Expression of microRNA in whole blood of active pulmonary tuberculosis patients with or without HIV co-infection at selected health centres in Oromia Region, Ethiopia

By

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ABBREVIATIONS AND ACRONYMS

AFB	Acid-Fast Bacilli
AGO	Argonaute
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunodeficiency syndrome
ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
ART	Anti-Retroviral Treatment
ATT	Anti-Tuberculosis Treatment
BCG	Bacillus Calmette–Guérin
CBC	Complete Blood Count
CIA	Chronic Immune Activation
DGCR8	DiGeorge syndrome Chromosomal Region 8
DABG	Detection Above Background
DCs	Dendritic Cells
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPHI	Ethiopian Public Health Institute
FDR	False Discovery Rate
HAART	Highly Active Antiretroviral Therapy
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
LTR	Long Terminal Repeat
hsa-miR	human miRNAs
ICL	International Clinical Laboratory
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IL	Interleukin
IRAK-1	IL-1 Receptor-Associated Kinase 1
LAM	Lipoarabinomannan
LPS	Lipopolysaccharides

LTBI	Latent Tuberculosis Infection
LU	Lund University
MAPKs	Mitogen-Activated Protein Kinases
miRNA	microRNAs
mRNA	messenger RNA
Mtb	*Mycobacterium tuberculosis*
MyD88	Myeloid Differentiation primary response gene 88
Mφs	Macrophages
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NKs	Natural Killer cells
NTC	Non Template Control
PBMCs	Peripheral Blood Mononuclear Cells
PFMCs	Pleural Fluid Mononuclear Cells
PRRs	Pathogen Recognition Receptors
PTB	Pulmonary Tuberculosis
QFT	QuantiFeron®-TB Test
QRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RIN	RNA Integrity Number
RISC	RNA-Induced Silencing Complex
SAM	Significance Analysis of Microarrays
SCIBLU	Swegene Centre for Integrative Biology at Lund University
SHIP1	Src Homology Inositol Phosphatase-1
TLR2	Toll Like Receptor 2
TNFα	Tumor-Necrosis Factor alpha
TRAF6	TNF Receptor-Associated Factor 6
UTR	Untranslated Region
WHO	World Health Organization
Xpert MTB/RIF	Gene Xpert *Mycobacterium Tuberculosis*/Rifampicin

ABSTRACT

Mycobacterium tuberculosis (Mtb) and human immunodeficiency virus (HIV) infections dysregulate the host immune response via interactions with multiple cells. However, the mechanism underlying the synergy between Mtb and HIV are not fully understood. Recently non-coding microRNAs (miRNAs), which are involved in many cellular processes by regulating the translation of coding mRNAs, have been suggested to be involved in the molecular pathogenesis of Mtb and HIV. But the expression profile of miRNAs in Mtb/HIV co-infection has barely been studied. In this study, we explored the differential expression of miRNAs in whole blood of patients with pulmonary tuberculosis (PTB) with or without HIV co-infection compared to uninfected healthy controls. The study included baseline samples from participants recruited from four health centres in Oromia Region, Ethiopia. The expression profile of miRNAs was measured using Affymetrix microarray. Using the significant analysis of microarray (SAM), we identified a total of 83 differentially expressed miRNAs comparing all the three groups (q=0). Hierarchical clustering analysis based on these miRNAs revealed clear separation among these groups. Compared with uninfected controls, the expression of 35 miRNAs was significantly altered in the PTB patients with HIV co-infection. There were only three miRNAs that distinguished PTB patients from uninfected controls. In contrast, a set of 77 miRNAs differentiated PTB patients from the PTB/HIV co-infected patients. Among these we found miR-150, miR-223, and family members of let-7, miR-27 and miR-26 that previously have been characterized as immune regulatory miRNAs during Mtb and HIV-1 infections. Overall, these results indicate that the expression of a large number of miRNAs are altered in PTB/HIV patients compared with the PTB cases without HIV infection, as well as the uninfected individuals, suggesting that HIV co-infection might have a profound impact on the dysregulation of miRNAs in whole blood of PTB patients. These findings provide, at least preliminary data, in support of further basic research and open new insights into the underlying mechanisms of miRNAs involvement in the pathogenesis of Mtb/HIV co-infection. Such studies may be used to develop biomarkers for the diagnostic purposes of TB/HIV co-infection.

Key Words: Ethiopia, Mtb/HIV co-infection, Microarray, MicroRNAs, Differential expression, Hierarchical clustering

1. INTRODUCTION

The emergence of new infectious diseases and re-emergence of earlier ones are creating major health challenges for millions of people in the world. Perhaps the two most intertwined health concerns since the last three decades are tuberculosis (TB) and human immunodeficiency virus (HIV); both of which are associated with high morbidity and mortality. Tuberculosis, caused by *Mycobacterium tuberculosis* (Mtb), is a disease that causes a great number of fatalities among HIV infected individuals (Getahun *et al.*, 2010).

TB is the second common cause of death from a single infectious disease in the world (after HIV/AIDS), with over 9 million new cases and 1.5 million deaths per annum worldwide (WHO, 2014). Of these 1.1 million TB cases and 360,000 deaths were reported among people living with HIV (WHO, 2014).

Even though eradicating TB was the goal of the 20th century, the co-epidemic situation particularly in Sub-Saharan Africa, where approximately 80% of the new dual TB/HIV cases were reported, made the goal unrealistic (WHO, 2014).

Ethiopia is one of the 22 high TB burden countries and ranks eighth in the world with the incidence of 224 cases per 100,000 populations in 2013. In the same year, among all forms of TB cases tested for HIV, 11% were dually infected (WHO, 2014). Actually, the incidence decreased by half when we compared with 23 years back due to the current integrated service of anti-TB and anti-retroviral therapy. However, it still remains high as it is possibly expanding to newer population groups and other geographical areas. Although HIV-related TB is both treatable and preventable, the interaction and overlapping of TB-HIV medication complicated the management of TB in HIV infected individuals (Pukenyte *et al.*, 2007; Marks *et al.*, 2009).

In addition to being two independent major health problems, the observed associations between TB and HIV infection also has greater prominence. Many studies in human Mtb/HIV co-infection have provided several important principles to understand how these distinct pathogens additively interact to accelerate the rates of disease progression (Siawaya *et al.*, 2007). Even though the precise mechanism of co-pathogenesis still remains elusive, it has also extensively been studied that both Mtb and HIV co-infection dysregulate the host immune homeostasis (Toossi, 2003; Bezuidenhout *et al.*, 2009).

In fact, an immune-competent individual with Mtb infection can contain the bacilli substantially in a latent state for life and minority portion of patients with active tuberculosis

can succeed in getting rid of infection and lead to an inactive tuberculosis (Ernst, 2012). Nevertheless, the mechanism is unclear and concurrent HIV infection changes these scenarios by disrupting the ability of the immune cells to contain TB infection (De Noronha *et al.*, 2008; Bezuidenhout *et al.*, 2009). HIV infection also significantly increases the risk of progression from latent TB infection to active disease as well as risk of recurrent TB, but how ongoing HIV infection facilitates Mtb progression is still ill-defined.

On the other hand, existing reports also suggest that Mtb exacerbates HIV infection by enhancing viral replication within activated cells or activation of latent HIV and entry into immune cell by causing alternations in signal transduction (Toossi, 2003; Diedrich and Flynn, 2011; Shankar *et al.*, 2014).

In recent past, studies have been focused on the immunological environments (Mihret *et al.*, 2014) since both pathogens share the same target cells in specific microenvironments. Also gene expression signatures (Dawany *et al.*, 2014) of the host during mono Mtb or Mtb/HIV-1 coinfection have been put forward with the aim to develop effective vaccines and reliable diagnostic tools as well as to achieve curative therapies. However such efforts are still in infancy (Shankar *et al*., 2014).

It is clear that the host pathogen interaction leads to the trigger of cell specific intracellular signalling cascades via the stimuli of pattern recognition receptors (PRRs). As a result, recent *in vitro* and *in vivo* studies have demonstrated that these signalling pathways are tightly regulated in signal dependent manner upon microbial stimulation by regulatory molecules, the so called 'microRNAs' (miRNAs) (Bartel, 2004; Chen *et al*., 2013).

MicroRNAs are small, non-coding RNA that regulate gene expression by targeting messenger RNAs (mRNAs). They are also regulate gene function and act in fine tuning various processes in eukaryotic cells including cell signalling, apoptosis and immune cells development and response to pathogens, by modulating protein expression at the posttranscriptional level (Baltimore *et al.*, 2008). Recently their involvement as critical effector molecular fragments has been reported in the regulation of host-pathogens interaction (O'connell *et al.*, 2012).

MiRNAs control signalling triggered by ligation of microbial components to receptors expressed by immune cells, and thereby influence pro-inflammatory cytokine release (Liu *et al*., 2009). *In vitro* and *in vivo* studies thus far have been mentioned that, Mtb and HIV-1 infections trigger changes in the cellular miRNAs expression profile that can independently modulate the expression of host proteins to the benefit of both pathogens. Though it's known that TB and HIV induce miRNA production, the global expression profile during such

infections can provide insights to understanding the mechanism of pathogenesis and identify biomarkers as well as selective therapeutic targets.

1.1. Mycobacterium tuberculosis

1.1.1. Mycobacterium tuberculosis Pathology

Mycobacterium tuberculosis, the etiological agent of tuberculosis, is globally the single greatest cause of mortality due to a bacterial pathogen. It is highly contagious facultative intracellular pathogen. A major feature of Mtb is the unique cell wall (lipid rich) structure, that provides an exceptionally strong impermeable barrier to noxious compounds and resistance to common antibiotic drugs and that plays a fundamental role in virulence (Sakamoto, 2012). The disease is transmitted from person-to-person through inhalation of small (1-5 μm) droplet nuclei containing Mtb, which are expelled by coughing, sneezing, or talking of another individual with cavitary tuberculosis. Once the tubercle bacilli are inhaled, it affects mainly the lungs (pulmonary tuberculosis) and epithelial cells of the lung (Lawn and Zumla, 2011). Although lung tissue is the primary site of Mtb infection, the bacilli can spread from the site of initial infection through the lymphatics or blood to regional lymph node and other parts of the body such as kidney, liver and brain (extra pulmonary tuberculosis) (Golden and Vikram, 2005).

Introduction of Mtb to the alveoli may lead to different outcomes, either immediate clearance of the organism, latent infection, the onset of active disease, or reactivation of the disease years later. Certainly, the development of the TB diseases is dependent on the individual immune status and the virulence of the Mtb strain.

1.1.2. Immune response to Tuberculosis

1.1.2.1. Innate Immune Response

Since the adaptive immune response to Mtb is delayed, innate immune cells, such as macrophages (Mφs), dendritic cells (DCs), natural killer cells (NKs) and neutrophils, mount the early immune protection against this intracellular pathogen. The first cells that initially encounter Mtb infection are alveolar macrophages, which reside in the lung. There, the microbes are rapidly phagocytised by the macrophages and role in confining the bacilli invasion and replication into the endosomes of macrophages. Dendritic cells also partake in the phagocytic process and mediates antigen presentation for activation of T cells with specific Mtb antigen (Natarajan *et al*., 2011).

The specific recognition of Mtb components such as lipoarabinomannan (LAM), through PRRs on the surface of phagocytes is the initial part of the host innate immune response activation (Hossain and Norazmi, 2013). Among others, TLRs are evolutionarily conserved and belong to a family of type I transmembrane PRRs, which exist as both cytoplasmic and transmembrane proteins and are involved in ligand recognition and intracellular signal transduction during Mtb infection (Hossain and Norazmi, 2013).

These host pathogen interaction through TLR-dependent triggering of intracellular signalling cascades, involves the activation of nuclear factor (NF-κB) signalling pathways and others in monocytes/macrophages as well as DCs (Sundaramurthy and Pieters, 2007; Yuk *et al.*, 2012). NF-κB is a transcription factor, which induces and regulates a large set of genes promoting cell innate immunity, inflammation, proliferation and apoptosis control in regulated way. This activation leads to secretion of pro-inflammatory cytokines such as tumor-necrosis factor alpha (TNFα) and interferon gamma (IFN-γ), chemokines, and antimicrobial molecules.

This also promotes influx of lymphocytes, fibroblasts, more DC and activated macrophages into the site of infection resulting in granuloma formation, crucial to the containment of Mtb (Guirado and Schlesinger, 2013).
Although subsequent stimulation of downstream signalling activates innate and adaptive immunity, in order to eliminate the bacteria and develop a long lasting protection, evidence indicates that Mtb is capable of modulating these cellular processes for its favour.

1.1.2.2. **Adaptive Immune Response**

Since Mtb is an intracellular pathogen, the persistence protection relies on the acquired immune response of cell-mediated immunity. Both CD4+ and CD8+ T cells subsets play an essential role in the pathogenesis of TB (Kwan and Ernst, 2011). Mtb activated CD4+ T cells are capable of containing the pathogen in the majority of Mtb-infected individuals. CD8 + T cells can also eliminate infected cells and contribute to production of cytokines, e.g. IFN-γ (Ernst, 2012).

The T cell response to Mtb is activated in the local lung-draining lymph node, where circulating naïve T cells recognise antigens through different receptors presented by infected DCs, critical at the interface of the innate and adaptive immune responses (Mendelson *et al.*, 2005). Activated T cells migrate to site of infection and interact with antigen presenting cells (APC's) such as DCs, that present mycobacterial antigens on major histocompatibility

complex (MHC) class II and which trigger a CD4+ T cell response to Mtb (Cooper, 2009). The outcome of the response to tuberculosis infection is determined by the type of professional CD4+ T cells subset; either T-helper type-1(Th1) or T-helper type-2 (Th2) response. Protective immunity against Mtb is predominantly based on CD4+ Th1 mediated cellular arm of the host immune response resulting in production of cytokine such as IL-2 and IFN-γ, which induce anti-mycobacterial activity in macrophages (Salgame, 2005). Whereas Th2 cell activation generally leads to TB disease progression. So that, the Th1-Th2 cell response against Mtb should be regulated for the effective containment of the infection within granulomas. Despite the fact that cell-mediated immune responses appear to be the main response to Mtb infection, studies have suggested that B cells and humoral immunity can modulate the immune response to Mtb infection (Kozakiewicz *et al.*, 2013; Chan *et al.*, 2014).

1.2. Human Immunodeficiency Virus type 1

HIV-1 is a retrovirus that has an RNA genome, which contains two copies of single-stranded RNA and enzymes needed for its productive infection such as reverse transcriptase, protease and integrase. The HIV-1 genome consists of nine genes (gag, pol, env, tat, rev, nef, vif, vpr, vpu) encoding proteins that make up the virion and regulate the life cycle of the virus. Its entry to the host cell is achieved through binding of the viral envelope glycoproteins to receptors, CD4 and chemokine receptors CCR5 or CXCR4, present on the surface of target cells, which causes fusing with the lipid outer layer of the cell and penetration of the virus into the target cell (Littman, 1998). Once inside the cell, the viral RNA undergoes reverse transcription by the viral reverse transcriptase to form double stranded viral DNA, which is transported to the nucleus for integration by the viral integrase as proviral DNA in to the host genome (Gummuluru and Emerman, 2002). Once integrated the provirus can remain latent until the target cell is activated. HIV-1 proviral genome transcription is regulated by the regulatory proteins, *tat* and *rev*, able to orchestrate complex interactions with the host cell transcription factors and machinery (Karn and Stoltzfus, 2012). In this way, a set of HIV-1 RNAs is transported from the nucleus to the cytoplasm, where RNAs can be translated. Finally, the viral proteins are assembled with the viral genomic RNA, after that the virion buds off from cell surface, turns mature by viral protease cleavage and is then able to infect another cell (Frankel and Young, 1998).

