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TESI DI DOTTORATO DI RICERCA

Identification of a Specific miRNA Profile in HIV Exposed Seronegative Individuals

Settore MED/04 - Patologia Generale

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To My Parents & Bayan

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SUMMARY

Objective: MicroRNAs (miRNAs) are small 20- to 24-nt non-coding RNAs involved in the post-transcriptional regulation of gene expression which play important defensive roles in several viral infections. Global expression profiles of cellular miRNAs have identified alterations in the expression of specific miRNA post-HIV-1 infection both in vitro and in different patient cohorts, suggesting a potential role for miRNAs in the pathogenesis and progression of HIV-1 infection. We therefore decided to verify if natural resistance to HIV-1 infection, observed in seronegative individuals repeatedly exposed to HIV-1 (HESN) through unprotected sexual intercourse, could be secondary to a different expression of their miRNA profile.

Methods: Expression level of 84 miRNAs was analyzed by RT-PCR array in plasma and unstimulated PBMC of 30 seronegative individuals repeatedly exposed to HIV (HESN), 30 HIV seropositive subjects (HIV+) and 30 healthy controls (HC). Results were confirmed by individual RT-qPCR in plasma, *in vitro* HIV infected PBMC and supernatants, isolated monocyte and in *in vitro* HIV infected monocyte derived macrophages (MDM). ABCA1, Dicer and Drosha mRNA expression was analyzed as well.

Results: Whereas Dicer and Drosha expression was comparable in HESN, HIV+ and HC, several miRNA were upregulated both in HESN and HIV+ compared to HC, suggesting that exposure to HIV modifies miRNA signature even in the absence of productive infection. MiRNA-29a and miR-223 were upregulated in both unstimulated PBMC and plasma of HESN alone; their expression was reduced upon in vitro HIV infection of HESN PBMC indicating that, upon infection, they are secreted in the extracellular milieu. These results were confirmed by individual qPCR.

Additionally, HESN miRNA profile in monocytes clustered with HC one, while, reduced susceptibility to HIV-1 infection in *in vitro* HIV infected MDM from HESN was associated with higher expression of all our tested miRNAs compared to HCs. Finally, the upregulation of miR-223 in basal PBMC, monocytes and HIV-1 infected MDM resulted into an increased expression of ABCA1.

Conclusions: Our studies demonstrate that HIV exposure modifies miRNAs expression. Because those miRNAs that are specifically increased only in HESN have been known to reduce HIV replication via the binding of viral mRNA 3' UTRs, the modulation of these miRNAs could represent an important mechanism in resistance to HIV.

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LIST OF ABBREVIATIONS

Ago: Argonaute
AIDS: Acquired immune deficiency syndrome
ADCC: Antibody dependent cellular cytotoxicity
APCs: Antigen presenting cells
APOBEC3G: Apolipoprotein B mRNA editing enzyme-catalytic polypeptide- like 3G
ART: Anti-retroviral therapy
bNAbs: Broadly neutralizing antibodies
BST-2: Bone marrow stromal cell antigen 2
CA: Capsid
CCR5: C-C chemokine receptor type 5
cDNA: Complementary deoxyribonucleic acid
CDS: Coding sequence
Ct: Cycle threshold
CTL: Cytotoxic t lymphocytes
CTLA: Cytotoxic t-lymphocyte antigen
CXCL: CXC-chemokine ligand
CXCR4: C-X-C chemokine receptor type 4
DCs: Dendritic cells
DGCR8: DiGeorge syndrome critical region protein 8
DNA: Deoxyribonucleic acid
dsRNA: Double-stranded ribonucleic acid
EC: Elite controllers
eIF4E: eukaryotic translation initiation factor 4E
ENV: Envelope
ES: Elite suppressors
ESN: Exposed seronegatives
Exp5: Exportin 5
FC: Fold change
GSK3β: glycogen syntetase kinase 3β
HESN: HIV-exposed sero-negatives
HDFs: HIV dependency factors
HDL: High-density lipoprotein
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigen
IDU-HESN: Injection drug users-HIV exposed seronegative
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
IN: Integrase
KIR: Killer cell immunoglobulin–like receptor
LAG: Lymphocyte activation gene
lncRNA: long non-coding RNA
LTNP: Long Term Non Progressor
LTR: Long terminal repeats
MACS: Multicenter AIDS Cohort Study
MDM: monocyte derived- macrophages
MEU: Multiple exposed uninfected

m^7G cap: mRNA 5`-terminal 7 methylguanosine- cap
MHC: Major histocompatibility complex class
miRNA: microRNA
MVs: Microvesicles
MYC: V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
MYOD1: myoblast determination protein 1
ncRNA: Non-coding RNA
Nef: Negative factor
NF-κB: nuclear factor-κB
NK: Natural killer
NLS: Nuclear Localization Signal
nt: nucleotide
PABP: poly (A)-binding protein
PACT: Protein kinase RNA activator
PAMP: Pathogen-associated molecular patterns
PBMCs: Peripheral Blood Mononuclear Cells
PCR: Polymerase chain reaction
PD: Programmed death
pDC: Plasmacytoid dendritic cell
PIC: pre- integration complex
piRNAs: Piwi interacting RNAs
POL: Polymerase
Pol II: RNA polymerases II
PR: Protease
PRR: Pattern recognition receptors
RNA: Ribonucleic acid
RT: Retro-transcriptase
RT-PCR: Real time - polymerase chain reaction
SIV: Simian immunodeficiency viruses
SNP: Single nucleotide polymorphisms
snRNAs: Small nuclear RNAs
snoRNAs: Small nucleolar RNAs
ssRNA: Single-stranded RNA
HRP: Horseradish peroxidase
TCRs: T cell antigen receptors
TGF−β: Transforming growth factor β
Th: T helper cell
Tim: T cell immunoglobulin domain and mucin domain
TLRs: Toll-like receptors
TNF: Tumor Necrosis Factor
TRBP: Transactivation response RNA binding protein
UNAIDS: United Nations programme on HIV/AIDS
Vif: Virion infectivity factor
vmiRNA: viral miRNA
Vpu: Viral protein unique
Vpr: Viral protein R
WHO: World Health Organization
Wt: Wild-type
ZEB: Zinc Finger E-Box Binding Homeobox

INTRODUCTION

1.1. Origin and History of HIV

The cross-species transmission of simian immunodeficiency viruses from chimpanzees to humans in West–Central Africa was the emergence of HIV-1 at the beginning of the 20th century (Worobey M. 2008).

With having infected far more than 60 million people, HIV-1 has been one of the most successful pathogens in human history (Osseo-Asare AD, 2007). In 1970s, the virus was only detected in east Africa (Bello G, 2008) and by 1980, in Europe and North America, although the actual introduction probably occurred decades earlier (Worobey M. 2008). In 1983 for the first time, the viral etiology was identified in a new retrovirus particle by Fracoise Barré-Sinoussi and Luc Montagne (for which they won the Noble Prize in 2008), later in 1984 the team of Robert C. Gallo confirmed to be the etiological agent of acquired immunodeficiency syndrome (AIDS) (Vicenzi at al., 2013).

The universal spread of HIV-1 during the latter half of the 20th century is generally attributed to expanded migration between countries as a result of globalization; increasing urbanization; warfare and ethnic conflict surrounding postcolonization independence of many African countries; and changing sexual practices (Vidal N, 2000). The cumulative total of individuals infected with HIV-1 and deaths due to AIDS since the pandemic began exceeds 60 million and 25 million people respectively (Cohen MS, 2008). At the end of 2007, the Joint United Nations Program on HIV/AIDS (UNAIDS) and the WHO (World Health Organization) estimated that there were 33.2 million people living with HIV-1, that 2.5 million individuals became newly infected in 2007 and in fact 2.1 million people died of AIDS in that year (Cohen MS, 2008).

Also, the state of profound immunodeficiency caused by HIV infection leads to the co-infection by other opportunistic pathogens, like Mycobacterium tuberculosis, hepatitis viruses and degeneration of central nervous system

as well as the development of tumors mostly Kaposi's sarcoma and B-cell lymphoma (Vicenzi at al., 2013).

1.2. HIV Virology

1.2.1. HIV Classification

HIV virus belongs to the viral Retroviridae family and the genus Lentivirus. There are two different types of HIV, which can cause infection and disease in humans, HIV-1 and HIV-2. HIV-1 arised from cross-species transmission of a chimpanzee virus to humans (Hahn B.H., 2000) and HIV-2 from cross-species transmission of a Sooty mangabey virus (Lemey P., 2003).

There are three groups of HIV-1, labeled M, N, and O, based on genome differences. Most HIV-1 infections are caused by group M viruses, and these are divided into 9 subtypes known as *clades* (A–D, F–H, J, and K). The DNA sequences of viruses in distinct clades can differ by 15%–20% (McCutchan F.E. 2000). The most common clade in the Americas, Europe, and Australia is clade B, whereas clade C predominates in the most heavily affected part of the world, southern Africa (McCutchan F.E. 2000).

In comparison with HIV-1, HIV-2 is much less prevalent, and individuals infected with HIV-2 are primarily found in West Africa and India. In addition, infection with HIV-2 is associated with a slower progression to immune deficiency, and the virus seems to be less efficiently transmitted, even from infected women to their offspring (Jaffar S., 2004).

1.2.2. HIV structure

An infectious HIV particle consist of a phospholipid bilayer envelope derived from the host cell membrane but containing virally encoded membrane proteins, which surround two identical single strands of RNA (ssRNA) packaged within a core of viral proteins (Fig. 1.1) (Abbas AK, 2010).

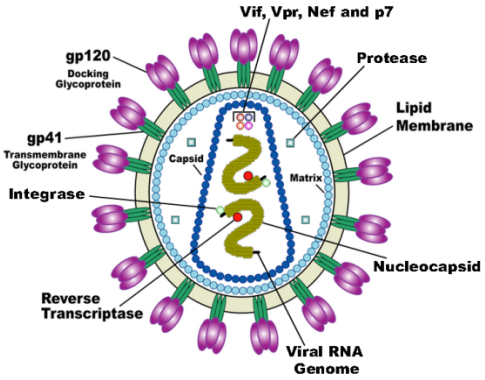


Figure1.1. *Structure of HIV-1 virus (Abbas AK, 2010).*

The HIV genome is 9.2kb long approximately and has the basic characteristic of retroviruses. At each end of the genome, there are long terminal repeats (LTR), which regulate viral gene expression and integration. Also, It encodes the envelope (Env) glycoproteins consisting of surface gp120 and transmembrane glycoprotein gp41 (Zhu, P. et al, 2006) which are required for HIV infectivity. The Gag sequences encode a 55-kD protein that is cleaved into the internal structure core proteins (p24, p9, p7 and p17). The Pol sequences encode the enzymatic polymerase proteins including, reverse transcriptase (RT), integrase (IN) and protease (PR) required for viral replication. In addition to these classic retrovirus genes, there are six other accessory proteins, namely, Vif, Vpr, Rev, Vpu, Tat and Nef (Turner et al., 1999).

Vif, virion infectivity factor, is considered as a prototype of viral antagonist of host-cell factors which can restrict or prevent virus infection or replication and it is essential for the production of highly infectious mature virions (Cohen EA, 1996).

Vpr, viral protein R, is a part of pre- integration complex (PIC) that mediate the transport of viral genome to the nucleus while undergoing conversion from RNA into DNA as result of the virion– associated reverse transcriptase enzyme. It includes a nuclear localization signal (NLS) that directs transport

even in the absence of mitotic nuclear envelope breakdown and it is essential for nuclear localization in non-dividing cells, such as macrophages (Gallay P, 1996). In addition at cellular level, Vpr induces cell cycle arrest in the G2/M phase but also apoptosis (Subbramanian et al., 1998).

Newly synthesized Env glycoproteins (gp160), which are later cleaved into gp120 and gp41, are sometimes held in the endoplasmic reticulum (ER) through interactions with newly synthesized CD4 molecules. The other important protein, viral protein unique (Vpu), promotes degradation of CD4 in these complexes, thus allowing Env transport to the cell surface for assembly into viral particles (Lamb RA, Pinto LH, 1997). Vpu is only present in HIV-1 and by preventing Bone marrow stromal cell antigen 2 (BST-2) or Tetherin, a host cell protein, plays an important role in budding and release of new virions in the final step of the virus life cycle (Andrew A, et al., 2010).

1.2.3. HIV life cycle

The Env complex has a trimeric structure of three gp120/gp41 pairs. An initial binding between gp120 subunits to the surface receptor CD4 molecule induces the conformational changes that expose secondary gp120 binding site for a second cell surface molecule, typically CC-chemokine receptor 5 (CCR5) (Rizzuto, C. D. 1998).

The HIV core includes integrase (IN), replication enzymes RT and the viral genomic RNA and it is encased by viral capsid (CA) protein (Briggs, J. A. 2006). Once an HIV virion enters a cell, the CA becomes disrupted and the RNA genome of HIV is transcribed into a double strand DNA by reverse transcriptase (Kohlstaedt, L. A. 1992). The integration of viral DNA is catalyzed by viral IN into the host genome. The integrated HIV DNA called provirus, with little or no production may remain transcriptionally inactive for months or years and in this way HIV infection can be latent (Abbas AK, 2010).

The transcription of the genes is regulated by LTRs upstream of the viral structural genes. The LTRs contain polyadenylation signal sequences, the TATA box promoter sequence and binding sites for two host cell transcription factors, NF-κB and SP1 (Liu R et al., 1996). Activation of the T cells by antigen or cytokines is linked to initiation of HIV gene transcription.

The first gene products of transcription are Nef, Tat and Rev proteins that are exported and translated into proteins in the cytoplasm and later, the structural components of the virus include pol, gag, and env are translated (Daugherty, M. D.,2010).

HIV-1 negative factor, Nef is expressed shortly after viral infection in high concentrations, and it increases viral replication and stimulates a decline in the number of CD4 receptors on the surface of the infected cells (Goldsmith, et al., 1995). Tat is a transcriptional activator and can enhance the production of viral mRNA transcript. It binds to nascent mRNA transcript and recruits cyclin T, the cellular protein, to enhance its affinity and increase the "processivity" of RNA polymerase by several hundred-fold (Wei et al, 1998). The major function of Rev is controlling the export rate of mRNA in order to regulate the expression of HIV proteins (M.Emerman and Malim, 1998).

Subsequently, the pol gene is cleaved to form reverse transcriptase, protease, ribonuclease, and integrase enzymes. The viral protease cleaves the gag protein proteolitically into p24, p17 and p15 polypeptides, which are the core proteins required for assembly of infectious viral particles (Turner et al., 1999). The gp120 and gp41 proteins, necessary for HIV binding to cells, are the product of env genes from cleaving of gp160 by cellular proteases (Wyatt and Sodroski, 1998).

The viral particle assembles by packaging viral RNA transcripts within a nucleoprotein complex which consist of the pol-encoded enzymes and gag proteins required for next integration. Later the assembled complex is enclosed within an envelope and released by budding from the plasma membrane of the infected cells (Greene et al., 2002). (Fig.1.2)

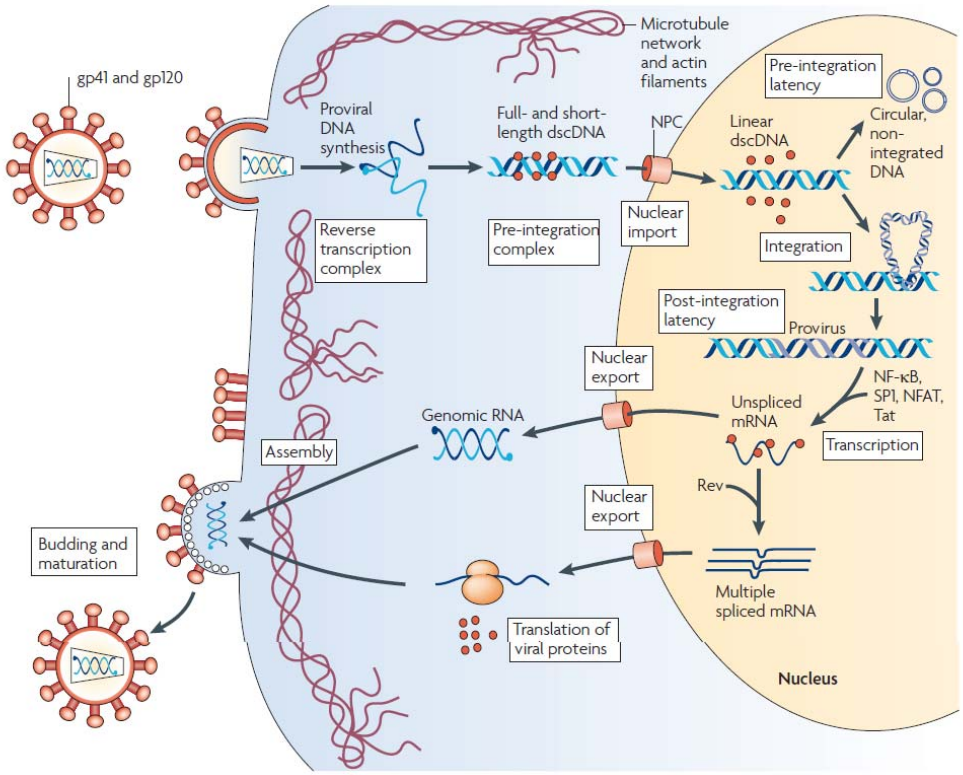


Figure 1.2 *HIV life cycle. Viral fusion and entry requires the binding of glycoprotein gp120 to CD4 receptors at the cell surface as well as to CC chemokine receptor type 5 (CCR5) or CXC chemokine receptor type 4 (CXCR4). The viral nucleocapsid enters the cytoplasm and uses cytoplasmic dynein to move toward the nuclear pore complex (NPC). The viral RNA is retrotranscribed into proviral double-stranded cDNA (dscDNA), which can stay in the cytosol, where it is highly unstable and exists in a transient, reversible pre-integration latent state, or can form a pre-integration complex consisting of dscDNA, viral proteins and some host cell proteins. When ATP levels are adequate, the pre-integration complex is transported into the nucleus through the NPC, and the dscDNA either circularizes as one or two long terminal repeat-containing circles or is integrated into a host cell chromosome. After integration, the provirus remains quiescent, existing in a permanent post-integration latent state. On activation, the viral genome is transcribed by the synergic interaction of cellular transcription factors (nuclear factor-* κ *B (NF-* κ *B), nuclear factor of activated T cells (NFAT) and specificity protein 1 (SP1)) and the viral transactivator, Tat. Rev, a viral protein, regulates the splicing and cytosolic transport of some of the viral mRNAs, which are translated into regulatory and structural viral proteins. New virions assemble and bud through the cell membrane, maturing through the activity of the viral protease (Card et al, Retrovirology 2013).*

1.3. HIV pathogenesis

1.3.1. HIV Transmission

Each HIV transmission event results from exposure to blood, a blood product, or bodily secretions contaminated with HIV virus. This may occur through sexual transmission (heterosexual or homosexual), parenteral transmission (injection drug use, receiving contaminated blood products), or vertical transmission (transmission of HIV from mother to child in utero or trough breast-feeding) (Simon V et al., 2006). The probability of a transmission event, whether from blood, sexual intercourse, or vertical transmission, broadly correlates with the concentration of HIV-1 in the host secretion (Quinn TC, 2000).

1.3.2. Disease progression

A majority of HIV infected individuals have acute HIV symptoms approximately, 2-4 weeks after transmission of the virus (Figure 1.3), characterized by flu-like clinical appearances combined with plasma viremia and frequently fever and lymphadenopathy (Gurunathan S, 1997). Other reported symptoms are myalgias, skin rash, headache, anorexia, and diarrhea (Gurunathan S, 1997), although the severity of these clinical symptoms may vary greatly between individuals.

During the early phase, plasma viremia peaks as high as 10 million viral copies per milliliter and the virus replicates aggressively in the absence of an immune response (Piatak M Jr, 1993). This step is followed by a sharp drop in viral load as a consequence of HIV- specific $CD8^+$ T cells appearance (Helz et al., 2006) and it is known as viral set point. The rate of disease progression in HIV- infected patients is determined by the level of the viral set point, as individuals with low viral set point tend to display slower progression to AIDS (Mellors et al, 1996).

The acute phase of infection is accompanied by a sharp decline in the $CD4^+$ T cell levels in the peripheral blood, which may rebound as viral replication is contained. This depletion of $CD4^+$ T cells may be associated with the wide depletion of $CCR5^+$ memory $CD4^+$ T cells in the gut-associated lymphoid tissue (GALT) leading to prompt dysregulation of gut immunity (Mattapallil et al, 2005).

Acute infection is followed by a much longer chronic phase, in which turnover of $CD4^+$ T cells is enormous and there is a gradual drop of $CD4^+$ T cells in the periphery. The onset of AIDS appears when the $CD4^+$ T cell count declines below 200 cells/μl of blood (Stevenson et al., 2003). At this point, the immune system is unable to cope with infectious challenges, and the host ultimately surrenders to opportunistic infections.

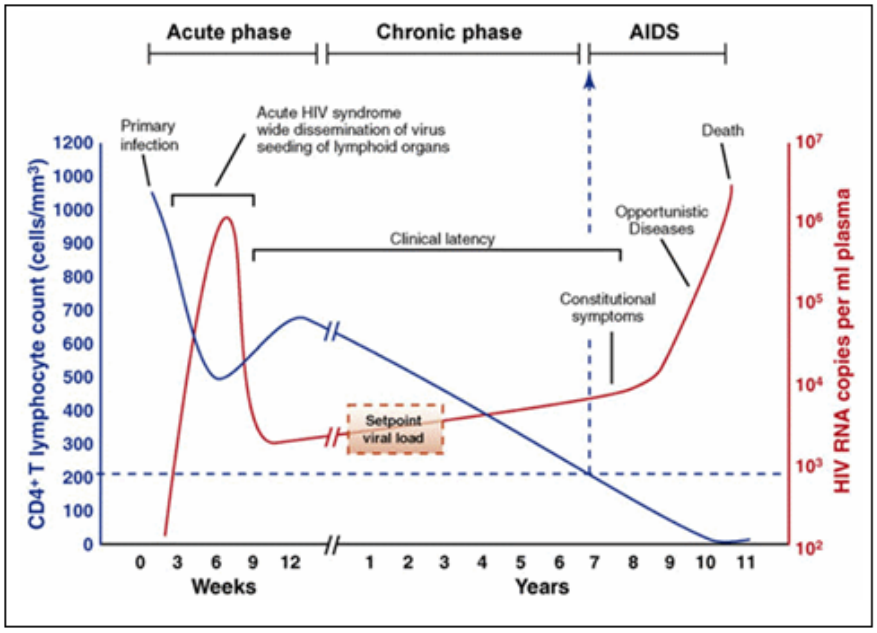


Figure 1.3. *Clinical and immunologic events associated with disease progression in untreated HIV-1 infection*

1.4. HIV immunology

1.4.1. Innate response to HIV infection

The innate immune system is the first line of defense against pathogens and consists of epithelial barriers, complement system, cells with phagocytotic and antigen presenting properties, such as granulocytes, macrophages, and dendritic cells (DCs) (Mogensen TH, 2009). Innate immune cells are capable to recognize the infection by pattern recognition receptors (PRRs) due to their ability to identify evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs).

Among PRRs, the family of Toll-like receptors (TLRs), with 10 different sub families has been identified. TLR1, 2, 4, 5, 6, and 10 are expressed at the cell surface and generally recognize hydrophobic molecules unique to microbes and not produced by the host. In the other hand, TLR3, 7, 8, and 9 are located almost only in endosomal compartments and are particular in recognition of nucleic acids (Medzhitov R, 2000). So in viral infections, the recognition is mediated by TLR9 recognizing DNA, as well as by TLRs 7, 8 and 3 detecting single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) respectively (Lund J, 2003).

Stimulation of TLR7, 8 induces the production of several antiviral and immunomodulatory cytokines. Recently, the cascade of cytokine production in the periphery has been thoroughly documented, showing an initial rapid increase in the level of interferon α (IFNα) followed by Interleukin- 15 (IL-15) and CXC-chemokine ligand 10 (CXCL10) which rise promptly but transiently. The other important cytokines like, IL-18, tumor necrosis factor α (TNFα), IFNγ and IL-22 also increase promptly and are sustained at high levels, whereas the increase in IL-10 is slightly delayed (Stacey, A. R. et al, 2009). The cellular sources of these cytokines include DCs, infected $CD4^{+}CCR5^{+}$ T cells, monocytes, macrophages and natural killer (NK) cells which are fundamental in shaping the immune responses that develop in acute or early HIV-1 infection (Cameron, M. J. et al. 2007).

IFNα acts to inhibit HIV replication at multiple stages in the viral lifecycle by different mechanisms including blocking the early steps of HIV replication, inhibition of HIV gene expression, and effects on viral assembly and budding (Beignon et al., 2005; Lee et al., 2006).

IFNα inhibits the replication of HIV through mechanisms mediated by the so called Interferon stimulated genes (ISG) such as ABOPEC3G, Mx2, OAS, IL8, IFNg ect. These effector molecules undermine viral replication at different levels inhibiting HIV gene expression, affecting viral nuclear import, assembly and budding. HIV-1 triggers the secretion of IFNα by activation of plasmacytoid dendritic cells (pDCs) via TLRs in acute HIV-infection (Beignon et al., 2005; Lee et al., 2006). Apolipoprotein B mRNA editing enzyme-catalytic polypeptide-like 3G (APOBEC3G), acts to restrict HIV replication in newly infected cells by editing C -> U in HIV DNA negative strand, introducing premature stop codons and inhibiting reverse transcription and chromosomal integration (Casartelli et al., 2010). HIV counters this form of host defence through virion infectivity factor (Vif), which targets APOBEC3G for proteasomal degradation. Howeve, it has been shown that IFNα is able to induce APOBEC3G expression by macrophages to overcome and abolish HIV Vif neutralization of APOBEC3G proteins and consequently decreases HIV replication markedly (Peng et al., 2006).

