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MicroRNAs as a Biomarker in Tuberculosis

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TABLE OF CONTENTS

Preface

This thesis is based on work carried out at the Centenary Institute under the supervision of Dr Bernadette Saunders and Professor Warwick Britton. The work carried out in this thesis was performed by the candidate, except where due acknowledgment has been made.

All work is original and has not been presented previously for the purpose of obtaining another degree.

Simone Barry

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Publications arising from this work

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Publications related to this work

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Abstract

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains a global health challenge and is one of the leading causes of death worldwide. New biomarkers that support rapid and accurate TB diagnosis, and are able to recognise early treatment failure as well as cure are urgently needed. MicroRNAs (miRNAs) are small non-coding RNAs that have recently come into prominence as promising diagnostic and prognostic biomarkers. They have been isolated from a number of tissues and body fluids and have been extensively studied and reviewed in both health and disease. This thesis explores the application of miRNAs as biomarkers in pulmonary tuberculosis and explores their expression in macrophages, the key innate immune cell involved in host defense against the bacterium.

As with messenger RNA (mRNA), measuring miRNA expression requires a consistently expressed and stable miRNA to act as a reference in order to control for non-biological variation. The suitability of 12 miRNAs, used in published studies to normalise miRNA expression, was examined in 24 TB patients, 12 patients with latent TB infection (LTBI) and controls sampled from Sydney, Australia and Yinchuan, China. Using geNorm and Normfinder software to analyse the data plasma-derived miR-93 was identified as a suitable reference miRNA and was used to normalise the large cohort study that examined the expression of miRNAs in patients from north-west China with pulmonary TB.

Using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) panels we investigated the expression of 175 miRNAs in a test set of 20 TB patients and their age and sex-matched healthy controls. These arrays identified 87

miRNAs that were differentially regulated between the two groups, including 18 that were up-regulated and 69 that were down-regulated. From this group, ten miRNAs were selected for targeted validation in a larger cohort of 100 patients with newly diagnosed pulmonary TB sampled before the commencement of antibiotic therapy and then at one, two and six months following treatment commencement. These miRNAs were chosen based on a mean fold change ≥ 1.5, a p-value < 0.01 and/or miRNAs previously reported in the literature to be regulated in the setting of TB, other infections or known to be important in macrophage function. miRNA expression levels were compared to those in 100 healthy controls. miRNA expression levels from plasma samples were analysed for their ability to detect and differentiate active pulmonary tuberculosis from healthy subjects and for their capacity to predict response to therapy. This is, to our knowledge, the largest miRNA profiling study in tuberculosis to date and also represents the first longitudinal study that examined miRNA expression over a time course.

From the ten miRNAs selected for the validation study, five were differentially regulated in newly diagnosed pulmonary TB subjects. miR-29a and -99b-5p were up-regulated in the setting of tuberculosis infection whilst miR-21-5p, -146a-5p and -652-3p were down-regulated. Following the TB patients over the course of treatment, this study observed that for most miRNAs there was a reduction in the mean difference between the TB and healthy subjects as treatment progressed. miR-99b which was significantly elevated in TB patients before the commencement of therapy returned to levels not dissimilar from healthy subjects following one month of therapy, whilst the levels of miR-29a-5p, which were also up-regulated in TB subjects, took far longer to return to healthy control levels with significant variation

noted up until treatment completion at six months. miR-146a-5p, which was significantly down-regulated in the TB subjects, fell further in the TB patients over the course of their therapy and miR-26a-5p which was not significantly regulated prior to the commencement of therapy became significantly down-regulated and remained so throughout the course of TB therapy.

The Chinese cohort consisted of two distinct ethnic populations, the Han and the Hui Chinese. Traditionally, these groups differ, not only ancestrally, with Hui being one of China's Islamic minorities, but also socioeconomically, with the Han generally residing in the more affluent city areas and the Hui living and working in the poorer farming regions of the area. Sub-group analyses noted significant variation in a number of plasma miRNAs, most notably, miR-146a-5p, -29a-5p and -221-3p that appeared to be secondary to ethnic or geographical differences. Other factors such as co-morbid illness or socio-economic status may have also influenced these observations.

Quite significant variation in miRNA expression was also noted between the sub-group that completed TB treatment successfully compared to those who did not. The overall trend was that those miRNAs that were down-regulated in the plasma of the patients with TB disease were more significantly down-regulated in those that inevitably failed therapy. This was particularly notable for miR-146a-5p, -26a-5p, -133a and -652-3p. These findings suggest that miRNA may be able to act as an early biomarker to predict treatment failure.

Given that plasma miRNA expression levels were clearly modulated by *M. tuberculosis* infection the capacity of *M. tuberculosis* to modulate miRNA expression in human macrophages was examined. Monocyte-derived macrophages were isolated from freshly collected human buffy coats from six healthy donors. Based on current literature together with the miRNAs that we found to be regulated in the plasma of TB subjects, seven miRNAs were chosen and examined in human macrophages with and without *M. tuberculosis* infection. The expression of miRNA in *M. tuberculosis* infected macrophages largely mirrored the findings from the plasma with a few exceptions. miR-146a and -21 which were significantly down-regulated in the plasma of TB subjects, were up-regulated in *M. tuberculosis* infected macrophages. So too, the expression of miR-221 which was significantly down-regulated in *M.* tuberculosis infected macrophages, was not differentially expressed in the plasma of the TB subjects compared to their matched healthy controls. The varying miRNA expression in different tissues is suggestive of likely varying miRNA targets and functional roles. Accompanying *in vitro M. tuberculosis* infection of human macrophages was a heightened secretion of pro-inflammatory cytokines.

Based on the findings from this work, miRNAs demonstrate great promise in their role as a potential biomarker for TB diagnostics and as early marker of treatment failure. Further work is needed to better understand the function of miRNAs in the biology of TB, and to better explore external factors that may alter their expression including ethnicity.