Although the majority of productive HIV-1 replication occurs in activated $CD4^+$ lymphocytes, HIV-1 also has the ability to infect relatively resting CD4 T cells and monocytes, which permit the latent HIV-1 infection. This latent viral infection supports ongoing low-level HIV-1 replication also during effective treatment, and is a major obstacle to a therapeutic cure of HIV-1 infection by anti-retroviral therapy (ART).

1.2.1. Immune Response to HIV Infection

Even though HIV-1 infection elicits strong innate and adaptive immune responses, these responses are too late to eliminate the infection and the virus establishes chronic infection. This may be related with the viral integration into the host genome and subsequent cellular latency, as well as virus genetic variability, which translates into constant immune escape. Despite the fact that an intricate molecular mechanism is at play between HIV and the host; it is evident that the organism is incapable of restricting and eradicating the invading pathogen, except for minor group referred to as elite controllers, which appear to control viremia in the absence of treatment.

HIV-1 mainly infects cells of the immune system the major targets are $CD4^+$ T cells, key regulators of the adaptive immune response. HIV-1 facilitates $CD4^+$ T cell depletion primarily *via* accelerated destruction, chronic immune activation (CIA) and also by impairing the regeneration of new T cells from existing T-cell precursors (Grossman *et al.*, 2002; Douek *et al.*, 2003). However, very early administration of ART may control viremia and prevent the killing of $CD4^+$ T cells, and thereby rescue strong HIV-1 $CD4^+$ T cell responses (Oxenius *et al.*, 2000). CD8 T cell also control the virus by either lysing the infected cell or inhibiting HIV replication and entry into target cells. This cellular response is strong in the early stage of the virus infection, however, the transition into long-term memory cells could be impaired (Sun *et al.*, 2004).

Neutralizing antibodies may also play role during HIV-1 infection by binding to the specific site on the viral envelop glycoprotein complex and preventing the interaction with the cellular receptors that leads to viral entry (Stamatatos *et al.*, 2009). However, it has also been shown that HIV-1 readily mutates and escapes the antibody response.

In addition to these cells, HIV-1 infects cells of the innate immunity such as monocytes, macrophages, DCs and NK cells. During acute HIV infection, NK cells can control HIV replication through cytolysis of virally infected cells and the production of antiviral cytokines

and chemokines (Ward *et al.*, 2007). In addition, they can interact with DCs and thereby influence T cell responses. DCs can also prime virus-specific $CD4^+$ and $CD8^+$ T cell responses upon HIV infection (Lubong Sabado *et al.*, 2009).

Previous reports have indicated that monocytes and macrophages have shown to harbour both productive and latent infections, serving as a part of the viral reservoir that could potentially be essential to chronic infection. However, it has been noted that certain microbial components are able to induce HIV-1 replication via NF-κβ-dependent mechanisms in latently infected, monocytes and macrophages, as well as resting $CD4^+$ cells (Brenchley *et al.*, 2006).

1.3. Mycobacterium tuberculosis and HIV-1 Co-infection

It is clear that both Mtb and HIV may dysregulate the immunological functions in the co-infected host. HIV coinfection also contributes strongly to the reactivation of latent Mtb infection and progression to active disease after infection, as well as exposes the host to new Mtb infection (Aaron *et al.*, 2004). The reasons for this may be linked to the exhaustion and dysregulation that HIV poses on the immune system that leads to immunosuppression and failure in controlling Mtb infection.

Immune cells, being targeted by both mycobacteria and HIV have been shown to be altered in relation to their immunological functions after co-infection. Cells belonging to myeloid origin, both DCs and macrophages, are susceptible to mycobacterial infection and their susceptibility to Mtb has been shown to be augmented upon exposure to HIV infection (Pathak *et al.*, 2010; Salte *et al.*, 2011). Moreover, coinfection with HIV-1 and Mtb seems to give rise to synergistic effects at the cellular level that mutually enhance the intracellular replication of both pathogens (Pathak *et al.*, 2010).

Infected macrophages and DCs present Mtb and HIV antigens to CD4+ and CD8+ T-cells. However, HIV infection results in systemic CD4+ T cell depletion and cytokine production abnormality, such as dysregulated TNF-α-mediated responses that affect Mtb protective mechanisms (Patel *et al.*, 2007; Chen *et al.*, 2009). HIV also disrupts the ability of immune cells to contain TB infection within granulomas, which leads to reactivation and progression to active disease (Diedrich and Flynn, 2011).

But, the mechanisms by which HIV disrupts function in both established and newly forming granulomas, leading to the increased morbidity and mortality of co-infected people compared to those of people with TB alone, remain to be determined.

On the other hand, studies have shown that Mtb induce HIV-1 replication in vitro, enhance the viral infectivity of monocytes and increase the transmission of HIV-1 from dendritic cells to lymphocytes. This in the presence of Mtb-infected macrophages and DCs increases the HIV *trans*-infecting ability to T cells than uninfected DCs in a bystander fashion (Mazurek *et al.*, 2012). The potential for TB to increase the HIV-1 load *in vivo* may be the chronic clinical course of active TB and the marked systemic immune activation that accompanies Mtb/HIV coinfection.

More interestingly, it has been shown that certain bacterial components such as Mtb cell wall are able to enhance HIV-1 replication via NF-κβ-dependent mechanisms in latently infected, resting $CD4^+$ cells and cells of monocytes lineage which drive HIV-1 LTR transcription (Mancino *et al*., 1997; Moriuchi *et al*., 2000). Thus, by means of cellular activation, Mtb may enhance HIV-1 replication in vivo.

In this way, both pathogens are capable of modulating a number of cellular processes such as transcription factors, antigen presentation and cytokine responses. However, the exact mechanism underlying the synergy between HIV and Mtb are still remain unclear. But recently miRNAs have been suggested to be involved in this since many of the cellular processes are under the control of miRNAs and it is possible that Mtb and HIV alters the expression of miRNAs to modulate the function of cellular processes for their own favour.

1.4. MicroRNAs

MicroRNAs are an evolutionarily conserved class of short, non-coding, endogenous, single stranded RNA molecules with a length of 18-25 nucleotides. Since their discovery in *Caenorhabditis elegans* nematode in controlling the expression level of lin-4 gene in 1993 (Lee *et al.*, 1993), these small RNA molecules have been shown to play critical regulatory roles in a variety of cellular functions. miRNAs are main regulators of gene expression at the posttranscriptional level by complementary binding to the coding mRNA 3'-untranslated region (UTR) in a sequence-specific manner, resulting in cleavage or translational repression of the target mRNA (Filipowicz *et al.*, 2008; Bartel, 2009). By affecting gene regulation, miRNAs are likely to be involved in many human biological and cellular processes such as cell proliferation, apoptosis, development, hematopoietic cell differentiation, metabolism,

stress response, oncogenesis and immune responses (Bartel, 2004; Kato and Slack, 2008). They are also involved as integral component with transcription factors in signalling pathways that maintain homeostasis and control cell functions in response to external stimuli (Tsang *et al.*, 2007; Leung and Sharp, 2010).

MiRNAs are found to be expressed in all eukaryotic cells and some viruses. In the human genome, more than 2500 mature miRNAs have been reported to date (miRBase, http://miRNAs.sanger.ac.uk/, release 20) and it is estimated that ~60% of all protein-coding genes are under the control of miRNAs (Friedman *et al.*, 2009; Siomi and Siomi, 2010). It is believed that a single miRNA can regulate hundreds of mRNAs and a single mRNA can be targeted by a number of different miRNAs, thus leading to a cell and context-specific network of miRNA: mRNA interactions (Lewis *et al.*, 2005). Aberrant miRNA expression is capable of disrupting the expression of several mRNAs and proteins and is involved in the initiation of many diseases such as cancer and immune disorder (Sonkoly and Pivarcsi, 2009; Di Leva and Croce, 2013).

1.4.1. MicroRNA Biogenesis

MiRNA biogenesis in the human cell requires a multistep complex process to yield the functional mature miRNA (Fig 1). The miRNA genes are initially transcribed by RNA polymerase II in the nucleus to form large capped (5') and poly adenylated (3') primary miRNA transcripts (pri-miRNAs). The primary miRNAs transcription occurs from distinct genomic locations; many are intergenic with independent promoters, others are clustered in polycistronic transcripts, or located within (~50%) introns of host genes (Bartel, 2004; Li *et al.*, 2007).

Pri-miRNA is subsequently cleaved in the nucleus by the RNase III enzyme Drosha, coupled with its binding partner DGCR8 complex, which generates 70-90 nts double-stranded, stem loop hairpin structure precursor miRNAs (pre-miRNAs) (Krol *et al.*, 2010). Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. In the cytoplasm, the hairpin precursors are cleaved by another RNase III endonuclease, Dicer, main miRNA processing enzyme and its binding partner the trans activator RNA-binding protein TRBP, into a dsRNA duplex, 22 nt product comprised of the mature miRNA guide strand and the miRNA* passenger strand (miRNA: miRNA*). The mature miRNA is then loaded into the RNA-induced silencing complex (RISC), which contains the AGO protein (Ago2) as a core component (Krol *et al.*, 2010), while the passenger strand is degraded.

The mature miRNA strand guides its associated RISC to target mRNAs containing complementary sequences to the mature miRNA. These mature miRNAs predominantly bind to the 3′-UTR region of the target mRNA, and repress its expression through mechanisms of both translational repression and mRNA degradation (Bartel, 2009). RISC may also direct miRNA repressed mRNAs to Processing bodies (P-bodies: small cytoplasmic granules for RNA degradation) for deadenylation, decapping and degradation, or alternatively for temporary storage and reuse of repressed transcripts (Eulalio *et al.*, 2007).

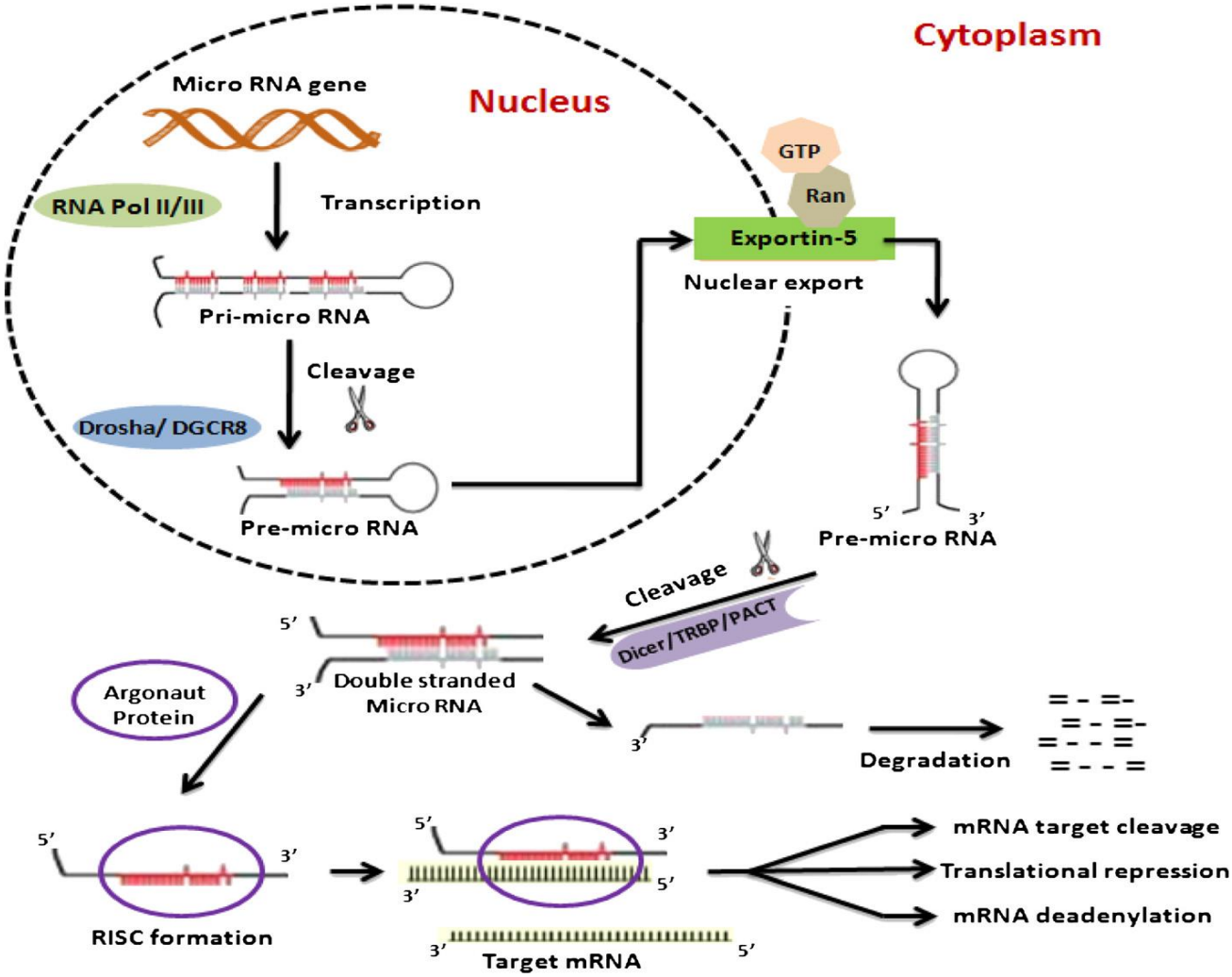

Figure 1: Schematic diagram on linear synthetic pathway of miRNA biogenesis and processing in human cell, adapted from (Singh *et al.*, 2013).

1.4.2. Mechanisms of Target Regulation

Even though striking progresses have been made to understand the miRNA biogenesis, the precise mechanisms that miRNAs use to regulate gene expression remain unclear. However evidences suggest that the translational inhibition and mRNA degradation occur depending on the degree of perfect or imperfect complementarity of miRNA-mRNA that results in inhibition of protein synthesis (Bernardo *et al.*, 2012).

The specific miRNA target recognition region is located on the 5'-end of the mature miRNA sequence, from bases two-to-eight termed as the "seed-sequence" (Bartel, 2004). Depending on the complementarity guidance of the miRNA seed sequence with its target mRNA, perfect base pairing homology between miRNA and mRNA sequences induces the RNA-mediated interference (RNAi) pathway leading to cleavage of the mRNA by Argonaute in the RISC. On the other hand, miRNAs regulate their target genes through imperfect binding to partially complementary sequences in the 3' UTR of mRNAs which promotes mRNA degradation or translational repression (Jackson and Standart, 2007). Additionally, miRNAs which guide RISC, are able to recruiting P body components to facilitate mRNA decay and/or inhibit translation of a particular mRNAs (Eulalio *et al*., 2007).

1.5. Role of miRNAs in Immune Rresponse

MiRNAs have multiple targets and regulate the expression of hundreds of genes that may impact on the immune cell development or functions. It is also obvious that the expression of the coding gene can determine the outcome of immune functions. Studies have demonstrated that miRNA-mediated gene regulation is involved in controlling different immune cell subsets during lineage differentiation and immune response (O'connell *et al.*, 2010; Rossi *et al.*, 2011). The role of miRNAs was first suggested by investigations in mice knockout for Dicer, an enzyme involved in miRNA biogenesis, which showed that lack of Dicer lead to lethality early in development (Bernstein *et al.*, 2003). Later studies have shown that specific deletion of Dicer in T cell lineage results in impaired T cell development and irregular T helper cell differentiation and cytokine production (Muljo *et al.*, 2005).

MiRNAs have not only been linked to development of immune cells but also in the regulation of the immune response against different pathogens or inflammation.

