and the Chi-square test there are not statistically significant differences in the age and male/female distributions between the two studied groups. The age distribution analysis is reported in Figure 14.

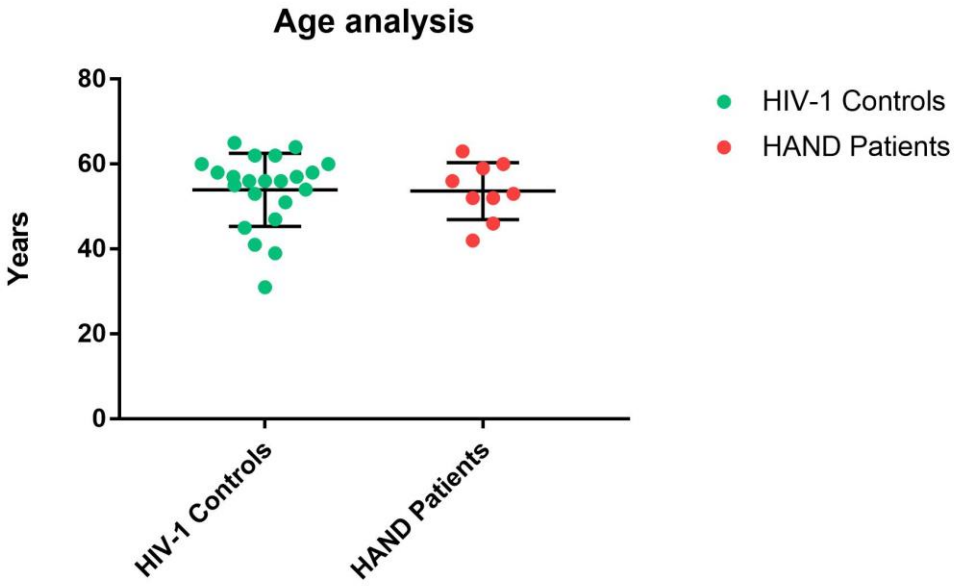

Figure 14: Age analysis of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders). The p-value obtained is 0.676.

The immunological and virological parameters are reported in Table 31.

Table 31: Immuno-virological characteristics of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive Disorders). The CD4+ T lymphocytes, viral load, and duration of HIV infection are reported as median and interquartile distance (IQR); co-infections are reported as number and percentage. Not all the data were available for the recruited subjects.

	HIV-1 Controls (n=12)	**HAND patients (n=6)**
CD4, median (IQR), lymphocytes/mm^3	603 (268-975.3)	77 (57.25-141.5)
	HIV-1 Controls (n=9)	**HAND patients (n=4)**
Plasma HIV RNA level, median (IQR), copies/mm^3	51 (10-166015)	199200 (152184-314850)
	HIV-1 Controls (n=17)	**HAND patients (n=8)**
HIV-1 infection, median (IQR), years	23 (16.5-31)	20,5 (5.25-29.75)
	HIV-1 Controls (n=17)	**HAND patients (n=8)**
Co-infections (HBV/HCV), n (%)	7 (41.2)	3 (37.5)

The Mann-Whitney test was applied to detect statistically significant differences in CD4+ T lymphocytes, Viral load, and duration of HIV-1 infection between the two studied groups. The analysis of the CD4+ T lymphocytes detected a statistically significant difference between HIV-1 controls and HAND patients (p-value = 0.0320) depicted in Figure 15. Moreover, applying the chi-square statistical test, the is not a statistically significant difference in the levels of co-infections (HCV or HBV) between the two studied groups. The recruited patients were all treated with antiretroviral therapy at the time of sampling.

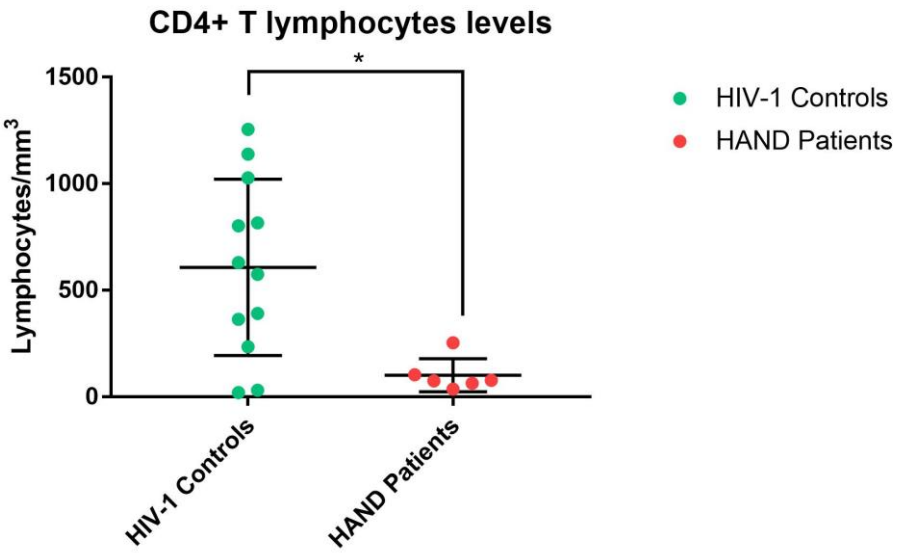

Figure 15: CD4+ T lymphocytes levels of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders). The p-value is indicated as follows: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001; **** = p-value ≤ 0.0001.

Statistically significant differences are not detected concerning viral load levels between the two studied groups (Figure 16).