Acute HIV-1 infection is also characterized by the activation and expansion of NK cells which might be the result of high levels of proinflammatory cytokines such as IL-15 and IFNα secreted by DCs and monocytes. In particular, a significant increase of NK cells before the development of any detectable antibody responses has been detected in acute HIV-1 infection (Alter et al., 2007). However, along with viral replication and disease progression, the initial expansion of NK cells becomes gradually defective.

1.4.2. HIV-specific B cells and antibodies

B cells producing antibody mediate the humoral immune response. Virus specific antibodies play an essential role in the viral infection by preventing infections of the host cells (Burton DR, 2002). The humoral immune response is activated following uptake and digestion of viral proteins by DCs into small peptides. DCs present the digested antigen on major histocompatibility complex (MHC) II molecules to $CD4^+$ T helper cells (Th) which produce B cells stimulating cytokines including IL-4, IL-5, IL-6, IL-10, and transforming growth factor β (TGF−β) to activate naïve B cells. Latter, B cells recognize specific epitopes or intact virus through their surface IgM and differentiate into plasma cells to produce large amounts of IgG, IgA, IgE antibodies and memory B cells. During HIV-1 infection antibodies against gp120, gp41, the nucleocapsid (p24) and the matrix (p17) arise few weeks to several months after infection. This process is commonly referred to as seroconversion (Wei X et al. 2003).

The virus neutralization is characterized by the interaction of specific antibodies with the viral envelope spikes. This interferes with virus attachment or viral entry in target cells and results in the inhibition of infection. Only a minority of anti-HIV Env antibodies, at any time, exerts immune pressure by autologous neutralization. However, the virus easily mutates and readily escapes from these potentially protective immune responses (Wei X et al. 2003). During the chronic course of infection only 20% of the infected individuals generate broadly neutralizing antibodies (bNAbs) capable to neutralize heterologous viruses (Stamatatos L. et, al. 2009). In addition to classical neutralization, antibodies can attach to HIV infected cells and kill them via antibody dependent cellular cytotoxicity (ADCC) mediated through their Fc moiety and NK (Aasa-Chapman MMI, 2005) (Hessell AJ, 2007).

1.4.3. HIV-specific T cells

Another important arm of adaptive immune system is the cellular immune response which consists of T lymphocytes which can be divided into two main clusters: T helper (Th) CD4 T cells and Cytotoxic CD8 T cells (CTL).

Following their activation by antigens and co-stimulatory signals, $CD4^+$ Th cells can differentiate into various distinct subsets of effector Th cells such as Th1, Th2 and Th17,

Th1 cells usually produce Type 1 cytokines such as IFNγ, TNFa and IL-2 required for the clearance of intracellular pathogens; whereas Th2 cells produce IL-4, IL-5 and IL-13, which activate humoral immune response against extracellular pathogens (Wilson, C. B., Rowell, E. & Sekimata, M. 2009).

CTL exert a cytotoxic activity and can detect and eliminate infected cells in different ways. MHC I molecules present the intracellular antigens on the surface of infected cells which can be recognized by precursor cytotoxic $CD8^+$ T lymphocytes. Also, antigen presenting cells (APCs) can induce $CD4^+$ T cells to produce IL-2, IFN-γ, and TNF-α which activate and differentiate the CTLs into memory or effector CTLs. Later, by producing perforines and granzymes, effector CTLs can directly kill infected cells (McMichael AJ, 2001). Additionally, after interaction of Fas ligand on CTLs with Fas receptor on infected cells, CTLs can promote apoptosis of the infected cells (Gehri R, 1996). Moreover, CTLs can exhibit a non-cytotoxic antiviral function by producing several cytokines, chemokines and $CD8^+$ T cell antiviral factors (CAF) (Saksena NK, 2008) (Figure 1.4).

HIV-specific $CD8^+$ T cells appear within the first few weeks of infection as peak viremia drops, and correlate with the development of viral escape mutant, suggesting a key role for $CD8^+$ T cells in controlling viral replication. Supporting this hypothesis , viral replication in vitro can be inhibited by isolated $CD8^+$ T cells from HIV-infected individuals (Saez-Ciriòn et al., 2007), and deficiency in $CD8^+$ T cells response in Simian immunodeficiency viruses

(SIV)-infected macaques results in a loss of viral control and rapid disease progression (Jin X et al., 1999) (Figure 1.4).

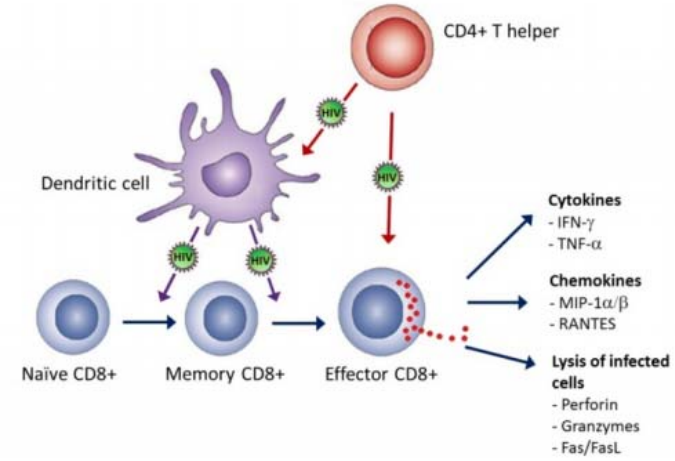


Figure 1.4. *HIV-induced T cell responses. HIV specific CD8+ effector cells produce chemokines and cytokines in order to eliminate infected cells. CD4+ T helper cells help to stimulate both dendritic cells and CD8+ T-cells to maintain a CD8+ T-cell memory response. HIV interferes with this supportive function of CD4+ T-cells. (Winni De Haes et al. 2012).*

The earliest $CD8^+$ T cell are generally Env- and Nef-specific while responses to other viral proteins, including Gag and Pol usually appear with viral control later in infection (Turnbull, E. L. et al, 2009). HIV infects and depletes $CD4^+$ T cells and HIV- specific memory cells, however strong HIV- specific $CD4^+$ T cell responses can be rescued by early administration of Anti-Retroviral Therapy (ART) (Rychert et al., 2007).

HIV-1 can escape from the host $CD8^+$ T cell recognition because of ongoing viral recombination and mutations. The continuous mutation in HIV-1 is the major obstacle to vaccine design and determines the big viral variety in a single HIV-1-infected individual as well as the significant sequence variation in circulating viral strains at the population level. However, it has been proposed that the capability of HIV-1 to escape immune response is limited and has some cost for the virus (Goepfert PA et al. 2008).

Exposure of HIV-specific T cells to persistent viremia leads to functional exhaustion during chronic infection. T cell exhaustion is a state of T cell

dysfunction defined by loss of proliferation capacity besides polyfunction and cytotoxic ability, followed by apoptosis (Wherry EJ, 2004). Up-regulation of different inhibitory molecules on the cell surface is the main feature of functional exhaustion, including programmed death 1 (PD-1), Cytotoxic T-Lymphocyte Antigen (CTLA)-4, lymphocyte activation gene (LAG)-3, T cell immunoglobulin domain and mucin domain (Tim)-3, 2B4 (CD244) and CD160 (Wherry 2011). PD-1 has been identified as a critical negative regulator in HIV infection (Zhang et al., 2007), and Tim-3 has been linked to elevated sensitivity to Treg-mediated suppression (Elahi et al., 2011).

1.5. Models of Natural Protection

1.5.1. HIV-exposed seronegative individuals (HESN)

Frequent and multiple exposures to the HIV do not necessarily result in HIV infection (Figure 1.5). Approximately 15% of HIV exposed seronegative individuals repeatedly resist infection, a phenomenon that has been observed in all investigated HIV-exposed cohorts. Despite this awareness, during the first years after AIDS discovery, little consideration was paid to the observation that mucosal exposure to HIV-1 would not consistently lead to infection, and none to the possibility that such putative noninfectious exposures might be secondary to inheritable factors (Shearer G, Clerici M, 2010).

Initial AIDS epidemiologic studies from the UCLA Multicenter AIDS Cohort Study (MACS) (Inagawa DT, 1989) demonstrated that, frequent unprotected sexual contacts in a limited percentage of homosexual men, did not automatically result in HIV sereoconversion.

Moreover, presence of a differential susceptibility to HIV infection was reported in a subset of repeatedly exposed female sex workers in Kenya (Plummer F, 19993) (Fowke KR, 1996). Interestingly, HIV-specific T-helper cell responses were shown to be present in these repeatedly exposed

seronegative individuals, suggesting immunologic and/or genetic protection (Clerici M et al. 1992).

Resistance to HIV infection has been subsequently described in intravenous drug users, healthcare workers accidentally exposed to HIV, homosexuals or heterosexual subjects that have unprotected sex with their seropositive partners and hemophiliacs who had received HIV-contaminated anticlotting factors (Miyazawa M, 2009). A particularly interesting cohort is represented by uninfected newborns of HIV-infected mothers. These infants are exclusive in that most of them are uninfected at birth (65–75%), despite having been continuously exposed to a HIV saturated environment during pregnancy; and are HIV seropositive, due to passively transferred maternal anti-HIV antibodies.

At the beginning HIV-exposed uninfected individuals were referred to as exposed seronegatives (ESNs), exposed uninfected or multiple exposed uninfected (MEU). Since the early 1990s, numerous research groups have studied such cohorts of ESN to define immunological and/or genetic correlates of protection to HIV infection, but none of the identified mechanisms have so far been able to completely explain this phenomenon. Furthermore, efforts to compare results collected in different cohorts of ESN and in diverse laboratories have been problematic for many reasons, including the lack of clear definition of who should be classified as ESN (Biasin M, 2013).

The first International Symposium on Natural Immunity to HIV held in Winnipeg, Canada was organized by the main investigators who had contributed to define the ESN/exposed uninfected phenotype in November 2009. The most important purpose was to definitely lay out the inclusion criteria of the ESN individuals and to compare the results of studies performed on different ESN cohorts. Later it was followed by a second meeting in June 2010 in Rockville, MD during which the decision was made to refer to these individuals as HIV-exposed seronegative individuals (HESN)

(Young JM et al, 2011). More recently, (October 2014) a new meeting held in Cape Town, South Africa enlightened that while several HESN cohorts have been identified worldwide and are currently being studied by different groups, key information is still lacking. For instance, the relative contribution of genetic and immune factors to determine the phenomenon of resistance to HIV is not yet clarified. Notably, the possibility to study the early dynamic of this phenomenon is inhibited by the definition of the resistance phenotype itself. In fact, the HESN status is assigned only after an individual has been able to efficaciously resist infection – typically over a period of years – despite multiple, frequent and repeated documented exposures to the virus. Thus, researchers can only observe and compare the consequences of potentially important immunological changes that probably occurred years before the HESN status could be defined (Biasin M, 2013). Even so, if a genetic profile is at least partially responsible for this phenomenon it should be possible to identify this/these factor(s) at any time after exposure. Furthermore, once a putative protective variant gene is associated to the resistant phenotype, analysis can be easily performed using ex vivo infection assays on cells isolated from healthy controls carrying that specific mutation; it is therefore worthwhile to focusing energy in this research field.

1.5.2 Genetic correlates of protection

There is a considerable component of heredity in susceptibility to HIV-1 infection. The first convincing evidence of this assumption dates back to 1996 when it was discovered that individuals homozygous for the allelicΔ32 variant of the chemokine receptor CCR5, the main coreceptor for R5-HIV-1 viruses, show a strong, although not absolute, protection to sexually transmitted HIV-1 infection (Piacentini, L. 2009) (Poropatich K & Sullivan DJ Jr. 2011). Based on this observation new anti-HIV drugs that block virus entry have been developed, and in 2009 a patient apparently recovered fromHIV-1 infection following transplantation of CCR5 Δ32/Δ32 stem cells

(Walker BD, 2007), thus pointing out the possibility of translating genetic research on HIV into effective therapies.

More recently, Draenert showed that, whereas monozygotic twins infected with the same viral strain are characterized by a similar rate of progression, time to AIDS differs if infection with the same strain occurs in fraternal twins (Clerici M, 1992). These observations encouraged the search for host mutant restriction factors that could influence susceptibility and resistance to HIV-1. As a consequence, the scientific community is investing substantial resources to genetically characterize individuals with non-classical responses to HIV disease. The two main criteria adopted to identify genetic variants responsible for HIV-infection include: candidate gene approach and genome wide studies (GWAs).

1.5.2.1. Candidate gene for protection

Candidate gene studies indicate a first knowledge of the gene function in HIV-1 pathogenesis (Shea et al., 2012) and consider only variants in those genes known to be directly implicated in the HIV-1 life cycle or in the immune response to the virus, thus excluding still unidentified genetic factors potentially able to interfere with HIV-infection. So far, the most AIDS restriction genes have been identified within the human leukocyte antigen (HLA) class I locus (An et al., 2010) (Figure 1.5). For instance, the protective effect of HLAB57 has been correlated with the ability of HLAB57-restricted CTLs to target multiple HIV peptides. Indeed, the expression of HLAB*27 has been illustrated to utilize a protective effect by preserving a high steadiness between the HLA B pocket and the arginine in position 2 of the gag peptide. In fact, mutations in this position lead to both instability of the B27-gag complex and faster progression to AIDS (Kelleher et al., 2007).

Recently, the association between HESN phenotype and the endoplasmic reticulum aminopeptidases 2 (ERAP2), another gene involved in the antigen presentation pathway, has been found (Cagliani et al., 2010). ERAP2 has

been target of long-standing balancing selection (Andrés AM, et al, 2010) (Cagliani R et al, 2010). In particular, this process has maintained two highly differentiated ERAP2 haplotypes at intermediate frequency in most human populations (Andres AM et al, 2010) (Cagliani R et al, 2010). The two haplotypes, referred to as HapA and HapB, differ at multiple variants in tight linkage disequilibrium. HapB harbours the T allele for rs2549782 (Asn392Lys) and the G allele for rs2248374; this latter has been shown to determine the activation of a cryptic splice site in intron 10 and the production of an alternatively spliced ERAP2 mRNA (ERAP2-AS) with an in-frame stop codon (Cagliani R et al, 2010) (Coulombe-Huntington J, 2009). Thus, the predicted protein product of HapB-derived transcripts is a truncated protein of 534 amino acids, whereas the canonical ERAP2 protein, consisting of 960 amino acids, is encoded by a full-length mRNA (ERAP2-FL) transcribed from HapA.

The G allele of rs2549782, which tags HapA, was found to be significantly over-represented in HESN, indicating that association between rs2549782 and HIV-1 protection follows a recessive model (Andres AM et al, 2010). Furthermore, genotype analysis in 104 HESN exposed to HIV through injection drug use (IDU-HESN) and 130 controls from Spain indicated that hapA protects from HIV infection. Meta-analysis with an Italian cohort of sexually-exposed HESN yielded a *p* value of 7.6×10^{-5}. *HLAB* typing indicated that the *HLA-B*57* allele is significantly more common than expected among HESN homozygous for haplotype A (homoA). Data obtained in a cohort of 139 healthy Italian controls showed that following in vitro HIV-1 infection the expression of ERAP2-FL and a number of genes involved in antigen presentation as well as of MHC class I on the surface of CD45+ cells was significantly increased in homoA cells; notably, homoA PBMC, but not isolated CD4+ cells, were less susceptible to HIV-1 infection (Biasin M et al, 2013). The data collected so far seem to validate the hypothesis that this variant is able to generate a peptide repertoire

qualitatively and/or quantitatively different, which in turn is able to confer natural resistance to infection.

Genetic studies also have established the association between NK activation and resistance to HIV infection. A higher frequency of inhibitory killer cell immunoglobulin–like receptor (KIR) genes in the absence of their associated HLA genes has been found in African HESN sex workers (Jennes et al. 2006). Some studies showed that NK activation exerts a defensive effect in HIV infection which derives also from studies on the activating receptor allele KIR3DS1. Indeed, an increase of homozygosis for this receptor in HESN compared to HIV-infected patients has been found (Guerini et al., 2011), while there is a strong protection in the presence of this allele in parallel with its presumed HLAB ligand(s) (Carrington et al., 2008).

Furthermore, HESN phenotype is associated with another molecular family including chemokines and chemokine receptors. Analysis of the relationship between CCL3L1 copy number and susceptibility to HIV-1 infection illustrated that CCL3L1 levels are inversely corresponded with CCR5 expression on $CD4^+$ T lymphocytes (Gonzalez et al., 2005). Additionally, the increase median copy number of CCL3L1 and the CCL3L1/CCL3 mRNA ratios has been detected in Human T-lymphotropic virus (HTLV)-2-infected individuals in HESN and Long Term Non Progressor (LTNP) compared to those seen in HIV-unexposed healthy controls (Pilotti E. et al, 2007).

The other protective factor toward HIV-1 infection is new genotypes at chromosome 22q12-13 (Kanari Y, 2005) and it has been shown that the APOBEC3 locus is present in the middle of this chromosome. The APOBEC3 locus includes 7 members (A, B, C, DE, F, G and H) which display different rate of anti-HIV activity. Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (A3G) (APOBEC3G) provokes G to A mutation on viral genome (Ross SR, 2009). Despite the fact that the HIV Vif protein can prevent A3G, data suggest that A3G may play a role in altering the natural course of HIV infection.

Among the other members of APOBEC family, one of APOBEC3H haplotype, Hap I is associated with protection from sexually transmitted HIV-1 infection. Hap I carries a protein-destabilizing variant as well as a residue conferring resistance to Vif-mediated degradation (Cagliani et al., 2011).

Candidate gene approaches have also been used to investigate polymorphisms in genes directly involved in the immune response to the virus. More remarkable results stemmed from the study of TLRs for bacteria and viruses. Interestingly, stimulation of PBMCs with agonists specific for TLR3 (poly I: C), TLR4 lipopolysaccharide (LPS), TLR7 (imiquimod), and TLR7/8 (ssRNA40) resulted in higher cell responsiveness in HESN compared to healthy controls (Biasin et al., 2010). The activation of TLR pathway in HESN has recently been associated to an increased expression of the full-length form of the adaptor protein MyD88, which acts as an intracellular negative regulator of this pathway. Furthermore, recent data obtained in two independent cohorts of HESN showed that a common TLR3 allele (rs3775291, Leu412Phe) confers immunologically-mediated protection from HIV-1 infection (Sironi M, et al, 2012) (Figure. 1.5).

More recently other genes involved in HIV-1 infection and replication have been considered in association with resistance to HIV-1 infection; among these MX2 and TIM-3 exhibited interesting results.

MX2 (myxovirus resistance 2) interferes with viral replication thus decreasing nuclear accumulation and integration of viral DNA. Mutations in the HIV-1 capsid protein, which is involved in nuclear import, can overcome the inhibition of MX2. Therefore it has been hypothesized that MX2 targets the viral capsid and that it interacts with other cellular proteins - such as Cyclofilin A - which block the nuclear import of HIV-1 (Haller O, 2013; Kane M, 2013).

Population studies have shown that natural selection operated on MX2 during recent human history: different selective events drove the frequency increase of two haplotypes in Asians and Europeans. The European

haplotype is tagged by rs2074560. Analyses performed on three independent cohorts of HESN showed that the ancestral allele of rs2074560 protects from HIV-1 infection with a recessive effect (combined p value= 1.55x10-4). The same allele increases MX2 expression levels in response to IFN-α. On the whole, this data exploit evolutionary information to identify a novel host determinant of HIV-1 infection susceptibility infection regardless of the transmission route (Sironi M et al, 2014).

TIM-3 (T cell immunoglobulin and mucin domain-containing molecule 3) is an immunoglobulin superfamily member encoded in humans by the HAVCR2 (hepatitis A virus cellular receptor 2) gene. Initially identified as a marker of IFN-γ-producing CD4+ Th1 and CD8+ Tc1 cells (Monney L et al, 2002), TIM-3 was more recently shown to be expressed by several other immune cell types including NK/NTK, macrophages/monocytes, and dendritic cells (Freeman GJ, 2010). The protein product of HAVCR2 can bind both galectin 9 (Gal-9) and phosphatidylserine (Koguchi K et al, 2006). Engagement of TIM-3 by Gal-9 on T cells induces cell death and promotes peripheral tolerance (Freeman GJ, 2010). Thus, TIM-3 plays an important role in the negative regulation of T-cell mediated responses, and abrogation of its signaling increases the secretion of IFN-γ by activated human T cells (Koguchi K et al, 2006). Recent evidences have indicated that expression of TIM-3 marks a population of exhausted CD8+ T cells during chronic viral infection (Freeman GJ, 2010). Specifically, in progressive HIV-1 infection TIM-3 defines an abundant population of CD8+ T cells and its expression correlates positively with viral load and inversely with CD4+ T cell counts (Jones RB et al, 2008). The loss of proliferative activity of HIV-specific TIM-3-expressing CD8+ cells is partially mediated by the interaction with Gal-9 on Treg cells and is modulated by HLA-B allelic status (Elahi S et al, 2011).

In a previous study we have reported that a variant located in the 3′UTR of HAVCR2 (rs4704846) has been a target of natural selection in human populations and suggested that the selective pressure is accounted for by

infectious agents (Forni D et al, 2013). In line with this view, a SNP (rs3087616) located 62 bp apart and in full linkage disequilibrium with rs4704846 (r2 = 1 in Europeans) has recently been shown to act as an expression QTL (eQTL) in CD14+ monocytes (Fairfax BP et al, 2014).

Furthermore, genotyping rs4704846 in three independent cohorts of HIV-1 exposed seronegative (HESN) individuals with different geographic origin (Italy and Spain) and distinct route of exposure to HIV-1 (sexual and injection drug use) the minor G allele at rs4704846 was more common in HIV-1 infected individuals than in HESN, with healthy controls showing intermediate frequency. Combination of the three association analyses through a random effect meta-analysis revealed no heterogeneity among samples (Cochrane's Q, p value = 0.89, I2 = 0) and yielded a p value of 6.8 ×10−4. The minor G allele at rs4704846 was found to increase HAVCR2 expression after in vitro HIV-1 infection. Thus, a positively selected polymorphism in the 3′ UTR, which modulates HAVCR2 expression, is associated with the susceptibility to HIV-1 infection (Sironi M et al, 2012).

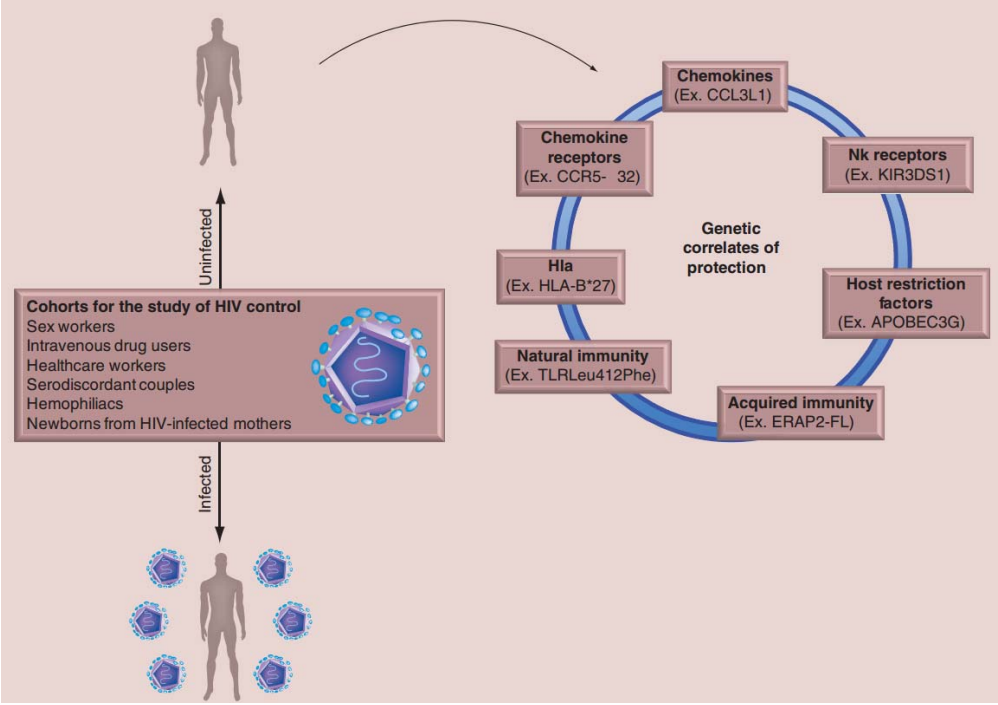


Figure 1.5. *Different cohorts that control HIV infection are used to assess the impact of hereditable factors on HIV-1 infection and/or progression. One of the genetic correlates of protection to HIV infection so far identified by the candidate gene approach is summarized herein ((Biasin M, 2013).*

1.5.3 Immune correlates of protection

Innate immunity is likely to contribute to protection upon primary exposure to HIV in HESN before adaptive responses have developed.

Accumulating functional evidence supports a role for NK activation in resistance to HIV infection (Montoya et al., 2006). Additionally, TLR stimulation of Peripheral Blood Mononuclear Cells (PBMCs) with TLR agonists resulted in higher cell responsiveness in HESN as demonstrated by elevated production of proinflammatory cytokines and chemokines (Biasin at al., 2010).