The recognition of the specific microbial components through PPRs such as TLR recruit adaptor proteins and activate downstream signalling cascades that activate NF-κβ and

mitogen-activated protein kinases (MAPKs), which lead to up-regulation of pro inflammatory cytokines that orchestrate innate and adaptive immunities to infection (Qian and Cao, 2013). However, this activation needs to be tightly regulated in order to avoid a prolonged inflammatory response and maintain immune homeostasis (Qian and Cao, 2013). Among others, Toll-like receptor (TLR) signalling pathways have been shown to be tightly regulated by miRNAs (O'neill *et al.*, 2011). NF-κβ activation may be turned on or off the miRNAs gene at the transcriptional level in signal dependent manner to influence the expression of signalling components that are crucial for the response of external stimuli (Sharbati *et al.*, 2011). Indeed, miRNAs are proving to be critical effectors in the regulation of host-pathogen interaction networks (O'connell *et al.*, 2012).

A number of miRNAs are also induced or repressed in response to bacterial or viral infections. In order to investigate their potential involvement in regulation of the innate immune response, Taganov *et al*. (2006) performed the expression profiling of 200 miRNAs in human monocyte cell line after TLR4 ligand stimulation by lipopolysaccharide (LPS). This study revealed the induction of miR-146a, miR-155, and miR-132 in response to LPS stimulation. Furthermore, miR-146a was highly up-regulated and it was induced in NF-κβ dependent manner. And so, IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) have been identified as the target genes of miR-146a posttranscriptional repression, a negative regulator for TLR and cytokine receptor signalling (Taganov *et al.*, 2006). In another study, miR-146a deleted mice were developing autoimmunity, were hypersensitive to LPS and demonstrated an exaggerated pro-inflammatory response when challenged with endotoxin (Boldin *et al.*, 2011). These studies suggest that immune cells require miRNAs to perform their normal functions and miRNAs dysregulation during infections may result in loss of immune homeostasis.

1.6. Role of miRNAs in Tuberculosis

Even though the mechanisms are not well understood, it has been revealed that Mtb has evolved a series of strategies to modulate its host immunity for its multiplication and survival. Recently revealed cellular miRNAs have altered expression and are able to modulate the host antibacterial pathways in response to Mtb infection. Such findings may provide insights to understand the mechanism for TB pathogenesis (Das *et al.*, 2013).

A number of miRNAs that target several genes that are important in tuberculosis immune response have been identified. For instance, miR-29 up regulation was reported in both animal model and clinical studies after mycobacteria infection (Fu *et al.*, 2011; Ma *et al.*,

2011; Yi *et al.*, 2011; Yi *et al.*, 2012). Moreover, miR-29 was demonstrated to inhibit IFN-γ production of T cells by direct targeting IFN-γ mRNA (Ma *et al.*, 2011). Likewise, miRNA-99b was found to be highly up-regulated in Mtb infected DCs and macrophage and involved in TNF-α modulation and inhibition (Singh *et al.*, 2011). Also, another study pointed out that miR-144* overexpression in active TB patients inhibits T cells proliferation (Liu *et al.*, 2011).

Human macrophage treated with virulent Mtb induced high miR-125b expression and low miR-155 expression with correspondingly low TNF production (Rajaram *et al.*, 2011). In this study the reverse was revealed when macrophage incubated with avirulent *M. smegmatis* (Rajaram *et al.*, 2011). In that way, Mtb hampered TNF biosynthesis by upregulating miR-125b while miR-155 enhanced TNF-α production. Thus, overall expression of miRNA may influence TNF-α induction.

In parallel, another study showed that a specific Mtb antigen, ESAT-6, induced miR-155 and by that diminished Mtb intracellular survival by inhibiting Bach1 (activator of the Mtb dormancy) and SHIP1 (required for Mtb survival) (Kumar *et al.*, 2012). In a very recent study, Kumar *et al*. (2015) has also shown the suppression of the miRNAs let-7f, which regulates the expression of the NF-κβ pathway regulator A20, in Mtb infected macrophage. These and other studies have demonstrated the involvement of miRNAs during Mtb infection and opened new dimension in tuberculosis research.

1.6.1. MiRNAs signatures in Tuberculosis

Today, global gene expression analysis based on microarray technology has facilitated new molecular marks for classification of cases in to groups according to their physiological and pathological conditions. Although thousands of miRNAs have been identified in humans, little is known about their signatures and functional roles in tuberculosis patients.

At present, some tuberculosis related studies have been reporting about miRNAs expression profiling in clinical specimen, animal model or on cell lines. Majority of them are focused on discriminating patients with active TB and latent TB as well as uninfected healthy controls from blood cells (peripheral blood mononuclear cells (PBMC)) or its derivatives and fewest in sputum samples.

miRNA profiling studies have identified 92 serum miRNAs that are aberrantly expressed in active pulmonary tuberculosis patients, of which 59 were upregulated and 33 were down regulated compared to uninfected controls (Fu *et al.*, 2011). Similarly, another comparative

study observed 97 (90 overexpressed) deferentially expressed miRNAs in pulmonary TB patient sera (Qi *et al.*, 2012). Further validation revealed that miR-361-5p, miR-889 and miR-576-3p were overexpressed in tuberculosis patients compared to control. Combination of three selected miRNAs was also distinguished TB patients from other microbial infected groups (Qi *et al.*, 2012).

The most recent study identified differentially-expressed miRNAs in serum of TB patients, individuals with LTBI, and healthy individuals, even with or without BCG inoculation. Comparisons between active TB groups with the 3 groups released 162 significantly altered miRNAs expression. Of which 24 miRNAs were up regulated in all the groups compared to TB patients, while 6 miRNAs were down regulated. They were also able to identify up- or down-regulated miRNAs in groups compared to each other. Further they performed validation analysis and identified hsa-miR-196b and hsa-miR-376c as markers for active TB disease (Zhang *et al.*, 2014).

Wang *et al.* (2011) conducted a study to compare miRNA expression in PBMC between active pulmonary tuberculosis patients and latent TB infected with healthy individuals. They found that a total of 17 miRNAs were differentially expressed between the three groups. This study was further supported by cluster analyses indicating a clear distinction between active TB group with LTBI or from healthy controls.

A recent in vitro study demonstrated miRNAs signatures in human monocyte-derived macrophages infected with virulent and non-virulent Mtb complex strains. Interestingly, this study tried to identify miRNAs (miR-155 and miR-146a) that previously are well-known in Mtb infection as well as not yet studied miRNAs (miR-145, miR-222*, miR-27a and miR-27b) in Mtb infection. At large, the finding of this study suggests these miRNAs are predicted to target important immune-related genes during intracellular Mtb infection (Furci *et al.*, 2013).

In a related but separate study, Meng *et al.* (2014) preformed miRNAs analysis to identify LTBI-related miRNA expression in macrophages expressing Mtb Hsp16.3 protein, PBMCs from subjects with LTBI and in healthy control individuals. They found four consistent miRNAs (miR-424-5p, miR-27b-3p, miR-377-5p, and miR-3680-5p) in PBMCs from LTBI and control subjects compared to in vitro macrophage models. In a different way, this study provides a better understanding on the role of miRNA homeostasis during intracellular infection.

On the other hand, studies have shown aberrant expression of miRNAs from sputum of tuberculosis infected individuals. For instance Yi *et al.* (2012) reported 95 miRNAs (43 up-regulated and 52 down regulated) were differentially expressed between tuberculosis infected and healthy individuals. MiR-3179 and miR-147 were overexpressed, while miR-19b was under expressed in tuberculosis patients. It has been suggested that miRNAs can be used as early diagnosis markers in extra cellular fluids such as sputum and serum due to their high stability (Xie *et al.*, 2010; Yu *et al.*, 2010).

1.7. microRNAs and HIV-1 infection

What make some viruses unique including HIV is that they can encode miRNAs like the host. Interestingly, they use these miRNAs to manipulate both host and viral gene expression. Some viral miRNAs can directly interact with host cell genes in order to enhance their replication and suppress the cellular function which may involve in antiviral immune response (Gatignol *et al.*, 2005). On the other hand, evidences have shown that cellular miRNAs can also inhibit viral replication by binding to the viral genome (Fowler and Saksena, 2013). Indeed as other RNA viruses, HIV-1 infection could have either a profound impact on the cellular miRNA expression profile or cellular miRNAs can affect its replication.

1.7.1. Impacts of HIV-1 on cellular miRNAs Expression

It has been reported that HIV-1 may interact directly or indirectly with cellular miRNAs in order to promote a favourable environment for its replication and survival. As part of the ongoing battle between host and virus, HIV-1 may produce a suppressor of RNA silencing protein to block host miRNA pathway. It has been suggested that HIV-1 trans activating protein Tat interact with Dicer, an enzyme engaged in miRNA processing, and inhibits its activity (Bennasser *et al.*, 2005; Hayes *et al.*, 2011). In addition to Tat, HIV-1 viral protein R (Vpr) has been reported to reduce Dicer expression levels upon HIV-1 infection of macrophages (Coley *et al.*, 2010; Klockow *et al.*, 2013).

On the other hand, one study demonstrated that knockdown of Dicer led to boosted HIV-1 replication in PBMCs from HIV-1-infected patients and in latently infected cells (Triboulet *et al.*, 2007). Subsequently, their group found that HIV-1 mRNAs binds and confines with proteins of RISC complex, required for miRNA-mediated silencing or effectors negatively regulate HIV-1 gene expression by preventing viral mRNA association with polysomes. Finally, this study confirmed that knockdown of some of these effectors led to virus

reactivation (Chable-Bessia *et al.*, 2009). However, the deletion of Dicer in turn affects the normal miRNAs maturation process.

1.7.2. Impact of host miRNAs on HIV-1 Infection

It's established that miRNAs play a role in regulating viruses-host gene expression and viral pathogenesis. During viral infection, miRNAs could be defined as virally encoded or host-encoded according to their source of biogenesis, since some viruses including HIV-1 encode their own miRNAs. Based on their function, they could be suppressors or activators of viral infection.

In this sense, several cellular miRNAs could have the potential to inhibit HIV-1 replication either directly through binding with the HIV-1 RNA or indirectly by targeting host factors (e.g. HIV Dependency Factors) that play a role in HIV-1 replication (Brass *et al.*, 2008). HIV-1-encoded viral miRNAs, vmiRNAs, might also modulate viral infectivity and replication; which may play a role in establishment of HIV-1 latency (Omoto *et al.*, 2004; Omoto and Fujii, 2005). Including proteins involved in miRNAs processing pathway such as Dicer and Drosha, miRNAs exert anti-viral role against HIV-1 infection. However, it has also shown that cellular miRNA expression may enhance HIV-1 infection (Han and Siliciano, 2007).

Even though HIV-1 infects and replicates in activated CD4+ T cells as well as in macrophage, it still exists in resting CD4+ T cells and monocytes in latent state. For instance, comparison of the miRNAs expression pattern in activated and resting primary CD4+ T cells infected with HIV-1 showed a cluster of cellular miRNAs such as miR-28, miR-125b, miR-150, miR-223 and miR-382 in resting CD4+ T cells. The study also found that the 3' UTR of HIV-1 mRNAs are targeted by these cellular miRNAs and contribute to HIV-1 latency (Huang *et al.*, 2007). The same analysis performed by Wang Xu *et al.* (2009) reported that these HIV-1 anti-viral miRNAs were enhanced in monocytes than their monocyte-derived macrophages. They also indicated that their down-regulation upon differentiation might contribute to the ability of macrophages to support productive HIV-1 infection. Another study, however, could only confirm that miR-223 is down-regulated in macrophages (Sisk *et al.*, 2012). This altered profile of miRNAs expression might influence viral replication and latency, as well as efficiency of host defences.

However, a recent *in vitro* study suggested that HIV only slightly influence the cellular miRNAs early after infection, and instead has evolved a mechanism whereby viral transcripts

avoid miRNA by adopting an extensive RNA secondary structure (Whisnant *et al*., 2013). So the role of miRNAs in modulating HIV-1 infection still remains uncertain.

1.8. Role of miRNAs in TB/HIV-1 Co-Infection

The dysregulation of miRNAs has been investigated in response to a range of bacterial and viral infections (Pedersen *et al.*, 2007; Singh *et al.*, 2013). It was also been shown that many miRNAs are involved in the regulation of immune responses to different pathogens, including expression of pro inflammatory cytokines (O'connell *et al.*, 2010). Furthermore, it has been shown that impairments of immune responses can be seen in Mtb/HIV co-infected individuals, response such as cytokine irregularities to Mtb in HIV-1 positive TB patients as compared to HIV negative TB cases (Mihret *et al.*, 2014). In addition, *in vitro* co-infection of monocyte-derived macrophages with HIV-1 and Mtb has been shown to mutually increase replication of both pathogens and inhibited cytokine response to Mtb in the presence of HIV-1 infection (Pathak *et al.*, 2010).

In a more recent in vitro study the expression of miR-146a in Mtb and HIV-1 co-infected macrophages was measured in order to characterize the mechanism behind the altered immune response to Mtb in HIV co-infected cells. In this experiment, the miRNA 146a was found to be up-regulated in macrophages co-infected with Mtb and HIV (Mazurek *et al.*, Unpublished data). This preliminary finding suggests that the expression of Mtb-triggered miRNAs and cytokines is linked and dysregulated by the presence of HIV-1.

One recent study included sera from Mtb/HIV infected subjects but did not focus on the comparative analysis to the mono-Mtb infected, rather the differences to between PTB cases and healthy or those with latent TB infection (Miotto *et al*., 2013). Thus, overall little is known on the alterations of miRNA expression profiles upon Mtb/HIV-1 co-infection *in vitro* as well as in the co-infected patients.

However, some overlapped miRNAs have been identified on data in the literature in Mtb and HIV-1 infection independently that could be of relevance for the pathogenesis of Mtb/HIV-1 co-infection. For instance, miRNAs such as miR-29a, 27b, 155, 150, 223 and 125b have been reported as altered miRNAs in each pathogen (either during Mtb or HIV-1 infection) (Huang *et al.*, 2007; Ma *et al.*, 2011; Ghorpade *et al.*, 2013; Monteleone *et al.*, 2015).These data suggests that host cells might respond to pathogens using similar miRNAs as part of the interactions between the pathogen and the host.

The aim of this study is to investigate the differential expression of the global miRNAs profile and miR-146a expression in whole blood of individuals with active pulmonary TB (PTB) co-infected with HIV and PTB mono-infected patients as compared to uninfected healthy individuals using Affymetrix microarray and qPCR, respectively.

1.9. Hypothesis

Distinct microRNA expression profiles can discriminate PTB cases being HIV-1 co-infected, PTB mono-infected and uninfected controls.

2. OBJECTIVES

2.1. General Objective

To explore differential miRNAs expression in patients with active pulmonary tuberculosis with or without HIV co-infection as compared to uninfected healthy individuals.

2.2. Specific Objectives

- To analyses miRNAs expression profiles, in whole blood of individuals with active PTB with or without HIV-1 co-infection, as compared with uninfected healthy controls.
- To identify overlapping differentially expressed miRNAs between the three groups.
- To establish hierarchical clustering among the three analysed groups.
- To measure *ex vivo* expression of miR-146a in patients with PTB and HIV co-infection, PTB mono-infection and uninfected healthy controls

3. MATERIALS AND METHODS

3.1. Study site

The study was conducted in four health centres (Adama, Geda, Dehra, and Wolenchiti) in East Showa zone of Oromia region, Adama, Ethiopia. This zone is located in the middle part of Oromia region with a population of about 600,000. Addis Ababa-Djibouti highway, which over pass through the zone is considered a high risk area for HIV transmission (Balcha *et al.*, 2014).