List of abbreviations

°C	degrees Celsius
AFB	acid-fast bacillus
AGO	Argonaute
ANOVA	one-way analysis of variance
APC	antigen presenting cell
ATP	adenosine triphosphate
AUC	area under the curve
BCG	Bacille Calmette-Guérin
bcl	B-cell lymphoma
Bcl-2	B-cell lymphoma 2
bp	base pair
CBA	cytometric bead array
CFP-10	culture filtrate protein-10
CFU	colony forming units
COPD	chronic obstructive pulmonary disease
Cq	quantification cycle
CT	computerised tomography
DEPC	diethylpyrocarbonate
DGCR8	DiGeorge Syndrome critical region 8
dL	decilitre
DMEM	dulbecco's modified eagle medium
DNA	deoxyribose nucleic acid
DOTS	directly observed treatment, short course
EDTA	ethylene-diamine-tetra-acetic acid
ESAT-6	early secreted antigenic target of 6 kDa
FBS	foetal bovine serum
FI	fluorescent intensities
g	acceleration due to gravity
GM-CSF	granulocyte macrophage colony stimulating factor
HDL	high-density lipoprotein
HIV	human immunodeficiency virus
hsa	homo sapiens

IFN-γ	interferon-γ
IGRA	interferon-γ release assay
IL	interleukin
IRAK	IL-1 receptor-associated kinase
LNA	locked nucleic acids
LPS	lipopolysaccharide
LTBI	latent tuberculosis infection
MAC	*Mycobacterium avium* complex
mcl	myeloid cell leukaemia
MDM	monocyte-derived macrophages
MDR	multi-drug resistant
mg	milligrams
miRNA	microRNA
mL	millilitres
mm	millimetres
mM	Millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
NF-κB	nuclear factor-kappaB
ng	nanogram
NGS	next generation sequencing
NHAR	Ningxia Hui Autonomous Region
NLR	negative likelihood ratio
nm	nanometre
NPV	negative predicitive value
nt	Nucleotide
NTP	National Tuberculosis Programme
OR	odds ratio
P2X	Purinoceptor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered sodium
PCR	polymerase chain reaction
PLR	positive likelihood ratio

POC	point-of-care
PPD	purified protein derivative
PPV	positive predictive value
PRC	Peoples Republic of China
qRT-PCR	real-time quantitative reverse transcriptase polymerase chain reaction
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNU6B	U6
ROC	receiver operating characteristic
RPM	revolutions per minute
RT	real-time
RT	room temperature
SNP	single nucleotide polymorphism
TB	tuberculosis
T_H1	type 1 T helper
TLR	toll-like receptors
TNF	tumour necrosis factor
TNFRSF-4	TNF receptor superfamily, member 4
TRAF	TNF receptor-associated factor
TST	tuberculin skin test
UTR	untranslated region
WHO	World Health Organisation
Xpert	GeneXpert®
µl	microliter
µm	micrometre

List of Figures

List of Tables

1 INTRODUCTION

1.1 Tuberculosis: an overview

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. Although substantial progress has been made in the treatment, management and prevention of TB, it remains a global public health problem and is still one of the most important infectious diseases worldwide (World Health Organization, 2013). Although the rates of TB in most Western countries which are inherently more wealthy, including Australia, are decreasing, migration and easy travel have allowed TB to re-emerge. In 2009, the UK reported a 75% increase in reported TB cases over the preceding 20 years (Abubakar et al., 2011).

In 2013 the World Health Organisation (WHO) reported nine million new cases of TB and approximately 1.5 million deaths attributable to the disease (World Health Organization, 2014). Approximately 13% (1.1 million) of the nine million people who develop TB each year are human immunodeficiency virus (HIV) co-infected (World Health Organization, 2014). The majority, about 60%, of the world's cases are found in China, India and Russian Federation (World Health Organization, 2014). The highest incidence rates are in sub-Saharan Africa (Figure 1.1) and are attributable to the high prevalence of HIV in that region (Figure 1.2).

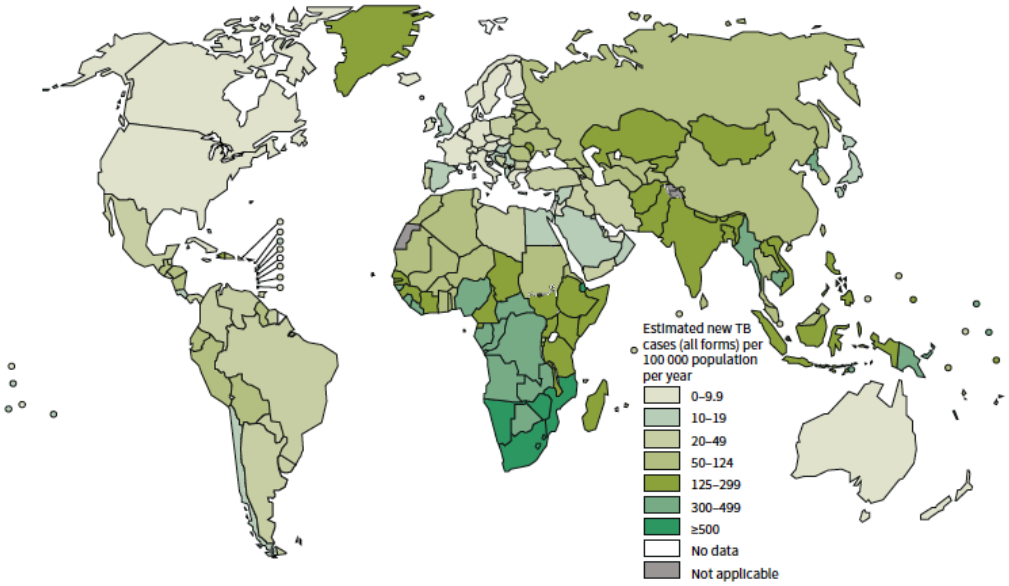

Figure 1-1. Estimated TB incidence (2013). Data from WHO report (World Health Organization, 2014).

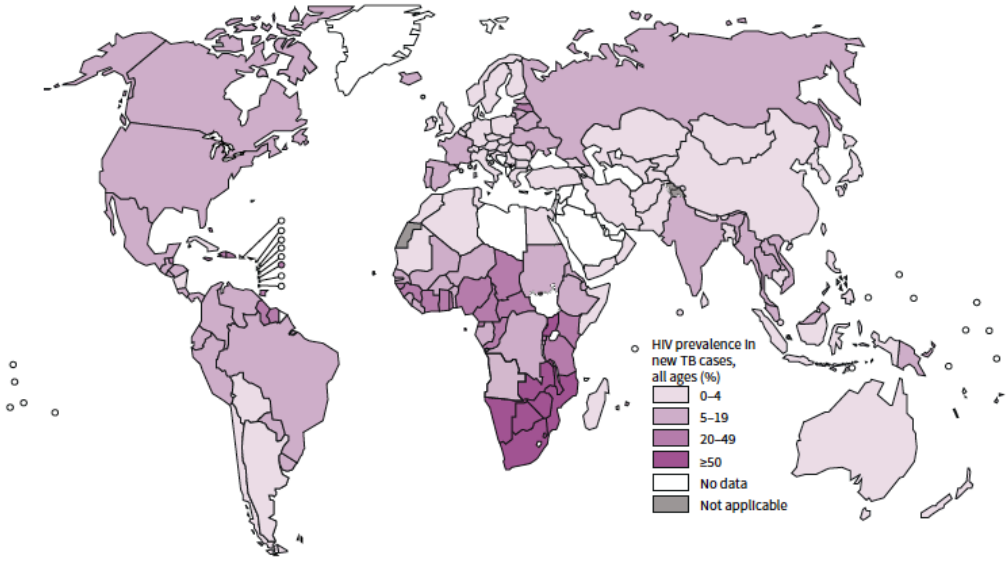

Figure 1-2. Estimated HIV prevalence in new and relapsed tuberculosis cases (2013). Data from a WHO report (World Health Organization, 2014).