Secreted factors have also been associated with reducing susceptibility to mucosal transmission of HIV. The β-chemokines macrophages inflammatory protein (MIP)-1α, MIP-1β and RANTES bind the HIV co-receptor CCR5,

thereby reducing HIV infection of target cells by outcompeting the virus for receptor usage. Peripheral production of β-chemokines was associated with resistance to HIV-1 infection (Tomescu et al., 2011), and elevated salivary of β-chemokines were associated with oral sexual behavior in HESN MSM (Hasselrot et al., 2010). Elevated IL-22 has also been associated with resistance to infection in HESN, and is thought to act by induction of acute–phase serum amyloid A, which downregulates CCR5 expression on target cells (Missè et al., 2007).

Multiple lines of evidences suggest that HIV-specific T cell responses are associated with resistance against HIV infection. HIV-specific T cells have been detected in PBMCs from commercial sex workers (CSW), men that have sex with men (MSM) discordant couples, occupationally exposed healthy care workers, injection drug users-HIV exposed seronegative (IDU-HESN) and prenatally exposed infants. Both $CD4^+$ and $CD8^+$T cell responses have been linked to HIV infection in HESN. HIV-specific CTLs have been described in several different HESN cohorts and many authors claim that these cells make a fundamental contribution at modulating resistance to HIV infection. HIV-specific CTLs have been observed and characterized in the Pumwani Kenyan cohort of sex workers both at systemic and mucosal levels; in injecting drug users; and in sexual partners of HIV-infected patients. These studies analyzed approximately 100 HESN individuals; HIV-specific CTLs were observed in the majority (>70%) of such HESN. The detection of HIV-specific CTLs in HESN individuals raises important scientific questions: only the successful infection of host cells, that is, infection resulting in at least one complete cycle of viral replication, allows the effective presentation of viral peptides within a binary complex with a human leukocyte antigen (HLA) class I molecule. The detection of HIV-specific CTLs in HESN individuals thus seems to indicate that HIV has managed to infect the host, but that its further propagation has been contained by immune mechanisms. The recent description of an alternative

mechanism of processing and presentation by HLA class I molecules of exogenous antigens known as cross-priming could, nevertheless, explain the presence of CTLs in HESN individuals in the absence of actual infection. According to this mechanism, dendritic cells can process the virus and present it to CTL precursors in the absence of viral replication. If this were the case, the presence of HIV specific CTLs in HESN individuals would be the consequence, not necessarily of an infection, but of a different and presumably more efficient processing pathway of HIV antigens within dendritic cells.

In particular, HIV-specific $CD8^+$ CTLs of the HESN individuals recognize HIV epitopes that are different from those recognized by cells of HIV-infected patients 32 and Gag-stimulated CD8 T lymphocytes isolated from HESN individuals are characterized by higher levels of intracellular perforin and granzymes than those isolated from the HIV-infected partners (n ¼30 HESN individuals studied) (Miyazawa et al., 2009, KAUL).

HIV-specific IgA have been isolated from the female genital tract (FGT) of HESN CSW as well as uninfected partners of discordant couples (Devito et al., 2002). However, similar antibodies were not detected in Gambian HESN CSW cohort and in Pumwany cohort there were HIV-specific IgA but no association was found between HIV-specific IgA and virus neutralization or shedding (Horton et al., 2009).

1.6. Non- coding RNAs (ncRNAs)

The term non-coding RNAs (ncRNAs) is usually used for RNAs that do not encode a protein, however this does not mean that such RNAs do not contain information nor have function, in fact they are molecules with biological relevant role. In prokaryotes, ncRNAs demonstrate only small proportion of the whole genome, which is generally represented by protein-coding sequence accounting for 80-95% of it (Mattick, J.S. 2004). In contrast, only a minority of the genome is occupied by protein- coding sequences in multicellular organisms. Indeed the proportion of ncRNAs rises with increasing complexity with an accompanying growth of the number of non-coding intergenic and intronic sequences, most of which are absolutely transcribed (Frith, M.C., 2005). These differences suggest the presence of a revolutionary shift in transcriptional output between micro and multicellular organisms from mainly protein-coding mRNAs to mainly non-coding RNAs (Mattick, J.S. 2006). As reported by the International Human Genome Sequencing Consortium, in the human genome the number of the protein-coding genes represents a range of 20.000- 25000 and corresponds to only 2% of the whole genome (International Human Genome Sequencing Consortium, 2004) while, the remaining of the human genome, previously assumed not to be functional and referred as “junk DNA”, has been recently classified as ncRNA (Calore F, 2013).

NcRNAs can be classified as small (~18–31 nt), medium (~31–200 nt) and long (from 200 nt up to several hundred kb) transcripts based on their size. The small ncRNAs group includes small interfering RNAs (siRNAs), microRNAs (miRNAs, miRs) and Piwi interacting RNAs (piRNAs), while medium ncRNAs represented by small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). Very little is known about the long non-coding RNA (lncRNA), which are not highly conserved at the primary sequence level but they have been estimated to be ~17,000 in human and ~10,000 in mouse genome.

Moreover, ncRNAs can be functionally divided into housekeeping ncRNAs such as ribosomal, transfer, snRNAs and snoRNAs which play essential roles in many cellular processes, and regulatory ncRNAs such as miRNAs, piRNAs, siRNAs and lncRNAs (Da Sacco L, 2012).

1.7. MicroRNAs

1.7.1. History of MicroRNAs

Historically, the small RNA revolution started with the detecting of an unexplained silencing phenomenon in floral pigmentation (J. Davidson-Moncada, 2010). The phenomenon was described “co-suppression” at the time, and it is the first example of what is now known as RNA interference but the molecular mechanism behind it remained unclear (Romano N, 1992). In 1993, the field of miRNA began with the identification in *Caenorhabditis elegans* of the *lin-4* microRNA gene by Victor Ambros and his colleagues. The authors reported that lin-4, a small RNA hitherto unknown as an miRNA, down-regulate expression of lin-14 by antisense complementarity to the 3’ untranslated regions (3’UTRs) of lin-14 (Cullen BR, 2004). This kind of RNA was then demonstrated not to be an isolated factor limited to nematodes, but rather a member of a large family of small regulatory RNA that is extensively expressed in many species. Actually, as reported by the microRNA database in 2012 (miRBase, www.miRbase.org), 2042 and 1281 mature microRNAs are documented in humans and in mice respectively (Kozomara A, 2011).

MicroRNAs are post-transcriptional regulators of gene expression that function by inhibiting translation of mRNAs (Bartel DP, 2004) (Berezikov E, 2011). They are endogenously encoded single stranded RNAs of 18-22 nucleotides in length that inhibit mRNA translation through imperfect base-pairing with sequences, which are generally located in the 3’UTR of mRNA transcript (Kim VN, 2006).

1.7.2. Genomics organization of microRNA

MicroRNA genes are scattered in all chromosomes in humans except for the Y chromosome. Most of them are transcribed independently from other neighboring genes so they are located in the intergenic regions of the genome. These microRNAs can be organized as monocistronic and possess their own promoter, or polycistronic (clustered), with a shared promoter. (Iorio MV, 2012).

MicroRNAs are also found in the introns of annotated genes, both protein-coding and non-coding. Similarly to the intergenic ones, also these microRNAs can be present as monocistronic or polycistronic and their expression is strictly linked to the transcription of the gene from which they originate. Also, some microRNAs proceed from spliced-out introns, and are basically equal to the pre-microRNAs and they are therefore called mirtron. They often overlap an exon and an intron of a non-coding gene and their maturation often excludes host gene function (Olena AF, 2010).

1.7.3. MicroRNA classification and nomenclature

MiRNA genes compose one of the most numerous gene families, and are extensively distributed in animals, plants and viruses. The latest release of the miRNA database (miRBase, www.miRbase.org) has classified 1881 precursors and 2588 mature miRNAs in humans, although the functional importance of many of these miRNA remains to be determine (Cammaerts S. et al. 2015).

MicroRNAs that possess identical sequences at nucleotides 2-8 of the mature form generally belong to the same “microRNA family”, for example the let-7 family, composed of 14 paralogs loci (microRNA sisters) (Bartel DP, 2009).

The nomenclature of miRNA genes is somewhat inconsistent. The genes detected in initial genetic studies were named after their phenotypes (for example, *lin-4*, *let-7* and *lsy-6*), whilst numerical names were given to most

miRNAs found from cloning or sequencing (for example, the *lin-4* homologues in other species are called *mir-125*). Genes encoding miRNA sisters (paralogs) are illustrated with lettered suffixes (for example, *mir-125a* and *mir-125b*). If the same mature miRNA is developed from multiple discrete loci, numeric suffixes are added at the end of the names of the miRNA loci (for example, *mir-125b-1* and *mir-125b-2*). Each locus generates two mature miRNAs: one from the 5′ strand and one from the 3′ strand of the precursor, designed as -5p and -3p respectively (for example, miR-223a-5p and miR-223a-3p). However, one arm (called the 'guide' strand) is usually much more prevalent (96–99% of the sum on average) and more biologically active than the other arm (the 'passenger' strand, which is known as miRNA*) (Ha M, 2014). Moreover, mature sequences and precursor hairpins differ by labeling with “miR” and “mir” respectively.

1.7.4. Biogenesis of MicroRNAs

MicroRNA genes are commonly transcribed from RNA polymerase II promoters and later processed into mature miRNAs through canonical or non-canonical miRNA biogenesis pathways (Figure 1.5). During canonical miRNA biogenesis, the primary miRNA (pri-miRNA) hairpin is digested to precursor miRNA (pre-miRNA) by Drosha, a member of the RNase III family. Non-canonical miRNA biogenesis varies at this step that pre-miRNAs are developed by mRNA splicing machinery, avoiding the requirement for Drosha-mediated digestion in the nucleus. In both pathways, the pre-miRNAs are exported to the cytoplasm via the nuclear export protein exportin 5 and further processed by a second RNase III enzyme, Dicer (Ha M and Kim VN, 2014).

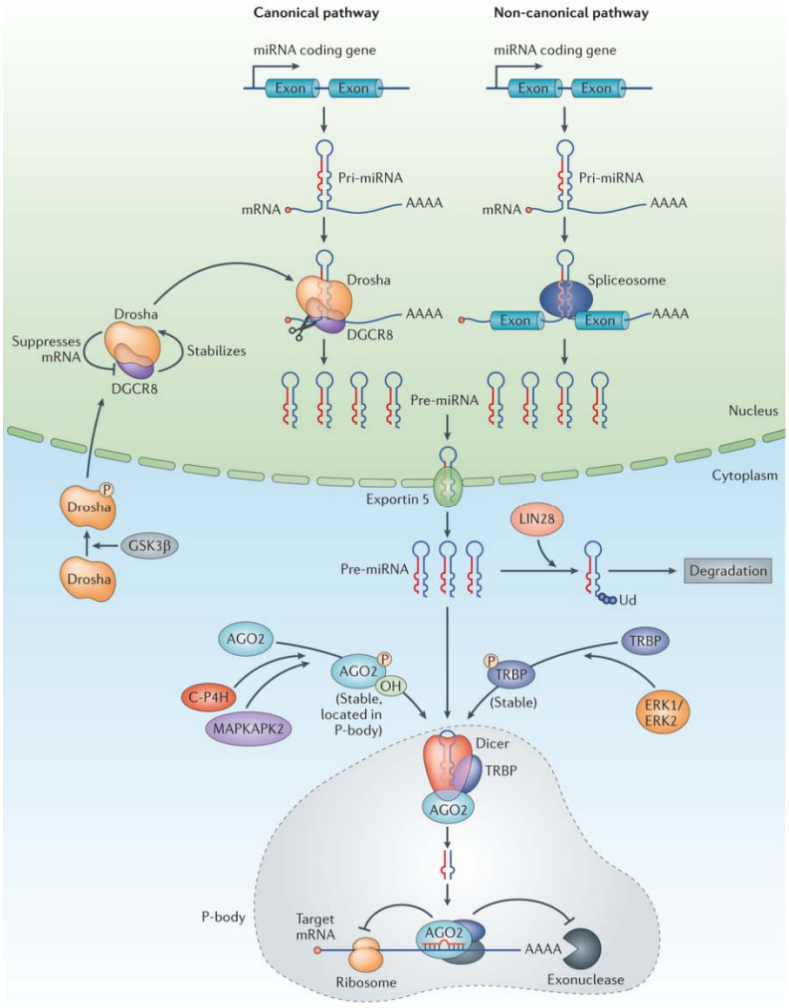


Figure 1.5: Canonical and non-canonical miRNA biogenesis pathways. *In the canonical pathway, microRNAs (miRNAs) are typically transcribed by RNA polymerase II to produce primary miRNA (pri-miRNA) hairpins, which are then processed by the Drosha–DGCR8 (DiGeorge syndrome critical region 8) complex to generate precursor miRNAs (pre-miRNAs). These molecules are transported by exportin 5 into the cytoplasm, where they are further processed by Dicer–TRBP (TAR RNA-binding protein 2) and loaded into Argonaute 2 (AGO2)-containing RNA-induced silencing complexes (RISCs) to suppress downstream target gene expression. miRNAs are also produced though non-canonical pathways, such as spliceosome-dependent mechanisms, as shown here. The miRNA biogenesis pathway is a tightly regulated process. For example, Drosha is dependent on phosphorylation by glycogen synthase kinase 3β (GSK3β) for proper nuclear localization; Drosha regulates DGCR8 expression by suppressing DGCR8 mRNA; DGCR8 stabilizes Drosha protein; AGO2 is hydroxylated by C-P4H and phosphorylated by MAPK-activated protein kinase 2 (MAPKAPK2), which stabilizes the protein and regulates its localization to processing bodies (P-bodies); and TRBP is stabilized by extracellular signal-regulated kinase 1 (ERK1) or ERK2 phosphorylation. miRNAs themselves are regulated by a number of modifications, including uridylation (Ud).(Zhonghan Li & TarqM Rana, 2014, Nat rev).*

1.7.4.1. Canonical Biogenesis of microRNAs

1.7.4.1.1. MiRNA Transcription

RNA polymerases II (pol II) and pol III are the two candidates for long primary transcript (pri-microRNA) transcription. Pol II produces the mRNAs and some noncoding RNAs, including the snoRNAs and four of the small nuclear RNAs (snRNAs) of the spliceosome, whereas pol III produces some of the shorter noncoding RNAs, including tRNAs, 5S ribosomal RNA, and the U6 snRNA (Ohler et al.2004). Pri-microRNAs are transcribed from non-coding regions of the genome by RNA pol II and hold the usual properties of class II genes transcripts, a 5́ m^7 G capping structure and 3́ poly (A) tails. They can be quite long, more than one 1 kb, thus longer than typical pol III transcripts (Lee Y, 2004).

The nuclear Ribonuclease III, Drosha, and the double-stranded RNA-binding domain protein DGCR8 (DiGeorge syndrome critical region protein 8) cleave the pri-miRNA and liberate a 60–70 nt stem loop intermediate, known as the miRNA precursor, or the pre-miRNA (Lee et al., 2002; Zeng Cullen, 2003).

1.7.4.1.2. Nuclear export

Exportin 5 (Exp5) a member of karyopherin family of nucleocytoplasmic transport factors, can recognize the pre-microRNAs and moreover, it serves as a nuclear export factor and is able to protect the pre-microRNA from digestion by nucleases (Yi R, 2003).

The exportins possess a RanGTP-binding site which circulate between nucleus and cytoplasm, and transfer cargo molecules from one side of the nuclear to the other. Exportins bind their cargoes at high RanGTP levels in the nucleus (Fornerod et al. 1997) and translocate as trimeric RanGTP-exportin-cargo complexes to the cytoplasm, where cargo and Ran are released upon GTP hydrolysis. The exportins can then return to the nuclear

compartment and mediate another round of transport ((Mattaj and Englmeier 1998). The presence of a >14-nt stem region along with a short 3′ overhang (1-8 nt) is a crucial requirement for pre-microRNA to be recognized by Exp5 (Gwizdek C, 2003).

Once in the cytoplasm, the pre-miRNA hairpin is further processed by Dicer, another type III RNase that produces a ~22-nucleotide miRNA duplex consisting of a 5p–3p strand. This final processing by Dicer is coupled to assembly of one of the two strands of the miRNA into the RNA-induced silencing complex (RISC) (Alwin Köhler, 2007).

1.7.4.1.3. Guide strands and passenger strands

Initially, it was proposed that one strand of RNA duplex remains in association with Argonaute proteins (Ago), the components of miRISC, as a mature miRNA (the guide strand or miRNA), whereas the other strand (the passenger strand or miRNA*) is degraded. But, later the importance of passenger strands was shown in some studies thus suggesting that the passenger strand can be actively incorporated into the miRISC and works as well. Therefore, it is not so accurate to use the name 'passenger strand or miR*' in these cases and it would be preferable to use a nomenclature rule which is based on the 5p–3p system since that does not prefer one strand over the other. Indeed, for many miRNAs, both strands are readily detected. Expression profiling shows that in some tissues, both strands can be equally abundant, whereas in other tissues there is a strong preference for one of the strands (Meijer HA, 2014). The exact mechanism for strand selection is not fully understood. Studies demonstrate that the relative thermodynamic stability of the two ends of the duplex indicates which strand is to be chosen. The strand with relatively unstable base pairs at the 5′ end usually remains in the miRISC (for example, G:U pair versus G:C pair) (Kim VN, 2005).

1.7.4.1.4. Assembly of MicroRNAs into miRISC complex (RLC)

Following the Dicer processing, mature microRNAs are incorporated into effector complexes known as 'miRNP' (miRNA-containing ribonucleoprotein complex), 'mirgonaute' or 'miRISC' (miRNA-containing RNA-induced silencing complex) (Kim VN, 2005).

The miRISC complex contains dsRNA binding proteins, including protein kinase RNA activator (PACT), transactivation response RNA binding protein (TRBP), Dicer and Argonaute (Ago) (Andrew D. Redfern et al, 2013). The main component of miRISC complex is Ago protein but all the other proteins were illustrated to be involved in strand selection. Four different Ago proteins have been identified and each of them is able to bind endogenous microRNAs, but only Ago2 exerts endonuclease activity to cleave complementary target mRNA sequences (Meister G, 2013). After micro RNA is loaded into the miRISC complex, the 2 – 8 nucleotides of the microRNA consist of the seed sequence recruit Ago protein to target mRNAs. If the base-pairing between miRNA and the 3′ UTR of the target mRNA is perfect the messenger is cleaved and degraded, whereas imperfect complementarity will result in translational silencing without mRNA degradation (Calore F, 2013).

1.7.4.2. Non-canonical Biogenesis of microRNAs

1.7.4.2.1. Drosha-independent pathways

The mirton pathway is the most important Drosha-independent microRNA biogenesis mechanism. The distribution of intron length has a sharp peak at around 60 nt in Drosophila and *Caenorhabditis elegans*, which is comparable to the size of pre-miRNAs. These Drosophila small RNAs originate from pre-miRNA-sized short introns with hairpin potential and termed "mirtrons". They can be spliced and disbranched into pre-miRNA

hairpins that are applicable for Dicer cleavage, thus bypassing the Microprocessor (Keita Miyoshi, 2010).

The first spliced intron product is not linear, but instead it is a lariat in which the 3' branch point is ligated to the 5' end of the intron. However, following resolution of this structure by lariat debranching enzyme (LDBR), the intron can adopt a pre-miRNA fold and be exported to the cytoplasm via Exportin-5 and later cleaved by Dicer to load into Ago for target regulation (Jakub O, 2011).

1.7.4.2.2. Dicer-independent pathways

Recently functional microRNAs with ability to bypass dicer activity have been discovered. Processing of the pre-miR-451 homologs from human, mouse, and zebrafish, has been shown to be independent of Dicer cleavage and occur by Ago2 slicer catalytic activity (Jr-Shiuan Yang, 2011).

At first, pri-mir-451 is cleaved by Drosha/DGCR8 to develop a short pre-microRNA with only~18 bp of duplex stem so too short to serve as a Dicer substrate. Instead, pre-mir-451 is loaded directly into Ago proteins. Ago2 binds the microRNA and cleaves the paired passenger strand 10 nt away from the 5' end of the Ago2 bound microRNA guide strand (Liu YP, 2013). Therefore, Ago2 has not only the cleavage capability of the complementary mRNA targets, but also the slicer activity on pre-microRNAs for the generation of functional mature microRNAs.

1.7.5. Principles of microRNA-mRNA interaction

The mechanistic details of the function of microRNAs in repressing protein synthesis are still poorly understood. MiRNAs may act through several mechanisms including translational repression, inhibition of translation initiation, inhibition of translation post-initiation and induction of mRNA destabilization and decay (Olsen, P. H. 1998) (Figure 1.6).

In plants, miRNAs generally base pair to mRNAs with nearly perfect complementarity. With few exceptions, a similar mechanism is used by vertebrate and viral miRNAs. However, in most cases, metazoan miRNAs pair imperfectly with their targets (Doench, J. G. 2004). Indeed, Binding of the miRISC complex to mRNA is mediated by a sequence of 2–8 nucleotides at the 5′ end of the mature miRNA, known as the seed region which is fundamental for selection of target mRNA (Lewis, B. P., 2005). However, the presence of non-canonical sites both in the 5'UTR and in the coding sequence (CDS) of mRNAs has been shown (Liu C, 2013). The perfect or imperfect complementarity interaction between the seed sequence and 3'UTR of target mRNA results in mRNA degradation or translational inhibition.

The mechanisms of microRNA-mediated repression are not completely understood. Translationally inactive eukaryotic mRNAs usually assemble into repressive miRISC that accumulate in cytoplasmic foci known as processing bodies (P-bodies, PBs) or GW-bodies for storage or mRNA decay (Sheth U, 2003). P-bodies are enriched in proteins that are involved in mRNA catabolism (deadenylation, decapping and mRNA degradation) and translational repression such as the mRNA decapping enzymes Dcp1/Dcp2, the 5' – 3' exonuclease Xrn1 (Eulalio, A., 2007) and the key P-/GW-body subunit, GW182 (Parker, R. 2007). They are highly dynamic structures, fluctuating in size and number during the cell cycle and in response to changes in the translational status of the cell.

Several recently published papers provide important mechanistic insights into translational repression both at the initiation step and at the post-initiation step (Filipowicz W, 2008). Two different models have been proposed for microRNA-mediated repression of translation at the initiation step. In the first model, it was suggested that microRNAs could hinder eukaryotic translation initiation factor 4E (eIF4E) recruitment to the mRNA 5`-terminal 7 methylguanosine- cap (m^7G cap), therefore inhibiting the

activity of this crucial translation initiation factor and the consequent access to mRNA by the translation machine (Pillai RS, 2005). In this model human Ago2 precisely binds to m^7G cap by its cap-binding motif similar to that one of eIF4E.

An alternative mechanism for miRNA action was proposed by Chendrimada et al. (Chendrimada, T. P. et al, 2007). In this model miRISC complex prevents the premature association of 60S with 40S ribosomal subunits by recruiting eIF6. Therefore, eIF6 and 60S ribosomal subunit proteins, co-immunoprecipitate with the AGO2–Dicer–TRBP complex and block the assembly of 80S ribosomal complex on target mRNA so, stop the translation initiation (Chendrimada, T. P. et al, 2007).

In addition to target the translation initiation, miRNAs can hinder translation at the post –initiation steps, in which miRNAs promoting ribosomes to drop off from mRNAs to premature termination of translation (Petersen, C. P. 2006).

Moreover, microRNAs could recruit GW182 to miRNA targets through direct interactions with Argonaute proteins and promote deadenylation of the poly-(A) tail of the target mRNA. Although the precise mechanism is not fully understood, GW182 proteins have been shown to interact with the cytoplasmic poly (A)-binding protein (PABP) followed by the decapping of the m^7G cap and subsequent mRNA decay (Fabian MR, 2009).