3.2. Study subjects and Eligibility Criteria

Three groups of individuals, including those with concomitant active PTB/HIV, active PTB mono-infection and uninfected healthy individuals were enrolled in this study. The following inclusion criteria were applied for all participants recruited to the study: both sexes, age 18 years or older, written informed consent, consented to HIV testing, no previous treatment with ART and/or anti-tuberculosis. All PTB patients were selected from the TB clinics and TB diagnosis was based on clinical presentation, chest radiography, and sputum smear microscopy for acid-fast bacilli (AFB) in the respective health centres. Patients with PTB/HIV co-infection (either smear-positive or smear-negative) confirmed by sputum culture and/or Xpert MTB/RIF were recruited. Uninfected healthy participants were enrolled from the voluntary counselling and testing clinic at the Adama health centres without any clinically relevant conditions. The presence of HIV infection was tested as part of routine care using rapid tests according to the national guide-line.

Exclusion criteria included having received anti-tuberculosis treatment (ATT) within the preceding 6 months and/or having received ATT for recent episodes of TB; confirmed or suspected extra pulmonary TB; presence of other significant medical conditions and pregnancy.

3.3. Operational definitions

PTB mono-infected patients: Individuals diagnosed positive for active pulmonary tuberculosis with sputum-smear and confirmed by culture before anti-TB treatment.

PTB/HIV co-infected patients: Individuals who are positive for HIV and diagnosed with active TB (with sputum smear confirmed by sputum culture and/or Xpert MTB/RIF, irrespective of sputum smear category) before ART and anti-TB treatment.

Uninfected healthy individuals/controls: Individuals who were negative for HIV and active TB disease as well as latent TB infection.

3.4. Sampling Work flow

All participant data was collected using a standardized questionnaire covering socio-demographic information, symptoms and physical signs, as well as current medications with particular focus on ART and ATT. All clinical examinations were done by regular staff involved in patient care, at the respective health centres, with the support of trained research team. Clinically relevant data to determine TB status (with relevant confirmatory exams), and HIV status was compiled for all participants during enrolment. Testing for active PTB for patients with concurrent PTB/HIV and active PTB, smear microscopy was performed on two morning sputum samples. Smear microscopy result was confirmed by liquid culture for PTB patients, whereas for patients with PTB/HIV, Xpert MTB/RIF test was performed as confirmatory test in addition to culture due to the difficulty to detect Mtb in HIV positive patients. Participants positive for TB confirmed by either of the two tests (irrespective of sputum smear) were selected for this study.

3.4.1. Blood Sampling

A total of 15 ml peripheral blood was drawn by professional nurses or lab technicians from the three study groups. For miRNA analysis 2x2.5 ml of whole blood was immediately transferred into PaXgene tubes containing RNA stabilizer solution. The remaining 10 ml whole blood was transferred to vacutainer tube containing EDTA as anticoagulant. In addition, 3 ml of blood sample was also collected from healthy participants for QuantiFeron®-TB test (QFT) to confirm latent TB infection. For this test 1 ml of the collected blood was added in to 3 different tubes with different antigens (Nil tube as control, TB Antigen tube for TB antigen, and Mitogen tube for positive control). The blood samples were directly transported from the health centres to the Adama Regional Laboratory, where further handling was done. There, 1 ml of whole blood was taken from the EDTA tube and CD4 cell and CBC counts were performed. From the remaining 9ml of blood, plasma was isolated and aliquotted in to Cryotubes for cytokine analysis (for further study but not included in this thesis) and viral load determination for PTB/HIV co-infected subjects. Tubes for QFT were centrifuged after 16-24 hrs incubation, and supernatants were aspirated and collected in cryotubes. All tubes were subsequently stored at -80°C until transferred to AHRI.

Samples were then shipped to AHRI on dry ice to maintain the constant temperature and stored in -80 ^{0}C freezer. Molecular laboratory method optimization and QFT test were performed at AHRI. Plasma HIV-1 viral load determination was assessed in samples from the PTB/HIV co-infected group at Ethiopian Public Health Institute (EPHI). Finally, half of the frozen samples were shipped to Lund University, Sweden to perform miRNAs array analysis.

3.5. Laboratory Methods

3.5.1. Culture and Xpert MTB/RIF

Liquid culture at International Clinical Laboratory (ICL), Xpert MTB/RIF and smear microscopy at Adama regional laboratory were performed. Details on the procedure for smear microscopy, liquid culture and Xpert are explained in independent published work (Balcha *et al.*, 2014).

3.5.2. Viral load determination

Viral load determination was conducted using Abbott m2000rt PCR instrument (Abbott Laboratories, USA) according to the manufacturer's recommendations. In brief, viral RNA was extracted from plasma samples using magnetic beads capture. The master mix was prepared containing 271µl of the HIV-1 Activation and 949 µl of the HIV-1 Oligonucleotide. The amplification master mix of 50 µl aliquots was dispensed into the 96-well plate. The plate was centrifuged at 1500rpm for 5 min and placed in the Abbott m2000rt instrument for amplification and detection. Detailed protocol for this assay is also attached in annex I.

3.5.3. QuantiFERON®-TB Gold In-Tube Test (QFT)

Enzyme-Linked Immunosorbent Assay (ELISA) was performed using Human IFN-γ ELISA Kit for interferon-gamma (IFN-γ) cytokine response. The assay was conducted in capture antibody-coated microtiter plates. Briefly, 50 µL of working strength conjugate was added to each well. Serially diluted 50 µL Standards (S1, S2, S3, and S4) were added in to the wells in triplicate. The plasma samples were mixed thoroughly and 50 µL of test samples were added to appropriate wells. After 2 hrs incubation, the wells were washed with 400µL working strength wash buffer for at least 6 cycles. 100µL of enzyme substrate solution was added and allowed for 30 min incubation at room temperature. Subsequently, 50µL of enzyme stopping solution was added to each well and then the Optical Density (OD) was determined and the OD value of the test samples was converted in to a concentration by means of a standard

curve from known concentrations of standard on each plate. Detailed materials and test procedure is also attached in annex II.

3.5.4. RNA extraction

Total RNA including miRNAs was extracted from frozen PAxgene whole blood after it was thawed at room temperature, using PAXgene Blood miRNA Kit (Qiagen) following the manufacturer instructions. Briefly, PAXgene blood RNA tubes were centrifuged for 10 min at 4000g to pellet the samples, which were then washed with 4 ml RNase-Free water and re-suspended in 350 µl Buffer BM1. The sample was transferred in to 1.5 ml microcentrifuge tube. 300 µl of Buffer BM2 and 40 µl of proteinase K were added to bind the RNA and digest the proteins in the samples, respectively, and then incubated for 10min at 55ºC in shaker incubator. The sample was passed in to PAXgene Shredder spin column by centrifuging at 17600g for 3 min. The entire supernatant was transferred to a new 1.5 ml micro centrifuge tube without disturbing the pellet in the processing tube. Isopropanol was added to the samples to optimize binding conditions, and the samples were then centrifuged through PAXgene RNA spin columns, where total RNA >18 nucleotides (including miRNA) was bound to the PAXgene silica-membrane. The bound RNA was incubated with 80 µl DNase and RDD Buffer mix to remove genomic DNA contamination for 15min at room temperature. Then, the spin columns were washed with 350 µl Buffer BM3 followed by 500 µl Buffer BM4. Finally, total RNA was eluted in 40 µl of Buffer BR5.The eluted RNA was incubated at 65°C for 5 min in shaker-incubator without shaking. The extracted RNA was immediately placed on ice and aliquots were stored at −80 °C until use.

3.5.5. RNA Quality and integrity measurement

Total RNA concentration and purity was assessed using the NanoDrop 2000® spectrophotometer (NanoDrop Technologies) for each sample to guide the selection of samples for the microarray analysis. From the total RNA elutes, 2 µL aliquot of RNA was pipetted on to the apparatus pedestal. RNA concentration, A260/280 and A260/230 absorbance ratios were automatically calculated. RNA concentration ranging between 22.1-273.5 ng/µL were obtained and samples with low RNA yield (<30ng RNA/µl) were concentrated in SpeedVac in order to yield adequate concentrations. All samples had the absorbance ratio of (A260/280) > 2 which met the required RNA purity. The absorbance ratio of 260nm/230nm was varied between 0.26 and 2.02 and considered acceptable for miRNA array analysis in this study.

RNA integrity was then evaluated using Agilent 2100 Bio-analyser (Agilent Technologies) to assess the RNA degradation. All chips were prepared according to the manufacturer's instructions. For this assay, 3μL RNA samples were loaded onto the Agilent chips which were then separated by capillary electrophoresis according to their molecular weight. The intensity of fluorescence on each sample's electropherogram represented the amount of RNA of a given size. An RNA integrity number (RIN) was generated for total RNA of each sample based on the ratio of ribosomal bands of (18S and 28S peaks) and also the presence or absence of degradation products on the electrophoretic image. Samples with a RIN greater than or ~ 7.0 were accepted, ensuring that only RNA of good integrity was used in this analysis.

3.5.6. miRNAs microarray run

The microarray run was performed using the latest version of Affymetrix Platform, GeneChip® miRNA 4.0 Array (Affymetrix Technology) based on miRNA database (miRBase) version 20 (v.20) at Genomic institute of Swegene Centre for Integrative Biology, Lund University (SCIBLU), Sweden.

Total quantified RNA samples were 3'-extended with a poly (A) tail using poly (A) polymerase followed by ligation of an oligonucleotide tag biotinylated signal molecule to the poly(A) tailed target RNA samples using FlashTag™ Biotin HSR RNA Labeling Kit (Genisphere, LLC, Hatfield, PA, USA) in accordance with the instructions of the manufacturer. In brief, 8 μL RNA aliquot was mixed with 2 μL RNA Spike Control Oligos to prepare 10 μL RNA/Spike Control Oligos. Five μL of Poly A tailing mix containing 10X Reaction Buffer (1.5 μL), 25mM MnCl2 (1.5 μL), 1 μL ATP Mix and poly (A) polymerase was prepared in a nuclease-free tube and 5 μL of this mix was added to 10 μL RNA/Spike Control Oligos. After 15 min incubation at 37°C, 4 μL of 5X FlashTag Biotin HSR Ligation Mix was added in to each sample followed by addition of 2 μL T4 DNA Ligase accordingly. Then, 2.5 μL HSR stop solution was added after 30 min incubation at room temperature.

The hybridization reaction mix containing 2X Hybridization Mix (50 μL), 27.5% Form amide (15 μL), DMSO (10 μL), 20X hybridization controls (5 μL), Control Oligo B2, 3nM (1.7 μL) and nuclease-free water was prepared and the mix was added to biotin-labelled sample in order to prepare the array hybridization cocktail. The reaction was incubated at 99°C for 5 min, then 45°C for 5 min. For hybridization, 100 μL RNA mix was aspirated and injected on to Affymetrix® GeneChip® miRNA 4.0 array plates. This Chip contains 6,658 probe sets for

human small RNAs which include mature miRNAs (n=2,578), precursor miRNAs (n=2025), other small RNAs (n=1,996), including internal and negative controls. The array was placed on to the hybridization oven for 16 to 18 hours at 48°C and 60 rpm. After hybridization, the slides were washed with wash buffer to remove unbounded RNA and stained with phycoerythrin conjugated streptavidin (SAPE) using an Affymetrix Fluidics Station 450. Finally, the fluorescence images were scanned using the Affymetrix® GeneChip® Scanner3000 7G which converted the fluorescence levels in to a set of number, resulting in a set of raw data that represent the expression levels of each genes.

3.5.6.1. Microarray data analysis

Microarray raw data were pre-processed using the Expression Console Software v1.4.1.46 (Affymetrix). Signal intensities for each spot were calculated by subtracting background levels. Probe sets having detection above back ground (DABG; $p>0.01$) were filtered out to exclude those genes that were not expressed or did not show any variation in at least 20% of the samples from further analysis. The expression gene data were normalized using quantile normalize method to make the distribution of intensities values the same across chips and then the signals were log2 transferred.

After that, the obtained average value for each gene were loaded to the TM4 Multiple Experiment Viewer (TMeV v4.0) software for t-test and significance analysis of microarrays (SAM) to identify miRNAs that show differential expression (DE) among the groups. The q-value of the SAM test which adapted for multiple-testing and a false discovery rate (FDR) value ($q=0$) was considered statistically significant and the P-values of the t-test were also calculated. The fold change was calculated by mean ratio between groups from the normalized values and the results were presented for each miRNAs.

The hierarchical clustering analysis was performed using Pearson correlation as a distance matrix for differentially expressed miRNAs to see their expression patterns across all samples.

3.5.7. miRNAs complementary DNA (cDNA) synthesis

The extracted miRNA was reverse transcribed into its complementary DNA using miScript II Reverse Transcription Kit (Qiagen). The reverse-transcription reaction mix (20 µl) was prepared using 4 µl of 5xHispec buffer (for selective conversion of mature miRNAs into cDNA), 2 µl of 10x nucleics mix containing dNTPs, rATP, oligo-dT primers and an internal synthetic RNA control [miRTC]), 2 µl of RT mix (containing poly (A) polymerase and

reverse transcriptase) and 7 µl RNase-free water. 20 ng of diluted total RNA (5µL) was added to the reaction mix. The negative controls with no template (NTC) were also included for the miRNAs. This mixture was incubated at 37°C for 60min and 95ºC for 5 min on a 7900HT GeneAmp thermal cycler (Applied Bio systems) and stored at -20°C until use.

3.5.8. Quantitative polymerase chain reaction (qPCR)

QPCR was carried out on StepOnePlus qPCR instrument (Applied Bio-systems) using miScript SYBR Green Kit and miRNA-specific Primer (Qiagen). The reaction was performed in a total volume of 15 µL containing 5 µl of 2x SYBR Green mix, 1.5 µl Universal primer, 1.5 µl miRNA specific primer, 3 µl RNase free water PCR reaction mix and 1.5 µl diluted cDNA template. QPCR reactions for each sample were run in duplicate. The cycling program were started at 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. U6 snRNA was used as an endogenous control for data normalization by measuring expression in each sample. After each PCR run, an additional melt curve analysis was performed to assess the Tm of the PCR amplicon; this verified the specificity of the amplification reaction. Quantifications of miRNA genes expression was calculated by the equation $2^{-\Delta\Delta Ct}$, where Ct is the cycle threshold and $\Delta CT = (Ct_{miRNA} - Ct_{U6})$; $\Delta\Delta CT = (\Delta CT_{disease} - \Delta CT_{contro1)}$.

3.6. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Comparisons between groups, in the case of 3 groups (PTB/HIV, PTB and HC), were analysed using Kruskal-Wallis, ANOVA, and Mann–Whitney test, t-test comparing from two groups (PTB/HIV and PTB or HC). Statistical analysis of the array data was performed as described above by bioinformatician at SCIBLU, Lund University. $P < 0.05$ was considered statistically significant.

3.7. Data and Laboratory quality assurance

Sample collection and participant screening at the study sites was accomplished by trained nurses and health officer with the help of the project team and main investigators. Socio-demographic and clinical data of participants were recorded and documented during enrolment.

Freeze-thawing of frozen samples was avoided to ensure the quality of the samples. Dry ice was used for sample shipment from study site to AHRI as well to the Swedish laboratory. Quality assessment of samples was performed after samples arrived at the Swedish laboratory in parallel with freshly collected samples. RNA quality control procedure was followed to ensure that only high-quality RNA sample was used for microarray analysis. The laboratory analysis was performed by the investigator, with the assistance of well trained and experienced laboratory professionals at the Lund University laboratory. Control measures were implemented at each step of the laboratory analysis to ensure good-quality data.