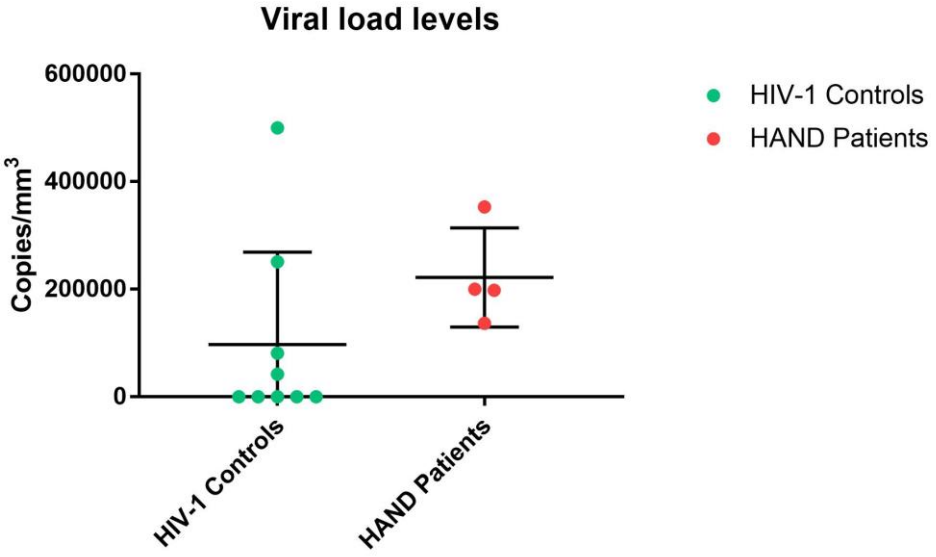

Figure 16: Viral load levels of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders). The p-value obtained is 0.100.

Statistically significant differences are not detected concerning the duration of HIV infection between the two studied groups (Figure 17).

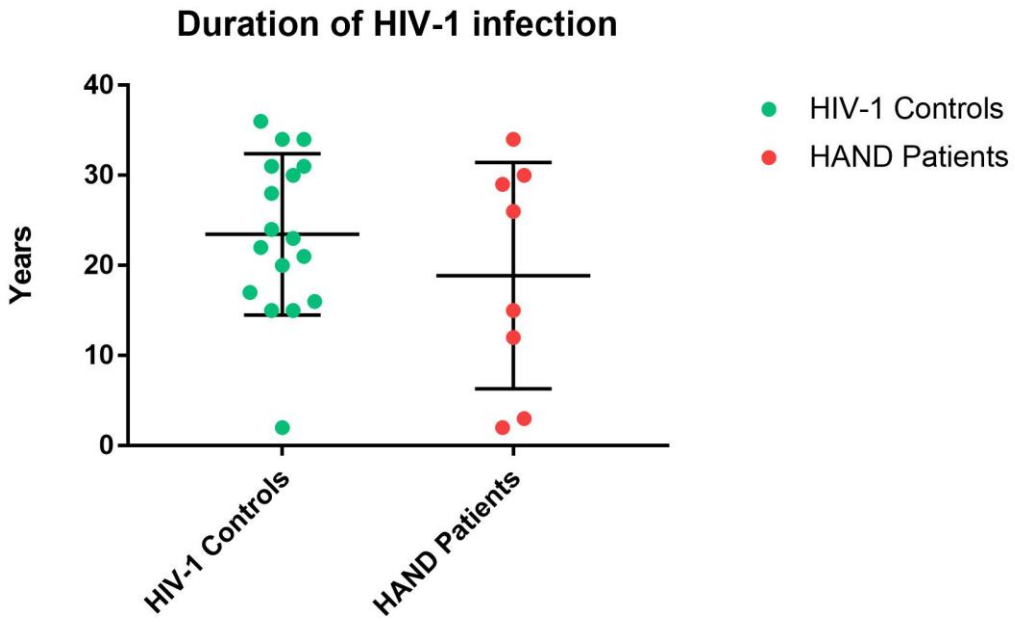

Figure 17: Duration of HIV-1 infection of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders). The p-value obtained is 0.365.

All 32 analyzed patients were subjected to cART therapy. In particular, the therapy of 25 patients includes at least one reverse transcriptase inhibitor (NRTIs and/or NNRTIs) and an integrase inhibitor or a protease inhibitor. Six patients have HIV-1 RNA levels below 50 copies/mm^3.

4.2.2 HLA-C genotyping and sequencing analysis

HLA-C genotype has been determined through 17 ASPCR reactions. An example of ASPCR results is reported in Figure 18 in which is depicted the results of the ASPCR performed on six samples for HLA-C*07:01/02/03 genotyping. Five out of six samples present the HLA-C*07:01/02/03 allele.

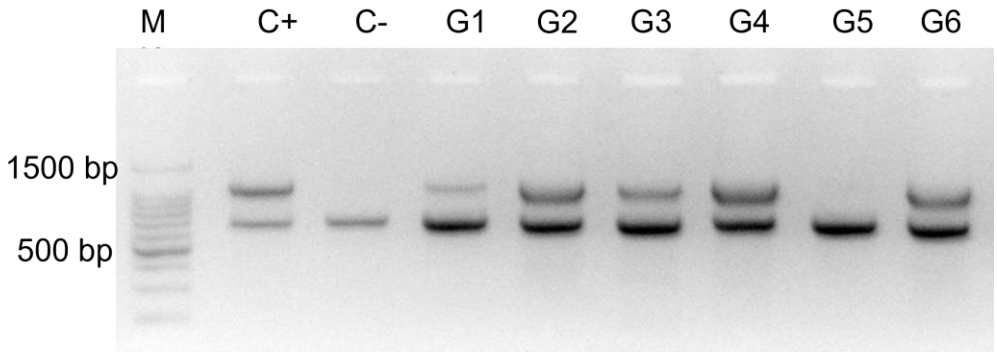

Figure 18: HLA-C*07:01/02/03 genotyping through ASPCR. M indicates Molecular weight marker (100 bp DNA Ladder Promega); C+ and C- indicate positive and negative controls. PCR bands represent HLA-C*07:01/02/03 (1062 bp) and internal control gene COL5A1 (667 bp).

As previously reported, when the HLA-C genotype couldn't be determined through ASPCR, Sanger sequencing has been performed to analyze HLA-C exons 2 and 3. In the example reported in Figure 19, there is a variation point represented by an in/del of a single nucleotide. The heterozygous genotype is marked by the electropherogram phase variation.