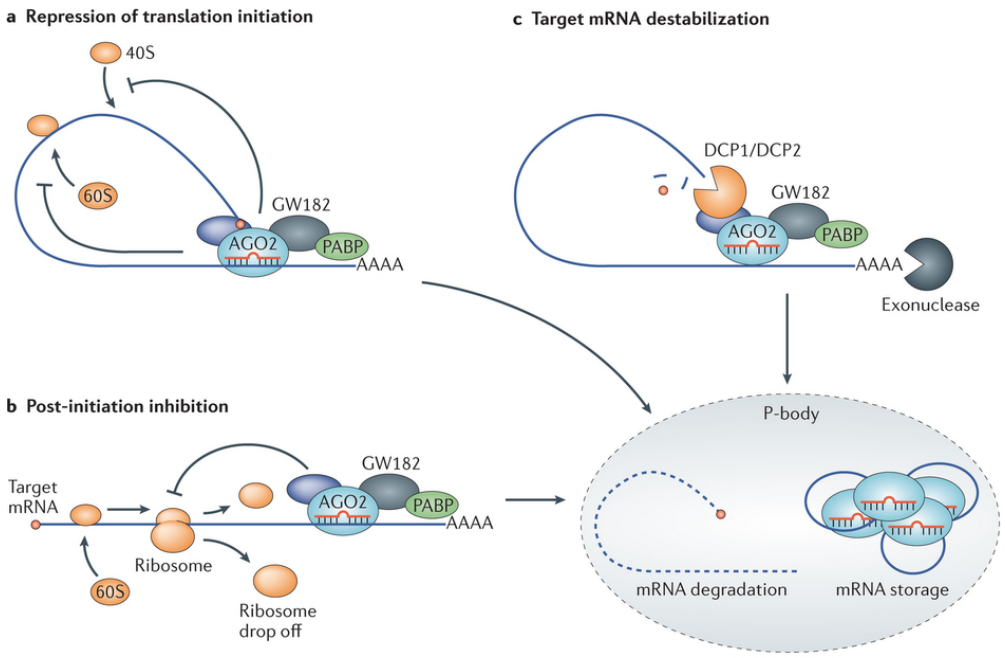


Figure.1.6. ***a*** *| Repression of translation initiation. MicroRNA (miRNA)-mediated silencing complexes (miRISCs) inhibit the initiation of translation by affecting eukaryotic translation initiation factor 4F (eIF4F) cap recognition, 40S small ribosomal subunit recruitment and/or by inhibiting the incorporation of the 60S subunit and the formation of the 80S ribosomal complex. Some of the target mRNAs bound by the miRISC are transported into processing bodies (P-bodies) for storage and may re-enter the translation phase when induced by exogenous signals such as stress.* ***b*** *| Post-initiation translational repression. miRISCs may inhibit the elongation of ribosomes, causing them to drop off the mRNAs and/or facilitate the degradation of newly synthesized peptides.* ***c*** *| Destabilization of target mRNAs. Binding of miRISCs to target mRNAs may recruit RNA decapping and/or deadenylating enzymes that lead to mRNA destabilization. P-bodies are the key cellular organelles for the degradation and storage of targeted mRNAs. AGO2, Argonaute 2; DCP1, mRNA-decapping enzyme 1; PABP, poly(A)-binding protein.(Zhonghan Li & Tariq M. Rana, 2014, Nat rev).*

1.7.6. Multistep regulation of microRNA biogenesis

Dysregulation of both miRNA biogenesis and function is usually combined with human disease, so it is not surprising that both these mechanisms are subjected to a narrow control.

1.7.6.1. Transcriptional regulation.

MiRNA transcription is carried out by RNA Pol II and is controlled by RNA Pol II-associated transcription factors and epigenetic regulators (Cai, X., 2004). Exact locations of the miRNA promoters have not yet been identified for most miRNA genes, some miRNA genes reside in the introns of protein-coding genes and, thus, share the promoter of the host gene. Also, miRNA genes often have multiple transcription start sites (Ozsolak, F. et al. 2008) and the promoters of intronic miRNAs are sometimes distinct from the promoters of their host genes (Monteys, A. M. et al. 2010). So far some transcription factors have been found, such as p53, V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (MYC), Zinc Finger E-Box Binding Homeobox 1 (ZEB1) and 2 (ZEB2), and myoblast determination protein 1 (MYOD1) which positively or negatively regulate miRNA expression (Krol, J., 2010). Epigenetic control, such as DNA methylation and histone modifications also contribute to miRNA gene regulation.

1.7.6.2. Drosha and Dicer processing regulation

There are different positive and negative processing factors that can alter miRNA biogenesis for instance, multiple mechanisms exist to control the expression level, activity and specificity of Drosha. The efficiency of Drosha-mediated processing is essential for determining miRNA abundance. Post-translational modifications can determine the protein stability, nuclear localization and processing activity of the Microprocessor. For instance, phosphorylation of Drosha by glycogen syntetase kinase 3β (GSK3β) is necessary for nuclear localization of Drosha (Tang X, 2010) and acetylation of Drosha by unidentified enzyme inhibits its degradation (Tang X, 2013).

Also, modifications on DGCR8 can influence the activity of the Microprocessor.

Moreover, RNA-binding proteins that selectively interact with Drosha and/or certain pri-miRNAs can control the Drosha-mediated processing including the helicases p68 (also known as DDX5) and p72, and receptor-activated SMAD proteins (R-SMADs) (Ha M, Kim VN, 2014). For instance, Drosha – mediated processing of miR-21 and miR-199a is stimulated by R-SMADs interaction with p68 and the stem of pri-microRNAs (Davis BN, 2008).

Some proteins can bind selectively to the terminal loop of pri-miRNA and affect the post transcriptional process, including heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) which promotes Drosha binding to the pri-microRNA, and the KH-type splicing regulatory protein (KSRP or KHSRP), which facilitates both Dicer- and Drosha- mediated processing (Ha M, Kim VN, 2014). Furthermore, Dicer cofactors including TRBP and AGO proteins are subject to post transcriptional modifications which influence their ability to regulate Dicer processing, RISC formation and miRNA activity.

1.7.6.3. MiRNA intrinsic regulation

There are many different ways to introduce changes in RNA molecule and these alterations in sequence and/or structure can affect the maturation and turnover of miRNAs. For instance, the conversion of adenosine to inosine (A-to-I) RNA editing, catalyzed by adenosine deaminases (ADAR) enzymes on specific pri-microRNAs determines the destiny of mature microRNAs in several ways. The processing of certain miRNAs can be inhibited by A-to-I editing in the hairpin region at Drosha and Dicer level and modify the miRNA biogenesis (Yang W, 2006).

Also, the presence of single nucleotide polymorphisms (SNPs) in miRNA genes can sometimes affect their biogenesis and/or change their target specificity (Ryan, B. M, 2010). For example, a C to T SNP in the first C of the CNNC motif in pri-miR-15a~16-1 reduces Drosha-mediated processing and

thereby lowers miR-16 production (Auyeung, V. C, 2013). Regulation through RNA tailing process which is adding untemplated nucleotididyl to the 3' end of RNA also can modify pre and mature miRNA to facilitate or inhibit miRNA decay (Heo I et al, 2008). Uridylation of the let-7 precursor (pre-let-7) has been most extensively studied. In that case LIN28 proteins induce terminal uridylyl tranferases TUT4 and TUT7 to enhance oligouridylation of pre-let-7 blocking Dicer processing and recruiting exonucleases that recognize the U-tail determining microRNA decay (Heo I et al, 2008).

Although so far very little is known about miRNA stability, the regulation of microRNAs turnover could be essential for their abundance in the cell (Ruegger, S. & Grosshans, H. 2013). Several nucleases have been proposed to cleave and degrade miRNAs, but it is unknown how they achieve substrate specificity and whether there is conserved machinery for miRNA decay. For example in *C. elegans*, degradation of unprotected mature microRNAs is performed by the 5'–3' exoribonuclease 1 and 2 (XRN-1 and 2) (Chatterjee S, 2009).

Moreover, it has been shown that the target mRNA can modulate the stability of miRNAs. Highly complementary targets induce miRNA degradation accompanied by tailing and trimming (Ameres, S. L. et al. 2010). By contrast, it has been reported in C. elegans that miRNAs are stabilized by their targets (Chatterjee S, 2009). Thus, further investigation is required to understand the mechanisms and physiological importance of target-mediated stability control.

1.8. Immune system and MicroRNAs

The immune system provides a complex but well-orchestrated defense mechanism against several pathogens. An infection may trigger an inflammatory response whose initiation, proliferation and resolution must be carefully coordinated and balanced. Lack of proper initiation or proliferation prevents the innate immune response; conversely, lack of suitable resolution can point to chronic disease states. Therefore, the immune response (both innate and adaptive) is extremely regulated and as recently demonstrated such control is largely dependent on micro-RNAs activity (Jan Davidson-Moncada, 2010).

MiRNAs in general participate in regulatory networks that control T cell activation, the expansion of the T cell population and effector T cell differentiation. MiRNA-deficient $CD4^+$ T cells showed a surprising increase in effector Th cell differentiation and cytokine production which indicates a key role for miRNAs in the maintenance of the naive T cell state (Muljo, S. A. et al. 2005).

Some miRNAs down-regulate T cell activation such as miR-29 which hinder the differentiation of particular Th cell subsets (Steiner, D. F. et al. 2011) or miR-125, seems to inhibit effector T cell differentiation in general. miR-125b is highly expressed in human naive $CD4^+$ T cells compared with various memory T cell populations, and it inhibits the expression of several genes involved in T cell differentiation, including IFNɣ, IL2RB, IL10RA (Rossi, R. L. et al, 2011).

One of the regulators of TCR signaling is miR-181 family that has important effects on T cell development and homeostasis. The inhibition of miR-181 in thymocytes reduces TCR sensitivity and diminishes positive and negative selection in the thymus. Conversely, the overexpression of miR-181a in mature T cells augments their sensitivity to peptide antigens in the periphery (Li, Q. J. et al. 2007).

Another essential miRNA in the regulation of TCR pathway is miR-146a which is a critical feedback regulator of nuclear factor-κB (NF-κB) signaling. It is abundantly expressed in human memory T cells and NF-κB p50 (also known as NFKB1) can further induce its expression following TCR engagement (Rusca, N. et al. 2012).

Some MiRNAs can mediate essential role in regulation of T cell proliferation and survival. The miR-17~92 cluster regulates various aspects of T cell activation as well as the consequent fate of the activated T cells and it comprises six miRNAs from four miRNA families. $CD4^+$ and $CD8^+$ T cells can express this miRNA cluster and the related miR-106a~363 and miR-106b~25 clusters (Bronevetsky, Y. et al, 2013). The overexpression of miR-17~92 in T cells causes lymphoproliferation and an autoimmune-like disease characterized by increased serum antibody levels and an increased production of autoantibodies (Xiao, C. et al. 2008) (Mogilyansky E, 2013). T cell proliferation can be enhanced in the absence of both miR-17 and miR-92. MiR-19, another miR-17~92 cluster member, positively regulates the survival of activated T cells. These miRNAs mediate their effects targeting directly many genes such as *Pten* and the pro-apoptotic BCL-2-interacting mediator of cell death (*Bim*; also known as Bcl2l11) (Xiao, C. et al. 2008).

Another phosphatidylinositol phosphatase, SH2 domain-containing inositol polyphosphate 5´ phosphatase 1 (*Ship1*; also known as phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1), is a validated direct target of miR-155 (Loeb, G. B. et al, 2012), highly induced following TCR stimulation (Thai, T. H. et al. 2007). miR-155 deficiency decreases T cell-dependent humoral and cellular immune responses, probably as a result of the absence of its activity on several mRNA targets (Thai, T. H. et al. 2007) (Vigorito E, 2013). Finally, miRNAs can have powerful effects on biological processes by regulating genes that control signaling thresholds. Several miRNAs function at multiple levels to regulate the development, the homeostasis and the activation of T

cells. These effects have important consequences on T cell function, and they are complemented by the miRNA-mediated regulation of particular T cell differentiation pathways and effector mechanisms (Figure 1.7).

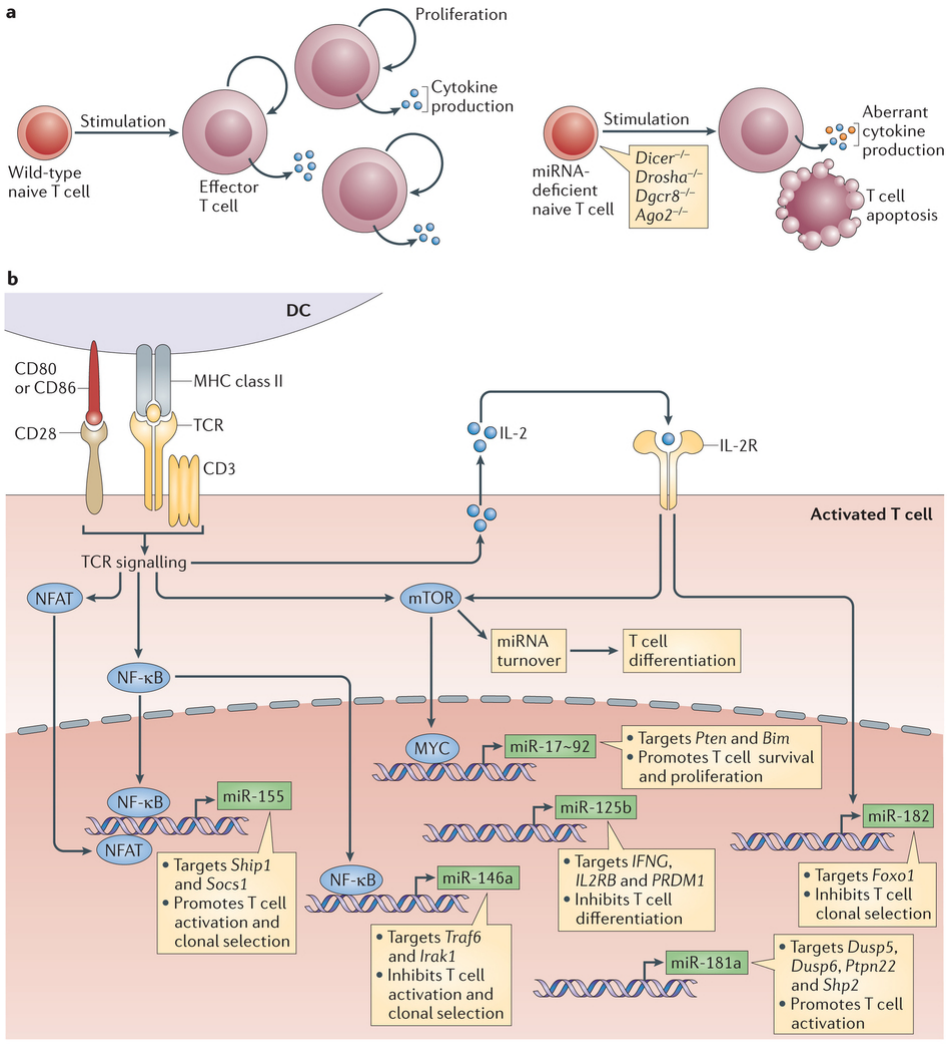


Figure .1.7. *a | MicroRNAs (miRNAs) are important regulators of effector T cell differentiation, including T cell activation, proliferation and the acquisition of effector functions such as cytokine production (left panel). The genetic ablation of key molecules of the miRNA biogenesis pathway in CD4+ T cells highlighted the importance of miRNAs in these*

processes. The activation of miRNA-deficient CD4+ T cells results in increased and aberrant cytokine production and decreased cell proliferation (right panel).

*b | A mechanistic overview of the participation of miRNAs in regulatory networks that control T cell activation, the expansion of the T cell population and effector T cell differentiation is shown. The T cell receptor (TCR) signalling cascade activates nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB), both of which induce the upregulation of miR-155 expression. miR-155 targets SH2 domain-containing inositol polyphosphate 5' phosphatase 1 (*Ship1*) and suppressor of cytokine signalling 1 (*Socs1*), and promotes T cell activation and population expansion. NF-κB signalling also induces the expression of miR-146a, which inhibits T cell activation and population expansion through a negative feedback loop involving the miR-146a target genes TNF receptor-associated factor 6 (*Traf6*) and interleukin-1 receptor-associated kinase 1 (*Irak1*). Mammalian target of rapamycin (mTOR) signalling results in accelerated miRNA turnover and promotes T cell survival and proliferation partly through MYC-induced miR-17~92 expression. The high levels of expression of miR-125b in human naive CD4+ T cells impede T cell differentiation by repressing T cell effector genes, including interferon-γ (*IFNG*),* IL2RB *and* PRDM1 *(the gene encoding B lymphocyte-induced maturation protein 1 (BLIMP1)). The downregulation of inhibitory phosphatases such as dual-specificity protein phosphatase 5 (*Dusp5*),* Dusp6*, protein tyrosine phosphatase non-receptor type 22 (*Ptpn22*) and SH2 domain-containing protein tyrosine phosphatase 2 (*Shp2*) by miR-181a increases TCR signalling. IL-2-induced miR-182 expression interferes with T cell population expansion by targeting the transcription factor forkhead box protein O1 (*Foxo1*). It is worth noting that specific miRNAs are both regulated targets and upstream regulators of signalling pathways that control T cell behaviour. AGO2, Argonaute 2;* Bim*, BCL-2-interacting mediator of cell death; DC, dendritic cell;* Pten*, phosphatase and tensin homologue. (Dirk Baumjohann and K. Mark Ansel, 2013, Nat rev.*

1.9. HIV-1 and MicroRNAs

MiRNAs are related to a plenty of pathological and physiological pathways in mammalian systems. For instance, the dysregulated expression of miRNAs and other RNAi components has been defined in cancers, metabolic disorders, and infectious diseases (Yeung M. L., Jeang K. T. 2011). In plants, RNAi serves as a host defense mechanism against viral infections (Pantaleo V. 2011). In mammals, the role of RNAi in regulating viral infection is less clear; however, recent studies showed the critical role of miRNAs in several viral infections, with different viral families expressing their own miRNAs, manipulating host miRNA expression, or displaying direct or indirect regulation by host or viral miRNAs (Roberts, et al. 2011).

The interactions between HIV-1 and the host cellular miRNAs have been studied, if not extensively, at least in some detail. However, it still remains a quite complex issue. It has been suggested that certain host cellular miRNAs can restrict HIV-1 replication and this in turn may play vital roles in host defense and in maintaining latency within resting $CD4^+$ T cells. Conversely, host cellular miRNAs have also been indicated to be fundamental for certain viruses to establish infection and different expression of cellular miRNAs in HIV-1 infected cells may also be a factor favoring viral replication (Sanjay Swaminathan, 2012).

MiRNAs involved in HIV-1 infection could be categorized as host-encoded (Cellular miRNAs) or HIV-1-encoded based on their source of biogenesis; or defined as suppressors or activators of HIV-1 infection based on their function. Also, they can be divided according to whether they directly or indirectly target HIV-1 transcripts by targeting host factors that are involved in the HIV-1 life cycle. An overview of the modulation of HIV-1 replication by cellular miRNAs is presented in figure 1.8 and table 1.1.

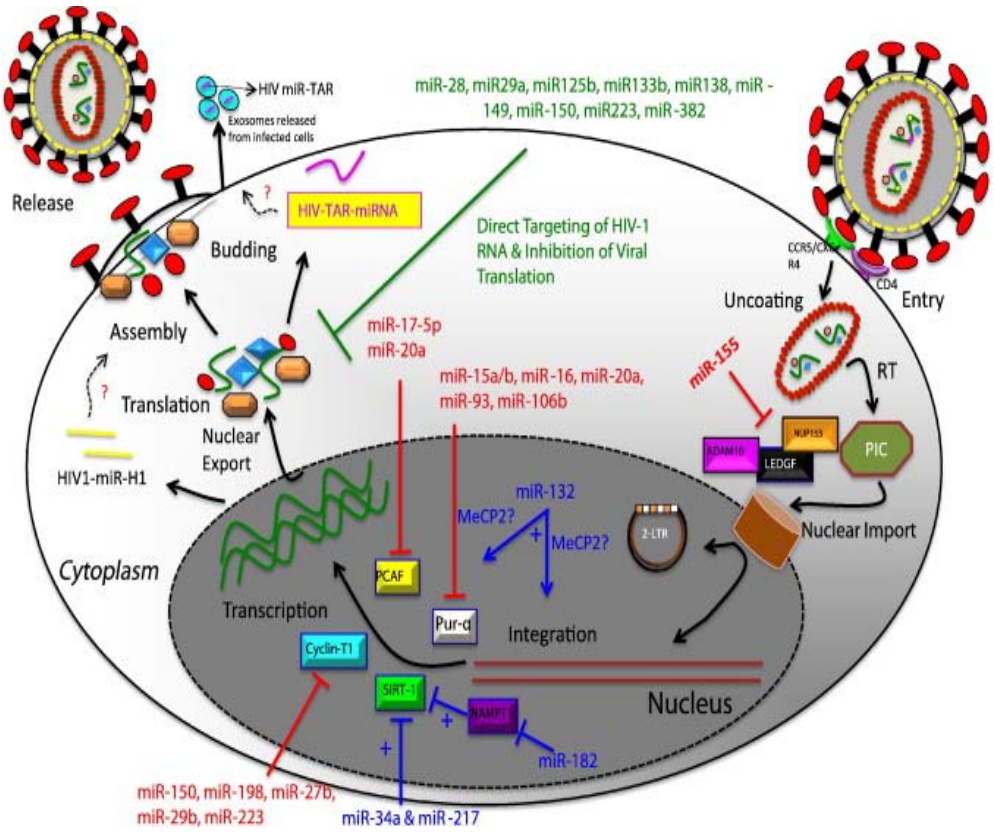


Figure 1.8. *Cellular miRNAs may modulate HIV-1 infectivity and replication. HIV-1 infection can be directly affected by cellular miRNAs that can target HIV-1 viral RNA through base pair complementarity (indicated in green). In addition, other miRNAs can inhibit host proteins that play an active role during productive HIV-1 infection and thereby indirectly inhibit HIV-1 replication (indicated in red). More recently, few miRNAs that can enhance HIV-1 infection have also been identified (indicated in blue). The mechanisms underlying these pro-HIV-1-miRNAs have not been well defined, but it is possible that these miRNAs can bind to and suppress negative regulators of HIV-1 infection. Finally, HIV-1-encoded vmiRNAs such as HIV1-miR-H1 and TAR-miRNA might also modulate viral infectivity. Further research is needed to elucidate the specific mechanisms by which these vmiRNAs and many cellular miRNAs might affect HIV-1 infectivity and replication (Gokul Swaminathan et al. 2014, J. Mol. Biol).*

1.9.1. Cellular miRNAs Regulate HIV-1 Expression

1.9.1.1. MiRNAs that directly target HIV-1

HIV-1 infection can be directly affected by cellular miRNAs that can target HIV-1 viral RNA through base pair complementarity (Fig. 1.8 and Table 1.1.). At first, Hariharan et al tried to search for cellular miRNAs that directly target sites in the HIV genome by using target prediction software (Hariharan et al, 2005). They determined five miRNAs (miR-29a, miR- 29b, miR-149, miR-324-5p, and miR-378) target sites in the HIV-1 genome with two of them located in the viral *nef* gene (Hariharan et al, 2005). Later, one of the five identified miRNAs (miR-29a) was confirmed to inhibit *nef* expression, thus blocking HIV replication in Jurkat cells (Ahluwalia J.K. et al. 2008). Recently Sun et al. reported that miR-29a and miR-29b can inhibit the HIV-1 infection; however, miR-29 access to HIV-1 is restricted by the complex RNA secondary structure surrounding the target site (Sun et al, 2012).

In this sense, a selected group of miRNA was illustrated to play an active role in modulating HIV-1 infectivity of resting $CD4^+$ T lymphocytes (Huang et al, 2007). Huang et al showed that 5 different cellular miRNAs, including miR-28, miR-125b, miR-150, miR-223 and miR-382, had the ability to bind to the 3′ UTR of viral mRNAs through sequence complementarity. They also demonstrated the down regulation of these miRNAs as result of activation of resting $CD4^+$ T cells, which correlated with enhanced HIV-1 susceptibility. Furthermore, it was proposed that translational repression is largely responsible for HIV-1 latency in resting $CD4^+$ T cells mediated by these “anti-HIV-1 miRNAs” (Huang et al, 2007) (Cobos Jimenez V, 2012).

Modulation of these anti-HIV-1 miRNAs can change HIV-1 infectivity in response to various stimuli for instance, cytokines and TLR ligands. It has been shown that the stimulation of TLR3 can induce an anti-HIV-1 effect in primary macrophages, partially through up-regulation of the set of anti-HIV-1 miRNAs mentioned above (Zhou Y. et al. 2010). Interestingly, up-regulation

of miR-28, miR-125b, miR-150 and miR-382 were also reported in macrophages treated with IFN-α and IFN-β, and was shown to partially contribute to inhibition of HIV-1 infection in primary macrophages (Cobos V. Jimenez et al. 2012).

Later, Wang et al. reported that this group of anti-HIV-1 miRNA was enriched in monocytes as compared to monocyte-derived macrophages and responsible for differences between monocytes and macrophages in their permissivity to HIV infection (Wang et al, 2009) (Wang X, 2011). They showed that these miRNAs were down regulated upon macrophages differentiation and that this down-regulation was associated with their capacity to support productive HIV-1 infection. However, more recently Sisk et al. published new results which only confirmed the down regulation of miR-223 in macrophages, while the other previously mentioned mirnas were either more abundant or unchanged in macrophages compared to monocytes (Sisk et al, 2012).

Furthermore, four new miRNAs (miR-133b, miR-138, miR-149, and miR-326) have been identified to target HIV-1. Houzet et al. were able to confirm the action of miR-29 as an anti-HIV miRNA (Houzet et al 2012).

Taken together, the findings suggest that, in divergent cells and in varying contexts, different miRNAs may selectively regulate HIV-1 infection through direct targeting of viral sequences.

1.9.1.2. MiRNAs that indirectly target HIV-1

Various groups have determined many host factors that seem to be necessary or at least to participate to a certain extent in, one or more steps of the viral life cycle by genome wide screenings (Brass A.L., 2008) (Bushman F.D., 2009). Since miRNAs play such an important role in modulating cellular protein expression, it is reasonable that miRNAs may regulate HIV-1 infection, indirectly, through the modulation of the levels of HIV dependency factors (HDFs).

One of the important components of eukaryotic RNA polymerase II elongation complex is cyclin T1 in association with cyclin-dependent kinase 9 (CDK9) and transcription elongation factor B (p-TEFb) (Zhou Q., Yik J.H., 2006) (Chiang K, 2012).

Many studies have described p-TEFb complex interaction with HIV-1 TAR factor and the viral protein Tat to facilitate viral transcription (Hoque M., 2011) (O'Keeffe B., 2000).