3.8. Ethical consideration

This study, as part of a larger project coordinated by the Swedish partner in collaboration with Armauer Hansen Research Institute (AHRI) and Oromia regional Laboratory, was ethically reviewed and approved at Lund University, Sweden; Armauer Hansen Research Institute (AHRI/ALERT) and National Ethics Review Committee, Addis Ababa, Ethiopia. It also obtained ethical approval from Addis Ababa University (AAU), College of Natural Science, Department of Biology Ethics and Research Committee. Written informed consent was obtained from participants prior to their enrolment in the study. For all over the laboratories procedures, the investigator strictly maintained the participant's confidentiality using study code.

4. RESULTS

4.1. Study subjects

Baseline samples from a total of 24 enrolled participants were analysed in this study. These study participants were patients with active PTB with HIV infection (n=8), active PTB without HIV infection (n=8) and uninfected healthy individuals/controls (n=8). Out of the 24 participants, 70.83% were female and 29.16% were males with median age of 30.5 years. There was significant difference in the age distribution between HIV positive PTB patients and uninfected controls with median age of 42 and 26 years respectively ($p<0.05$). But, there was no significant difference between the PTB/HIV and PTB, as well as the PTB and uninfected control groups in terms of the median age ($P>0.05$) and also gender distribution.

All PTB/HIV co-infected patients were ART and ATT naïve with a median CD4 count of 249 Cells/mm3 (55-756 Cells/mm3) and median viral load of 44,672 RNA Copies/ml (150-2,206,027). Participants with only active PTB had a median CD4 count of 590 Cells/mm3 (257-1087 Cells/mm3) and the median CD4 count of uninfected controls was 818.5 Cells/mm3 (671-2924). As expected, PTB/HIV co-infected patients had significantly lower CD4 counts in comparison with those of uninfected controls ($p=0.0071$), while there was no significant difference in CD4 counts between PTB and uninfected individuals (Fig 2). The uninfected individuals were also screened for latent TB infection and we found that five individuals were positive for LTBI, but these individuals were excluded from further analysis. The demographic and clinical characteristics of all study participants are shown in table 1.

Table 1. Demographic and clinical characteristics of PTB/HIV, PTB and uninfected control study participants which blood samples were analysed by miRNAs microarray technology.

Participants ID	Status	Sex	Age (yrs)	CD4count (cells/m m^3)	MTB diagnosis	Viral load (copies/ml)
ET1	PTB/HIV	F	45	310	Cult+, Smr-,Xpert-	65779
ET2	PTB/HIV	M	42	697	Cult+, Smr- & Xpert+	11673
ET10	PTB/HIV	F	45	188	Cult+, Smr- & Xpert+	2206027
ET11	PTB/HIV	F	27	55	Cult-, Smr+ & Xpert+	125788
ET17	PTB/HIV	F	46	146	Cult-, Smr+ & Xpert+	11339
ET18	PTB/HIV	M	42	756	Cult-, Smr- &Xpert+	150
ET19	PTB/HIV	F	32	328	Cult+, Smr- & Xpert-	159790
ET25	PTB/HIV	M	30	91	Cult+, Smr- & Xpert+	23564
ET3	PTB	F	40	453	Smear & Culture(+)	—
ET4	PTB	F	23	685	Smear & Culture (+)	—
ET12	PTB	F	25	572	Smear & Culture(+)	—
ET13	PTB	F	43	377	Smear & Culture(+)	—
ET21	PTB	M	35	609	Smear & Culture(+)	—
ET27	PTB	F	31	1087	Smear & Culture(+)	—
ET28	PTB	M	28	842	Smear & Culture(+)	—
ET29	PTB	F	45	257	Smear & Culture(+)	—
ET6	Control	F	28	658	QFT-	—
ET7	Control	M	25	2924	QFT-	—
ET8	Control	F	23	686	QFT-	—
ET14	Control	M	27	1161	QFT-	—
ET22	Control	F	20	2924	QFT-	—
ET23	Control	F	23	671	QFT-	—
ET24	Control	F	36	809	QFT-	—
ET31	Control	F	28	957	QFT-	—

Key: Cult+/- (culture positive/Negative), Smr-/+ (Smear Negative/Positive), Xpert-/+ (Xpert MTB/RIF Negative/Positive); Smear & Culture (+) (both smear and culture positive), QFT^- (QantiFeron Test Negative)

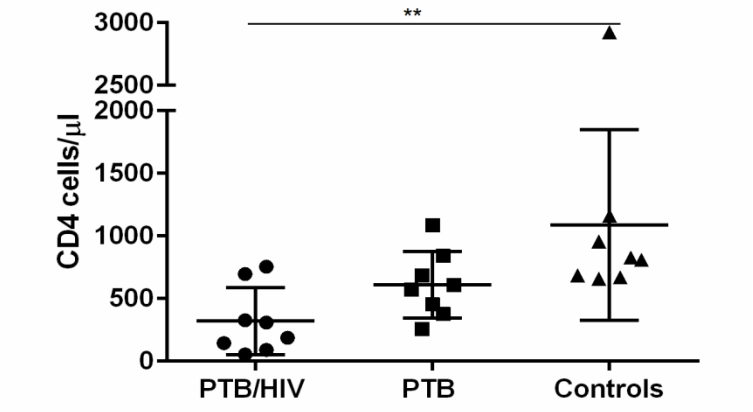

Figure 2: CD4+ T-cell count comparing PTB/HIV, PTB and uninfected controls

4.2. Deferentially expressed miRNA profiles in PTB and PTB/HIV co-infected patients compared to uninfected individuals

Using the significant analysis of microarray (SAM) algorithm, we identified a total of 83 differentially expressed mature miRNAs comparing all three groups together at q value=0, the lowest False Discovery Rate (FDR) at which that gene is the most significant.

Expression levels of all these miRNAs was apparently differentiated comparing PTB/HIV and PTB groups with uninfected controls. Compared with uninfected healthy controls, the expression of 35 miRNAs was significantly altered in the PTB/HIV co-infected patients. Of these, 29 miRNAs were downregulated and 6 miRNAs were upregulated with the fold change ranged from 1.05-5.69 (Table 2). There were only three miRNAs (miR-1275, miR-150-5p, miR-629-5p) that distinguished PTB patients from uninfected controls with the fold change ranged from 1.63-1.79. Two of these miRNAs (miR-150-5p and miR-1275) were down regulated, while only one miRNAs (miR-629-5p) was upregulated (Table 3). A complete list of differentially down and up regulated miRNAs sorted by fold-change and p value is shown in tables 2 and 3.

Table 2. List of miRNAs significantly differentially expressed in PTB/HIV co-infected patients comparing with uninfected healthy controls (Ctrl vs PTB/HIV)

Ctrl vs PTB/HIV (Down regulated miRNAs in PTB/HIV co-infected patients)		
Human miRNAs	**Fold change**	**P-value**
hsa-miR-3609	5.69	0.001
hsa-miR-27b-3p	5.18	**0.001**
hsa-miR-26b-5p	5.05	**<0.001**
hsa-miR-374b-5p	4.42	0.005
hsa-let-7d-3p	4.09	0.003
hsa-miR-22-5p	3.24	0.003
hsa-miR-103a-2-5p	3.01	0.006
hsa-miR-942-5p	2.95	0.001
hsa-miR-128-3p	2.74	0.003
hsa-miR-6513-3p	2.68	0.006
hsa-miR-345-5p	2.64	0.006
hsa-miR-5010-3p	2.59	0.001
hsa-miR-361-5p	2.55	0.004
hsa-miR-505-3p	2.34	0.002
hsa-miR-671-3p	2.32	0.004
hsa-miR-183-5p	2.27	0.007
hsa-miR-6747-3p	2.26	0.006
hsa-miR-23b-3p	2.17	0.002
hsa-miR-182-5p	2.14	0.004
hsa-miR-26a-5p	2.06	**0.001**
hsa-miR-23a-3p	2.06	**0.001**
hsa-miR-550a-3p	2.06	0.005
hsa-miR-423-3p	1.97	0.006
hsa-let-7f-5p	1.83	0.006
hsa-miR-1275	1.63	0.001
hsa-miR-181a-2-3p	1.59	0.005
hsa-miR-25-3p	1.49	0.003
hsa-miR-151a-5p	1.38	0.007
hsa-miR-486-5p	1.05	0.003

Ctrl vs PTB/HIV (Up regulated miRNAs in PTB/HIV co-infected patients)		
Human miRNAs	**Fold change**	**P-value**
hsa-miR-939-5p	1.65	<0.001
hsa-miR-1268b	1.82	**<0.001**
hsa-miR-4516	1.87	<0.001
hsa-miR-4689	1.89	0.001
hsa-miR-4463	2.07	<0.001
hsa-miR-1224-5p	2.36	**0.001**

Table 3. Differential miRNA expression in PTB patients comparing with uninfected healthy controls (Ctrl vs PTB)

Human miRNAs	Fold Change	P value	Expression
hsa-miR-1275	1.63	0.003	Down
hsa-miR-150-5p	1.79	0.002	Down
hsa-miR-629-5p	1.75	**0.001**	UP

4.2.1. Deferentially expressed miRNA profiles in PTB patients compared to PTB/HIV co-infected patients

We also identified a set of 77 miRNAs that were differentially expressed in PTB patients compared with PTB/HIV co-infected patients with fold-change ranged from 1.53-6.45. Among these, 60 miRNAs were down regulated and 17 miRNAs were up regulated (Table 4).

Table 4. Differential miRNA expression in PTB mono-infected compared to PTB/ HIV co-infected patients (PTB/HIV vs PTB)

PTB/HIV vs PTB (Down regulated miRNAs in PTB patients)

Human miRNAs	Fold change	P-value
hsa-miR-6075	2.69	**0.001**
hsa-miR-6800-5p	2.30	**0.001**
hsa-miR-4530	2.28	0.001
hsa-miR-6790-5p	2.27	0.001
hsa-miR-4741	2.20	0.002
hsa-miR-1224-5p	2.19	0.01
hsa-miR-3135b	2.18	0.004
hsa-miR-149-3p	2.18	0.002
hsa-miR-4758-5p	2.07	0.003
hsa-miR-371b-5p	2.04	0.006
hsa-miR-1225-5p	2.03	0.003
hsa-miR-2861	2.02	**0.001**
hsa-miR-4745-5p	1.98	0.001
hsa-miR-4651	1.98	0.002
hsa-miR-5001-5p	1.97	0.001
hsa-miR-937-5p	1.96	0.004
hsa-miR-7108-5p	1.95	0.001
hsa-miR-6068	1.94	0.002
hsa-miR-6088	1.94	0.001

hsa-miR-6716-5p	1.94	0.002
hsa-miR-1227-5p	1.92	0.003
hsa-miR-1915-3p	1.90	0.001
hsa-miR-6724-5p	1.90	0.001
hsa-miR-8069	1.89	0.002
hsa-miR-6786-5p	1.89	0.002
hsa-miR-6821-5p	1.89	**0.001**
hsa-miR-638	1.88	0.001
hsa-miR-6729-5p	1.87	0.002
hsa-miR-6727-5p	1.86	0.002
hsa-miR-4687-3p	1.85	0.002
hsa-miR-4763-3p	1.84	0.005
hsa-miR-3187-3p	1.84	0.003
hsa-miR-4463	1.84	0.002
hsa-miR-4689	1.83	**<0.001**
hsa-miR-4497	1.83	**0.001**
hsa-miR-1268b	1.82	0.001
hsa-miR-150-3p	1.81	**<0.001**
hsa-miR-6125	1.81	0.002
hsa-miR-6858-5p	1.80	0.006
hsa-miR-4734	1.79	0.003
hsa-miR-3656	1.78	0.002
hsa-miR-1268a	1.77	0.003
hsa-miR-4516	1.74	0.002
hsa-miR-4466	1.73	0.003
hsa-miR-3665	1.71	0.002
hsa-miR-4707-5p	1.70	0.003
hsa-miR-6789-5p	1.70	0.006
hsa-miR-4787-5p	1.69	0.005
hsa-miR-6850-5p	1.68	0.002
hsa-miR-6756-5p	1.66	0.003
hsa-miR-6749-5p	1.66	0.004
hsa-miR-3185	1.64	0.004
hsa-miR-6090	1.63	0.002
hsa-miR-7704	1.62	0.005
hsa-miR-6089	1.60	0.003
hsa-miR-6869-5p	1.60	0.002
hsa-miR-3960	1.60	0.004
hsa-miR-6087	1.58	0.007
hsa-miR-939-5p	1.54	0.007
hsa-miR-3162-5p	1.53	0.006

Table 4 continued

PTB/HIV vs PTB (Up regulated in PTB patients)		
Human miRNAs	**Fold change**	**P-value**
hsa-miR-148b-3p	1.96	**0.001**
hsa-miR-26a-5p	2.13	**<0.001**
hsa-miR-550b-3p	2.21	0.001
hsa-miR-197-3p	2.28	**<0.001**
hsa-miR-23a-3p	2.36	**<0.001**
hsa-miR-625-5p	2.53	**0.001**
hsa-miR-223-3p	2.64	0.001
hsa-miR-23b-3p	2.74	**<0.001**
hsa-miR-128-3p	2.92	0.001
hsa-miR-219b-5p	3.33	0.002
hsa-miR-505-3p	3.51	**0.001**
hsa-miR-146b-5p	4.47	**<0.001**
hsa-miR-30e-3p	4.74	**<0.001**
hsa-miR-374b-5p	5.52	**<0.001**
hsa-miR-27a-3p	5.99	**<0.001**
hsa-miR-27b-3p	6.27	**<0.001**
hsa-miR-26b-5p	6.45	**<0.001**

4.3. miRNAs deferentially expressed comparing both PTB/HIV co-infected with the PTB patients and the uninfected individuals

Among the miRNA, listed to be differentially expressed in the above tables, we identified miRNAs that appeared as either up- or down-regulated both comparing the PTB/HIV co-infected group and the uninfected individuals as well as in the comparison between the PTB/HIV and the PTB only patients. These included 13 miRNAs (miR-128-3P, miR-23a, miR-23b, miR-26a-5P, miR-26b-5P, miR-27b-3P, miR-505-3p, miR-550b-5P, miR-1224-5P, miR-1268b, miR-4463, miR-4516 and miR-4689). Eight of these miRNAs (miR-128-3P, miR-23a, miR-23b, miR-26a-5P, miR-26b-5P, miR-27b-3P, miR-505-3p and miR-550b-5P) were shown to be down regulated in PTB/HIV co-infected patients compared with uninfected controls, while up regulated in PTB patients compared to PTB/HIV co-infected subjects with similar fold change of >2. Conversely, the other 5 miRNAs (miR-1224-5P, miR-1268b, miR-4463, miR-4516 and miR-4689) were up and down regulated in PTB/ HIV patients compared to uninfected controls and PTB subjects' respectively $p<0.001$(Fig 3). Moreover, these five

miRNAs were found to be among the miRNAs that were most significantly differentially expressed (Table 2 and 4).

The miRNAs miR-26b and miR-27b were identified as the most downregulated in co-infected patients compared to uninfected controls and upregulated in PTB patients relative to co-infected subjects with fold change 5 and 6 respectively (Fig 3). Only one miRNA (miR-1275) was identified as a differentially expressed miRNA found in PTB/HIV co-infected and PTB mono-infected patients compared with uninfected group (1.63 fold changes).