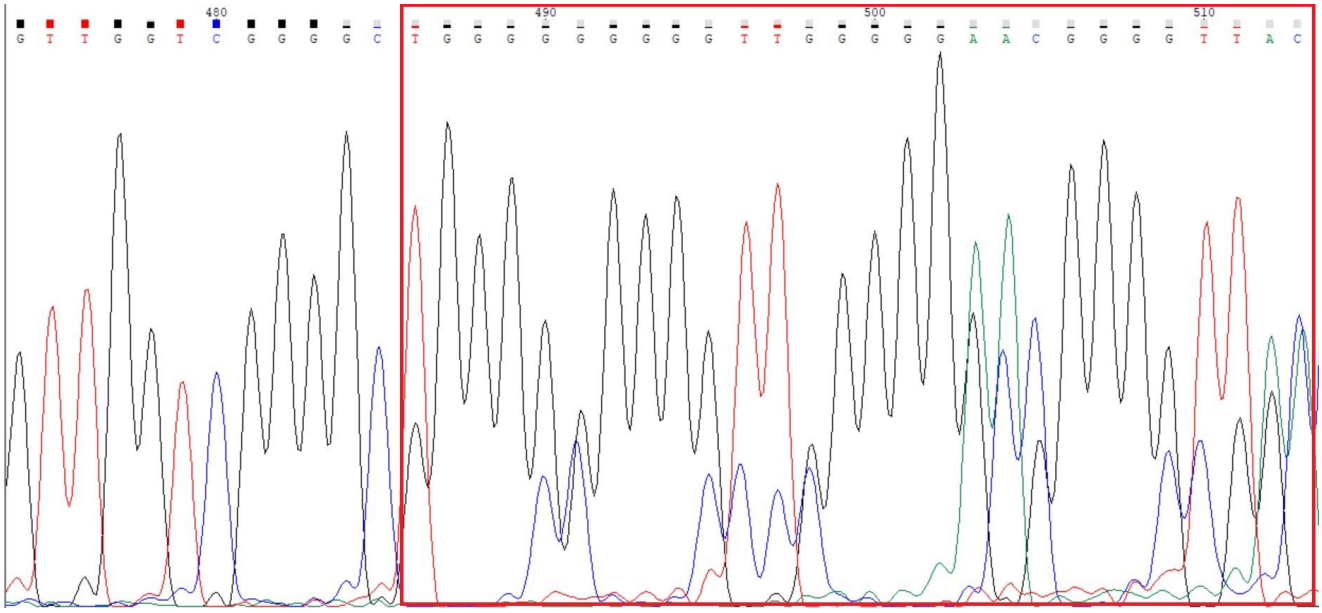

Figure 19: Representation of an obtained electropherogram, the in/del of a single nucleotide is marked by the red box

After the annotation of all the variation points present on the electropherograms, the putative individuals' genotype was examined with a bioinformatic approach based on sequence alignments

using the entire database of all known HLA-C alleles. The HLA-C alleles frequencies results are indicated in Table 32.

Table 32: HLA-C allele frequencies results reported as number of alleles found and percentage of alleles of the total in each category. HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders

	HIV-1 Controls alleles (n=46)	**HAND Patients alleles (n=18)**
HLA-C*01, n (%)	0 (0)	2 (11.1)
HLA-C*02, n (%)	3 (6.4)	1 (5.6)
HLA-C*03, n (%)	2 (4.3)	2 (11.1)
HLA-C*04, n (%)	7 (15.4)	2 (11.1)
HLA-C*05, n (%)	2 (4.3)	1 (5.6)
HLA-C*06, n (%)	2 (4.3)	2 (11.1)
HLA-C*07, n (%)	16 (35)	6 (33.2)
HLA-C*08, n (%)	1 (2.2)	0 (0)
HLA-C*12, n (%)	8 (17.4)	1 (5.6)
HLA-C*14, n (%)	1 (2.2)	0 (0)
HLA-C*15, n (%)	4 (8.7)	0 (0)
HLA-C*16, n (%)	0 (0)	1 (5.6)
HLA-C*17, n (%)	0 (0)	0 (0)
HLA-C*18, n (%)	0 (0)	0 (0)

4.2.3 HLA-C association with HIV-1 neurocognitive disorders development

To assess the correlation between HLA-C stability and HIV-1 associated neurocognitive disorders development, HLA-C genotyping was performed on the 32 enrolled subjects. The result of the Chi-square test indicates that there is not a statistically significant association between HLA-C unstable alleles and HANDs development as represented in Figure 20. Even if HLA-C unstable alleles are more represented in HAND patients.

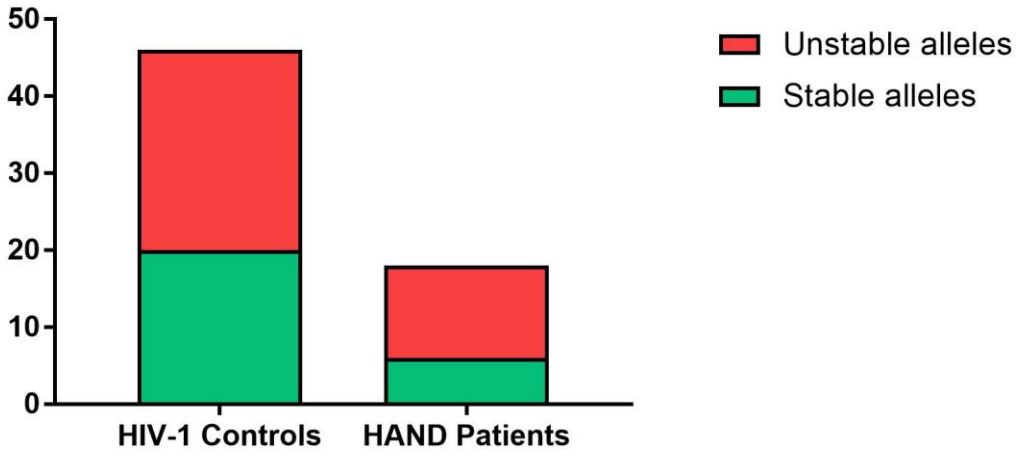

Figure 20: Representation of the HLA-C stable or unstable alleles of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders). The p-value obtained is 0.457.