Sung et al. proposed an anti-HIV-1 role for miR-198 which targets the cyclin T1 mRNA; at first they detected a negative correlation between cyclin T1 levels and miR-198 in monocytes and macrophages: subsequently, miR-198 binding to the 3` UTR of cyclin T1 mRNA was reported and demonstrated to reduce its protein level (Sung et al, 2009). Moreover, the same authors showed that miR-198 over-expression hindered HIV-1 replication in macrophages, supporting the idea that miRNAs apply cell-type-specific effects (Sung et al, 2009). It has been shown that miR-198 is expressed at very low levels in resting CD4+ T cells and is not modulated upon activation (Chiang et al. 2012). Chiang et al. also detected other miRNAs capable to regulate cyclin T1 expression (e.g. miR-27b, miR-29b, miR-223, and miR-150), potentially explaining expression differences of HIV in resting versus activated T cells. They reported that the down regulation of these miRNAs, is a consequence of CD4+ T cells activation and correlates with a Cyclin T1up-regulation and enhanced HIV-1 susceptibility (Chiang et al. 2012).

Independently, another group found that miR-17-5p and miR- 20a can target p300/CBP associated factor (PCAF), another cellular cofactor of the viral Tat protein. Specifically, the over-expression of miR-17-5p and miR- 20a resulted in decreased mRNA levels and protein output of PCAF. Of note, HIV-1 infection actively suppressed these miRNAs to augment its infectivity by arrogating the p300/PCAF complex for efficient viral translation (Triboulet, R. 2007).

Furthermore, six miRNAs (miR- 15a, miR-15b, miR-16, miR-20a, miR-93, and miR-106b) were found to target purine-rich element binding protein α (*Pur-α*) mRNA. Pur-α protein is a sequence-specific DNA and RNA binding protein which facilitates viral transcriptional up-regulation by binding to the HIV-1 TAR component and viral Tat protein in the nucleus of infected cells (Gallia GL et al. 1999) (Shen CJ, 2012). In a recent study, higher expression levels of miR-15a, miR-15b, miR-16, miR-20a, miR-93 and miR-106b was associated with significantly lower expression of Pur-α and lower susceptibility to HIV-1 infection in monocytes compared with monocyte-derived DCs (Shen CJ et al, 2012).

Targeting	miRNAs	Target	Function
DIRECT	**miR-29a**	Nef	Decrease HIV infectivity
	miR-28 **miR-125b*** **miR-150** **miR-223** **miR-382**	3′ LTR	Latency in primary resting $CD4^+$ T cells Restrict HIV-1 replication in monocytes
	miR-133b **miR-138** **miR-149** **miR-326**	3′ LTR**	Decrease HIV replication
INDIRECT	**miR-17-5p** **miR-20a**	PCAF	Decrease HIV replication
	miR-198	Cyclin T1	Restrict HIV-1 replication in monocytes
	miR-27b	Cyclin T1	
	miR-29b **miR-150** **miR-223**	Cyclin T1***	Restrict HIV-1 replication in resting $CD4^+$ T cells
	miR-15a **miR-15b** **miR-16** **miR-20a** **miR-93** **miR-106b**	Pur-α	Restrict HIV-1 replication in monocytes

Table 1.1. *miRNAs involved in the regulation of HIV-1 expression.*

** miR-125b was involved in HIV-1 restriction in resting T cells only.*

*** This study specifically examined only the miR-326 target site.*

**** Indirect regulation of cyclin T1 by miR-29b, miR-150, and miR-223. (Zachary Klase et al. JBC, 2012)*

1.9.1.3. MiRNAs that enhance HIV-1 infection

Very few studies have reported miRNA-mediated enhancement of HIV-1 infection however, recently HIV-1 infection was shown to be increased by miR-132 (Chiang K, Liu H, Rice AP, 2013). Activated $CD4^+$ T cells were reported to have significantly higher miR-132 expression and also ectopic miR-132 expression in Jurkat T cells was shown to increase HIV-1 infection (Chiang K, Liu H, Rice AP, 2013). However, the mechanisms underlying this effect are not completely clear. Preliminary data showed that miR-132 overexpression decreased the expression of a cellular protein MeCP2, a methyl CpG binding cellular protein. siRNA- mediated inhibition of MeCP2 can enhance HIV-1 replication, however, the specific role of miR-132 and MeCP2 in HIV-1 replication has not been identified (Leoh LS et al. 2012).

In another study, sirtuin 1 (SIRT1) was shown to mediate regulation of HIV-1 Tat transactivation. SIRT1 is a class III deacetylase dependent on nicotinamide adenine dinucleotide that can regulate HIV-1 transcription by deacetylating Tat (Pagans S et al, 2005). In a recent study, significant up-regulation of miR-217 and miR-34a was detected upon Tat exposure and it was shown that they have the ability to bind the 3′ UTR region of SIRT1 mRNA and hinder its expression, so increasing HIV-1 Tat-mediated transactivation (Zhang HS, 2012).

1.9.2. HIV-1-encoded MiRNA

Certain families of viruses encode their own miRNAs known as viral miRNA or vmiRNAs which can influence viral replication (Kincaid and Sullivan, 2012; Nair and Zavolan, 2006). The majority of vmiRNAs belong to DNA viruses, while the expression of functional vmiRNAs by retroviruses, especially HIV-1, remains controversial. Through bioinformatic prediction algorithms, 5 candidate vmiRNAs (Bennasser et al., 2004) were predicted to be encoding by the HIV-1 genome. Moreover, vmiRNA (Nef-U3-miR-N367) was found in

the HIV-1 Nef coding region (Omoto and Fujii, 2005; Omoto *et al*., 2004) and, the 3′ end of the HIV-1 RNA was shown to encode for another vmiRNA: HIV-1-miR-H1 (Kaul et al*.,* 2009) (Zhang Y, 2014). While these studies all illustrated proof for functional vmiRNAs within the HIV-1 RNA genome, other groups have found contradictory results and failed to detect vmiRNA expression post-HIV-1 infection. More recently, a study utilized sensitive deep sequencing technology and found that HIV-1 does not seem to express any functional vmiRNA in infected cell lines, PBMCs, and primary macrophages (Whisnant et al., 2013).

1.9.3. MiRNA Profiles in HIV-1-Infected Patient Cohorts

One of the first reports on profiling miRNA expression was published by Houzet et al in HIV-1 seropositive individuals (Houzet L, 2008). They grouped the patients into four categories based on differential $CD4^+$ T cell counts and viral load and compared miRNA expression profiles in PBMCs. They explored that, of the 63 miRNAs that were commonly changed, 4 miRNAs were up-regulated and 59 were down-regulated in all seropositive individuals including miR-150, miR-191, miR-223, miR-16 and miR-146b (Houzet L, 2008).

Witwer et al characterized global miRNA expression profiles in PBMCs from HIV-1-infected viremic patients and Elite suppressors (ES), to determine if differential miRNA expression profiles in ES might shed light on their resistance to virus-induced disease progression (Witwer et al, 2012). ES also known as elite controllers (EC) are a group of HIV-1-infected patients that are able to preserve excessively low to undetectable viral loads in blood (50 copies/ml, below the limit of detection of commercial assays) without antiretroviral therapy (ART) for many years and generally do not show any clinical signs of disease progression (Blankson JN. 2010).

Noticeably, they were able to identify specific miRNAs that were uniquely expressed in PBMCs from ES when compared to viremic patients and uninfected controls. Furthermore, anti-HIV-1 miRNAs including miR-125b, miR-150 and miR-29 were down-regulated in both ES and viremic patients (Witwer et al, 2012) and, miR-155, which inhibits HIV-1 infection in macrophages, was reported to be significantly higher only in viremic patients as compared to ES or healthy controls (Swaminathan G, 2012).

More recently Reynoso et al., identified 3 miRNAs, miR29b, miR-33a and miR-146a that are more expressed in plasma of ES than HIV chronic infected patients. Moreover, they discovered that, after an HIV-1 infection assay, the overexpression of miR-33a and miR-146a drastically reduce viral replication in infected PBMC. These results suggested that these miRNAs might be used in developing therapeutic strategies against HIV especially considering that miR-29b is known to target Nef and alterations in Nef functions have been associated with slower progression to AIDS (Reynoso et al., 2014).

Human miR-29b has been previously described as one of the key components characterizing ES miRNA profile as well as the one of infected patients with low viral load. Gorrono˜ et al found that PBMC from ES showed increased levels of miR-29b but also others miRNAs like miR- 221, miR-27a, hsa-miR-27b compared to viremic patients. On the other hand, the expression of 19 miRNAs was significantly down regulated in ES. Out of these miRNAs, miR-146a and miR-155 are the most important as they play a central role in a wide spectrum of immune function (L. Egaña-Gorroño, et al 2014). In summary, the published works reveal a differential expressed miRNA profile in Elite Suppressors that is similar to non-infected individuals and differs from Viremic Patients.

Long-term non-progressors (LTNP) are HIV-1 seropositive individuals that naturally control viremia, but in detectable low levels (10,000 copies of

RNA/ml of blood) without ART, have normal $CD4^+$ T cell counts and do not show the typical disease progression of HIV-1-infected individuals (Poropatich K, Sullivan DJ. 211). Mechanisms underlying resistance to or efficient control of HIV-1 infection in LTNP and HESN cohorts have not been well characterized. To this end, MiRNA expression profile has been evaluated by Bignami et al. in $CD4^+$ T cells from LTNP, naïve healthy and HESN individuals (Bignami et al. 2012). Twenty-three miRNAs were differentially expressed between HESN and naïve healthy individuals. For instance, resting $CD4^+$ T cells from HESN had significantly lower levels of miR-28-5p, miR-125b and miR-223 while miR-155, was expressed at higher levels in LTNP than in HESN. Furthermore, miRNA biogenesis proteins such as Dicer and Drosha were significantly down-regulated in CD4+ T cells derived from HESN individuals (Bignami et al. 2012).

Finally, other study argued against the conclusions of previous work reveling lower levels of previously characterized miRNAs (miR-125b, miR-31, miR-146b and miR-29a) (Witwer and Clements JE. et al. 2012). The controversial results may in part relate to differences in the cell types studied or in the technology and/or approaches used to detect and confirm miRNA expression. Future studies will be crucial in resolving such controversy.

1.9.4. Cholesterol metabolism and MiR-233

Cholesterol is critical for membrane integrity, signal transduction, and in general cellular physiology. There is a close relationship between lipid metabolism and antiviral responses. Many viral pathogens indeed exploit cellular lipid metabolism to ensure a correct amount of cholesterol for the release of functional virions (Matsumiya et al., 2013).

Cholesterol homeostasis is accomplished through a complex network of sensing and effector mechanisms (Espenshade PJ, 2007). Liver control the

plasma cholesterol levels since it is responsible for the production of lipoproteins and the removal of excess systemic cholesterol through reverse cholesterol transport, cholesterol uptake, and sterol biliary excretion (Cuchel M, et al. 2010) (Ishibashi S, et al. 1993). In particular, the production of the 25-hidroxy cholesterol (25-HC), a natural oxysterol that is produced through a cholesterol-25 hydroxylase (CH25H)-mediated enzymatic reaction, lead to the activation of liver X receptor (LXR) family. This event cause to the activation of ATP-binding cassette A1 (ABCA1) that promotes the efflux of cholesterol excess's from the cells. (Park K et al., 2010).The connection between virus and cholesterol has been widely established in the last few years. According to this observation, studies have provided evidence that activation of LXRs results in the inhibition of viruses replication, including HIV and HCV. Regulation of cholesterol metabolism is largely mediated through interdependent regulatory modules, and miRNAs have appeared as critical components of this network (Moore KJ, 2010).

Many genes associated with lipid and cholesterol metabolism are regulated by MiR-223 (Vickers KC, et al. 2013). Moreover, it has been established that miR-223 transcription and its mature levels are sensitive to intracellular cholesterol changes. MiR-223 regulates cholesterol biosynthesis, uptake, and efflux, thus demonstrating as a critical posttranscriptional regulatory coordinator of cholesterol metabolism (Vickers KC, et al. 2014).

HIV-1 infection can impair both in vitro and in vivo reverse cholesterol transport (Djuranovic S, 2012) (Leung AK, 2010). In *in vitro* HIV-1 infected macrophages, the virus inhibits cholesterol efflux by Nef-mediated down-regulation of adenosine triphosphate–binding cassette transporter A1 (ABCA1) (Mujawar Z, 2006). ABCA1, also known as cholesterol efflux regulatory protein, is a protein encoded by the gene *ABCA1* and is a major regulator of cellular cholesterol and phospholipid homeostasis as well as high-density lipoprotein (HDL) metabolism. It has been illustrated that ABCA1 mRNA and protein levels are significantly elevated with miR-223

overexpression (Vickers KC, et al. 2014) and the mechanism of action was shown to be indirect. Sp1 is a known transcriptional activator of ABCA1 while Sp3 is an antagonist of Sp1. Both mRNAs harbor putative miR-223 target sites, but only Sp3 mRNA levels were reduced by miR-223 overexpression, and in luciferase reporter assays, the putative target site was confirmed as functional. Thus, the proposed scenario is that the reduction in Sp3 by miR-223 allows Sp1 greater positive sway over the ABCA1 promoter, with more efflux-promoting ABCA1 protein resulting. Consistent with this scenario was that when Sp3 itself was knocked down by siRNA, there was significant upregulation of ABCA1 gene expression, and with miR-223 overexpression, Sp3 nuclear binding activity decreased (Vickers KC, et al. 2014). In HIV-1 infected cells, HIV induces a switch of cholesterol trafficking from physiological efflux to virus-controlled transport, thus reducing the ability of a cell to export excessive cholesterol.

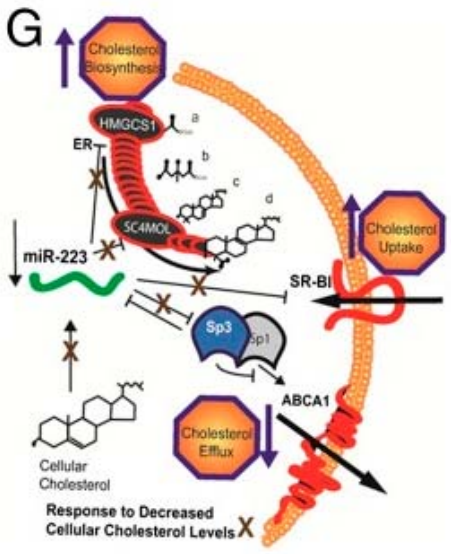


Figure 1.9. *Schematic of miR-223 targets and regulatory modules associated with cholesterol metabolism. Cholesterol biosynthesis intermediates: a, acetoacetyl-CoA; b, β-hydroxy-β-methylglutaryl-CoA; c, 4,4-dimethylcholesta-8(9),24-dien-3β-ol; and d, 4-methyl,4-carboxycholesta-8(9)24-dien-3β-ol. (Vickers KC, et al., PNAS 2014).*

1.9.5. Extracellular and Circulating miRNA

Several studies have investigated the association of host cellular miRNAs in HIV-1 replication however; our knowledge of extra cellular and circulating miRNAs is limited. In 2008, miRNAs were detected in blood including plasma, platelets, erythrocytes, and nucleated blood cells. Plasma miRNAs were found to be surprisingly stable even under conditions as severe as boiling, low or high pH, long-time storage at room temperature, and multiple freeze-thaw cycles (Mitchell PS, 2008) (Lawrie CH, 2008) (Chen X, 2008).

Recent studies have shown that to prevent their degradation, miRNAs can be packaged in microparticles (exosomes, microvesicles, and apoptotic bodies) (Zernecke A, 2009) (Valadi H, 2007) or associated with RNA-binding proteins (Argonaute2 [Ago2]) (Arroyo JD, 2011) or lipoprotein complexes (high-density lipoprotein [HDL]) (Vickers KC, 2011) (Figure 1.10).

The presence of miRNAs in microparticles has also led to suggest that miRNAs are selectively targeted for secretion in one cell and taken up by a distant, target cell, possibly to regulate gene expression (Zernecke A, 2009).

1.9.5.1. Plasma miRNAs in Microparticles

Plasma RNA is protected from degradation by its inclusion in protein or lipid vesicles (El-Hefnawy T, 2004) which are known as exosomes, microvesicles (MVs), or apoptotic bodies depending on their size and mode of release from cells (Kosaka N, 2010) (Figure 1.10).

Valadi et al found that some miRNAs were at approximately higher levels in exosomes than in their donor cells, which suggests an active mechanism by which selected miRNAs are promoted toward exosomes (Valadi H, 2007). In fact it has been confirmed that cells can indeed select some miRNAs for cellular release while others are retained (Pegati et al, 2010).

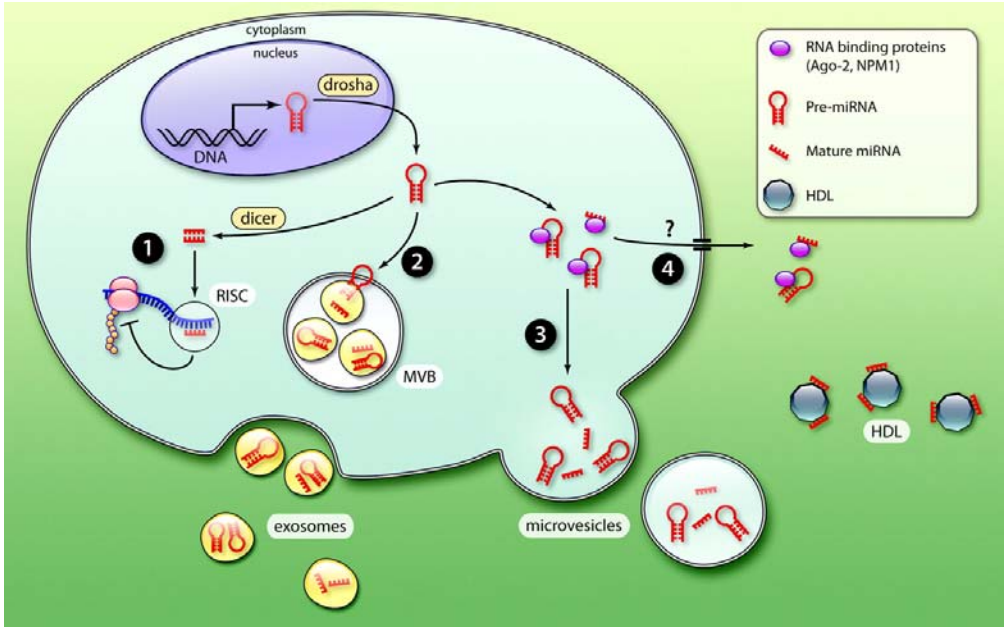


Figure 1.10. *Cellular release mechanisms and extracellular transportation systems of miRNAs. In the nucleus, miRNAs are transcribed from DNA. A precursor hairpin miRNA (pre-miRNA) is formed after cleavage by the RNase III enzyme Drosha. After being transported into the cytoplasm, the pre-miRNA can be further cleaved into 19- to 23-nucleotide mature miRNA duplexes. One strand of the miRNA duplex can be loaded into the RNA-induced silencing complex (RISC), where it can guide the RISC to specific mRNA targets to prevent translation of the mRNA into protein (1). The other strand may be degraded or released from the cell through export mechanisms described below. In the cytoplasm, pre-miRNAs can also be incorporated into small vesicles called exosomes, which originate from the endosome and are released from cells when multivesicular bodies (MVB) fuse with the plasma membrane (2). Cytoplasmic miRNAs (pre-miRNA or mature miRNA) can also be released by microvesicles, which are released from the cell through blebbing of the plasma membrane (3). MiRNAs are also found in circulation in microparticle-free form. These miRNAs can be associated with high-density lipoproteins or bound to RNA-binding proteins such as Ago2. It is not known how these miRNA-protein complexes are released from the cell. These miRNAs may be released passively, as by-products of dead cells, or actively, in a miRNA-specific manner, through interaction with specific membrane channels or proteins (4). Although pre-miRNAs have been detected in exosomes and microvesicles, and mature miRNAs have been found in complex with Ago28 and HDL, the exact proportion of mature and pre-miRNAs in the different extracellular compartments is not known (Creemers et al, Circ. Res, 2012).*

1.9.5.2. MiRNAs Associated With Protein Complexes

Recent studies showed that the majority of miRNAs are not found inside vesicle but rather are bound to RNA-binding proteins. Arroyo et al reported that almost up to 90% of miRNAs in circulation are present in a non–membrane-bound form, whereas the minority of circulating miRNAs is vesicle-associated (Arroyo JD, 2011) (Figure 1.10).

Protein K digestion of plasma specifically destabilizes these non–vesicle-associated miRNAs, which demonstrates the existence of a miRNA-protein complex as a mechanism for their stability in the RNase-rich circulation. Further characterization indicated that a considerable portion of circulating miRNAs were associated with Ago2, the key intracellular effector protein of miRNA-mediated RNA silencing (Arroyo JD, 2011). Although, the exact mechanism is not clear, it has been proposed that the Ago2-miRNA complexes are passively released by death or apoptotic cells and remain in the extracellular space because of the high stability of the Ago2 protein. It is also possible that cell membrane–associated channels or receptors exist that allow for the specific release of these Ago2-miRNA complexes (Creemers et al, 2012).

1.9.5.3. Circulating miRNAs Associated With HDL

Recently, Vickers et al showed that endo-genous miRNA can be transported by high-density lipoprotein (HDL) (Vickers KC, 2011). They have an average size of 8 to 12 nm and are smaller than exosomes. Furthermore, HDL contains lipids, such as phosphatidylcholine which form stable ternary complexes with nucleic acids (Janas T and Yarus M. 2006). The mechanism of loading miRNAs into HDL is not known exactly; however, biophysical studies propose that HDL simply binds to extracellular plasma miRNAs through divalent cation bridging (Vickers KC, 2011).

1.9.5.4. MiRNAs in Cell-to-Cell Communication

Zhang et al reported that miR-150 can be packed into MVs by human blood cells and directly delivered to cultured endothelial cells, reducing c-Myb protein levels, a validated target gene of miR-150 in target cells (Zhang Y, 2010). Likewise, HDL from patients with hypercholesterolemia increased levels of miR-105, whereas HDL from HCs had no effect. HDL from familiar hypercholesterolemia patients induced significant gene expression changes in hepatocytes. This study provides evidence that HDL can deliver miRNAs to cells and alter gene expression (Vickers KC, 2011). These researches demonstrate that secreted miRNAs can be delivered effectively to cultured cells, where they can function as endogenous miRNAs.

1.9.5.5. Circulating miRNAs as Biomarkers

Mitchell et al. used a mouse prostate cancer model to study the presence of tumor- derived circulating miRNAs in plasma and found a strong correlation between miRNAs obtained from plasma and serum. Moreover, they were able to distinguish healthy subjects from patients with prostate cancer by measuring miR-141 level in serum (Mitchell PS, 2008). In the other study, the up-regulation of plasma miRNAs was detected in acute phase of HIV-1 infected subjects and was associated with the progression of central nervous system disease (Witwer KW, 2011). Remarkably, MiRNAs appear as encouraging biomarkers to develop new miRNA- based therapies and diagnostic tools (Edwards JK. 2010).

AIM OF THE STUDY

The variability in susceptibility to HIV infection has been intensively studied and was found to correlate with a variety of viral, genetic, immunological and socio-behavioral variables which influence host cellular gene expression leading to increased antiviral activity (Clerici M et al. 1992).

MicroRNAs are small 20-24nt non-coding RNAs that modulate gene translation of mRNAs by binding to complementary sequences in the 3'UTR of target transcripts. MicroRNAs are known to play essential functions in cellular differentiation, proliferation and apoptosis, and are also involved in human diseases, the modulation of innate and adaptive immunity, and host-virus interaction (Bartel DP, 2004) (Zhao Y, 2007). In particular, several studies examined the effects of HIV infection on miRNAs expression. MiRNAs have been shown to directly and indirectly modulate HIV replication via their interaction with cellular genes that influence such replication. Actually, 5 cellular miRNAs (miR-28, miR-125b, miR-150, miR-223and miR-382) were shown to target the 3' LTR region of the HIV genome, thus contributing to the maintenance of latency in resting $CD4^+$ T cells and monocytes (Huang et al, 2007). Despite several studies characterized the role played by different cellular miRNAs in HIV-1 infection in *ex vivo* and *in vitro* studies, only a limited number of reports have tried to analyze global miRNA profile in HESN and HIV-1-infected patients. Moreover, to our knowledge plasma miRNA profile of HESN cohorts in comparison to HIV-1 infected and HCs has not been investigated so far. Since a deeper understanding of miRNA profile in HESN individuals could open a new window to explore the mechanism behind the secret of natural resistance to the HIV-1 virus in HESN subjects, we profiled the expression of 84 miRNAs with a known antiviral and/or immunological activity in PBMC, monocytes, macrophages, plasma and supernatants of HIV-infected cells from a well-characterized Italian HESN cohort.

MATERIALS AND METHODS

3.1. Sample Collection

3.1.1. Sample population

Blood samples were collected from 30 HESN and 30 HIV-1 positive individuals who are part of a serodiscordant cohort of heterosexual couples recruited at the S. Maria Annunziata Hospital in Florence, Italy followed since 1997 (Mazzoli, 1997). Thirty age-matched HCs, without known risk factor for HIV infection, were also included in the study. The whole groups are Italian of Caucasian origin. Inclusion criteria for HESN was a history of multiple unprotected sexual episodes for more than 4 years at the time of the enrolment, with at least 3 episodes of at-risk intercourse within 4 months prior to study entry, and an average of 30 (range, 18 to >100) reported unprotected sexual contacts per year. All individuals (HESN and HIV-1 infected) have been longitudinally followed for >4 years before the study by the Department of Obstetrics and Gynecology of the S. M. Annunziata Hospital.; which allowed us to exclude HESN and HIV-1 infected patients with sexually transmitted diseases or other reported pathologies during the time of study. The range of CD4 cell counts in HIV-1 infected patients was 71-927 cells/ml, and viral loads were <50- >750,000 copies/ml. All the patients were receiving antiretroviral therapy (ART) at the time of the study. Written and informed consent was obtained from all subjects.