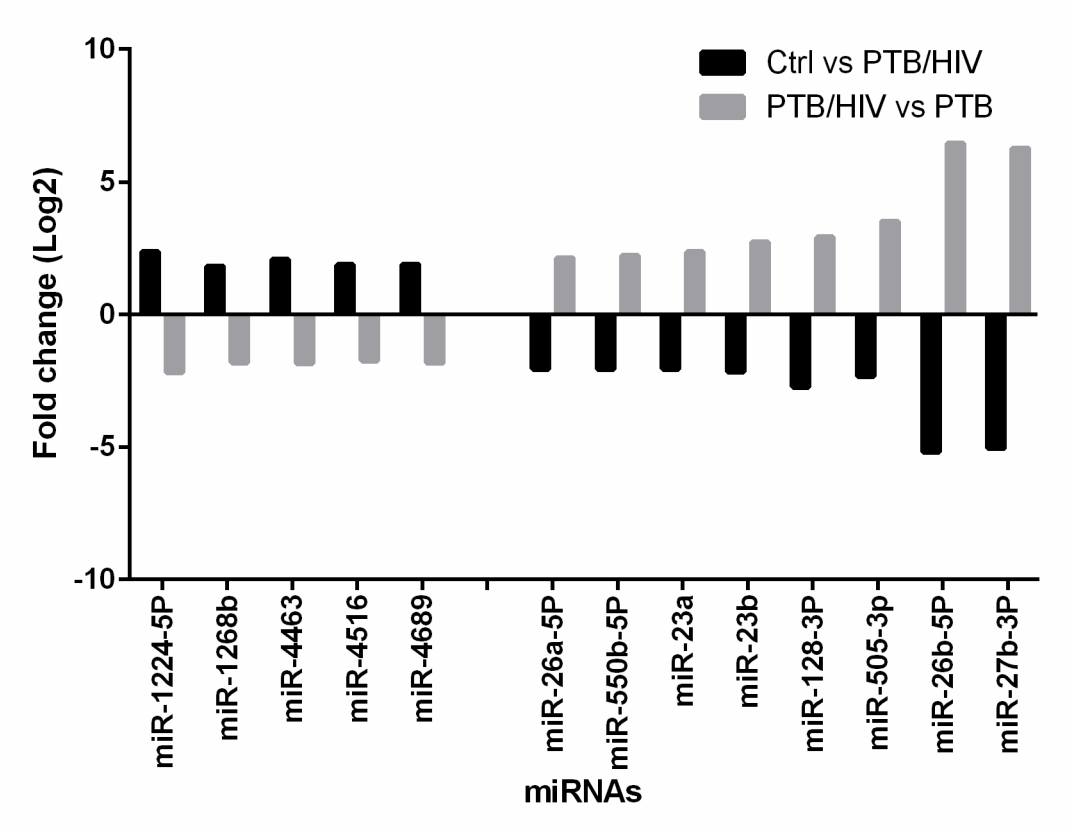

Figure 3 miRNAs that appeared differentially expressed in several two-group comparisons

4.4. Hierarchical Clustering Analysis

The expression profile of the 83 mature miRNAs that were found from the previous analysis was subjected to unsupervised hierarchical clustering using the Pearson correlation. This analysis was performed without prior knowledge of the link between samples and infection status classification while clustering; hence it is called an unsupervised clustering. Hierarchical clustering analysis based on these differentially expressed miRNAs revealed clear separation of the three groups, the PTB/HIV co-infected (Group 1), PTB mono infected (Group 2) and uninfected healthy control (Group 4) groups, in the condition tree (Fig 4a). Also, the expression signature of the miRNAs of PTB patients appeared more related to

uninfected healthy controls as depicted by the color-coding as compared to the PTB/HIV co-infected subjects. Remarkably, these miRNAs were comparable with the miRNAs listed in tables 2 to 4 based on their expression.

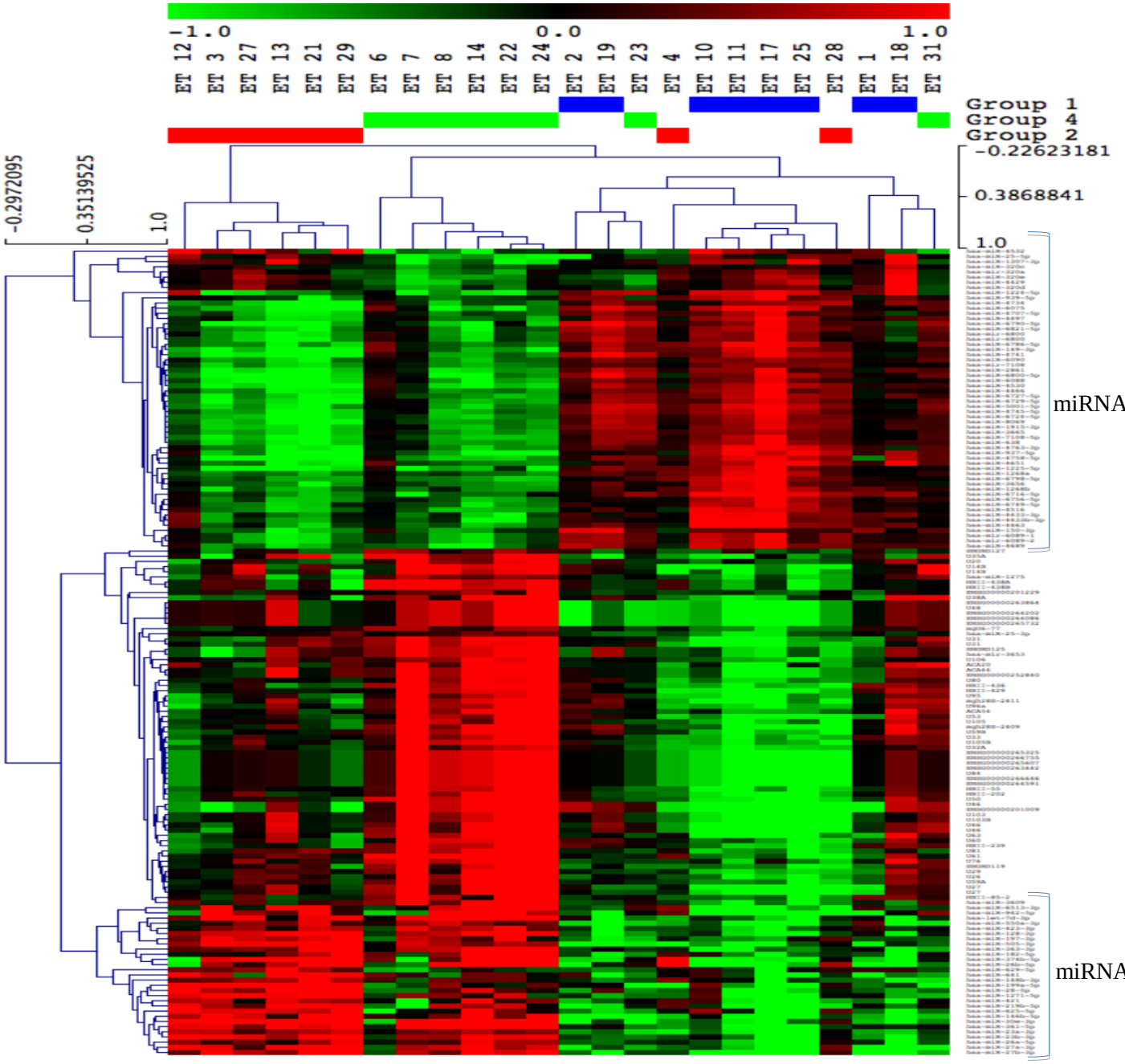

Figure 4a: ***Unsupervised Heat map showing the cluster of human small RNA among the three groups.*** *Columns represent individual patients, rows respective miRNA and other small RNA expression levels. Expression level of each gene in a single sample is depicted according to the colour scale. Red indicates relative overexpression while green values represent relative under expression with intensity according to the expression level. Black values represent central expression values. In the edge next to the left hand of the dendrogram, subjects with PTB/HIV indicated by blue (Group 1), subjects with active PTB indicated by red (Group 2) and uninfected controls indicated by green (Group 4).*

Beside this, hierarchical clustering was also constructed for the miRNAs expression profiles comparing two groups (controls vs PTB/HIV and controls vs PTB) using supervised learning methods. Out of 35 miRNAs that differentiated PTB/HIV co-infected patients from the uninfected controls, 29 and 6 miRNAs were down and up regulated, respectively (Table 2). There were two under and one over expressed miRNAs that differentiated PTB patients from uninfected individuals (Table 3). Hierarchical clustering revealed that these miRNAs differentiated uninfected individuals from PTB/HIV patients ($p<0.01$) (Fig 4b) as well as uninfected controls from PTB patients ($p<0.01$) (Fig 4c).

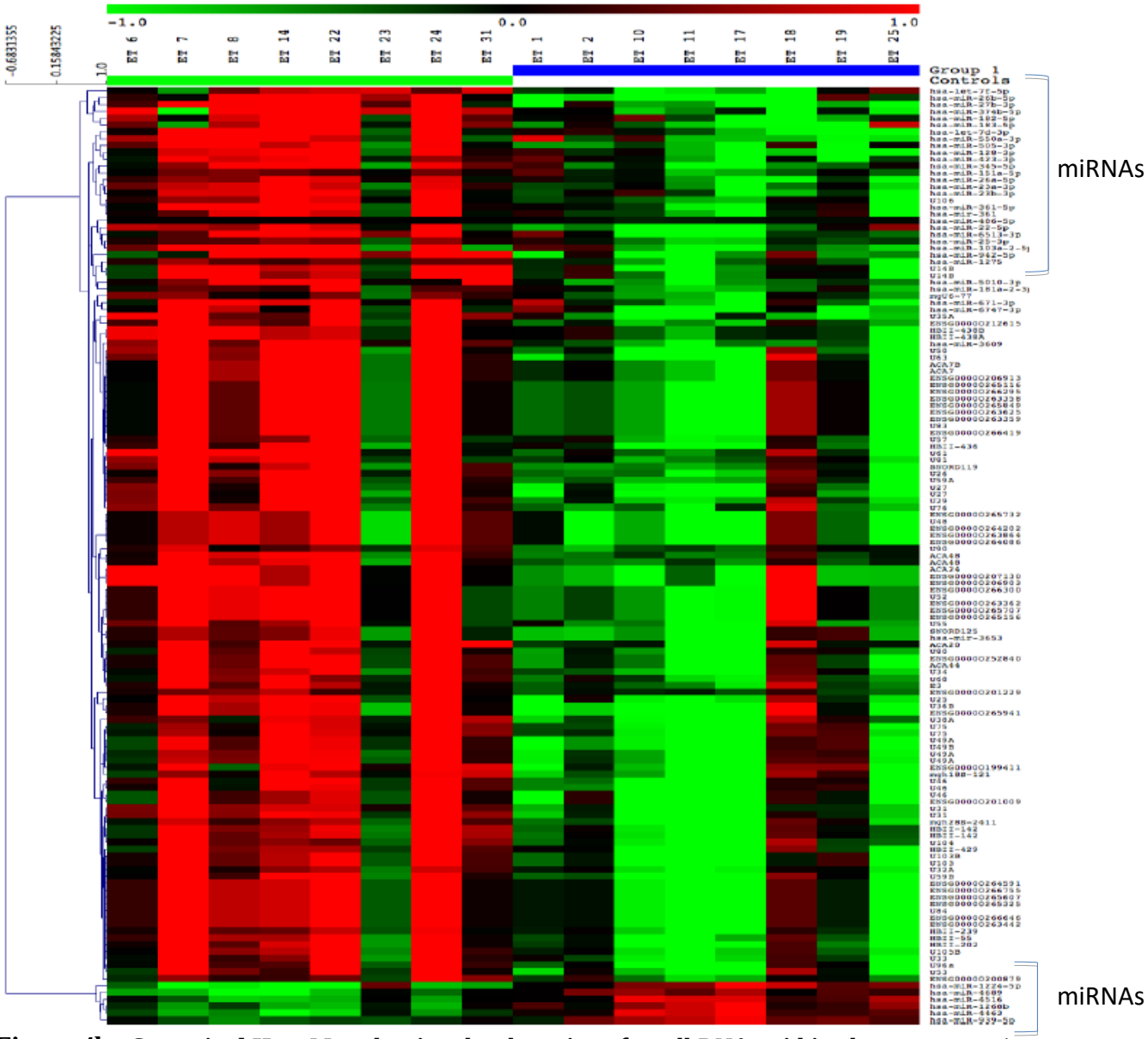

Figure 4b: ***Supervised Heat Map showing the clustering of small RNAs within the two groups*** *i.e. PTB/HIV (Group 1, blue) and Uninfected controls (Controls, green)*

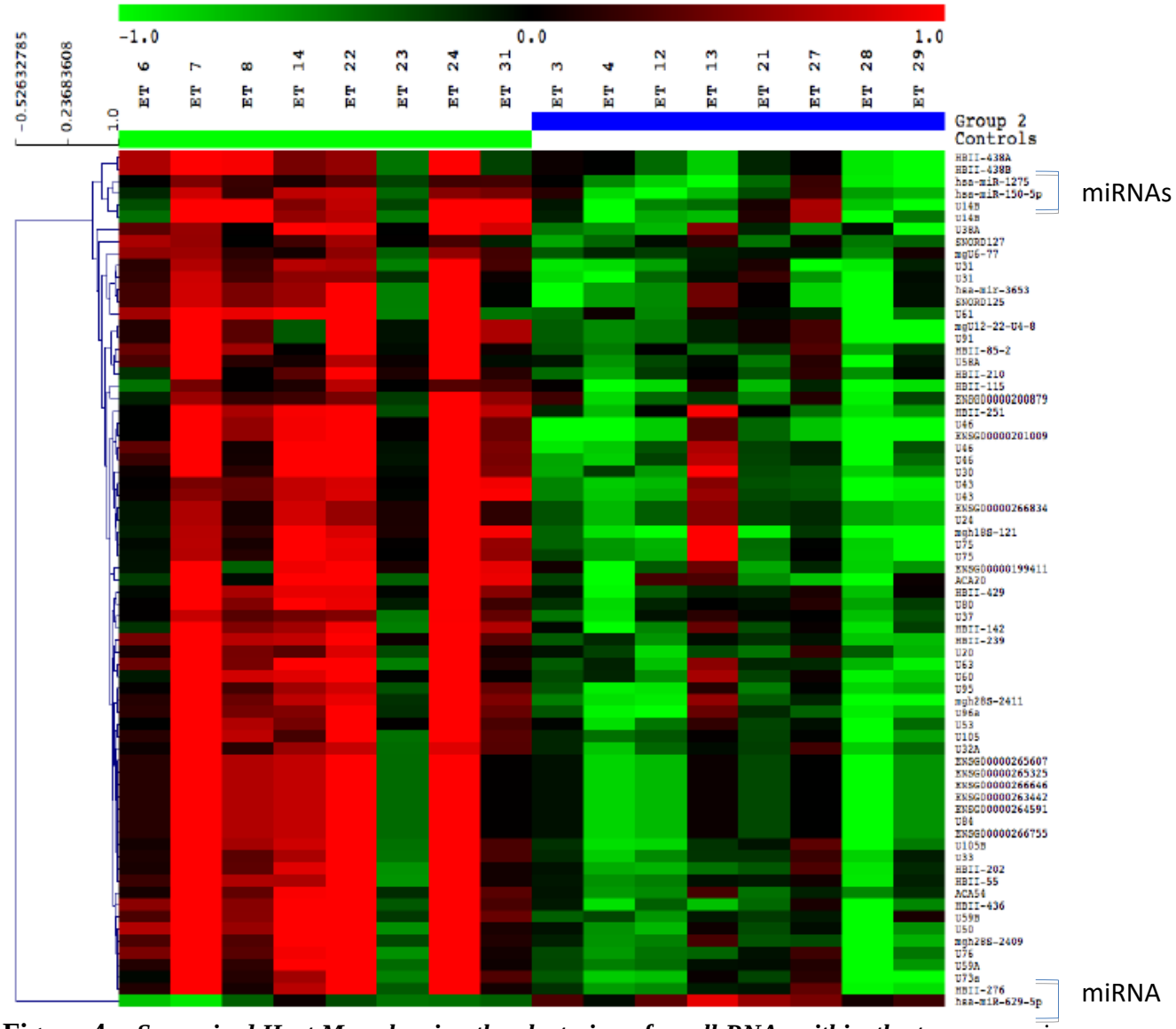

Figure 4c: ***Supervised Heat Map showing the clustering of small RNAs within the two groups*** *i.e. PTB (Group 2, blue) and Uninfected controls (Controls, green)*

Note: Since the chip included all human small RNAs (sRNA), including miRNAs, other sRNAs were evaluated and incorporated into the hierarchical clustering analysis and illustration.