4.2.4 APOE genotyping and its association with HANDs development

The ASPCR approach was also used for APOE genotyping. In this case, all three ASPCR reactions are performed with a Touchdown thermal profile. An example of the obtained results is represented by Figure 21 in which is reported APOE2 genotyping of eleven samples, two of which possess APOE2 haplotype (sample G4 and G11).

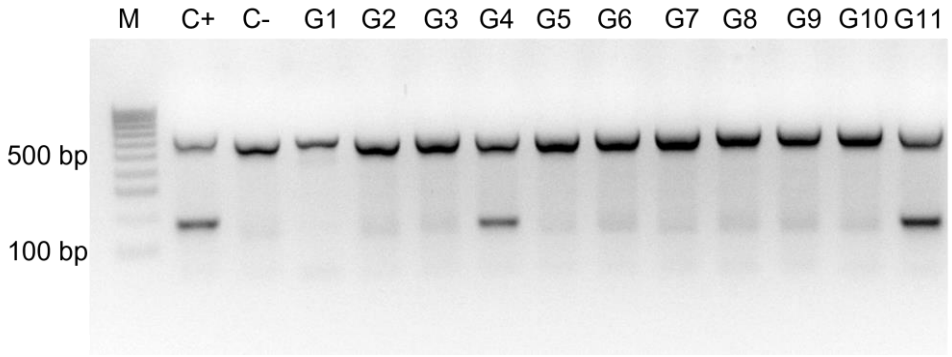

Figure 21: APOE2 genotyping through ASPCR. M indicates Molecular weight marker (HyperLadder 100 bp, BioLine); C+ and C- indicate positive and negative controls. PCR bands represent APOE2 (170 bp) and internal control gene COL5A1 (667 bp)

The APOE genotype results obtained through ASPCRs are indicated in Table 33.

Table 33: APOE genotyping results reported as number and percentage of haplotypes found. HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders

	HIV-1 Controls haplotypes (n=46)	**HAND Patients haplotypes (n=18)**
APOE2, n (%)	3 (6.5)	2 (11.1)
APOE3, n (%)	41 (89.1)	14 (77.8)
APOE4, n (%)	2 (4.4)	2 (11.1)

To verify a possible statistical association between the APOE4 variant and the development of HANDs the chi-square statistical analysis has been performed. The result shows that there isn't a statistical correlation between APOE4 and HANDs development (p-value: 0.315).

4.2.5 B2microglobulin plasma levels assessment and its influence on HANDs development

To assess if there is a statistically significant difference in β2m between HANDs patients and HIV-1 positive subjects without neurological impairment the β2m plasma levels were measured. The β2m plasma level parameters are reported in Table 34.

Table 34: Beta2microglobulin plasma level parameters reported as median and interquartile distance (IQR). HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders

	HIV-1 Controls (n=23)	**HAND patients (n=9)**
β2m, median (IQR), mg/L	1.94 (1.52-2.31)	2.22 (1.83-3.84)

As depicted in Figure 22 applying the Mann Whitney test, there isn't a statistically significant difference in the β2m plasma levels of the two studied groups (p-value: 0.130). Two HAND samples possess high values of β2m plasma levels. These patients (categorized respectively as HAD and ANI) possess also high NFL plasma levels and one of them has an HLA-C unstable genotype.

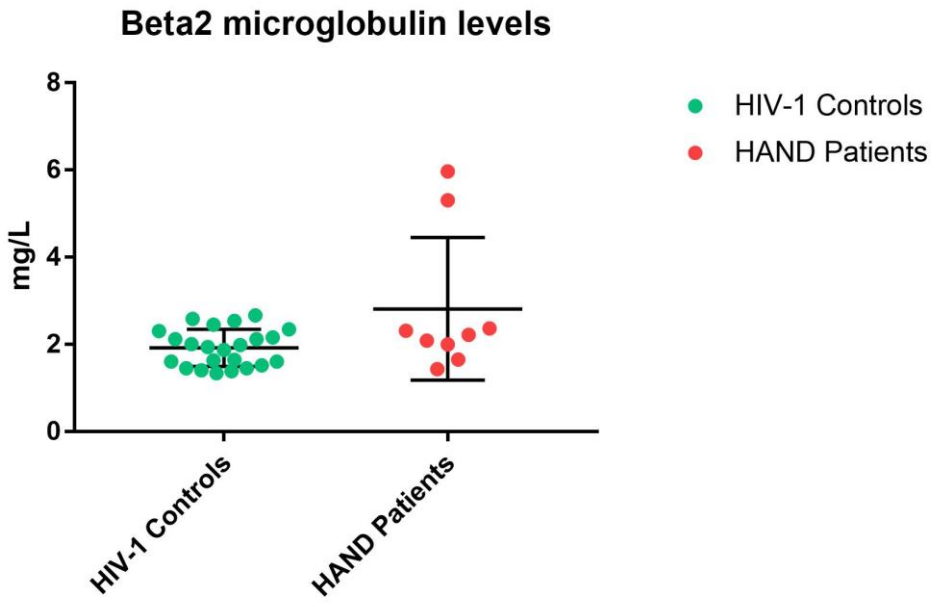

Figure 22: B2microglobulin plasma levels of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders). The p-value obtained is 0.130.

4.2.6 Neurofilament light chain plasma levels evaluation and its influence on HANDs development

To evaluate if there is a statistically significant difference between NFL levels between HIV-1 positive subjects and HAND patients, the quantification of NFL plasma levels was performed. The NFL plasma level parameters are reported in Table 35.

Table 35: NFL plasma level parameters reported as median and interquartile distance (IQR). HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders)

	HIV-1 Controls (n=23)	**HAND patients (n=9)**
NFL, median (IQR), pg/mL	7.34 (6.47-8.59)	10.74 (5.71-27.54)

The results of NFL plasma level quantification are reported in Figure 23.