3.1.2. Isolation of PBMC

Peripheral blood mononuclear cells (PBMCs) were separated by whole blood on lymphocyte separation medium (Lympholyte-H, Cederlane Laboratories, Burlington, NC, USA). Briefly, whole blood was centrifuged for 25 minutes at 2300 RPM, without brake, on a Ficoll discontinuous density gradient.

After the centrifugation step, the blood sample was separated showing the following layers from top to bottom plasma and platelets, the PBMCs band

situated at plasma/Ficoll interface, Ficoll and red blood cells, covered by a granulocyte layer.

The PBMCs layer were carefully removed from the tube and transferred to a new conical tube. The PBMCs were then washed with phosphate buffered saline (PBS) and cell number and cellular vitality were determined.

3.1.3. Cell count

The number and viability of the cell were determined by automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Corea). ADAM-MC automatic cell counter measures total cell numbers and viabilities by cutting edge detection technologies. Two sensitive fluorescence dye staining solutions, AccuStain Solution T (Propidium Iodide/lysis solution) and AccuStain Solution N (Propidium IodideI/PBS) are used in ADAM instead of tryphan blue staining which can lead to inaccurate data. AccuStain Solution T allows plasma membrane disruption and nucleus staining for measurement of total cell concentration. AccuStain Solution N allows staining of non-viable cells, thus leaving viable cells completely intact. A 532 nm optic laser is automatically focused onto the cell solution inserted into a disposable microchip and cell analysis is made by a CCD detection technology.

3.1.4. Monocyte isolation and Macrophages differentiation

In order to calculate the percentage of monocyte in basal PBMC, 250,000 cells were resuspended in PBS, and stained for surface antibodies: CD36FICT, CD4PC5, CD14PECY7 (Beckman-coulter, Fullerton, CA). After 15 minutes cells were washed and fixed in 1% paraformaldehyde in PBS. After determining the percentage of monocyte at flow cytometer, 500,000 monocyte/well were resuspended in RPMI 1640, seeded in a 12 wells monocyte attachment plate and incubated for 1 hour at 37°C with 5% CO_2. Non-attached cells were removed by vigorously swirling the medium in the

well and aspirating the non-attached cells. One well for each subject was used for basal monocyte analysis and RNA was immediately extracted as described below (section 3.4.1).

Furthermore, two wells for each HESN and HC subject were used for macrophages differentiation. In this step, after washing the wells with PBS, cells were cultured in RPMI 1640 containing 20% FBS and 100ng/ml of macrophages colony-stimulating factor (MCSF) at 37°C plus 5% CO_2 for 5 days.

3.2. In Vitro HIV-1 Infection

3.2.1 HIV-1 strains

The R5 tropic HIV-1_{BaL} (contributed by Drs. S. Gartner, M. Popovic and R. Gallo, courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program) was used to perform In-vitro HIV-1 infection. The virus was provided through the EU programme EVA center for AIDS Reagents (The National Institute for Biological Standards and Control NIBSC, Potter Bars, UK).

3.2.2. HIV-1 infection of PBMC

Four x10^6 PBMCs isolated from HESN and HC were cultured in RPMI 1640 containing FBS (20%), with or without 0.5ng/1x10^6 cells HIV-1_{Ba-L} virus and incubated for 24 hours at 37°C and 5% CO_2. Cells were then washed and resuspended in medium containing IL-2 (15ng/ml)(R&D systems, Minneapolis, Minnesota, USA) and PHA (1µg/ml)(Sigma–Aldrich, Saint Louis, Missouri, USA). Two days later cells were washed, resuspended in complete medium with IL-2, plated in 24-well tissue culture plates and incubated at 37°C and 5% CO_2. One and 7 days post-infection 1x10^6 PBMC were analyzed for miRNA and gene expression while p24 antigen ELISA

was performed on 7-days post infection supernatants. MiRNA expression was analyzed on 7-days post infection supernatants as well.

3.2.3. HIV infection assay of monocyte derived- macrophages (MDM)

Following PBMCs isolation, monocytes were seeded in a 12 wells monocyte attachment plate for macrophages differentiation according to section 3.1.5. After differentiation assessment, macrophages from HESN and HC subjects were cultured in RPMI 1640 containing 20% FBS and 5ng/5 $x10^5$ cells HIV-1_{Ba-L} and incubated for 3 h at 37°C and 5% CO_2. The attached macrophages were washed with PBS and cultured in complete medium containing 20% FBS for 9 days. Half of the medium was changed 3 and 5 days post infection. Nine days post infection supernatant was collected for p24 antigen ELISA while the attached macrophages were lysed with RNAZOL to extract RNA as described below (section 3.4.1).

3.3. ELISA Assay

3.3.1. P24 ELISA

HIV-1 p24 Elisa assay kits (XpressBio, Frederick, MD, USA) were used to measure viral p24 on 7-days post infection PBMC and 9- days post infection MDM supernatant according to the manufacturer's protocol.

Microtitration wells were pre-coated with murine high specificity and affinity anti-HIV-1 P24 capture antibody. Five serial dilution (1:50 dilution) were prepared from a positive control to generate a linear standard curve. Controls and specimens were seeded together with a lyses buffer and incubated at 37°C for 60 minutes. All controls and samples were tested in duplicate.

After washing the plate with a wash buffer (Tris buffered saline containing 0.05% Tween 20) a detector antibody (anti-HIV p24 conjugated to biotin) was added into each well and incubated at 37°C for 60 minutes.

Subsequently, Streptavidin HRP conjugate (Streptavidin conjugated to horseradish peroxidase enzyme containing 0.01% Bromonitrodioxane) was added into wells and incubated for 30 minutes at room temperature (18-25°C). Substrate solution (Tetramethlbenzidine) was added into the plate and incubated at dark and room temperature (18-25°C) for 30 minutes. Reaction was stopped by adding a stop solution (1 N H_2SO_4) to all wells.

Plates were immediately read at 450 nm, using the IMark microplate reader equipped with Microplate Manager® 6 software (both from Biorad, Hercules, CA, USA). The absorbance of each microplate well was calibrated against the absorbance of an HIV-1 p24 antigen standard curve. Samples with absorbance values equal to or greater than the cutoff factor were considered initially reactive, but were retested in duplicate to determine whether the reactivity is reproducible.

3.4. Total RNA extraction and miRNA isolation

3.4.1. Total RNA extraction

Total RNA was extracted from unstimulated, HIV-1 infected PBMC, $CD4^+$ T cells, Monocyte and Monocyte- derived macrophages by using the acid guanidium thiocyanate-phenol- chloroform method. Cells were lysed by RNAzol B reagent (Duotech, Milan, Italy), a monophase solution containing phenol and guanidine thiocyanate, and later chloroform (20% of initial volume of RNAzol B) was added to separate the lysate into aqueous and organic phase. After centrifuging the samples (at 12,000g 15 minutes at 4°C) DNA and proteins were separated from the aqueous phase containing RNA. Total RNA was obtained from the aqueous phase by the isopropanol precipitation and washing with 75% ethanol and dissolved in RNase-free water (RQ1 DNase; Promega, Madison, WI, USA). The quality of RNA was tested using Nanodrop Spectrophotometer and only RNAs with high quality were used for further analysis.

3.4.2. Preparation of miRNAeasy Serum/Plasma Spike-In Control

For quality control and to monitor the extraction efficiency of miRNA, 3.5µl ($1.6X10^8$ copies/µl) of synthetic cel-miRNA-39 (Qiagen, Venlo, Limburg, Netherlands) was spiked-into plasma and supernatant samples. The miRNAeasy Serum/Plasma Spike-In Control is a C. elegans miR-39 miRNA mimic and reconstitute by adding RNase-free water, resulting in a $2x10^{10}$ copies/µl stock.

3.4.3. MiRNA extraction from Plasma and cell culture medium

MicroRNAs were isolated from 600µl of HESN, HIV+ and HC plasma sample and 300 µl supernatants of HIV-infected PBMCs by NucleoSpinMiRNA Plasma Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. Each sample was incubated with MLP buffer (Guanidiniumthiocyanate) at room temperature (RT) for 3min. Protein precipitation Buffer (MPP) and cel-miRNA-39 (Qiagen, Venlo, Limburg, Netherlands) (MPP) plus synthetic cel-miRNA-39 (Qiagen, Venlo, The Netherlands), for quality control and to monitor the extraction efficiency, were added to each sample. After centrifugation at 11,000 x g for 3min, the supernatant was collected and transferred to a new tube containing isopropanol. The total mixture was loaded to the NucleoSpinMiRNA column and after centrifuge for 30 s at 11,000 x g we discard the flow-through. The column was washed 3 times by adding wash buffer provided by kit (buffer MW1 and MW2). MicroRNAs were then eluted in 25µl of standard elution buffer and stored at -80˚C for further analysis.

3.5. miScript Reverse transcription II RT

One microgram of total RNA was reverse transcribed into first-strand cDNA in a 20µl final volume using miScript II RT Kit (Qiagen, Venlo, Limburg,

Netherlands) according to the manufacturer's protocol. Each reaction contained: 10x miScript Nucleic mix, RNase free water, miScript reverse transcriptase mix and 5x miScript HiSpec buffer. The reverse transcription master mix was added to the RNA template and incubated for 60 minutes at 37ºC; later the sample was incubated at 95ºC for 5 minutes to inactivate miScript reverse transcriptase. The reaction was diluted by adding 200µl RNase free water provided by kit and stored at -20ºC for further analysis.

3.6. miScript PCR Array analysis

Expression level of 84 miRNAs with antiviral and/or immunological function was evaluated in unstimulated PBMC and plasma samples using a miRNA PCR Array (MIHS-111Z) (Qiagen, Venlo, Limburg, Netherlands) (Table 3.1). Reactions were performed using a 2x QuantiTect SYBR Green PCR master mix (Qiagen, Venlo, Limburg, Netherlands).

SYBR Green specifically binds double-stranded DNA by intercalating between base pairs, and fluoresces only when bound to DNA. Detection of the fluorescent signal occurs during the PCR cycle at the end of either the annealing or the extension step when the greatest amount of double-stranded DNA product is present. By recording the amount of fluorescence emission at each cycle, the PCR reaction was monitored during exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. Reactions containing a greater amount of the initial template substrate generate a detectable amount of product (a detectable signal) earlier than reactions containing a smaller amount of template. Appearance of detectable fluorescence at an earlier cycle number indicates a greater amount of initial template substrate. The detectable amount of fluorescence, a signal significantly greater than background, is known as the threshold. The cycle during which a reaction emits that threshold level of fluorescence is known as the threshold cycle,

abbreviated Ct. A Ct value of 40 or higher means no amplification and this value was not included in the calculations.

In summary, SYBR Green PCR master mix, 10x miScript universal primer, RNase-free water and template cDNA were mixed to prepare one reaction mix. Experiments were run on all of the subjects included in the study pooled into a unique HESN HC and HIV+ sample. Thus, the results represent the mean value of the different targets analyzed in HESN, HC and HIV+ subjects. The arrays were performed on CFX ConnectTM Real time PCR system (BIO RAD, Hercules, CA, USA) using the following running protocol: Initial activation step at 95°C for 15 minutes, 40 cycles at 94°C for 15 seconds (denaturation) and 55°C for 30 seconds (annealing), followed by 1 step at 70°C for 30 seconds (extension). Undetermined raw Ct values were set to 40. Expression profile was analyzed using the PCR Array Gene Expression Analysis Software (SABiosciences, Frederick, MD, USA). For each miRNA, Ct values were transformed into relative quantities using as normalization factor the arithmetical mean of the reference miRNAs available in the arrays (SNORD68, SNORD72, RNU6-2 for basal PBMC and miR-93 and RNU6-2 for plasma samples). Controls were also included on each array for genomic DNA contamination, RNA quality, and general PCR performance. Fold regulation of ± 2.5 was considered as significant.

T-Cell Differentiation	
Double Negative (CD4-/CD8-):	let-7d-5p, let-7e-5p, miR-126-3p, miR-128, miR-146b-5p, miR-15a-5p, miR-17-5p, miR-17-3p, miR-181c-5p, miR-191-5p, miR-9a-3p, miR-199a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miR-222-3p, miR-223-3p, miR-28-5p, miR-29c-3p, miR-138-5p, miR-342-3p, miR-423-5p, miR-93-5p, miR-98-5p.
Double Positive (CD4+/CD8+):	let-7b-5p, miR-181a-5p, miR-181b-5p, miR-181d, miR-19b-3p.
CD4+ Naive:	miR-132-3p, miR-146a-5p, miR-182-5p, miR-184, miR-25-3p, miR-326, miR-92a-3p.
CD8+ Naive:	let-7a-5p, let-7c, let-7f-5p, let-7g-5p, miR-130b-3p, miR-139-5p, miR-142-3p, miR-150-5p, miR-155-5p, miR-15a-3p, miR-16-5p, miR-26a-5p, miR-26b-5p, miR-29b-3p, miR-30b-5p, miR-30c-5p, miR-30d-5p.
CD8+ Effector:	miR-147a, miR-148a-3p, miR-18a-5p, miR-27a-3p, miR-27b-3p.
CD8+ Memory:	let-7i-5p, miR-106b-5p, miR-142-5p, miR-15b-5p, miR-17-5p, miR-21-5p, miR-23a-3p, miR-23b-3p, miR-24-3p, miR-29a-3p, miR-31-5p.
B-Cell Differentiation:	
Naive:	let-7a-5p, let-7b-5p, let-7d-5p, let-7g-5p, let-7i-5p, miR-101-3p, miR-132-3p, miR-142-3p, miR-142-5p, miR-150-5p, miR-181c-5p, miR-195-5p, miR-204-5p, miR-214-3p, miR-221-3p, miR-222-3p, miR-223-3p, miR-29b-3p, miR-30e-5p, miR-331-3p, miR-92a-3p.
Germinal Center:	miR-106b-5p, miR-130b-3p, miR-132-3p, miR-148a-3p, miR-15a-5p, miR-15b-5p, miR-16-5p, miR-17-5p, miR-17-3p, miR-181a-5p, miR-181b-5p, miR-191-5p, miR-19a-3p, miR-19b-3p, miR-210, miR-23b-3p, miR-25-3p, miR-28-5p, miR-30d-5p, miR-93-5p,
Memory:	miR-100-5p, miR-125b-5p, miR-145-5p, miR-146a-5p, miR-155-5p, miR-21-5p, miR-23a-3p, miR-24-3p, miR-26a-5p, miR-26b-5p, miR-27a-3p, miR-27b-3p, miR-29a-3p, miR-29c-3p, miR-30b-5p, miR-30c-5p, miR-34a-5p.
Differentially Expressed in Tregs:	miR-100-5p, miR-125b-5p, miR-146a-5p, miR-181c-5p, miR-20b-5p, miR-21-5p, miR-31-5p, miR-335-5p, miR-365a-3p, miR-99a-5p.
T-Cell Activation:	let-7e-5p, let-7i-5p, miR-106b-5p, miR-139-5p, miR-142-5p, miR-146a-5p, miR-146b-5p, miR-155-5p, miR-15a-5p, miR-15a-3p, miR-181a-5p, miR-181c-5p, miR-195-5p, miR-20b-5p, miR-214-3p, miR-223-3p, miR-23a-3p, miR-23b-3p, miR-25-3p, miR-26a-5p, miR-26b-5p, miR-27a-3p, miR-27b-3p, miR-29a-3p, miR-29b-3p, miR-29c-3p, miR-30a-5p, miR-30b-5p, miR-30e-5p, miR-342-3p, miR-346, miR-574-3p, miR-98-5p

Table 3.1 – Complete list of T-Cell & B-Cell Activation miScript miRNA PCR Array analyzed (SABiosciences)

3.7. Individual miScript Real-Time PCR

Target miRNAs showing marked differences between HESN, HC and HIV+ patients in both unstimulated PBMC and plasma were retested by RT-PCR on each individual sample (PBMC, plasma, supernatant, monocyte, MDM and $CD4^+$ T cells). Samples were amplified using the miScript SYBR Green PCR Kit with the same running protocol used for array analyses.

The primers (Qiagen, Venlo, Limburg, Netherlands) used were: hsa-miR-15a-5p, hsa-miR-16-5p, hsa-miR-17-5p, hsa-miR-21-5p, hsa-miR-26a, has-

miR-28-5p, hsa-miR-29a-3p, hsa-miR-29b-3p, hsa-miR-29c-3p, hsa-miR-34a-5p, hsa-miR-92a-3p, hsa-miR-125b-5p, hsa-miR-138-5p, hsa-miR-146a-5p, hsa-miR-150-5p, hsa-miR-155-5p, hsa-miR-190b, hsa-miR-223-3p, hsa-miR-326, hsa-miR-382-5p. Endogenous controls used to normalize the relative miRNA expression were: small RNA U6 for isolated cells (PBMC, monocyte, MDM and $CD4^+$ T cells) and miR-425 and miR-93 for extracellular miRNA (plasma and supernatant samples) as used by others (Sung et al, 2009) (Thapa, 2014) (Zhu W, 2009). Synthetic cel-miRNA-39 was also used as quality control of miRNA extraction for the measurement of extracellular miRNA (Thapa, 2014). Melting point or dissociation curve analysis was performed for amplicon identification for all miRNAs.

Experiments were performed in duplicates for each sample; reverse transcriptase-negative controls were included in each batch of reactions. The fold changes in miRNAs were calculated by the equation $2^{-\Delta\Delta Ct}$ as ratios between the target gene and the housekeeping miRNAs.

3.8. cDNA synthesis and retrotranscription (RT)

One microgram of RNA from unstimulated PBMCs was reverse transcribed into first-strand cDNA in a 20µl final volume containing 1 µM oligonucleotide, 1 µM random hexanucleotide primers and 200 U Moloney murine leukemia virus reverse transcriptase (Promega).

Total RNA was dissolved in RNase-free water, and purified from genomic DNA with TURBO DNAse (Applied Biosystems/Ambion, Austin, TX, USA), a genetically engineered form of bovine DNase I with greater catalytic efficiency than conventional DNase I at higher salt concentrations and lower DNA concentrations. One reaction mixture, containing 1µg of RNA, Turbo DNasi 1U and TURBO DNasi Buffer, was incubated at 37°C for 30 minutes. Then DNase inactivation reagent (Applied Biosystems/Ambion, Austin, TX, USA), was added to the mixture to inactivate the DNase, since it can bind

and remove the divalent cations from DNase. Subsequently, a reaction mixture containing 1 μM random hexanucleotide primers, 1 μM oligo dT and the RNA was heated at 70°C for 5 minutes to melt secondary structure within the template. The mixture was immediately cooled on ice to prevent reforming secondary structures. Finally, dNTPs mix, 200U Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 20U Recombinant RNasi inhibitor and M-MLV 5X reaction buffer were added to each sample (Promega, Fitchburg, WI, USA). The reaction mix were incubated at 42°C for 60 minutes and then heated 5 minutes at 95°C to inactivate the RT mix.

3.9. Real time PCR and gene expression analysis

Real time PCR was performed for Dicer, Drosha, ABCA1 and GAPDH on CFX ConnectTM Real time PCR system (BIO RAD, Hercules, CA, USA) using a SYBR Green PCR mix (BIO RAD). Results were calculated by $2^{-\Delta\Delta Ct}$ equation as ratios between the target and GAPDH as a housekeeping gene. Reactions were performed according to the following thermal profile: an initial 95°C for 15 minutes (denaturation) followed by 40 cycles of 15 sec at 95°C (denaturation), 1 min at 60°C (annealing) and 20 seconds at 72°C (extension). Melting curve analysis was also performed for amplicon identification. Ct value of 40 or higher not included in the calculations. All the samples were analyzed in duplicate. The primers are summarized in Table 3.2.

GAPDH	For Rev	CGGATTTGGTCGTATTGGG GCTTCCCGTTCTCAGCCTTG
DICER	For Rev	GGGGTGTGTGAAGGGGGATA ACAGCAAGGCAGGACCAAGC
DROSHA	For Rev	CCTGCGTCTGCTGTTCGTCT GGCTGGGGTGTTTTGAGTGG
ABCA1	For Rev	CCCAAGATGACAAGTAACATGCTC GGAAGCGCTCCTGTTGCAG

Table 3.2. List of primers used to assess the expression of DICER, DROSHA and ABCA1 genes. GAPDH was used as an endogenous housekeeping gene.

3.10. Flow cytometric analysis

Gallios flow cytometer (Beckman-Coulter, Miami, FL), supplied with a double 15-mW argon ion laser operating at 456 and 488, was used to perform all the cytometric analyses. Data were analyzed using Kaluza software (Beckman Coulter). Monocyte percentage was checked by staining with the FITC- labelled anti-human CD14 monoclonal antibody (clone RMO52, Beckman Coulter). For each analysis, we acquired 20,000 events. Green fluorescence from FITC (FL1) was gathered through a 525-nm band-pass filter. Linear amplifiers were utilized to collect the data for forward and side scatter and logarithmic amplifiers for FL1.

3.11. Statistical analysis

The relative expression levels of miRNA and target gene were calculated using the comparative $\Delta\Delta C_t$ method as described previously. The fold changes were calculated by the equation $2^{-\Delta\Delta Ct}$. Data were analyzed using Student's T or ANOVA test by GRAPHPAD PRISM version 5 (Graphpad software, La Jolla, Ca, USA), and p-values of 0.05 or less were considered to be significant.

RESULTS

4.1. Differential miRNA expression in unstimulated PBMC

In order to verify if natural resistance to HIV-1 infection is associated with a specific miRNA profile we evaluate the expression of 84 miRNAs (Table 3.1) endowed with immunomodulant and/or antiviral properties. Therefore, a Real time PCR array was performed on unstimulated PBMC of 30 well characterized HESNs (Biasin 2010), 30 HIV-1 positive and 30 HC individuals. At first, we decided to examine PBMC miRNA expression rather than focusing on subsets of cells, since PBMCs include the major circulating targets of HIV infection and knowledge of overall expression is the logical first step towards characterizing cell subtype expression. Individual samples were grouped together in these initial assays to create 3 different pools and the expression level of miRNAs was evaluated using a miRNA PCR Array (MIHS-111Z). Array results were calculated relative to SNORD68, SNORD72, and RNU6-2 expression.

Results showed that, whereas the expression of 50 miRNAs was comparable in the three groups, 1) 10 miRNAs mir-26b, mir-29b, mir-29c, mir-34, mir-146, mir-148a, mir-155, mir-195, mir-326 and mir-365b) were significantly increased in both HESN and HIV+ individuals compared to HC; 2) 9 miRNAs (mir-9a, mir-17, mir-18a, mir-20b, mir-21, mir-106b, mir-126, mir-130b and mir-182) were upregulated and 3 miRNAs (mir-138, mir-150 and mir-190) were down regulated only in HIV+ compared to HC; and 3) the expression of 12 miRNAs (mir-15a, mir-16, mir-26a, mir-28, mir-29a, mir-92, mir-99a, mir-125b, mir-128, mir-142, mir-223 and mir-423) was significantly augmented in HESN alone compared with HC (Figure 4.1A).

4.2. Differential miRNA expression in plasma

The plasma concentration of the same miRNAs was measured next in our samples, once again grouped into 3 pools. The expression level of miRNAs was evaluated using a miRNA PCR Array (MIHS-111Z) and results were

calculated relative to miR-93 and RNU6-2. Also in this case, whereas 53 miRNAs did not show any different modulation between three groups, the concentration of 31 miRNAs distinguished them. Thus: 1) the concentration of 13 miRNAs (mir-23b, mir-28, mir-29a, mir-29b, mir-29c, mir-30c, mir-146, mir-150, mir-155, mir-190, mir-346, mir-326 and mir-382) was similarly increased in HESN and HIV-infected individuals compared to HC; 2) 6 miRNAs (mir-16, mir-17, mir-34, mir-92, mir-210 and mir-31) were increased only in HIV+ patients compared to HC; and 3) 12 miRNAs (mir-15a, mir-21, mir-25, mir-26a, mir-98, mir-125b, mir-138, mir-139, mir-147a, mir-184, mir-214 and mir-223) were augmented in plasma of HESN alone compared to HC (Figure 4.1B).

4.3. Validation of miRNA expression in unstimulated PBMC by individual Real Time PCR

To further confirm these results, the expression of the 20 miRNAs (miR-17, miR-21, miR-26, miR-28, miR-29a, miR-29b, miR-29c, miR-34a, miR-92, miR-125b, miR-133b, miR-138, miR-146, miR-149, miR-150, miR-155, miR-190, miR-223, miR-326 and miR-382) that were augmented in unstimulated PBMC and/or in plasma of HESN and HIV-infected individuals was validated by individual real time PCR. Results were calculated relative to snRNA U6 as an endogenous control to normalize miRNA expression and they are shown as fold change expression. Results obtained in unstimulated PBMC confirmed that all these miRNAs are differently expressed in HESN and HIV-infected individuals compared to HC (Figure 4.2A and 4.2B), with statistically significant differences being observed in 5 cases (miR-29a, miR-138, miR-150, miR-190, and miR-223) (Figure 4.2B). Notably, amongst these 5 molecules 3 miRNAs (miR-138, miR-150 and miR-190) play a role in HIV-1 latency: the expression of these 3 miRNAs was significantly reduced in HIV-infected patients alone. The expression of the 2 remaining miRNAs (miR-

29a and miR-223) was significantly increased exclusively in HESN ($p<0.01$ for all comparisons) (Figure 4.2B): these miRNAs are known to suppress HIV replication.