As it is shown in the hierarchical clustering analysis (Fig 4a), majority of the samples were grouped under the respected study groups according to their expression. However, expression pattern of six samples (ET18 from group 1; ET4 and ET28 from group 2 and ET6, ET23 and ET31 from group 4) did not relate with the expression pattern of other groups. To produce clear clustering pattern, we repeated the analysis by removing these samples. Accordingly, the profiles of differentially expressed miRNAs were increased and another heat map was

generated comparing the three groups (data not shown). It was also noted that the miRNAs with large differences in expression between the groups overlapped between the two analyses, i.e. appeared as differentially expressed in the analysis containing all samples as well as in the analysis after these samples were excluded. There was no known association linked to the samples with unfit (outlier) expression patterns, except that the one sample from Mtb/HIV co-infected group (ET18) with low viral load and relatively high CD4 count was the outlier sample in this group. Importantly, in both analyses we observed distinct and noticeable cluster among the three groups.

4.5. miR-146a expression

In a previous *in vitro* study, miR-146a had been shown to be elevated in macrophage infected with both Mtb and HIV (unpublished data) compared to uninfected cells. On the basis of this result we established *ex vivo* investigation to examine the expression of miR-146a in whole blood samples of patients with PTB/HIV, PTB without HIV and in uninfected individuals. Among the here tested samples no significant difference was found ($P>0.05$) in whole blood comparing patients with PTB/HIV co-infection, PTB patients and uninfected controls (Fig 5). miR-146a was also not identified to be among the miRNAs with the strongest differential expression between the groups ($q=0$), while in the comparison of PTB/HIV versus control samples the q-value was found to be rather low ($q=0.08$), in our microarray miRNAs profiling study.

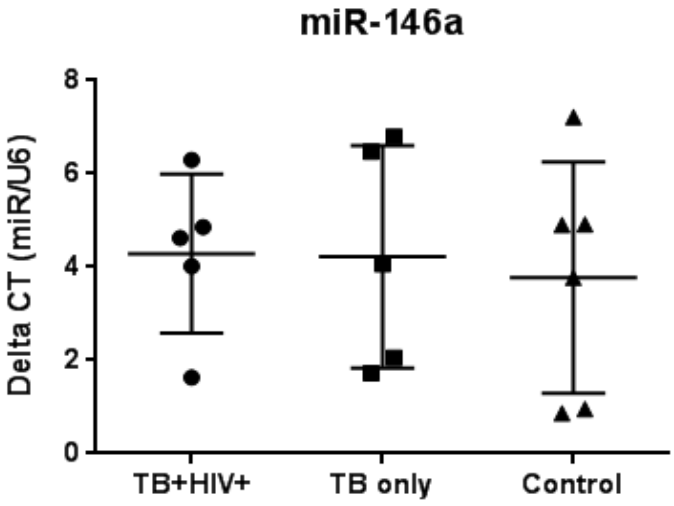

Figure 5: miR-146a expression in PTB/HIV, PTB patients and uninfected control individuals

5. DISCUSSION

The aberrant expression of miRNAs during infections is correlated with disease progression due to their major regulatory role for gene expression and immune cell function (Singh *et al*., 2013). This indicates that cellular miRNA expression is influenced by microbial infection, which can be attributed to both host antimicrobial defences and alteration of the cellular environment to favour microbial replication. In line with this, several studies have also addressed the dysregulation of miRNAs in response to mono-infections with Mtb and HIV-1 (reviewed in Harapan *et al*., 2013; Swaminathan *et al*., 2014).Whilst the importance of miRNAs modulation during Mtb and HIV-1 mono-infections has been reported (Kleinsteuber *et al*., 2013; Huang *et al*., 2007), little is known on the alteration of miRNAs expression profiles upon Mtb/HIV co-infection that could be of relevance for the pathogenesis of these diseases.

Here we conducted a comprehensive miRNA expression profiling in peripheral blood from PTB cases with or without HIV infection (PTB vs PTB/HIV) compared to uninfected individuals to find out if miRNAs were deferentially expressed among these groups. Our exploratory analysis of miRNAs indicated that PTB/HIV co-infection altered several miRNA expression compared to uninfected individuals. We found that 35 miRNAs were significantly dysregulated in PTB/HIV co-infected patients; most of them (29/35 miRNAs) showing downregulated expression compared to uninfected individuals. This observation was in line with studies by Houzet *et al*. (2008) and Zhu *et al*. (2013) who showed a major down-regulation of miRNAs in PBMCs of HIV-1 seropositive individuals compared with uninfected individuals. Our observation suggested that HIV-1 co-infection might cause a profound impact on the dysregulation of miRNAs in the PTB/HIV co-infection setting. On the other hand, differentially expressed miRNAs in PTB mono infected subjects were limited to only three miRNAs compared to uninfected individuals. Therefore HIV co-infection might be responsible for the large number of cellular miRNAs found to be dysregulated, as compared with PTB disease without HIV infection.

To compare the differential expression of miRNAs between the case groups (PTB mono and PTB/HIV co-infected patients), we analysed the miRNAs expression profiles from these two groups. We identified 60 down and 17 up regulated miRNAs in PTB patients relative to co-infected subjects. Likewise, the majority of the miRNAs released in this analysis were also

down regulated and these include the miRNAs that have not previously been reported among Mtb or HIV-1 mono-infections. Some of these miRNAs may represent new molecular markers for patients with PTB/HIV. Furthermore, twice the number of miRNAs were differentially expressed when PTB mono-infected were compared with co-infected than when the co-infected group was compared with uninfected individuals. This observation implies that the additive interaction of both pathogens in human cells might be the cause for increased changes in pattern of miRNAs expression profile.

In this study, only three miRNAs (miR-1275, miR-150 and miR-629) were identified in peripheral blood of PTB mono-infected patients compared to uninfected individuals. The minimum number of differentially expressed miRNAs identified by previous studies in whole blood of PTB patients was 21 (Latorre *et al*., 2015). The most probable reason for the discrepancy on miRNAs signature among studies could be the difference in methods used for miRNAs quantification and normalization since no standard platforms and data normalization protocol for miRNAs analysis is established. Here it should be considered that different methods used to detect miRNA expression may produce different profiles, as reported by comparison of microarray methods and array methods based on qRT-PCR (Chen *et al*., 2009b). Other factors such as ethnicity or genetic background (Miotto *et al.*, 2013), age & gender, and cell types employed for miRNAs analysis (Ajit, 2012) can influence miRNA expression levels and profiling results. Thus, several factors pose potential problems and should be considered in miRNAs profiling studies.

However, in the other comparisons, we found significantly differentially expressed miRNAs in whole blood of PTB and PTB/HIV patients that have previously been reported in connection with Mtb and HIV-1 mono infection (Chiang and Rice, 2012; Ueberberg *et al*., 2014). Importantly, some of these miRNAs such as miR-150, miR-223, and family members of let-7, miR-27 and miR-26 appeared in this study has been the most characterized miRNAs as immune regulatory during tuberculosis and HIV-1 infections. This suggests that specific miRNAs functions, defined by the genes it targets, are altered during infection and influence the disease state (Sharbati *et al*., 2011).

In the current study, miR-150 was found to be down regulated in PTB patients compared to uninfected controls. Similar result was reported in PBMCs (Ghorpade *et al*., 2013) and whole blood (Latorre *et al*., 2015) of tuberculosis patients. It is also well-established that miR-150 contribute by regulating the transcription factor c-Myb, involved in a multitude of

hematopoietic processes during B cell development and the immune response of both B and T lymphocytes (Zhou *et al*., 2007; Xiao *et al*., 2007). Its down-regulation results in increased proliferation of cells and uncontrolled inflammation. Our observation taken together with the available evidence points out that the down regulation of miR-150 upon PTB infection might be one of the mechanisms of Mtb immune modulation to create favourable environment for its survival.

On the other hand, miR-150 and miR-223 have been reported as "anti-HIV-1 miRNAs" by targeting 3'UTR of HIV-1 mRNA and inhibiting its transcription in resting CD4+ T cells and monocytes (Huang *et al.,* 2007; Wang *et al*., 2009). It's also demonstrated that activation of resting CD4+ T cells and differentiation of monocytes to macrophages resulted in down-regulation of these miRNAs. Accordingly, these anti-HIV-1 miRNAs could play a role in controlling HIV-1 latency and their manipulation could potentially contribute to the purging of viral reservoirs (Huang *et al.,* 2007).

Furthermore, lower levels of miR-150 and miR-223 were reported in PBMCs of HIV-1 patients compared to healthy controls (Houzet *et al*., 2008). In our profiling, miR-150 and miR-223 were found to be differentially expressed between the case groups (PTB/HIV vs PTB). As was noted in the PTB mono infected group compared to the uninfected controls, miR-150 was also down-regulated in the PTB patients compared to PTB/HIV co-infected group, suggesting that the down-regulation of these miRNA could be an indicator for TB disease progression.

In contrast, miR-223 appeared only differentially expressed comparing PTB mono and PTB/HIV co-infected groups and was shown to be upregulated in PTB mono-infected patients. This miRNA is involved in regulating myeloid cell differentiation and limiting inflammation to prevent collateral damage during infection. In relation to tuberculosis, Dorhoi *et al*. (2013) identified over expression of miR-223 in the blood and lung tissues of TB patients.

It has also been reported that upregulation of miR-223 and miR-150 inhibits HIV replication by binding to the viral 3'UTR and downregulation of these miRNAs result in enhanced HIV-1 replication (Huang *et al*., 2007). On the other hand, Mtb infection resulted in down regulation of miR-150 and over expression of miR-223 (Dorhoi *et al*., 2013). Our profiling, although comparing PTB mono with PTB/HIV co-infected patients, showed down and up regulation of miR-150 and miR-223 respectively. Thus, it is difficult to suggest which

pathogen (either Mtb or HIV) that could be the cause for altered expression of these miRNAs in PTB/HIV co-infection setting since we did not perform their deferential expression in HIV mono infected individuals.

Generally, it is possible that the conditions triggered by one pathogen may help the replication and pathogenesis of the other pathogen by modulating these miRNAs. As a result, the response of cellular miRNAs to a single infection might be altered during coinfections that in turn might cause the replication and disease progression as seen in the context of PTB/HIV co-infection. Further validation and functional study should be performed on the relevance of these miRNAs in PTB/HIV co-infected individuals.

It was previously demonstrated that intracellular pathogens induce miRNAs expression in a cell type-dependent manner and promotes downregulation of the *let-7* family, an actor of innate anti-microbial defence and potential tumour suppressor (Chen *et al*., 2007, Schulte *et al*., 2011). This evidence is supported by our profiling that showed the down regulation of let-7 family (let-7d and f) in PTB/HIV co-infected patients compared to uninfected healthy individuals. In addition, the let-7 family of miRNAs has been shown to be downregulated in PBMCs and CD4+ T cells of HIV-1 infected patients (Swaminathan *et al*., 2012) and in Mtb infected macrophage (Kumar *et al*., 2015). It was further demonstrated that the down regulation of let-7 miRNAs during HIV-1 infection results in an increased expression of IL-10, anti-inflammatory cytokine and identified that let-7 miRNAs target IL-10 mRNA. According to the latter study (Kumar *et al*., 2015), let-7f targets A20 (TNF-a-induced protein 3), a feedback inhibitor of NF-kB signalling, suggesting that Mtb-enhanced suppression of let-7f to facilitate its survival. Interestingly, our profiling demonstrated the down regulation of let-7d and let-7f in peripheral blood of PTB/HIV co-infected patients, suggesting that the let-7 family may also be involved in the regulation of immune responses in PTB/HIV cases.

In the human genome several miRNA genes are located in close vicinity and encoded as clusters and multiple miRNAs can be transcribed and processed from a single transcription unit (Altuvia *et al*., 2005; Inomata *et al*., 2009). Evidence indicated that clustered miRNAs are transcribed as polycistrons and have similar expression patterns (Seitz *et al*., 2004; Baskerville and Bartel, 2005), suggested that the expression of miRNAs in a cluster is co-regulated, and that they play a role in a common molecular process. In our study, we observed that some miRNAs such as miR-23a, miR-23b, miR-26a, miR-26b, miR-27b, miR-128-3P, miR-505 and miR-550b showed similar level (fold change) of up- and down regulation

between the three groups. We found these miRNAs down regulated in PTB/HIV patients compared to uninfected individuals, while up regulated in PTB patients relative to co-infected subjects. Of these, miR-23a, miR-23b, miR-27a and miR-27b were recently identified as polycistronic miRNA transcribed from the same miR-23/27/24-1 gene cluster and their down regulation was regarded as a marker of disease progression (Chen *et al*., 2014; Goto *et al*., 2014). Similarly, these miRNAs were found to be down regulated miRNAs in profiling studies of PBMCs from HIV-1 seropositive individuals (Houzet *et al*., 2008; Bignami *et al*., 2012). Houzet *et al*. (2008) also noticed these miRNAs as polycistronic miRNAs clusters. These findings might suggest their co-regulation in PTB/HIV patients, and therefore would cluster gene analysis and validation of these miRNAs be of interest, in order to confirm such claims.

In line with this, the miR-26 family members, miR-26a and miR-26b, which share the same seed region for target recognition but differ by two nucleotides in the remaining sequence, exhibited similar expression trends in the current study.

Furthermore, the two miRNAs, miR-26b and miR-27b showed the highest fold change difference between diseased and healthy individuals in our study. MiR-26b has been shown to be down regulated in several cancers and auto-immune diseases and is involved as an inhibitor of different pathways, including NF-κB, to control excessive cell proliferation and inflammation (Zhao *et al*., 2014b; Sun *et al*., 2015). Beside these, miR-26b has been found to be decreased in hepatitis C (HCV) and B (HBV) infections (Kasama *et al*., 2014, Zhao *et al*., 2014a). Furthermore, miR-26b was reported by Xu *et al*. (2013) as a target of highest numbers of mRNAs in their miRNA-mRNA target analysis in PBMCs of TB patients, but its role either in Mtb or HIV-1 infections has not been described so far. Our results showed that miR-26b was the most downregulated miRNA in PTB/HIV co-infected patients, indicating its involvement during such infections.

However, its family member miR-26a was also shown to be deferentially expressed in our profiling and it has been reported during Mtb and HIV-1 infections (Kleinsteuber *et al*., 2013; Ni *et al*., 2014). miR-26a was also found to be down regulated in PBMCs of HIV-1-infected patients (Houzet *et al*., 2008). In relation to tuberculosis, miR-26a was reported to be down regulated in CD4 T cells and whole blood of Mtb infected patients (Kleinsteuber *et al*., 2013). In contrast, miR-26a was shown to be upregulated in monocyte derived macrophage upon Mtb infection (Ni *et al*., 2014). Further investigation disclosed that miR-26a as a negative

regulator of transcriptional activator p300, a component of the IFN-γ signalling pathway that mediates transcriptional responses to IFN-γ in macrophages (Ni *et al*., 2014). This may be one of the mechanisms of action during human Mtb infection in which capacity of macrophages to be activated by IFN-γ is decreased (Cavalcanti *et al*., 2012). As a comparative study, our result showed that the down regulation of miR-26a in whole blood of PTB/HIV co-infected patients and its upregulation in PTB patients. As suggested by Ni *et al*. (2014), this could be one mechanism by which Mtb modulates innate immune cells by altering cellular miRNA expression. Overall, the deferential regulatory role of miR-26a/b warrants further investigation including target identification in co-infected cases.