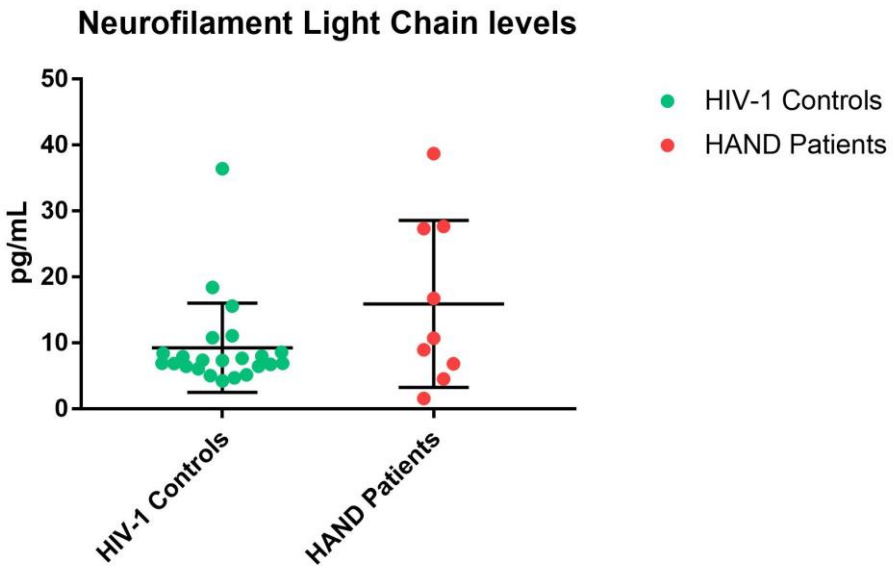

Figure 23: Neurofilament Light Chain (NFL) plasma levels of the studied groups of the HIV-1 patients studied groups (HIV-1 Controls indicates HIV-1 positive subjects without neurological impairments whereas HAND indicates patients suffering from HIV-associated Neurocognitive disorders). The p-value obtained is 0.224.

Despite a tendency of the HAND group to higher values, the Mann-Whitney statistical analysis didn't detect a statistically significant difference in NFL plasma levels between the two studied groups (p-value: 0.224).

4.3 ACOT8 influence on HIV-1 infectivity

The effect of ACOT8 cellular protein on HIV-1 infectivity is still under investigation. Here are reported the results obtained so far including the production of Hek293T and TZM-bl ACOT8 KO cell lines using CRISPR/Cas9 technique and the preparation of HIV-1/VSV-G pseudotyped virus exploiting two different systems. This study aims to elucidate if ACOT8/Nef interaction influences HIV-1 infectivity.

4.3.1 Production of Hek293T and TZM-bl ACOT8 KO cell lines through CRISPR/Cas9

To develop ACOT8 knock-out cell lines (Hek293T and TZM-bl) CRISPR/Cas9 system has been employed. After the isolation of single edited clones performed by limiting dilution, ACOT8 knock-out was verified through Western Blot analysis (Figure 24).

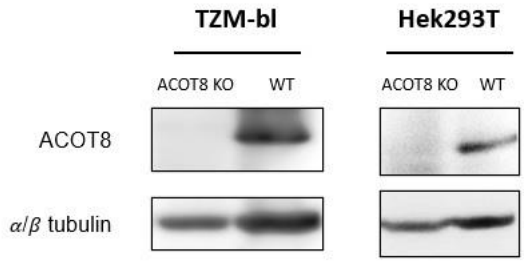

Figure 24: Western Blot analysis to verify ACOT8 knock out: on the left TZM-bl, on the right Hek293T. α/β tubulin was used as a control

As reported in Figure 24 ACOT8 expression occurs only in the wild type TZM-bl and Hek293T indicating that ACOT8 knock-out was obtained successfully on the selected clones. α/β tubulin was used as control.

4.3.2 ACOT8 has an impact on HIV-1 pseudotyped virus production

In the first experimental set, the production of HIV-1/VSV-G pseudotyped virus was performed in Hek293T and Hek293T ACOT8 KO cell lines using PSG3$^{\Delta Env}$ vector and plasmids coding for QHO/VSV-G envelopes. As previously reported PSG3$^{\Delta Env}$ was designed to prevent Env protein expression, whereas all the other HIV-1 proteins are expressed (H. Wei et al., 2020). The subsequent infection was performed using 300 pg of quantified p24 on TZM-bl and TZM-bl ACOT8 KO cell lines. The obtained results indicate that ACOT8 influences HIV-1 pseudotyped virus production (p-value = 0.0011). HIV-1 pseudotyped viruses produced in Hek293T are more infectious than those produced in Hek293T ACOT8 KO (Figure 25A). Instead, ACOT8 does not affect VSV-G pseudotyped virus production and/or infection steps (Figure 25B)