4.4. Validation of miRNA in plasma by individual Real Time PCR

Plasma concentration of the same 20 miRNAs was validated as well by individual real time PCR using miScript SYBR Green PCR Kit. MiRNA expression level was analyzed using miR-93 and miR-425 to normalize the relative miRNA. Also, since it is not possible to test the quality of MiRNA using Nanodrop Spectrophotometer, spiked-in Ce-miR-39-1 was used to monitor the extraction efficiency of obtained miRNA. Only miRNAs with the approximately same Ce-miR-39-1 Ct values were used for further analysis.

Results confirmed that all the miRNA analyzed are differently expressed in the three groups (Figure 4.3A); furthermore, the concentration of 10 of these miRNAs (miR-28, miR-29a, miR-29b, miR-29c, miR125b, miR-146, miR-150, miR-155, miR-190 and miR-382) is increased in both HESN and HIV+ individuals compared to HC (Figure 4.3B), indicating the presence of a "retroviral exposure signature" that is shared between these two groups of individuals, and occurs even in the absence of a productive infection. Plasma expression of miR-138 and miR-223, however, was increased in HESN alone compared to either HIV patients (miR-138 and miR-223: $p<0.03$) or HC (miR-138: $p<0.03$; miR-223: $p<0.0001$) (Figure 4.3B). As the PCR normalizers used in the array and in the individual PCR are different, the replication of the results strengthens the key role played by these miRNAs in natural resistance to HIV-1 infection.

4.5. HIV-1 p24 and miRNA expression analyses following in vitro HIV infection of PBMC

To verify if miRNA expression is altered following HIV-1 infection, the same 20 miRNAs were analyzed in PBMCs from HESN and HC in vitro infected with R5 HIV virus. MiRNA expression was analyzed on PBMC at 7 days post-infection while p24 antigen was quantified on supernatants of *in vitro* HIV infected PBMCs.

HIV-1 p24 ELISA confirmed that PBMC from HESN display a better control of HIV-1 infection, in fact HC individuals sustain significantly higher HIV-$1_{Ba\text{-}L}$ replication compared with that of HESN individuals (Figure 4.4A). No differences in miRNA expression were observed 1 day post infection in HESN compared to HC (data not shown). Seven days post infection; nevertheless, all the 20 analyzed miRNAs were down regulated to various extents in HESN compared to HC (Figure 4.4B); these differences reached statistical significance for miR-28 ($p<0.006$), miR-29a ($p<0.04$), miR-125b ($p<0.05$), miR-150 ($p<0.02$) and miR-223 ($p<0.05$) (Figure 4.4C). Our results suggest that following HIV-1 infection these miRNAs are released in the extracellular milieu where they could display a paracrine control over neighboring cells.

4.6. Differential miRNA expression in supernatant from HIV-infected PBMC

To verify whether miRNAs are indeed released in the extracellular milieu by HIV-1 infected cells, the expression level of the 20 selected miRNAs that were down-regulated in *in vitro* HIV-infected PBMCs (see above) was analyzed next in the supernatant from the same PBMCs.

Results showed that this was indeed the case as the expression level of all the analyzed miRNAs was higher 7-days post infection in supernatants of HESN compared to those of HC (Figure 5.5A). These differences reached

statistical significance for miR-28 ($p<0.05$), miR-29a ($p<0.04$), miR-125b ($p<0.05$), miR-150 ($p<0.05$) and miR-223 ($p<0.05$) (Figure 5.5B).

4.7. Differential miRNA expression in isolated Monocytes

We then decided to analyze miRNA profile in fractionated PBMC starting from basal monocyte. Ten miRNAs were analyzed in basal monocyte based on their role in HIV-1 infection in monocytes (Wang et al, 2009) (Sung et al, 2009): miR-28, miR-29a, miR-29b, miR-125b, miR-150, miR-155, miR-190, miR-198, miR-223 and miR-382. Likewise to miRNA signature in basal PBMC, HESNs profile in monocytes clustered with HCs ones (Figure 4.6A); conversely HIV-1 infected patients showed a significant reduction in anti HIV-1 miRNA expression (miR-29a, miR-28, miR-125b and miR-150) suggesting that a decline in these miRNA increases susceptibility to HIV-1 infection (Figure 4.6B). However, the expression of miR-198 and miR-223 was significantly augmented only in HESN subjects compared to both HIV-1+ patients ($p<0.01$) and HC ($p< 0.05$) (Figure 4.6A). The other tested miRNAs showed the same trend and had higher miRNA expression in HESN individuals and HCs (Figure 4.6B). PCR results were calculated relative to snRNA U6 to normalize miRNA expression.

4.8. HIV-1 p24 and miRNA expression analyses following in vitro HIV infection of MDM

Macrophages derived from monocyte (MDM) from HESN and HC subjects were infected with HIV-$1_{Ba\text{-}L}$ virus and miRNA expression profile was evaluated 9-days post infection. HIV-1 p24 ELISA established that MDM from HESN are able to better cope with HIV-1 infection compared with HC ones ($p<0.01$) (Figure 4.7A). Furthermore, reduced susceptibility to HIV-1 infection in HESN MDM was associated with higher expression of all our tested miRNAs compared to HCs (Figure 4.7B); these differences reached

statistical significance for miR-29a, miR-150, miR-155, miR-190, miR-198, miR-223 and miR-382. Thus strengthening the protective role exerted by these miRNAs in resistance to HIV-1 infection (Figure 4.7C).

4.9. Analyses of ABCA1 expression level

As Vickers et al. showed that the overexpression of miRNA-233 elevates ABCA1 mRNA and protein levels (Vickers KC, et al. 2014), we analyzed its expression in unstimulated PBMCs as well as in monocytes and HIV-infected MDM isolated from all the individuals included in the study (Figure 4.8). Interestingly, ABCA1 mRNA level was higher in both PBMC (Figure 4.8 A) and monocytes (Figure 4.8 B) from HESN and HIV+ subjects compared with HC (PBMC: HESN vs HC $p<0.001$; HIV+ vs HC $p<0.05$. Monocytes: HESN vs HC $p<0.05$) showing the highest expression in HESN individuals (PBMC: HESN vs HIV+ $p<0.01$. Monocytes: HESN vs HIV $p<0.05$). Likewise, the expression of ABCA1 was significantly increased even in HIV-1 infected MDM from HESN compared to HC ones ($p<0.01$). These results confirmed that in HESN subjects the up-regulation of miR-223 is accompanied by an increased expression of ABCA1 (Figure 4.8).

4.10. Analysis of Dicer and Drosha expression level

As increased expression levels of miRNAs could be secondary to higher levels of Dicer and Drosha, two essential enzymes in miRNA biogenesis, these enzymes were analyzed in unstimulated PBMCs of all of the subjects included in the study.

However, our results did not show any appreciable difference in the expression level of Dicer and Drosha in PBMCs of HESN compared to HC and HIV+ individuals (Figure 4.9).

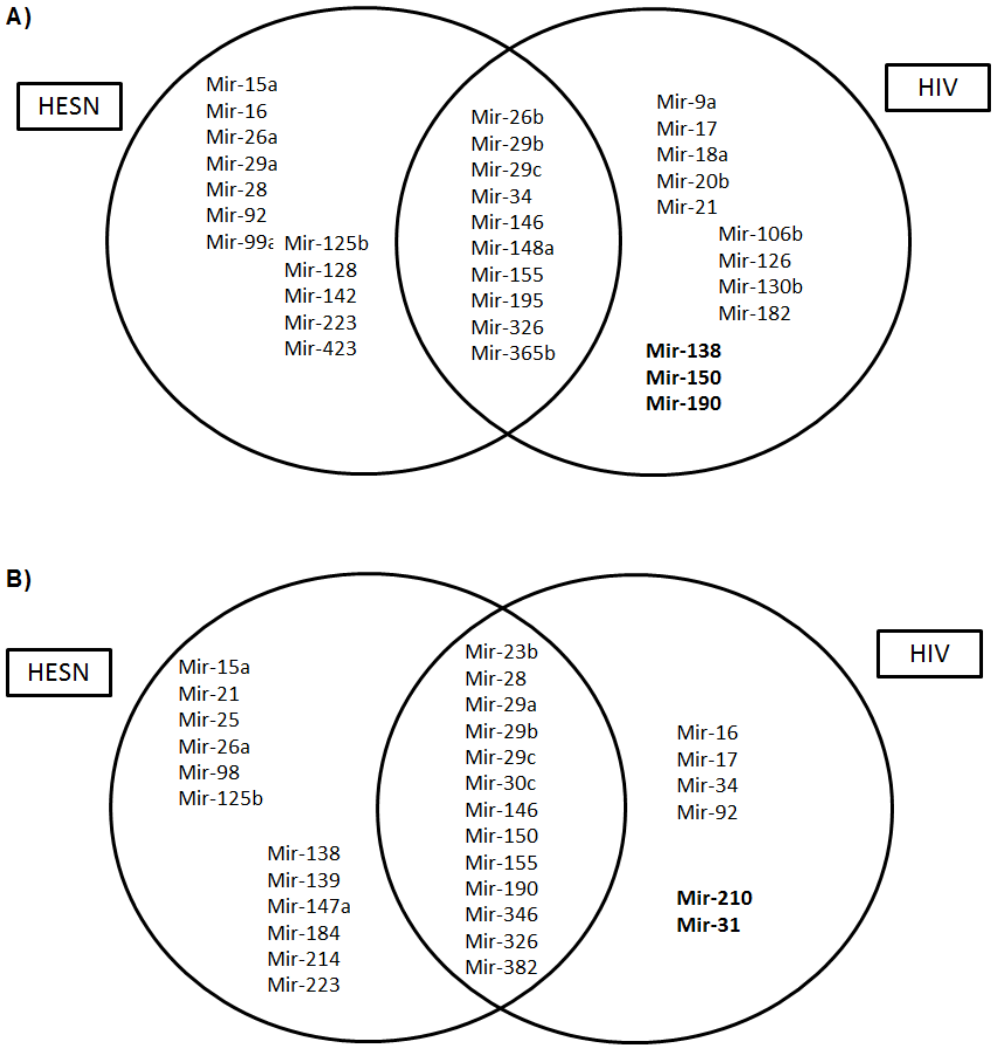


Figure 4.1. *Vann diagram of miRNA profile in unstimulated PBMC* ***(A)*** *and plasma samples* ***(B)*** *of HESN and HIV+ individuals. The miRNAs differentially expressed in HESN and HIV+ samples compared to HC are shown in two overlapping circles.* ***(A)*** *Results were calculated relative to SNORD68, SNORD72, RNU6-2 expression.* ***(B)*** *Results were calculated relative to Ce_miR-39_1, miR-93 and RNU6-2. Fold regulation of ±2.5 was considered as significant. The miRNAs with fold-regulation values less than -2.5 (downregulation) are indicated in bold.*

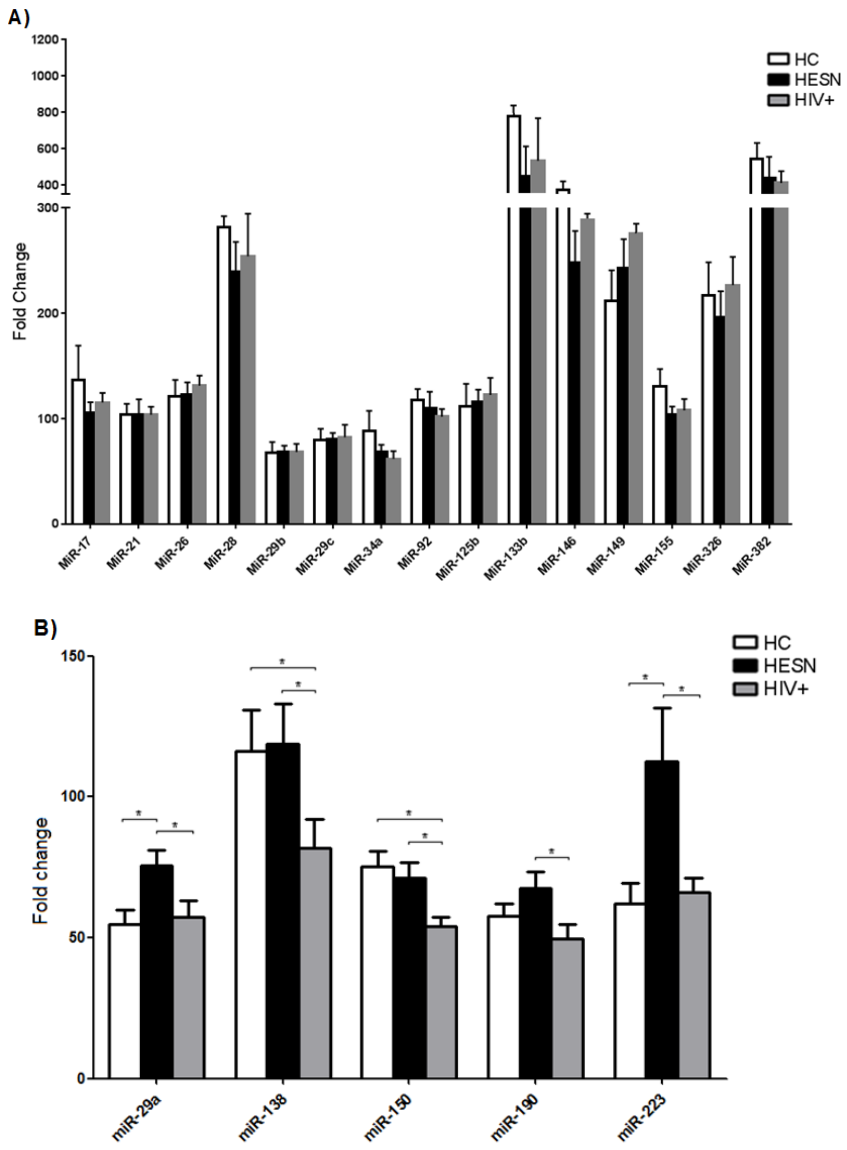


Figure 4.2. *MiRNA expression analysis in unstimulated PBMC by individual real time PCR. Analyses were performed on unstimulated PBMCs of 30 HC (white bars), 30 HESN (black bars) and 30 HIV+ (gray bars) individuals. Results were calculated relative to snRNA U6 as an endogenous control to normalize miRNA expression. Results confirmed that all these miRNAs are differently expressed in the 3 groups* ***(A)****, with statistically significant differences being observed for 5 miRNAs* ***(B)****. Values are expressed as mean ± SE. Significance is indicated as follows: * = p<0.05.*

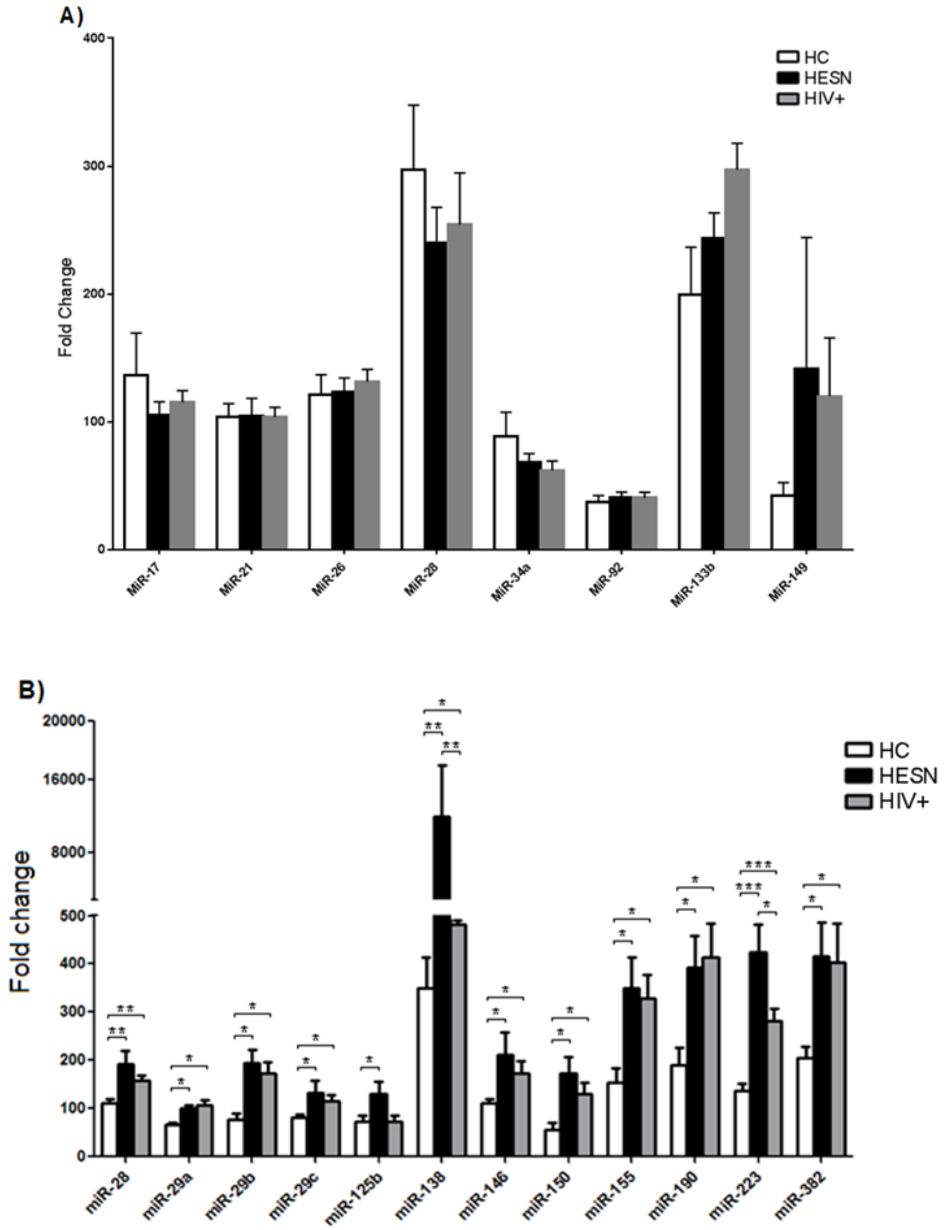


Figure 4.3. *MiRNA expression analysis in plasma samples by individual real time PCR. Plasma samples were collected from 30 HC (white bars), 30 HESN (black bars) and 30 HIV+ (gray bars) individuals. MiRNA expression level was analyzed using miR-93, miR-425 and Ce-miR-39-1 to normalize the relative miRNA and to monitor the miRNA extraction efficiency.* ***(A)*** *Results confirmed that all the miRNA analyzed are differently expressed in the three groups; with statistically significant differences being observed for 12 miRNAs* ***(B)****. Values are expressed as mean ± SE. Significance is indicated as follows: * = p<0.05, **= p<0.01, ***= p<0.001.*

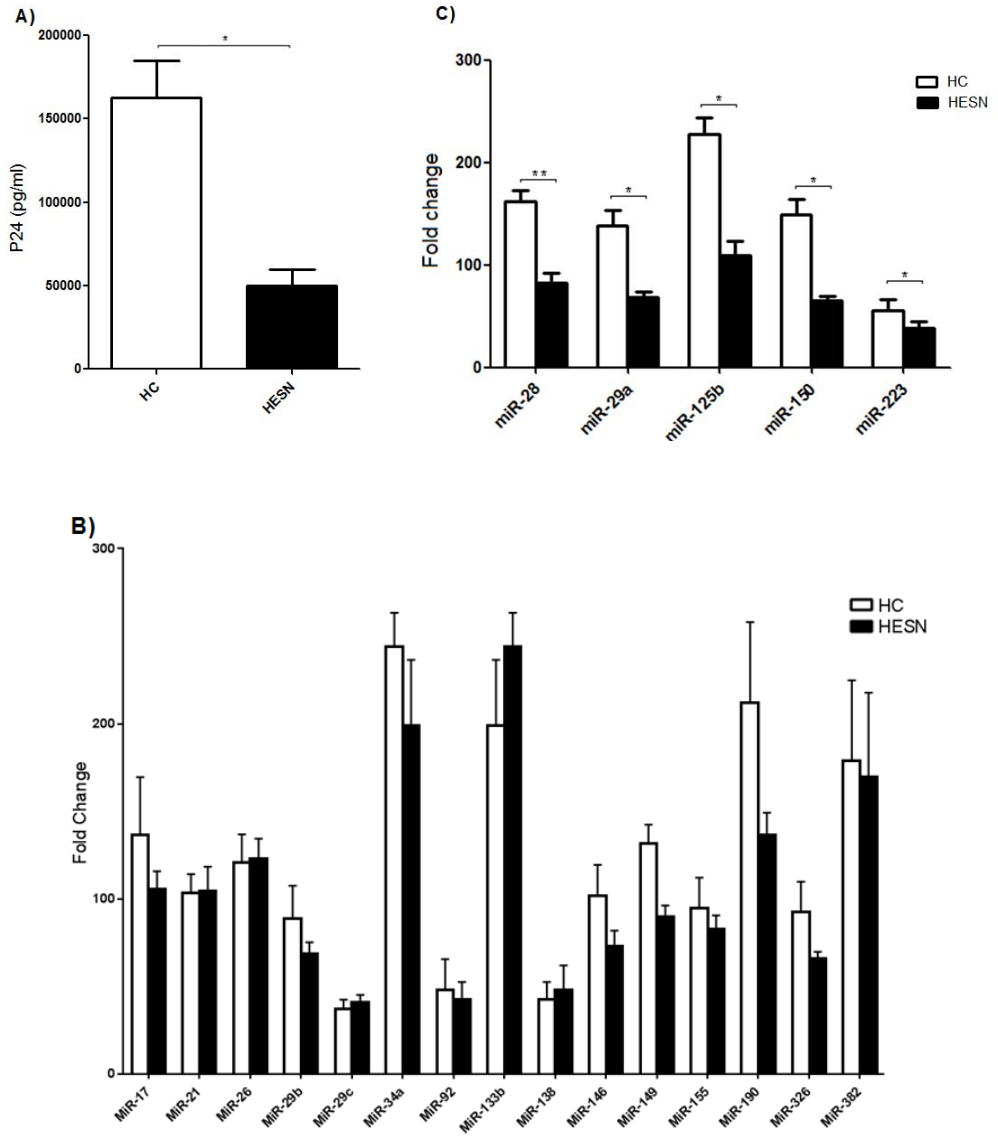


Figure 4.4. *PBMC from 30 HESN (black bar) and 30 HC (white bar) were in vitro infected with HIV-1$_{Ba-L}$ for 7 days.* ***(A)*** *P24 levels were measured from HIV-1$_{BaL}$-Infected cells. Seven days post infection; all the 20 analyzed miRNAs were down regulated to various extents in HESN compared to HC* ***(B)****; these differences reached statistical significance for 5 miRNAs* ***(C)****. RNA U6 was used as endogenous control to normalize the relative miRNA expression. Results are shown as mean ± SE. Significance is indicated as follows: * = p<0.05; **= p<0.01.*

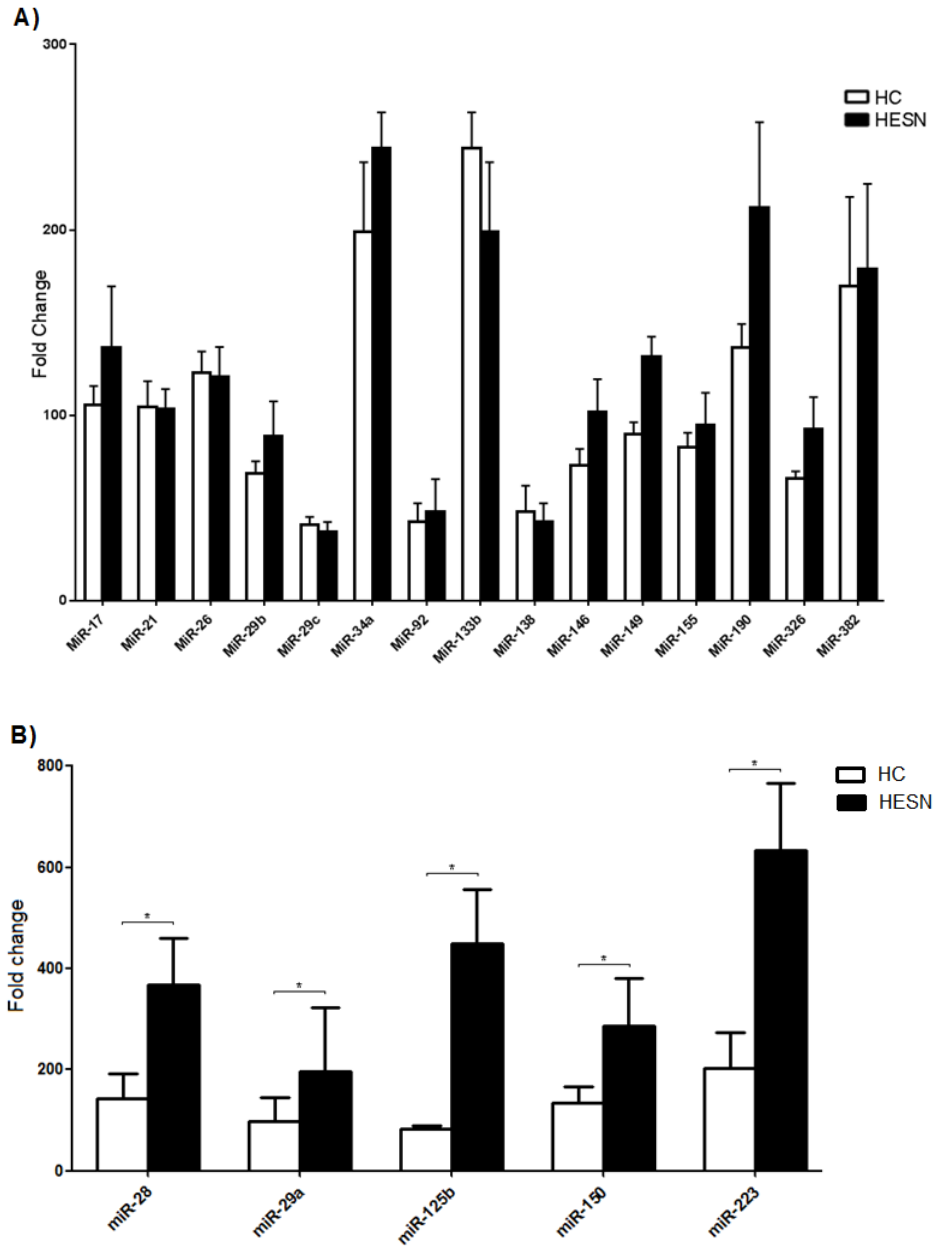


Figure 4.5. *PBMCs isolated from 30 HC (white bars) and 30 HESN (black bars) individuals were infected with HIV-1_{Ba-L} and miRNA expression level was analyzed on 7 day post infection supernatant. Expression level of all the analyzed miRNAs was higher 7-days post infection in supernatants of HESN compared to those of HC* ***(A)****. These differences reached statistical significance for 5 miRNAs* ***(B)****. MiR-93, miR-425 and Ce-miR-39-1 were used to normalize the relative miRNA and to monitor the miRNA extraction efficiency. Values are mean ± SE. Significance is indicated as follows: * = p<0.05.*