Typically TB affects the lungs (pulmonary) but it can also affect other organs (extra-pulmonary*)*. TB is spread through the air by droplet infection from a person with pulmonary disease expelling the bacteria, usually by coughing. Approximately one third of the world's population is latently infected with *M. tuberculosis* (World Health Organization, 2014). About 10% of those infected will go on to develop disease at some stage during their lives, with most reactivation occurring within two years of infection (Tiemersma et al., 2011). The rate of progression is substantially increased in those co-infected with HIV (Tiemersma et al., 2011). Reactivation is also influenced by other factors such as *M. tuberculosis* strain type, genetic background, degree of immunosuppression, geographical location, concurrent medical illnesses such as diabetes or renal impairment, and other risk factors (World Health Organization, 2010a).

TB, although a curable disease, remains a major health problem. Without proper treatment mortality rates are high. The WHO estimates that in 2009 almost ten million children were orphaned as a result of parental deaths caused by the disease (Skogen et al., 2011). There has been a marked increase in the number of multi-drug resistant, extensively drug resistant and totally drug resistant TB strains and in such cases treatment is prolonged and often requires more intensive intravenous therapy (World Health Organization, 2010b). Compliance with therapy for drug-resistant disease is made more difficult as many of the drugs used have unpleasant side-effects and are often poorly tolerated. Hospitalisation of patients who cannot be treated with conventional drugs in the community ties up resources and exposes otherwise healthy patients and staff to nosocomial cross-infection of resistant organisms. There is also a huge economic cost associated with treating drug resistant TB due to the

prolonged and often more expensive antibiotic therapy required and significant lost workplace productivity.

The usual treatment consists of multi-drug chemotherapy given for a period of at least six months. Efforts to control the disease are hampered by difficulties in diagnosis, prevention, treatment and the emergence of drug resistance.

1.2 An historical perspective

M. tuberculosis is the causative agent of TB in humans and is a member of the *Mycobacterium tuberculosis* complex. The *M. tuberculosis* complex strains include seven species and subspecies – *M. tuberculosis*, *Mycobacterium africanum*, *Mycobacterium canettii, Mycobacterium pinnipedii*, *Mycobacterium microti*, *Mycobacterium caprae* and *Mycobacterium bovis*. It is a family of very closely related mycobacteria, with each species being adapted to cause tuberculosis disease in a specific host species or group (Smith et al., 2006). It is possible, however, for inter-species transmission to occur.

Robert Koch was the first to identify *M. tuberculosis* as the causative agent of TB in 1882 (Daniel, 2006). It was originally thought that cattle were the source of TB disease and that *M. tuberculosis* evolved from *M. bovis*, a zoonotic infection predominantly affecting cattle and other livestock (Michel et al., 2010). However, recent findings indicate that *M. tuberculosis* first emerged in humans and was then transmitted to animals (Wirth et al., 2008).

There is evidence to suggest that the common ancestor of the *M. tuberculosis* complex emerged about 70,000 years ago in East Africa (Comas et al., 2013). Approximately 10,000 – 20,000 years later two independent groups evolved, one resulting in *M. tuberculosis* lineage in humans and the other diversifying into other *M. tuberculosis* complex species including *M. bovis* (Michel et al., 2010). Using sensitive polymerase chain reaction (PCR)-based deoxyribose nucleic acid (DNA) techniques, *M. tuberculosis* DNA was able to be identified from a human male who lived approximately 2,200 years ago and was suffering Pott's disease (Taylor et al., 2005).

TB became a major problem in the Western world when the industrial revolution created crowded living condition that favoured the spread of the bacillus. Termed "the white plague", TB arose in the seventeenth century, peaking in the nineteenth century when TB was the most common cause of death in Europeans (Dubos, 1987). As Europeans migrated to more distant lands TB became endemic worldwide, firstly in the Americas and later in North and sub-Saharan Africa. It was not until the emergence of HIV infection in the late twentieth century that TB reached epidemic proportion in the African continent (Good et al., 1993).

1.3 Immunopathology of tuberculosis

M. tuberculosis is an obligate intracellular pathogen and although it can infect several different animal species, humans are the principal host. It is an aerobic, acid fast, non-encapsulated, non-motile, non-spore forming bacillus. It grows best in tissues with high oxygen content such as the lungs. The cell wall is lipid rich and is relatively impermeable to basic dyes unless combined with alcohol. It is neither

Gram positive nor Gram negative but it is described as acid fast. *M. tuberculosis* has a very slow replication rate of 15-20 hours. This very slow doubling rate and its ability to persist in the latent state means that duration of therapy for both treatment and prevention is long (Lawn and Zumla, 2011).

TB is transmitted via inhalation of aerosolised *M. tuberculosis* from patients with active TB disease (Lawn and Zumla, 2011). Although pulmonary TB is by far the most common presentation, the disease can also affect other sites (extra-pulmonary) (Tiemersma et al., 2011; Lawn and Zumla, 2011). Upon inhalation of bacilli, most of these particles settle in the upper respiratory epithelium and are expelled by the mucocilliary escalator. Only a few droplets reach the respiratory bronchioles and alveoli in the peripheral lung tissue.

Innate immunity plays a critical role in host defences against *M. tuberculosis* with the first step being the recognition of the bacteria by the innate immune system. Phagocytic cells, in particular alveolar macrophages, play a key role in the initiation and orchestration of the adaptive T-cell response to infection. They do this by presenting mycobacterial antigens on their cell surface and also by secretion of co-stimulatory signals and cytokines (Van Crevel et al., 2002).

Mycobacteria that arrive in the lung parenchyma are phagocytosed by alveolar macrophages and are either killed or survive (Behar et al., 2011). Mycobacteria that are able to evade destruction, multiply leading to the activation of the macrophage with release of several cytokines. When this occurs, monocytes and other inflammatory cells are recruited to the lung. The monocytes differentiate into

macrophages, which ingest but may not destroy the *Mycobacterium*. Two-to-three weeks after initial infection, T-cell immunity develops with antigen-specific T lymphocytes proliferating and activating macrophages to kill the intracellular mycobacteria. This inhibits bacterial growth and can aid development of latency.

One of the hallmarks of the immune response to *M. tuberculosis* infection is the formation of the granuloma. The granuloma is made up of aggregates of infected macrophages together with other innate and adaptive immune cells. The granuloma effectively contains the *Mycobacterium*, which facilitates the persistence of latent TB infection. It also provides a local milieu where inflammatory cells can communicate with each other, either directly or via the production of cytokines and chemokines. During active disease, the integrity of the granuloma is compromised, releasing the bacteria resulting in dissemination of the bacteria and clinical symptoms of TB disease (Ehlers et al., 2013).