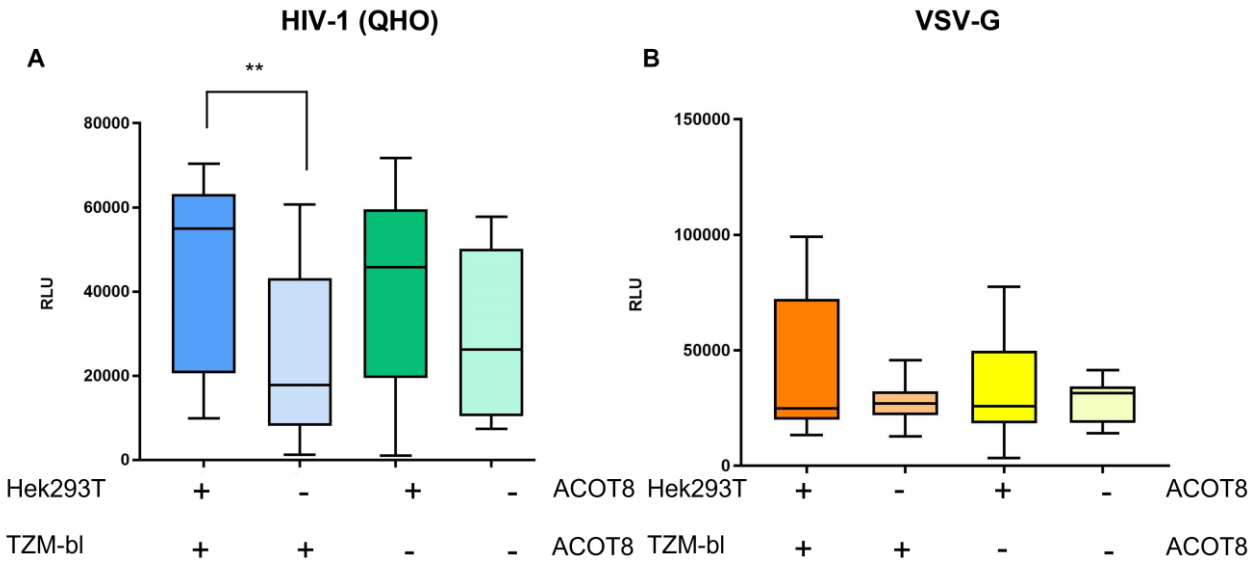

Figure 25: (A) HIV-1 pseudotyped virus produced in Hek293T WT cell line (blue and green); produced in Hek293T ACOT8 KO cell line (light blue and light green) which were used to infect TZM-bl WT cell line (blue and light blue) or TZM-bl ACOT8 KO cell line (green and light green). (B) VSV-G pseudotyped virus produced in Hek293T WT cell line (orange and yellow); produced in Hek293T ACOT8 KO cell line (light orange and light yellow) which were used to infect TZM-bl WT cell line (orange and light orange) or TZM-bl ACOT8 KO cell line (yellow and light yellow). The p-value is indicated as follows: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001; **** = p-value ≤ 0.0001.

These results underline ACOT8 importance in the HIV-1 pseudotyped virus production step and how this mechanism doesn't occur in the production of VSV-G pseudotyped virus.

4.3.3 ACOT8 has an impact on HIV-1 pseudotyped virus production and infection

In the second experimental set, the production of HIV-1/VSV-G pseudotyped virus was performed in Hek293T and Hek293T ACOT8 KO cell lines using p8.91 backbone plasmid coding for HIV-1 gag and pol genes in which HIV-1 Vif, Vpr, Vpu, and Nef were deleted (Brennan et al., 2018; Zufferey et al., 1997), pCSFLW packaging vector (Sampson et al., 2021) and plasmids coding for QHO/VSV-G as envelopes. The subsequent infection was performed using 625 pg of quantified p24 on TZM-bl and TZM-bl ACOT8 KO cell lines. The obtained results indicate that ACOT8 influences both HIV-1 pseudotyped virus production and infection steps. HIV-1 pseudotyped viruses produced in Hek293T are more infectious than those produced in Hek293T ACOT8 KO as reported in Figure 26A in which the production effect is highlighted. This effect is evident in both TZM-bl target cell lines: pseudotyped virus produced in Hek293T and in Hek293T ACOT8 KO infecting TZM-bl cell line (p-value < 0.0001); pseudotyped virus produced in Hek293T and in Hek293T ACOT8 KO infecting

TZM-bl ACOT8 KO cell line (p-value < 0.0001). The same data are reported in Figure 26B to point out ACOT8 influence on the infection step: pseudotyped virus produced in Hek293T infecting TZM-bl WT and TZM-bl ACOT8 KO cell lines (p-value < 0.0005); pseudotyped virus produced in Hek293T ACOT8 KO infecting TZM-bl WT and TZM-bl ACOT8 KO cell lines (p-value = 0.0051).

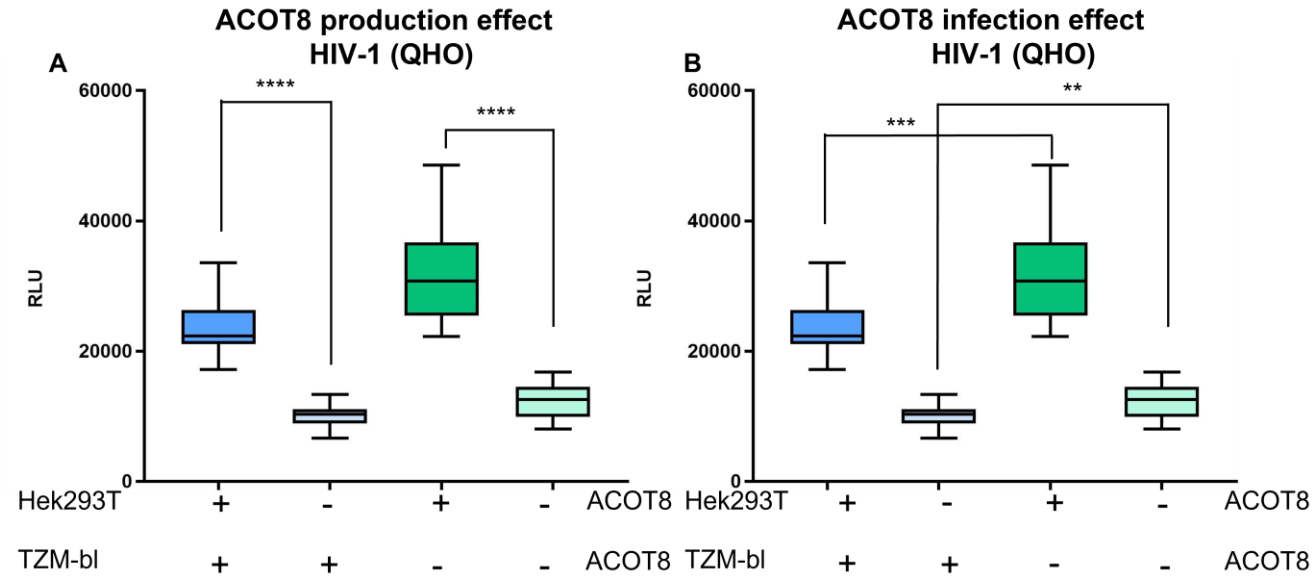

Figure 26: (A and B) HIV-1 pseudotyped virus produced in Hek293T WT cell line (blue and green); produced in Hek293T ACOT8 KO cell line (light blue and light green) which were used to infect TZM-bl WT cell line (blue and light blue) or TZM-bl ACOT8 KO cell line (green and light green). The p-value is indicated as follows: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001; **** = p-value ≤ 0.0001.