In our miRNAs profiling miR-27a and miR-27b were shown to be down regulated in peripheral blood cells from PTB/HIV co-infected individuals. MiR-27b has been identified as one of the important miRNAs involved in Mtb persistence within macrophages (Meng *et al*., 2014). One study identified miR-27a/b induction in virulent Mtb H37Rv infected macrophage and is predicted to target important immune related genes (Furci *et al*., 2013). Interestingly, Gorrono and his colleagues showed overexpression of miR-27a and miR-27b in HIV-1 uninfected individuals and elite controllers (maintain undetectable levels of viral load for long time without ART) and down-regulation in HIV-1 viremic progressors (Egaña-Gorroño *et al*., 2014). Moreover, miR-27b has been shown to inhibit HIV-1 replication by repressing the Cyclin T1 expression, an important cellular factor required for Tat-mediated transactivation of HIV-1 LTR-directed gene expression and replication, in CD4+ T cells (Chiang *et al*., 2012). These results suggest a regulatory role of miR-27 in Mtb and HIV-1 infections and its down regulation during co-infection might be related with the activation and pathogenesis of these infections.

In the current study, other miRNAs showing differential expression and fold changes in different two-group comparisons were also identified. These included miR-1224-5P, miR-1268b, miR-4463, miR-4516 and miR-4689 as being significantly up regulated in the co-infected group relative to the uninfected individuals, while downregulated in the PTB group compared to the PTB/HIV group. Currently, there is no published report regarding the signature and functions of these miRNAs in relation to TB or HIV, as well as other infections, while limited information in cancer exists. The deferential expression of these miRNAs in our profiling might indicate their involvement in such infections.

Only a single miRNA (miR-1275) appeared to be differentially expressed with the same direction of change in both the PTB/HIV co-infected and PTB mono-infected groups compared to uninfected individuals. MiR-1275 is described to play an important role as tumor cell proliferation suppresser and has been shown to be deferentially expressed in different cancers (colorectal liver metastases, glioblastoma) (Kahlert *et al*., 2011; Agrawal *et al*., 2014). In relation to infections Das *et al*. (2013) recently reported that miR-1275 expression was elevated in macrophage cells infected with virulent than avirulent Mtb strains. Similarly, it was found to be upregulated in PBMCs of HIV-1 seropositive individuals in comparison to seronegative (Duskova *et al*., 2013). In contrast to these reports, our study demonstrated that miR-1275 was down-regulated in both PTB/HIV co-infected and PTB mono-infected individuals compared to uninfected controls. Further characterization of this miRNA in different compartments, *in vitro* and *in vivo*, is likely to provide insights into its role in the pathogenesis of tuberculosis and HIV-1 co-infection.

To further confirm the consistency of the deregulated miRNAs profiles found in this study, we established the hierarchical clustering among the three groups and across the groups. Unsupervised cluster analysis based upon 83 miRNAs that were significantly deferentially expressed among the three groups generated three descriptive clusters containing PTB/HIV co-infected; PTB mono-infected and uninfected groups. Furthermore, we showed that the expression pattern of miRNAs in the co-infected group was distinctly segregated from the PTB mono infected and uninfected groups, while the miRNAs profile in Mtb mono-infected group is closely related with uninfected groups. These findings indicate that specific miRNA expression profiles could differentiate individuals based on the infections status (Houzet *et al*., 2008). This might make it possible to distinguish PTB/HIV co-infected patients from PTB mono-infected using these miRNAs.

Comparable with the findings of the miRNA expression data, the relative expression of miRNAs for each group of individuals was also depicted in the hierarchical clustering. The expression profile of 35 miRNAs showed a clear separation between PTB/HIV co-infected and uninfected groups. The same was true comparing PTB group with that of controls even with 3 miRNAs difference. This is also in accordance with other studies that performed miRNAs expression profiling in active TB patients compared with latent infection and

uninfected individuals and successfully clustered and separated the individuals based on miRNAs expression (Wang *et al*., 2011; Xu *et al*., 2013).

Even though the majority of samples clustered under their respective group, we also noted that six samples were outliers, i.e. did not cluster with any of the groups, in our study. The reason for this finding is not clear; expect that the outlier in the PTB/HIV co-infected group (ET18) was the patient with the lowest HIV-1 viral load and highest CD4 count in this group. This result is supported by other studies (Duskova *et al*., 2013; Houzet *et al*., 2008). They conducted miRNAs profiling in HIV-1 infected individuals with distinct viral load and CD4 counts and demonstrated class specific miRNAs and a separate cluster based on miRNAs expression profile. From this finding, we can anticipate that further studies may link specificity of miRNAs to distinct disease conditions.

The other outlier samples, that we couldn't identify the rationale for the non-relatedness with any of the groups, might be due to other infections or chronic diseases that were not identified at enrolment, as we didn't screen all types of infections, such as HBV and HCV or cancer, which are well-known cases for miRNAs aberrant expression (Lu *et al*., 2005; Shwetha *et al*., 2013).

Another part of our miRNA study was qPCR quantification of miR-146a. Here significant differences in miR-146a expressions were not found in whole blood of PTB/HIV or PTB patients compared to uninfected individuals. Still, miR-146a differential expression has been reported in *in vitro* Mtb and HIV-1 infections as well as in infected patients. miR-146a was found to be down regulated in alveoli macrophage of PTB patients (Liu *et al*., 2014). Similarly, in the Spinelli *et al*. (2013) study miR-146a was found to be down regulated in PBMCs and Pleural fluid mononuclear cells (PFMCs) of tuberculosis patients. miR-146a was also found to be overexpressed in HIV-1 infected human microglial cells (brain macrophage) (Rom *et al.*, 2010). In accordance to these findings, another in vitro study by Mazurek *et al*. (unpublished data) showed increased expression of miR-146a in human macrophages infected with both Mtb and HIV-1.

Unlike the studies by Rom *et al.* (2010), Spinelli *et al*. (2013) and Liu *et al*. (2014), we couldn't find any difference in miR-146a expression among the analysed samples in the current study. Taken together, the different results presented by our study and others indicated that miRNA quantification studies, representing *in vitro*, *ex vivo* or *in vivo* systemically activated cells, of different subsets and compartments, in PTB and PTB/HIV co-infections

using varying protocols might produce different results and should be considered when interpreting future similar studies.

6. CONCLUSIONS

For the first time we revealed miRNAs signature profiles in whole blood of Ethiopian PTB patients with or without HIV infection, as compared to uninfected individuals. The findings presented here demonstrate that distinct miRNA expression profiles can discriminate the different study groups. Large number of miRNAs was dysregulated (mostly downregulated) in PTB/HIV patients compared with PTB patients without HIV infection and uninfected individuals. This trend was also observed in clustering analysis where the uninfected individuals and PTB mono-infected group clustered separately with similar expression patterns as compared to the co-infected individuals. Among miRNAs that previously been characterized in relation to immune cells responses to Mtb and HIV-1 infections we noted that miR-150, miR-223, and the family members of let-7, miR-27 and miR-26 were differentially expressed in PTB/HIV co-infected patients compared to the PTB mono-infected patients and the uninfected control group. These findings provided at least preliminary data for further basic research and open new insights into the underlying mechanisms of miRNAs involvement in Mtb/HIV-1 pathogenesis in co-infected patients. Furthermore, these finds may provide keys to the development of biomarkers that could be used for diagnostic purposes.

7. RECOMMENDATIONS AND FUTURE PERSPECTIVES

- The miRNAs profiling results found in this study should be validated using quantitative PCR to confirm the consistency of miRNAs changes.
- Future dedicated, focused research in this field is imperative to ascertain the potential role of miRNAs as novel diagnostic, prognostic and therapeutic targets for Mtb/HIV-1 co-infection.
- Functional validation of all miRNAs reported to be dysregulated in Mtb/HIV-1 infections, including the predictions and identification of their target genes is also important to further elucidate their role in the pathogenesis of Mtb/HIV-1 mono and co-infections.
- Further evaluation of the identified miRNAs in larger case-control and cohort studies is necessary to validate the preliminary findings, and to further elucidate the feasibility of developing circulating miRNA assays specific for individual infections (Mtb, HIV, and Mtb/HIV) as clinically useful tools. Moreover, studies representing population heterogeneity, both genders and all ages with defined case/control, considering different cell types, analytical settings, and data evaluation will be vital to ensure comparability of results.
- It will be important to study the ability of systemic miRNA signatures to distinct other infections or related infections (e.g Mycobacterial species) to identify disease-specific miRNAs.
- Measuring the miRNAs levels with clinical parameters (CD4 counts) and the possible cytokine response after and before treatment will be very informative for the management of patients with PTB/HIV co-infections.
- In addition to miRNAs, other small RNAs will be important tools as new descriptive markers for TB/HIV-1 disease as it is depicted in our clustering.

Overall, our ongoing studies emanating from this study and other future studies will endorse investigations to clarify the issues mentioned above, and validate the preliminary findings described throughout this work.

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Annex I: Viral Load Determination Assay

Materials

- 1.5 ml screw top microfuge tubes
- 5 ml reaction Tubes
- Magnetic rack
- Vacuum sucker
- Vortex mixer
- Aerosol barrier pipette Tips (200 µL and 1000 µl)
- Abbott Optical 96-well reaction plate
- Abbott Optical Adhesive cover

Abbott Real time HIV-1 Amplification Reagent Kits

- Abbott m2000rt Sample Preparation Kit (Lysis buffer, Micropartical beads, Wash buffers 1&2 and Elution Buffer)
- Abbott m2000rt Control kit (Negative Control, Low Positive Control and High Positive Control)
- Abbott m2000rt Calibrator Kit (Calibrator A and Calibrator B)
- Abbott m2000rt Amplification Kit (HIV-1 Internal Control, rTth Polymerase Enzyme and HIV-1 Oligonucleotide)

Procedure

1. Thaw human plasma specimens
2. Thaw internal control, controls and calibrators
3. Label all tubes (24 tubes per one run)
4. Add 500 µl internal control in to one bottle of lysis buffer and mix gently
5. Add 100 µl of microparticles to each lysis tube
6. Add 2.5ml of Lysis buffer and IC mix to each lysis tube containing the microparticles.
7. Add 600 µl of Controls, calibrators and samples to lysis tubes
8. Incubate at 50 ^{o} C for 20 minutes.
9. Place tubes in the magnetic capture stand for 2 min

10. Carefully remove the lysate from each tube using sterile disposable pasteur pipettes

11. Add 700 µl of wash buffer 1 to each tube and resuspend the magnetic particles by aspiration

12. Transfer wash fluid and particles to a labeled 1.5ml screw top tube

13. Place 1.5 ml tubes in a magnetic capture stand (blue) for 1 min and remove the supernatant

14. Repeat steps 11to 13 one more time

15. Add 700 µl of Wash Buffe 2, place in magnetic rack and resuspend the magnetic particles by aspiration

16. Repeat step 15 one more time

17. Add 25µl of Elution Buffer

18. Incubate at 75 º C for 20 minutes.

19. Add 63 µl of Wash 2 and resuspend the magnetic particles by aspiration

20. Place 1.5 ml tubes in a magnetic capture stand and remove the Eluate and transfer to a fresh RNAse free 1.5ml tube.

Master Mix Preparation and Amplification

21. Add 271µl of the HIV-1 Activation in the Thermostable rTth DNA Polymerase Enzyme bottle

22. Add 949 µl of the HIV-1 Oligonucleotide in the Enzyme bottle

23. Pipette the master mix from the enzyme bottle into RNase/DNase-free tube and vortex to mix

24. Prepare 96-Well Optical Reaction Plate

25. Dispense 50 µl aliquots of the amplification master mix into the 96-well plate

26. Transfer 50 µl of sample eluate to the 96-well plate and mix by pipetting up and down 3-5 times

27. Seal the 96-well plate using Abbott m2000rt Optical Adhesive Cover

28. Centrifuge the 96-well plate at 1500rpm for 5 min

29. Place the plate in the Abbott m2000rt instrument and Run according to the instructions

Annex II: QuantiFERON®-TB Gold ELISA Test

Materials

- Multichannel pipette
- Incubator (37^{o}C)
- Microplate shaker
- Microplate washer
- Distilled water/dH_2O
- Microplate reader with 450nm filter and 620 to 650nm reference filter

QuantiFERON®-TB Gold IT blood collection tubes

- Nil Control tube (Grey cap)
- TB Antigen tube (Red cap)
- Mitogen control tube (Purple cap)

QuantiFERON®-TB Gold ELISA kit

- Microplate strips, 24 x 8 well
- Human IFN-γ Standard, lyophilised, 1 x vial
- Green Diluent (GD), 1 x 30mL

Conjugate 100X Concentrate, lyophilised, 1 x 0.3mL

Wash Buffer 20X Concentrate, 1 x 100mL

Enzyme Substrate Solution, 1 x 30mL

Enzyme Stopping Solution, 1 x l5mL

Specimen collection and handling

1. Collect 1mL of blood by venepuncture directly into each of the QuantiFERON®-TB Gold IT blood collection tubes
2. Mix by vigorously shaking the tubes up and down 10 times
3. Label tubes appropriately
4. Incubate tubes at 37°C for 16 to 24 hours
5. Isolate plasma by centrifuging tubes for 15 minutes at 2500rpm

Human IFNγ ELISA

Standard Preparation

- Reconstitute the freeze dried Kit Standard with the volume of dH_2O indicated on the vial. Mix thoroughly.
- Label 4 tubes S1, S2, S3, S4
- Add 150 µL GD to tube S1 and 210 µL GD to tubes S2, S3, and S4
- Add 150 µL of reconstituted standard to tube S1 and mix well.
- Transfer 70 µL of S1 to tube S2 and mix thoroughly.
- Transfer 70 µL of S2 to S3 and mix thoroughly.
- S4 is GD alone

Conjugate preparation

- Reconstitute freeze dried Conjugate 100X Concentrate with 300 µL of dH_2O.
- Add 60 µL of 100X reconstituted Conjugate to 6ml of Green Diluent per plate.

Procedure

1. Mix plasma thoroughly
2. Add 50 µL of Working Strength conjugate to each well
3. Add 50 µL of test plasma samples to appropriate wells
4. Add 50 µL Standards (S1, S2, S3, and S4) in triplicate.
5. Cover plate and mix by shaking on a microplate shaker for 1 minute.
6. Incubate at room temperature for 120 ± 5 min
7. Prepare Working Strength wash buffer by 1 part Wash Buffer 20X Concentrate with 19 parts dH_2O.
8. Wash wells with 400µL Working Strength wash buffer for at least 6 cycles. Soak 5sec between cycles. Tap plate face down on absorbent tissue paper.
9. Add 100µL of enzyme Substrate Solution. Mix with microplate shaker
10. Incubate at room temperature for 30min.
11. Add 50µL of Enzyme Stopping Solution to each well
12. Measure the Optical Density (OD) within 5 min of stopping the reaction with 450nm filter and 620nm to 650nm reference filter
13. Use QTB Gold Analysis Software to analyse data

DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in this or any other University for the fulfilment of academic requirement and all the resources of materials used for the thesis have been duly acknowledged.

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