Toll-like receptors (TLR) are one of the primary receptors in the macrophage that recognise *M. tuberculosis*. Following this recognition, their signals activate nuclear factor-kappaB (NF-κB) that then induces pro-inflammatory cytokine release. Cytokines released by several immune cells including macrophages play an integral role in both the innate and adaptive response to TB, either directly or by means of stimulating other immune cells and thereby facilitating a coordinated response against the invading pathogen. Of critical importance in TB are tumour necrosis factor (TNF) and interferon-γ (IFN-γ).

TNF has a central role in controlling the growth of *M. tuberculosis* and is important in the establishment of the latent state of the infection (Stenger, 2005). TNF deficient mice have much higher mycobacterial loads and succumb more quickly to *M. tuberculosis* infection (Flynn et al., 1995). Anti-TNF therapies have provided effective treatment for several debilitating diseases such as inflammatory bowel disease and other autoimmune pathologies (Seymour et al., 2001; Peyrin-Biroulet, 2010). Associated with their use is an increased risk of reactivation from LTBI to active TB disease (Harris et al., 2010). Interestingly, reactivation of TB associated with TNF-blockers, not uncommonly follows an unusual course with extra-pulmonary and disseminated disease far more common among patients using these therapies (Keane et al., 2001). Interestingly, the pattern of disease is not dissimilar to TB encountered in other immune deficiency settings such as HIV (Keane et al., 2001). The general consensus is that TNF inhibitors disrupt the integrity of the granuloma, which normally contains latent bacilli (Harris et al., 2010). The increased risk of TB reactivation associated with these therapies highlights the important roles of TNF in maintaining the bacterium in the latent state.

IFN-γ is a crucial cytokine in adaptive immunity to infection. T-cell mediated immune responses to *M. tuberculosis* are critical to the host's control of the disease. The ability of $CD4^+$ T cells to produce IFN-γ is crucial to contain the intracellular bacteria and afford protection against the disease (Ottenhoff et al., 2005). Type 1 T helper (T_H1) cells and the IFN-γ that they produce are critical to protection against the disease. This is evident by the vastly increased risk of developing disease in individuals who have deficiency in IFN-γ or interleukin (IL)-12, which acts to

promote T_H1 cell differentiation, and the association between the depletion of $CD4^+$ T cells in HIV infected persons and their increased risk of TB (Lawn et al., 2009).

There are several other important cytokines in *M. tuberculosis* infection, including the pro-inflammatory IL-1β, IL-6, IL-12 and IL-18 and anti-inflammatory cytokines IL-10 and TGFβ. Furthermore there are number of key chemokine and chemokine receptors which have been found important in the setting of TB such as IL-8 (CXCL-8), MIP-1α (CCL-3) and IP-10 (CXCL10). IL-8 (CXCL8) is a potent neutrophil, monocyte and T-cell attractant that is secreted by macrophages following infection with *M. tuberculosis* (Monin et al., 2014).

The role of macrophages and the cytokines that they secrete in *M. tuberculosis* infection will be explored during Chapter Five.

1.4 Current diagnostics

Two approaches can be taken in order to reduce the burden of TB. The first approach is to diagnose active TB rapidly and institute prompt and effective treatment to those with the disease. This will reduce transmission and thus reduce the number of persons who will develop active TB later on. However, as presented below, there is often a significant delay in diagnosis of active disease that results in on-going disease transmission. The second approach is to diagnose and then treat persons with latent TB infection (LTBI) to prevent the later development of disease.

1.4.1 LTBI diagnostics

LTBI is a sub-clinical infection with *M. tuberculosis* without clinical, bacteriological or radiological evidence of disease. Diagnosing LTBI is important for overall control of the disease. Offering anti-tuberculosis chemoprophylaxis to those with LTBI substantially reduces their risk of developing active disease. The most currently used regime is isoniazid for nine-months which has an estimated efficacy of 90% (Society, 2000).

The most widely used test to diagnose TB is the tuberculin skin test (TST), also commonly referred to as the mantoux test (Huebner et al., 1993). This test involves the intra-dermal injection of purified protein derivative (PPD), which results in injection site induration that peaks at 48-72 hours. The PPD comprises of numerous antigens that are found in *M. tuberculosis* and other non-tuberculosis mycobacteria including from immunisation with the vaccine strain *M. bovis* bacilli Calmette-Guérin (BCG). The major short fall of the TST is therefore its lack of specificity, especially in BCG-vaccinated persons, and its low sensitivity in the setting of immune-compromise (HIV or other immune suppression). Immune-compromised patients are at the greatest risk of developing TB, but the poor sensitivity limits its utilization in this subgroup (Pesanti, 1994). The absence of a gold standard to diagnose TB, makes it difficult to establish its exact sensitivity and specificity.

Recently commercially available immune-based blood tests were developed that measure IFN-γ release in response to stimulation of sensitized T-cells by specific *M. tuberculosis* antigens, specifically early secreted antigenic target of 6 kDa (ESAT-6) and culture filtrate protein-10 (CFP-10) (X. Q. Liu et al., 2004). The two available

tests are the QuantiFERON®-TB Gold test (Cellestis Ltd., Victoria, Australia) and T-SPOT-TB assay (Oxford Immunotec Ltd., Arbingdon, United Kingdom). These tests have the advantage of greater specificity, as results are not influenced by previous BCG vaccination. Their use in immunocompromised and children however have not been verified and further studies are needed to determine their utility (Mazurek et al., 2010).

1.4.2 Active TB diagnostics

There has been substantial progress in global TB control, however these gains are threatened by the global spread of drug resistant TB. Early diagnosis and treatment are both essential for effective TB management and control. Delays in diagnosis and hence treatment negatively affect both the individual and the community. Most transmission of infection occurs between the onset of cough and the initiation of treatment (Storla et al., 2008). A meta-analysis by Storla *et al.* found that the diagnostic delay, as defined by the onset of clinical symptoms to the notification of disease, to be in the range of 60-90 days (mean ± standard deviation: 72 days ± 28 days) with the longest delays (>120 days) in some endemic countries (Storla et al., 2008). Factors associated with a delay in diagnosis including HIV co-infection, extra-pulmonary TB, sputum smear negative TB, healthcare seeking behaviour of patients, low access to healthcare and poor health care infrastructure and facilities.