Regarding VSV-G similar results were obtained also using the three-vector system for pseudotyped virus preparation, as reported in Figure 27. ACOT8 does not influence VSV-G pseudotyped virus production and/or infection steps.

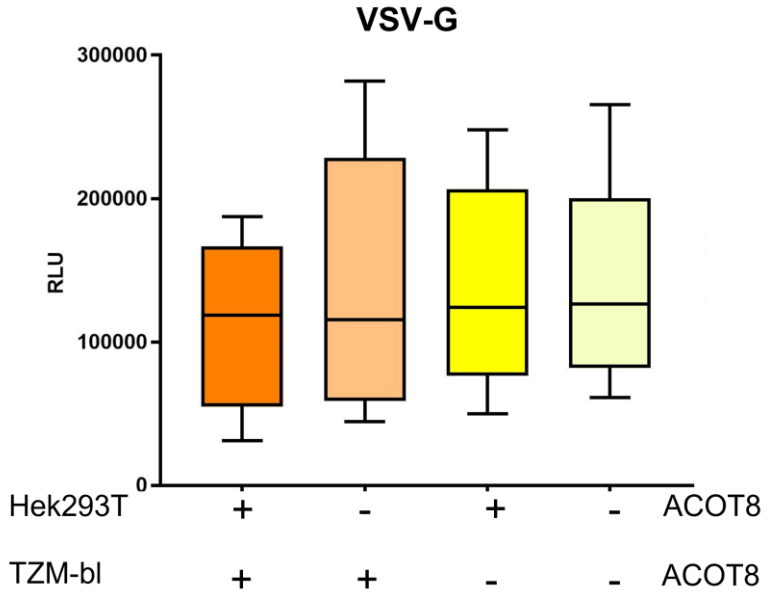

Figure 27: VSV-G pseudotyped virus produced in Hek293T WT cell line (orange and yellow); produced in Hek293T ACOT8 KO cell line (light orange and light yellow) which were used to infect TZM-bl WT cell line (orange and light orange) or TZM-bl ACOT8 KO cell line (yellow and light yellow).

5. DISCUSSION

This thesis analyzed different aspects of host-HIV interactions, pointing out the role of some host factors in AIDS progression, HIV-1-associated neurocognitive disorder development, and HIV-1 infectivity. Consequently, the discussion is organized in three different sections dedicated to each topic: the association between HLA-C and AIDS progression, host factors influencing HANDs development, and the role of ACOT8 in HIV-1 infectivity.

5.1 Association between HLA-C and AIDS progression

Since its pivotal role in the immune response, the role of HLA-C in viral infections is widely studied. For example, some HLA-C alleles were associated with HCV, HBV, HPV, and Lassa virus-positive patients (Song et al., 2013; Ursu et al., 2020; Wauquier et al., 2019; Yengo et al., 2020). The association between HLA-C alleles and HIV infectivity has been extensively analyzed (Apps et al., 2013; Parolini et al., 2018). The hypothesis of this work was to assess if there is a correlation between HLA-C stability and AIDS progression. To this aim, a cohort of 96 patients was recruited and divided in three categories based on their AIDS progression status. Progressors were characterized by a higher HIV-1 viral load and decreased CD4+ T lymphocytes level and possessed a more rapid progression. Whereas Long Term Non-Progressors and Elite Controllers have lower HIV-1 viral load and higher CD4+ T lymphocytes levels, and AIDS proceeds slowly. The analysis of the HLA-C genotype of this cohort underlined a statistically significant correlation between HLA-C unstable alleles and a more rapid AIDS progression (p-value = 0.0143). This result is in line with what is reported in the literature. HLA-C could be subdivided into stable or unstable alleles depending on the binding stability to the β2m/peptide complex (Sibilio et al., 2008). The stability of HLA-C was previously reported as HIV-1 infectivity increasing factor. In a previously published paper (Serena et al., 2017) demonstrated that HIV-1 Env could associate with HLA-C free chains (i.e. no longer bound to β2m/peptide complex) on the cell membrane, thus enhancing HIV-1 infectivity. Another work published by our group shows that HIV-1 pseudotyped viruses possess increased infectivity if produced in the presence of HLA-C unstable variants (Parolini et al., 2018). Another factor that can contribute to HIV-1 infectivity is HLA-C expression levels. In other previously published papers, higher HLA-C expression was associated with better control of HIV-1 infection (Apps et al., 2013). Moreover, HLA-C expression levels are regulated by the binding of miR-148a. In miR148a binding site was discovered a SNP which destroys the binding site, leading to a higher expression of HLA-C alleles possessing this SNP (Kulkarni et al., 2011). Since HLA-C

alleles which possess higher expression levels are also the more stable ones, we hypothesize that these phenomena are linked together. These previously published works corroborate our results in which HLA-C unstable alleles are associated with a rapid AIDS progression, indicating that HLA-C unstable alleles are linked to a worse HIV-1 infection control which could lead to a faster disease progression.