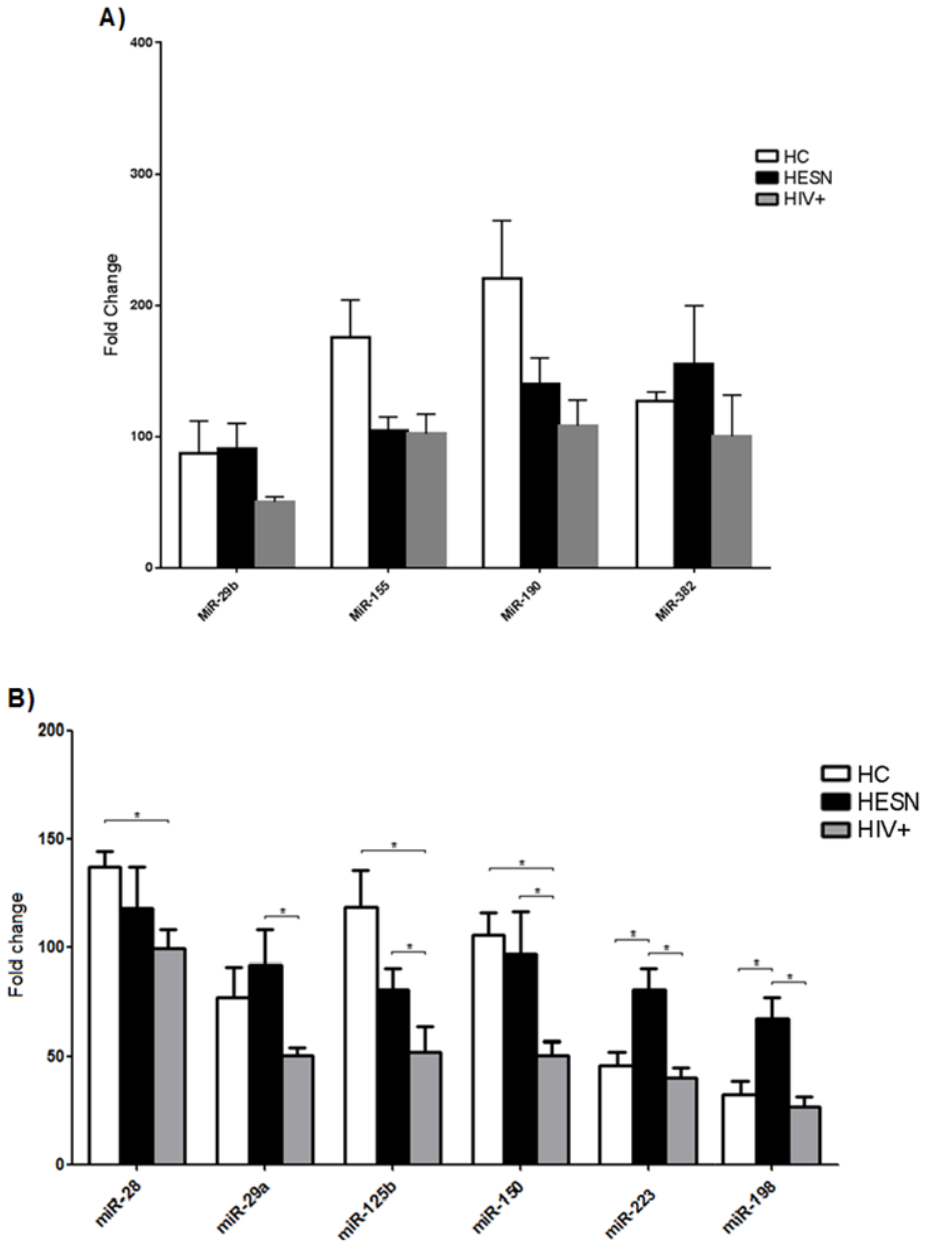


Figure.4.6. *Monocytes were isolated from 30 HC (white bars), 30 HESN (black bars) and 30 HIV+ (gray bars) individuals. MiRNA expression was calculated relative to snRNA U6 as an endogenous control to normalize miRNA expression.* ***(A)*** *HESNs profile in monocytes clustered with HCs ones;* ***(B)*** *statistically significant differences were observed for 6 miRNAs. Values are shown as mean ± SE. Significance is indicated as follows: * = p<0.05.*

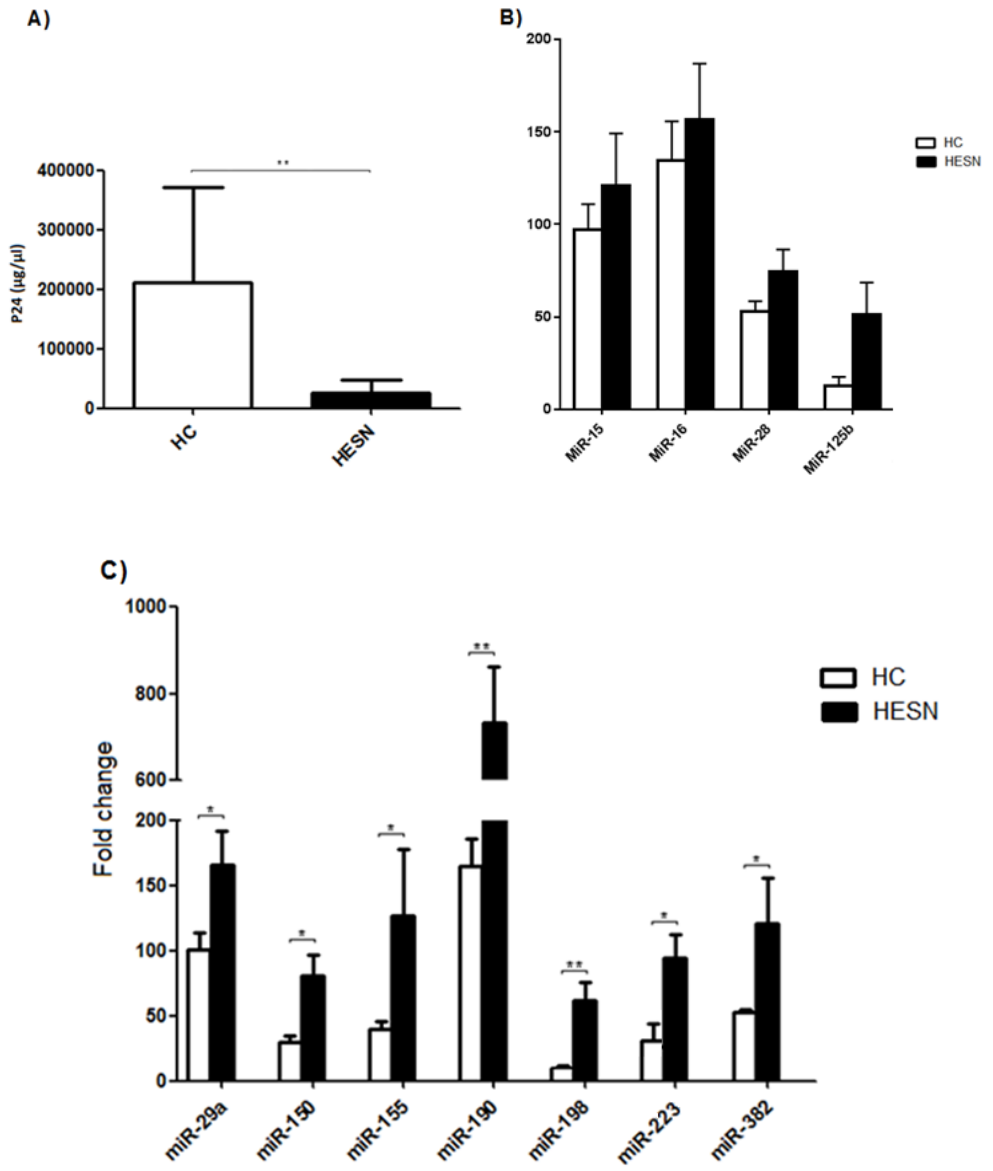
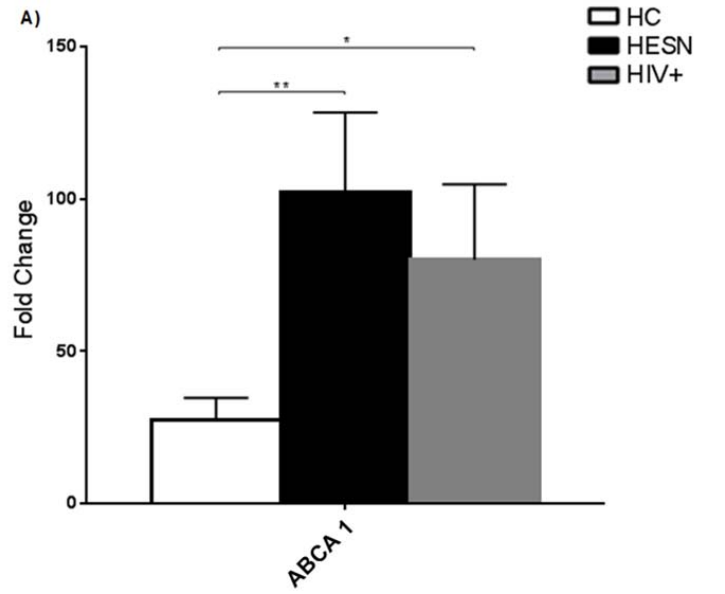


Figure 4.7. *MDMs of 30 HC (white bars) and 30 HESN (black bars) individuals were infected with HIV-1$_{Ba-L.}$* ***(A)*** *P24 levels measured from HIV-1BaL-Infected cells.* ***(B)*** *Reduced susceptibility to HIV-1 infection in HESN MDM was associated with higher expression of all tested miRNAs compared to HCs;* ***(C)*** *these differences reached statistical significance for 6 miRNA. RNA U6 was used as endogenous controls to normalize the relative miRNA expression. Results are shown as mean ± SE. Significance is indicated as follows: * = p<0.05; **= p<0.01.*


A)
HC
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HIV+
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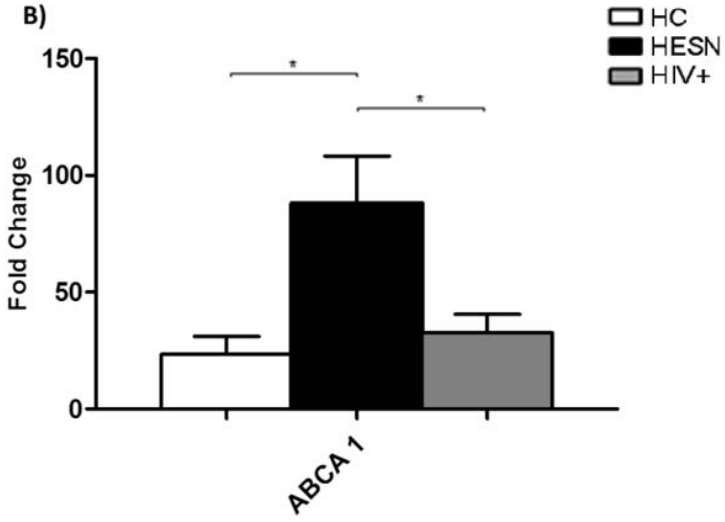
B)
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HESN
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ABCA 1

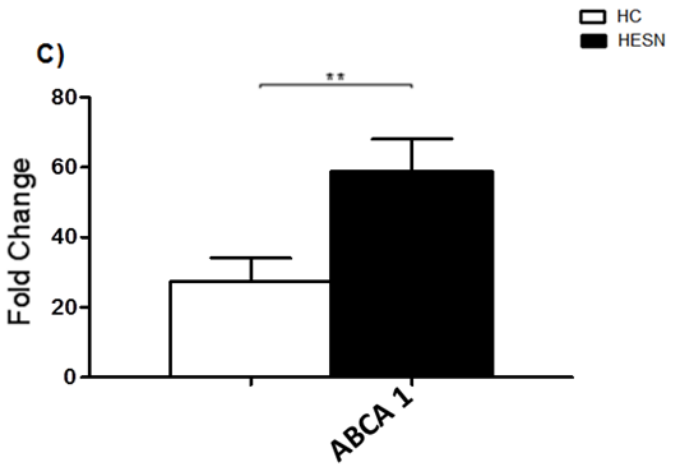

Figure 4.8. *Cells were isolated from 30 HC (white bars), 30 HESN (black bars) and 30 HIV+ (gray bars) individuals. Results were calculated relative to GAPDH expression and they are shown as fold change expression. ABCA1 mRNA expression in basal PBMC(A) monocytes (B) and MDM (C) by real time PCR. Values are mean ± SE. Significance is indicated as follows: * = p<0.05; **= p<0.01.*

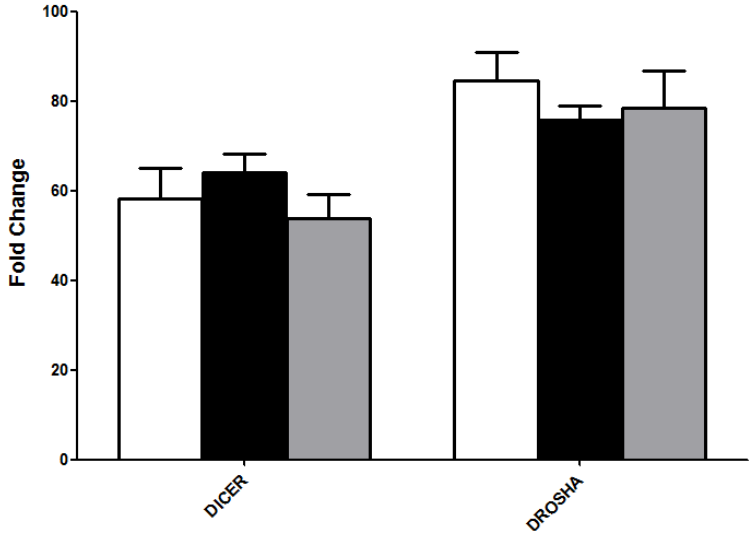

Figure 4.9. *Dicer and Drosha mRNA expression in basal PBMC by real time PCR. Basal PBMCs were isolated from 30 HC (white bars), 30 HESN (black bars) and 30 HIV+ (gray bars) individuals. Results were calculated relative to GAPDH expression and they are shown as fold change expression. Values are mean ± SE.*

DISCUSSION & CONCLUSIONS

Eighty-four miRNAs which are mainly expressed by lympho-monocytes and involved in the regulation of immune responses and/or in host virus interaction (Bartel, 2009) (Guo, 2010) were analyzed to verify whether a particular miRNA profile could be identified in HESN; results were validated by analyses on 20 of these miRNAs by individual real time PCR. The decision to study miRNA profile in PBMC was based on their accessibility and on the fact that PBMC include the major circulating targets of HIV-1 infection, and therefore represent the logical first step towards characterizing cell subtype expression. Findings herein show that a number of miRNAs is upregulated both in HESN and HIV-infected individuals, indicating that exposure to HIV impairs the miRNA profile independently of the establishment of overt infection. Additional results indicated that the expression of miR-29a and miR-223 was significantly augmented in HESN alone. Because these two miRNAs are known to hamper HIV replication the detection of their up-regulation in both plasma and cell culture medium from HIV-1 infected PBMC of HESN suggests a protective role for these molecules in resistance to HIV-1 infection. Finally, results showing that the expression level of Dicer and Drosha, two enzymes playing essential roles in miRNA biogenesis, was comparable in HIV-infected individuals, HESN and HC suggest that exposure to HIV changes miRNAs profiles through mechanisms that are independent of Dicer and Drosha. Yet, in the light of recent acquisition suggesting that Drosha protein level and nuclear localization can be altered by molecular mechanisms such as ubiquitination, acetylation (Tang X et al, 2013) and phosphorylation (Tang X et al, 2010), without affecting mRNA levels, we cannot rule out that differences in miRNA expression observed in our cohort are dependent on these mechanisms. Further analyses are needed to elucidate this aspect".

MicroRNA-29a has been shown to play a crucial role in thwarting HIV replication since it is able to bind both the 3' UTR sequence and the HIV-1 encoded protein Nef, hence preventing viral replication (Ahluwalia JK et al,

2008). Recent studies have also shown that reduced expression levels of miR-29a have been seen in HIV-1 infected patients and have been associated with the presence of a secondary structure within the miR-29a binding region in HIV-1 LTR (Sun G et al, 2012). MiR-223, on the other hand, is part of a selected group of cellular miRNAs known to play an active role in modulating the ability of HIV-1 to infect resting CD4+ T lymphocytes (Huang J et al, 2007). This effect was demonstrated to depend on the ability of miR-223 to bind the 3' UTR of viral mRNA through sequence complementary. Notably, activation of resting CD4+ T cells, a process known to trigger HIV replication and to enhance HIV-1 susceptibility is associated with the down-regulation of this miRNA. Two other independent studies confirmed miR-223 to be down regulated upon differentiation from monocyte to macrophages, thus shedding light on the mechanisms associated with the productive HIV-1 infection, taking place in monocytes (Wang X et al, 2009)(Sisk JM et al, 2012). Finally, recent results showed that miR-223 indirectly modulates cyclin T1 expression, resulting in lower HIV-1 replication (Chiang K et al, 2012), and plays a critical role in systemic cholesterol regulation by coordinated post-transcriptional control of multiple genes in cholesterol metabolism (Vickers KC et al, 2014).

In particular, it has been illustrated that adenosine triphosphate–binding cassette transporter A1 (ABCA1) mRNA and protein levels are significantly elevated following miR-223 overexpression (Vickers KC, et al. 2014). Furthermore, in *in vitro* HIV-1 infected macrophages, it has been demonstrated that the virus inhibits cholesterol efflux by Nef-mediated down-regulation of ABCA1 (Mujawar Z, 2006).These data are in line with the observation that in our HESN samples miR-223 overexpression is associated with increases in ABCA1 mRNA compared to HCs and HIV+ samples in PBMC as well as monocytes and MDM. Further analyses are needed to prove a cause-effect relation between these two variables but these preliminary results let us to speculate that higher Mir-223 levels could

contribute to ABCA-1 increase which in turn could regulate the intracellular cholesterol metabolism, essential for the correct assembly and release of functional virions.

Notably, our results are in conflict with older data showing a down-regulation of miR-223 in resting CD4 T cells from HESN (Bignami F et al, 2012) However because our PBMC samples have not been fractionated into cellular subset we reasoned that some of the differences in miRNA signature could be explained by in/out flux of miRNAs from different cell types.

The above findings allow the speculation that an initial exposure to sub infectious amounts of HIV is sufficient to modify the expression of specific miRNA in individuals in whom subsequent exposures to the same virus will not result in infection. Alternatively, it is possible that, higher levels of these 2 miRNAs are "naturally" present in HESN prior to exposure, and that the augmented amounts of these miRNAs are protective or indicative of the presence of alternate mechanisms of protection. To further investigate these hypotheses we next analyzed miRNAs expression profiles following in vitro HIV-1 infection of PBMC from HESN and HC. Unexpectedly, the expression of all the analyzed miRNAs was down-regulated to various extents in HESN compared to HC after *in vitro* infection, and differences reached statistically significance for miR-28, miR-29a, miR-125b, miR-150 and miR-223. We wondered how to reconcile the reduced susceptibility to HIV-1 infection in HESN PBMC, as shown by p24 analyses, with the observed decreased expression of miRNAs with described anti-HIV properties. Advances in the understanding of miRNA physiology, showing that miRNAs can be released upon cellular activation, offer a plausible explanation to these results. Indeed, recent data indicate that the release of miRNAs in association with exosomes, or in association with protein complexes, is not a passive phenomenon, but is actually a regulated active process, as shown by the fact that exosomal RNA content is not a mere reflection of what is found within the intracellular milieu (Zhu W et al, 2009) (Zhang Y et al, 2010).

Quantitative analyses hence show a selective enrichment of some miRNAs when exosomes are compared to cells. These data suggest that the controlled release of miRNAs into exosomes represents an additional layer of post-transcriptional regulation for miRNAs that, in this form, can have a rapid and effective paracrine effect on target genes of neighboring cells.

Our working hypothesis indeed is based on the assumption that when the immune system is activated upon viral exposure, cells that participate in the response will release a detectable quantity of miRNAs into the bloodstream. These miRNA will modulate gene expression in paracrine cells, thus controlling HIV-1 infection and replication. MicroRNAs release following lymphocyte activation is dependent on the magnitude of immune response (De Candia P, 2014). Our cohort of HESN is characterized by an immune activated phenotype (Mazzoli S et al, 1997) (Biasin M, 2010) it is therefore possible to speculate that increased miRNAs release by HESN cells could be the logical consequence of a greater responsiveness of their immune system to viral exposure. Interestingly, the expression of the miRNAs of interest was increased in both cell culture medium of HIV-infected PBMC and plasma of HESN. Actually, as Fabbri at al. demonstrated that secreted miRNAs, (mainly miR-29a), may act as paracrine agonists of TLRs (Fabbri M et al, 2012) and TLR activation has been associated with resistance to HIV-1 infection (Biasin M, 2010), it is tempting to speculate that these miRNAs could be internalized by neighboring cells and modify their susceptibility to infection.

The differences observed in miRNA expression when in vivo and in vitro infected PBMCs of HESN were analyzed could be due to the viral load used in the experimental procedure. Although the viral burden used in the in vitro assay was relatively low, cells were exposed in vitro to concentrations of HIV that likely are much higher than those encountered upon natural exposure; this could have speed up the release of those miRNA that might have a protective effect in controlling HIV infection. An alternate possible

explanation is that, whereas PBMC were infected in vitro to an HIV lab strain (HIV-1_{Ba-L}), HESN are exposed to primary HIV strains whose virulence is likely different.

In the last step, analyzing miRNA profile in fractionated subpopulations, the expression level of miRNA in basal monocyte of HESN clustered with HC subjects while HIV+ patients showed lower level of anti HIV-1 miRNA expression specifically miR-28, miR-29a, miR-125b and miR-150. Interestingly, the expression of miR-198 was exclusively upregulated in HESN individuals. It has been demonstrated that although, both monocytes and macrophages express anti HIV-1 miRNAs, their level vary from monocyte to macrophages, a characteristic that could be correlated with their susceptibility to HIV-1 infection (Xu Wang, 2009). Indeed freshly isolated monocytes express higher levels of miR-28, miR-150 compared to MDM and this increase has been associated with inhibition of HIV-1 replication (Xu Wang, 2009). Furthermore, Xu Wang et.al found that the expression of these miRNA decrease in vitro when monocyte differentiates into macrophages.

In our study, MDM from HESN were less susceptible to HIV-1 infection 9-days post infection and this reduction was associated with significantly increased expression of miR-29a, miR-150, miR-155, miR-190, miR-198 and miR-382 compared to HC subjects. These results suggest that the higher expression of these miRNAs in both monocyte and MDM of HESN individuals could play important roles in resistance to HIV-1 infection in HESNs. Also, monocyte and MDM permissivity to HIV infection could be affected by the presence of these anti HIV-1 miRNAs.

Since abnormal miRNA expression seems to characterize many diseases, miRNA expression profiles are likely to become important diagnostic and prognostic tools as well as potential therapeutic targets.

The mechanism for miRNAs up regulation in HESN remains elusive. Nevertheless if HIV-1 exposure truly affects miRNA transcription and

maturation in particular subsets of PBMC, such knowledge might be useful for the design of novel HIV-1 therapies and for diagnosis, prognosis and treatment response parameters.

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