The lifetime risk of developing active disease after being infected is about 10% (Lawn and Zumla, 2011). The actual risk is influenced by a number of factors including geographical location, *M. tuberculosis* strain type, genetic background, immunocompetency and other risk factors. Most immune-competent people infected with *M. tuberculosis* either eliminate the bacterium or contain the bacterium in a

latent state. LTBI is a state where equilibrium is established between host and pathogen. The host's immune response maintains *M. tuberculosis* in a quiescent phase thus preventing active replication and surrounding tissue damage. During this time *M. tuberculosis* bacilli are present within the host but there are no signs or symptoms of disease activity and no possibility of disease spread.

Reactivation can occur at any time in an infected person's life, though it is greatest within the first two years of infection (Lawn and Zumla, 2011). Risk factors for the development of active disease include a waning immune system, either due to diseases such as HIV, chronic kidney disease, malnutrition and diabetes or the use of immune modulating therapy including corticosteroids, TNF inhibitors and other immunosuppressive agents. Genetic factors also contribute to increased risk.

There are complex interactions between *M. tuberculosis* and both environmental and host genetic factors which play a critical role in TB development. Host genetic factors explain, at least in part, why some people are more or less susceptible to infection. Recent studies, including twin studies, genome-wide linkage studies and recently published genome-wide association studies demonstrate that host genetics strongly influence susceptibility to TB. As an example, the TLR2 variant, R753Q, appears to influence the progression of LTBI to active TB disease in children (Dalgic et al., 2011). Another single nucleotide polymorphism (SNP) of TLR2 (597T-C) was found to be strongly associated with TB meningitis and miliary TB in Vietnam (Thuong et al., 2007). Purinoceptor $(P2X)_7$ is responsible for mediating adenosine triphosphate (ATP)-induced killing of *Mycobacterium* and a polymorphism in the

$P2X_7$ receptor (1513 A-C) has been associated with increased susceptibility to extra-pulmonary TB (Fernando et al., 2007).

Diagnosis of TB can be difficult. Sputum smear microscopy remains one of the most common ways to diagnose pulmonary TB, especially in resource-poor settings. Accuracy of this test varies and is reported to be 20-80% using fluorescence microscopy methods (Steingart et al., 2007). It is often insensitive in children (as they are often unable to give a good sample) and the immunocompromised who are more likely to be sputum smear negative (Steingart et al., 2007; McNerney et al., 2012). It also is unsuitable for use in extra-pulmonary TB as there is no disease within the lung parenchyma.

Ultimately, however, definitive TB diagnosis is reliant on the isolation and later culture of *M. tuberculosis* from the sputum or other tissue of infected patients coupled with radiological and clinical findings. Corbett *et al*. examined the rates of sputum smear and culture positivity in a population of HIV infected and uninfected individuals in Zimbabwe with active pulmonary TB. Of the 31 cases in non-HIV infected individuals, 14 (45%) were both sputum and culture positive and nine (29%) were culture positive meaning that 26% (eight cases) were both smear and culture negative. In the HIV population the smear and culture rates were even lower (Corbett et al., 2010). This low rate of sputum and/or culture positivity delays diagnosis and hence treatment and impedes the discovery of potential drug resistant strains. This increases the rate of person-person transmission and treatment failure.

Extra-pulmonary disease often requires invasive investigations and in some instances several diagnostic procedures in order for a diagnosis to be made (Solovic et al., 2013). For example, those with TB meningitis often require cerebral spinal fluid analysis. Such invasive investigations require a skilled practitioner and are not feasible to be performed in resource-poor settings. Given that extra-pulmonary TB accounts for about 30% of all TB, this represents a large pool of TB that is hard to diagnose and effectively treat (Lawn and Zumla, 2011).

In recent times there have been several new diagnostic tests that have become available. The most significant of these being the recently developed GeneXpert® (Xpert). Xpert is a fully automated platform that performs real-time (RT) PCR to amplify *M. tuberculosis* nucleic acid. With a turnover time of two hours, it is able to detect the *M. tuberculosis* complex whilst simultaneously detecting the most common mutation responsible for rifampicin resistance (a reliable proxy for multi-drug resistant (MDR)-TB [as defined by resistance to both first-line agents, isoniazid and rifampicin]) (Piatek et al., 2013). The sensitivity of Xpert is superior to sputum smear microscopy with a case detection rate of 73% in Xpert compared with 28% using smear microscopy (McNerney et al., 2012). Xpert has no role to play in monitoring response to treatment as it is purely a diagnostic tool.

The implementation of Xpert in a clinical setting presents many hurdles and it is not a panacea. Although performing Xpert is relatively simple and involves minimal specimen preparation and manipulation, major challenges particularly related to cost and infrastructure exist that inhibit its rollout, particularly in resource-poor settings where it is most needed. To successfully implement Xpert into standard diagnostic

practice, operational requirements would include uninterrupted power supply, ambient temperature no higher than 30°C, adequate biosafety to handle potentially infectious material, adequate storage for test kits at temperatures no higher than 28°C, waste disposal system for cartridges, secure location to protect equipment from theft, trained laboratory and clinical staff and yearly calibration of the machine (Piatek et al., 2013). Additional programmatic requirements would also be required. Such a comprehensive list of requirements would not be feasible in many areas where TB is endemic, as the systems to support such technology would not be in place (Piatek et al., 2013).

Of other concern relating to Xpert, a number of false-positive cases of rifampicin resistance have been detected and as such it cannot be reliably used as a definitive point-of-care (POC) test (Boehme et al., 2010). The unforeseen negative implications of a falsely positive test could be the unwarranted initiation of MDR-TB therapy, which is highly intensive with considerably more potential serious side effects for the patient coupled with the unnecessary cost of implementing the MDR-treatment regime. These issues are currently being investigated so that a resolution can be identified and implemented (Piatek et al., 2013).

1.4.3 More rapid and accurate diagnostics needed

The WHO estimates that in the African Region 40% of active TB cases are detected. This means that over half of all active cases remain undetected and continue to transmit infection (World Health Organization, 2010b). These figures are often worse in HIV-TB co-infected subgroups. In addition it is estimated that only 7% of the estimated 500,000 MDR cases each year are being identified. This translates to

people receiving ineffective treatment thereby further amplifying drug resistance and the on-going transmission of multi-drug resistant strains to close contacts (World Health Organization, 2010a).

1.4.4 Ideal criteria for diagnostic test

Failure to halt the spread of TB is in part due to the inability to detect active cases in the early phase of disease. TB diagnostics lack an accurate and rapid POC test. The current gold standard diagnostic tests for TB are primarily laboratory based. It is not uncommon for numerous tests to be carried out over a prolonged period before a diagnosis is made.

The ideal test would be simple, cheap and robust and would be a POC, on the spot, accurate diagnostic test of active disease for both pulmonary and extra-pulmonary disease. Ideally it would be able to detect resistance profiles to all first line agents so as to avoid inappropriate antibiotic use. It should also be able to distinguish active disease from LTBI. In addition, there needs to be a better test than the surrogate marker of sputum culture conversion to monitor response to treatment.