5.2 Host factors influence on HIV-associated neurocognitive disorders

This study aimed to find clinical biomarkers and genetic factors linked to HIV-associated neurocognitive disorders. Since HAND diagnosis is difficult, finding biomarkers and genetic factors linked to its development could represent useful diagnostic tools. A cohort of 32 HIV-1 positive patients was recruited. All the subjects underwent a battery of neurocognitive tests and then were divided into two groups: HAND patients and HIV-1 positive patients without neurocognitive impairment. Since HLA-C unstable alleles were previously linked to HAND development (Zipeto et al., 2018), the HLA-C genotype was evaluated also in this patient cohort. Unfortunately, in this case, there is not a statistically significant association between HAND development and HLA-C unstable alleles. Another genetic marker considered in this study is APOE due to the association between the APOE4 allele and Alzheimer's disease. The role of APOE4 on HAND onset is still a matter of debate since controversial results can be found in previously published works: a study published by (Morgan et al., 2013) didn't find an association between APOE4 and HAND; instead, the APOE4 allele was linked memory impairment in HIV-1 positive patients (Hoare et al., 2013; Wendelken et al., 2016). In this study, there is not a statistically significant connection between APOE4 and HAND. Moreover, the plasma levels of two soluble biomarkers were examined. Beta2microglobulin, given its ability to form amyloid aggregates, was previously correlated to Alzheimer's development (Dominici et al., 2018). Furthermore, beta2microglobulin levels were connected to the AIDS dementia complex (Brew et al., 1992). Also, in this case, unfortunately, a relationship between beta2microglobulin plasma levels and HAND wasn't detected. Neurofilament light chain plasma levels were also analyzed as a potential biomarker of HAND development. NFL levels are linked to neuronal injury and were previously correlated to CNS injury in HIV-infected patients (Peluso et al., 2013; Peterson et al., 2014). Despite HAND patients showing a tendency to higher NFL levels, a statistically significant correlation between NFL levels and HAND was not determined. However, this study analyzed only 32 subjects and just 9 of them possess HIV-associated neurocognitive impairments. This poor recruitment reflects the difficulties that we

managed during the COVID-19 pandemic since enrolling new subjects in the study was hard due to the medical emergency. Expanding the patient cohort could certainly benefit our analysis on finding new biomarkers and genetic factors linked to HAND development. These non-significant results were expected since we did not reach the number of patients that we planned to enroll (i.e. nearly 100 subjects per group).

5.3 ACOT8 influence on HIV-1 infectivity

ACOT8 was initially discovered as a Nef interacting partner. This work aimed to better understand the influence of Nef-ACOT8 interaction in HIV-1 infectivity. To this aim, we produced Hek293T and TZM-bl ACOT8 knock-out cell lines exploiting the CRISPR/Cas9 system. Subsequently, we used these four cell lines to perform the production and infection of the pseudotyped virus. In the first experimental set, we used a two-vector system for pseudotyped virus production in which Nef protein was encoded by PSG3$^{\Delta Env}$ plasmid (H. Wei et al., 2020). The obtained results underlined an HIV-1 dependent mechanism, there aren't effects on production/infection steps using VSV-G env. Moreover, we observed an effect on the HIV-1 production step: pseudotyped viruses produced in the Hek293T wild-type cell line were more infectious than those produced in the Hek293T ACOT8 knock-out cell line. It is known that Nef influences virion assembly, which could lead to an increased HIV-1 infectivity. Several hypotheses on how Nef could mediate this effect were proposed all suggesting a Nef-dependent virion composition. For example, it was proposed that Nef could assist Env incorporation and p17 phosphorylation (Qi & Aiken, 2008). Moreover, CD4 inhibits the separation between gp120 and gp41, thus Nef-dependent CD4 downregulation could lead to increased HIV-1 infectivity (Lundquist et al., 2004). The obtained results underlined how Nef-ACOT8 interaction is important for pseudotyped virus infectivity, in the presence of both Nef and ACOT8, pseudotyped viruses are more infectious. In the second experimental set, a three-vector system was used to produce a pseudotyped virus. In this case, Nef wasn't encoded by any of the utilized plasmids (Brennan et al., 2018; Zufferey et al., 1997). The obtained results confirmed the HIV-1 dependent mechanism previously observed. Also, in this case, effects on production/infection using VSV-G Env protein weren't noted. The effect on pseudotyped virus production was also observed in this second experimental set: pseudotyped viruses produced in Hek293T wild-type cell line were more infectious than those produced in the Hek293T ACOT8 knock-out cell line. Moreover, in this second experimental set, the effect on the infection step was evident: TZM-bl ACOT8 knock-out cell line possesses a higher infection rate than TZM-bl wild-type

cell line. Since Nef, in this case, is not present, this unexpected result could point out an ACOT8 dependent mechanism, which can influence pseudotyped virus infectivity. Furthermore, pseudotyped virus produced in the first experimental set were more infectious than those produced in the second one. Nevertheless, the difference in the production is higher in the second experimental set.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The role of host-retrovirus interactions represents the common thread of this thesis which analyses different aspects of this wide field. The first examined aspect is the association between HLA-C unstable alleles and AIDS progression. In this case, a statistically significant correlation was found (p-value = 0.0143) demonstrating that the HLA-C genotype could influence the speed of AIDS progression. Considering this obtained result, a manuscript reporting these data is in preparation. Finding new biomarkers linked to the development of HIV-associated neurocognitive disorders is the aim of the second part of this work. Unfortunately, an association between the analyzed biomarkers *(i.e.*, HLA-C and APOE genotype, beta2microglobulin, and neurofilament light chain plasma levels) and HAND development could not be determined. However, due to the COVID-19 emergency, the subject's recruitment was difficult, so we have analyzed only 32 patients of the 100 initially planned to reach the statistical power. Therefore, the study will proceed in the coming months by recruiting more patients. By increasing the number of the analyzed subjects, we will try to find new biomarkers linked to HAND development. Another aspect analyzed in this thesis is how the interaction between HIV-1 Nef protein and the cellular protein ACOT8 could impact HIV-1 infectivity. Using pseudotyped virus system an effect on production and infection steps was noted. Moreover, this mechanism seems to be HIV-1 specific since this effect was not observed using VSV-G pseudotyped virus. To better elucidate this phenomenon, we will produce pseudotyped virus using other HIV envelopes in addition to the one tested to check if this phenomenon occurs also using other envelopes. Moreover, we will produce HIV-pseudotyped virus in the presence of ACOT8 mutants and different Nef proteins. Furthermore, through a cytofluorimetric analysis, we will clarify the impact of Nef-ACOT8 interaction on CD4 and the MHC-I internalization process. In conclusion, this work tries to analyze different aspects of host-retrovirus interaction: how HLA-C genotype could influence AIDS progression, the importance to find new biomarkers linked to HAND development, and how Nef-ACOT8 interaction could influence HIV-1 infectivity. This preliminary work will be the basis for future developments.

7. ACKNOWLEDGMENTS

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