1.5 Biomarkers in tuberculosis

A biomarker is defined as a characteristic that can be objectively measured and assessed as an indicator of normal processes, pathological processes or pharmacological responses following therapeutic intervention (McNerney et al., 2012). They can either be host or pathogen specific. A biomarker may provide

information about the pathological processes including the current health status and the long-term prognosis of the disease state.

An effective and reliable biomarker in tuberculosis is most important in three key areas:

(1) to predict durable (non-relapsing) treatment success in patients with active disease,

(2) to determine reactivation risk in those with latent disease and in those without active TB, and

(3) to indicate protection from the disease by new vaccines (Wallis et al., 2010).

1.5.1 Biomarkers predicting non-relapsing cure

The current marker used to predict response to treatment and likelihood of cure is sputum culture status following two months of treatment. Wallis et al. found that sputum culture conversion at two months was reliable to determine the effect of new treatment on relapse rates (Wallis et al., 2010). This supports the role of two-month sputum culture conversion as a viable endpoint, which therefore has the potential to fast track new drug development, which is particularly important in drug-resistant disease. Sputum culture conversion however has not been shown to be a good prognosticator for the individual. Although a positive sputum culture at two months was an independent predictor of relapse for an individual (hazard ration 2.8, 95% CI 1.7-4.7), both its positive predictive value (18%) and sensitivity (50%) were low (Benator et al., 2002). This may be explained by a small sub-population of drug-resistant bacteria that are not readily detected in sputum by culture at the time of assessment.

In pulmonary TB, obtaining suitable sputum specimens is sometime problematic. Many patients have difficulty in producing a sputum suitable for examination, this is particularly the case in the paediatric population. Accompanying this, is the need to have competent technical staff to examine the sputum in a well set-up laboratory. This may not be possible, especially in developing countries or non-urban settings. Compounding these shortfalls, sputum culture takes time and the results of the culture are often not available for several weeks. During this time there is the real risk of amplifying drug resistance in cases where treatment is not effective and therefore continues to propagate the on-going spread of potentially drug-resistant TB.

In cases of extra-pulmonary disease, monitoring response to treatment and predicting relapse is even more difficult. This is due to the fact that two-month culture is not performed on extra-pulmonary cases because the procedures are often invasive, difficult and typically low yielding. In extra-pulmonary disease and culture negative cases, clinical judgement and possibly radiological assessment are often the only tools available to monitor treatment response.

Studies have been undertaken during the implementation of new drug regimes that examine *M. tuberculosis* colony counts on agar at varying time points, in addition other studies have measured the time to culture conversion as a means of monitoring therapy (Dorman et al., 2009; Kolibab et al., 2014). However, these studies are still in their infancy and further evaluation of their effectiveness needs to be assessed. A

limitation of these potentially new biomarkers is the need for positive culture to exclude extra-pulmonary and paucibacillary disease.

1.5.2 Determining risk of reactivation

The principle strategy with regards to TB management aims to reduce the incidence of infection with *M. tuberculosis.* This is achieved through three main interventions: the first being to treat active cases of TB promptly, the second is vaccination with BCG before acquisition of infection with the aim of reducing the risk of progression from sub-clinical latent infection to active disease should infection occur and lastly to treat sub-clinical, latent *M. tuberculosis* with preventative chemotherapy.

The diagnosis of LTBI is made based on either a positive TST (usually greater than 15 mm) or a positive IFN-γ release assay (IGRA) coupled with no clinical evidence of active disease and normal chest radiography. Treating LTBI is very effective and reduces the risk of reactivation by 90% (Menzies et al., 2011). However, not all that are infected will go on to develop active disease. In fact, the lifetime risk is thought to be around 10%(Society, 2000). Prophylactic treatment is prolonged (up to nine months) and there are many serious side effects from the medications, most notably hepatitis that can become fulminant. Therefore careful selection of patients to offer chemoprophylaxis to is important.

There are certain risk factors that are known to increase a persons risk of reactivation including immunosuppression especially HIV, those receiving immunosuppressive agents, especially TNF inhibitors, chronic kidney disease, diabetes, silicosis, poverty and malnutrition and residing in a high TB burden country (Lopez et al., 2006).

Although these are known risk factors that increase the risk of reactivation, there still are a large population with no identifiable risk factors that develop active disease (Lönnroth et al., 2009). These patients would benefit from chemoprophylactic therapy. Recently there have been a number of studies that have tried to determine the risk of reactivation by means of measuring TB-specific antigens and IFN-γ.

Recently, studies have examined IFN-γ levels and their correlation with progression from latent to active disease. There is a suggestion that increasing concentrations of IFN-γ may be associated with the development of active TB (Diel et al., 2008; Aichelburg et al., 2009; Diel et al., 2011). Other measures of TB-specific antigens such as ESAT-6 and CFP-10 have also been investigated as prognosticators for active TB development, though at this stage studies have been limited to animals and further work needs to occur in human studies (Weldingh et al., 2008). Both ESAT-6 and CFP-10 are specific *M. tuberculosis* complex antigens that are used as part of the IGRA. IGRA detects the level of IFN- γ that is released in response to these antigens. Being better able to predict those most likely to develop active disease following infection would allow a more tailored approach to chemopreventative therapy and would limit the need to use such treatment in patients who if left untreated would never develop active disease.

1.5.3 Biomarkers to predict drug and vaccine efficacy

Currently there are no known biomarkers that can predict the efficacy of a TB vaccine candidate. This means that vaccine studies involve lengthy and costly trials.

Our understanding of *M. tuberculosis* and its interaction with the human host is incomplete. These knowledge gaps hinder the development of biomarkers that can distinguish active from latent disease and predict those that will progress from latent to active TB. These knowledge gaps also impede new drug and vaccine development. Small non-coding ribonucleic acids (RNA) termed microRNAs (miRNAs) have recently been identified as a potential biomarker for other human diseases such as malignancy (Chen et al., 2012) and this research project aims to study miRNAs in the setting of *M. tuberculosis* infection with the aim of developing a suitable biomarker.

1.6 MicroRNAs

MicroRNAs (miRNA) are short, 19-24 nucleotides in length, non-coding RNAs that play an important role in post-transcriptional regulation of gene expression through targeting messenger RNA (mRNA). It is estimated that more than 60% of mRNAs are targeted by at least one miRNA (Mause et al., 2010). miRNAs are therefore key regulators of cellular processes. It has been suggested that miRNAs play a role in nearly all aspects of cell physiology, including cell proliferation, differentiation and apoptosis (Tomankova et al., 2010). Circulating miRNAs exist in a highly stable cell-free form and recent literature has demonstrated their varying expression in diseased states such as malignancy, schizophrenia, diabetes, heart failure and sepsis (He et al., 2004; Oak et al., 2011; Mastronardi et al., 2012). Circulating miRNAs have potential as a diagnostic, prognostic and predictive markers. The lung has been shown to have a very specific miRNA profile across mammalian species (Williams et al., 2007).

Biomarker discovery, not only for TB, but also for other inflammatory and infectious disease has featured prominently in the literature. There is emerging evidence that miRNAs play a crucial role in pathogen-host interactions. Circulating miRNAs have been shown to be consistently expressed and are stable in blood and tissue (Chen et al., 2008; Kroh et al., 2010; Turchinovich et al., 2011). miRNAs have been found to be useful prognosticators in the setting of malignancy and their use in clinical medicine as a biomarker, particularly in malignancy, is increasing (Ng et al., 2009; Yamamoto et al., 2012; Y. Lu et al., 2012; Yuxia et al., 2012). At the commencement of this body of work, little was known about the role of miRNAs in TB. However, knowledge in this area is increasing and there has been an exponential rise in miRNA-related research over the last decade.

1.6.1 Discovery of miRNAs

In 1993 the first miRNA precursor, *lin*-4, was discovered. *lin*-4 was shown to regulate worm (*Caenorhabditis elegans*) development (R. C. Lee et al., 1993). The *lin*-4 miRNA was found to be necessary for development beyond an early larval stage; interestingly, examination at that time of its sequence revealed a 22-nucleotide RNA sequence with no open reading frame. This was found to interact with the three prime untranslated region (3'-UTR) of its negatively regulated protein, *lin*-14, thereby inhibiting protein synthesis (T. H. Lee et al., 2011). It was not until several years later, with the discovery of *let*-7, that miRNAs were found to be highly conserved across mammalian species (Pasquinelli et al., 2000). It is now understood that humans express >2000 miRNAs (as listed in microRNA database, www.mirbase.org) that can regulate the translation of mRNA and can silence genes at the DNA level (Friedman et al., 2009).

1.6.2 miRNA biogenesis

The canonical biogenesis pathway for most animal miRNAs is summarised in Figure 1.3. miRNAs are derived from precursor transcripts known as primary miRNA (pri-miRNA) that originate in the nucleus as single long transcripts up to 1000 nucleotides in length. They are processed by Drosha, an RNase III enzyme and DiGeorge Syndrome critical region 8 (DGCR8), an RNA binding protein, into a pre-cursor hairpin structure, 70-100 nt long, known as "pre-miRNA".

Next, pre-miRNA is then transported to the cytoplasm via Exportin 5 where it undergoes further processing by Dicer into mature miRNA and a miRNA strand, which is later degraded. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC). Once incorporated into the RISC, the mature miRNA will guide the RISC to the target mRNA. For those with known characterized gene targets, miRNAs have been found to regulate gene expression through translational repression and mRNA degradation (He et al., 2004).

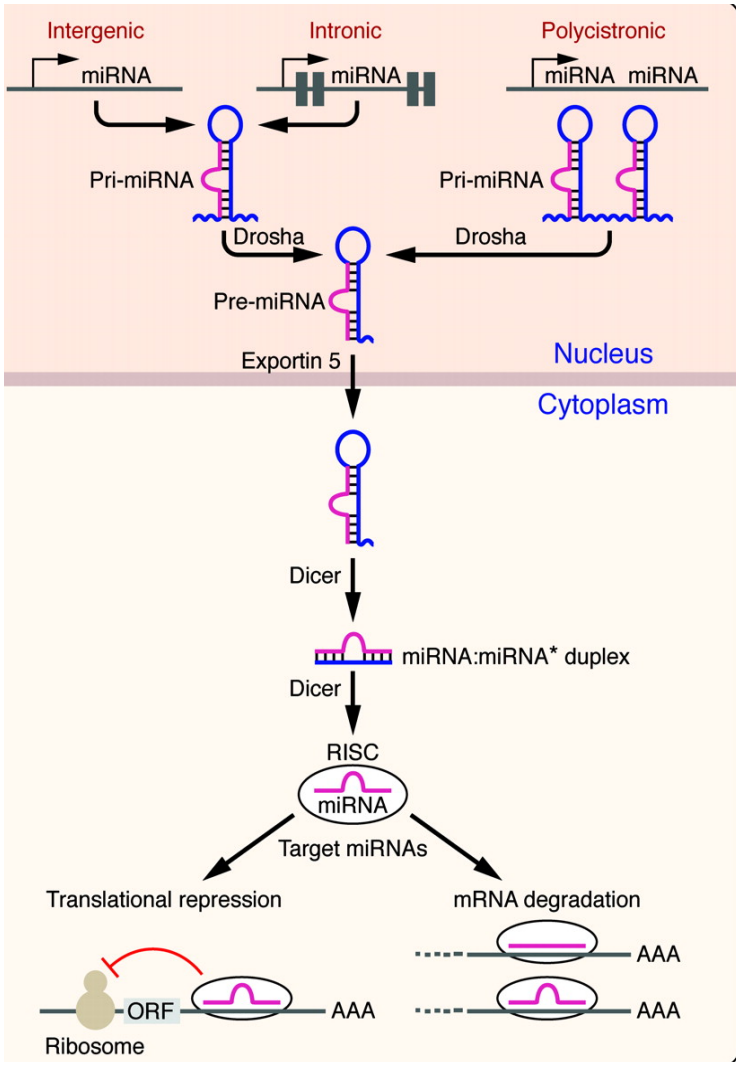

Figure 1-3. miRNA biogenesis pathway. Illustration of miRNA biogenesis pathway showing the primary precursor molecules (pri-miRNA) which are processed by Drosha to 70 nt long precursor hairpins (pre-miRNA) and exported to the cytoplasm by Exportin 5. Dicer then cleaves the pre-miRNA hairpins to generate 20 bp long miRNA duplexes, one strand of which gets incorporated into RNA induced silencing complex (RISC) to bind to target mRNAs, leading to gene silencing. Figure adapted from van Rooij (van Rooij, 2011).

1.6.3 Mechanisms of miRNA action

Following its synthesis and incorporation into the RISC complex, the mature miRNA interacts with its target mRNA. miRNAs target mRNA through the second to seventh bases of the mature miRNA sequence known as the miRNA seed (Lewis et al., 2005). This miRNA seed has complementarity to target sites in the 3'-UTR of

mRNA transcripts (Lewis et al., 2005). The binding of miRNA to mRNA via this mechanism allows miRNAs to regulate hundreds of genes, as the miRNA is capable of binding to any mRNA with sufficient seed region complementarity (Lim et al., 2005).

In humans the most common method of miRNA:mRNA interaction is first through translational repression followed by deadenylation and decay of the mRNA, leading to mRNA degradation and a decrease in protein expression (Friedman et al., 2008; Meijer et al., 2013). Translational repression is where a decrease in protein product is found that is greater than the observed decrease in mRNA, therefore the mRNA is not being transcribed. This occurs initially through miRNAs binding to mRNAs in a complex with Argonaute (AGO) proteins 1-4, forming the initial translational repression at the 3'-UTR. This complex then recruits one of the trinucleotide repeat-containing proteins and binds to a secondary structure in the 5'-UTR causing deadenylation. In other methods of miRNA control, mRNAs are formed with the addition of a 3'poly(A) tail which increases the stability of the mRNA structure, thus limiting degradation. miRNAs can cause degradation of this poly(A) tail (deadenylation), and therefore destabilising of the mRNA (Djuranovic et al., 2012; Meijer et al., 2013).

1.6.4 miRNA nomenclature

miRNAs are named in three parts as illustrated in Figure 1.4. The initial classification relates to the species they are observed in, with "hsa" referring to human miRNAs, and "rno" referring to rat miRNA and "mmu" referring to mouse miRNA. They are then named as such with a prefix, miR, followed by a number. The

number relates to the miRNA's time of discovery and is sequential; meaning more recently discovered miRNAs receive a higher number. miRNAs with a similar structure are distinguished by a lower case letter following the number. For example, miR-146a and miR-146b are very similar in sequence. Some miRNAs are transcribed from two different genetic loci and are named by a dash and a second number. For example, miR-250-1 and miR-250-2. Finally, miRNAs originating from the same pre-miR, but originating from the 3' or 5' end are denoted with -3p or -5p suffix.

There are a few exceptions that exist for miRNAs that were described before the advent of the naming system, such as *lin-4* and *let-7* from model *C. elegans*.

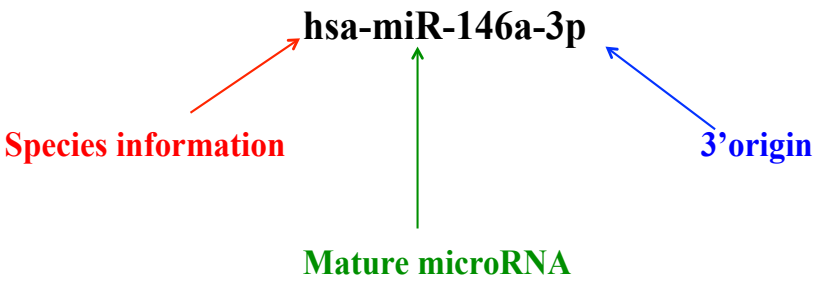

Figure 1-4. Nomenclature of miRNAs.

1.6.5 MicroRNA as a biomarker

An ideal biomarkers needs to fulfil a number of criteria. It should be accessible through reasonably non-invasive measures and specific to the disease of interest; it should be a reliable means to detect disease before clinical symptoms appear; it should be sensitive to changes in pathology, either disease progression or therapeutic response; and it should be easily translatable from the bench to the patient. In terms of TB, a good biomarker would be able to detect those that are most likely to

progress from latent TB to active TB. It would also be able to detect non-response to therapy earlier than the current standard 2-month sputum culture and would be able to detect drug resistance in those with active disease. Finally, it should be able to be used as a marker of vaccine or new drug efficacy. It is likely that no single biomarker on its own would be able to fulfil these criteria and that a combination of biomarkers will need to be used in conjunction.

Most of the current biomarkers are blood based and measure levels of specific proteins, for example, troponin, for ischaemic cardiovascular problems, carcinoembryonic antigen for colorectal malignancy and prostate-specific antigen for prostate pathology (Duffy, 2001; Rao et al., 2008). The development of protein-based biomarkers is sometimes problematic in part due to the relatively low abundance of many proteins of interest in blood (Drucker et al., 2013). Furthermore, often biomarkers with high sensitivities have low specificity, meaning that over diagnosis is often made (Drucker et al., 2013). Those that are more specific inversely have low sensitivities.

miRNAs have many requisite features of a good biomarker. They are stable in various body fluids, the expression of some miRNAs are specific to tissues and the level of miRNAs can be easily assessed by various methods including PCR. The changes in miRNA expression in plasma, serum, saliva and urine have been associated with different diseases. Serum miRNAs have been shown to be modulated in cases of interstitial fibrosis in smokers, expression of *let-7* and miR-181b have been associated with chemoresponse in colon cancer, and expression of a number of miRNAs in urinary sediment was found to predict both the presence and severity of

IgA nephropathy (Nakajima et al., 2006; G. Wang et al., 2010; Y. Huang et al., 2012).

1.6.6 Circulating miRNAs

It wasn't until 2008 that miRNAs were discovered to be present in the blood, where they were detected in platelets, plasma, erythrocytes and nucleated red blood cells (Mitchell et al., 2008; Xi Chen et al., 2008). Interestingly plasma-based miRNAs are remarkably stable and this is in contrast to synthetic miRNAs added to samples, which degrade quickly. This is thought to be due to the effects of RNase activity in the plasma (Mitchell et al., 2008; Xi Chen et al., 2008). This stability of endogenous miRNAs suggests that plasma miRNAs are protected. Recently it has become apparent that miRNAs achieve this through two mechanisms; miRNAs can be packaged within small microvesicles (exosomes, microvesicles or apoptotic bodies) (Valadi et al., 2007; Zernecke et al., 2009) or alternatively be associated with lipoprotein complexes or RNA-binding proteins such as Ago2 (Vickers et al., 2011).

1.6.6.1 miRNAs packaged in microvesicles

One of the ways that plasma-derived miRNAs are protected from degradation is to be packaged into protein or lipid vesicles. Exosomes (50-100 nm) originate from the endosome and are released into the extracellular environment when multivesicular bodies fuse with the plasma membrane.

Exosomes can transfer molecules, such as miRNAs, from one cell to another via membrane-vesicle trafficking (Raposo et al., 2013). Valadi et al. discovered that

mast cell-derived exosomes carry approximately 121 different miRNAs (Valadi et al., 2007). Mesenchymal stem cells have also been shown to secrete miRNA exosomes, however these miRNAs were secreted as pre-miRNA and not in their mature form (Chen et al., 2010).

Microparticles are larger than exosomes at 0.1-1 μm that are released from cells through outward budding and fission of the plasma membrane. Once shed, microparticles can travel large distances which enables packaged miRNA to act at distant sites (Muralidharan-Chari et al., 2010). Diehl and colleagues examined miRNA in microparticles from human platelets in the setting of coronary artery disease. They observed that platelet microparticles contained numerous miRNAs and that these microparticles acted as transport vehicles for a large number of miRNAs (Diehl et al., 2012).

Apoptotic bodies are the largest microparticle measuring 0.5-2 μm and are shed from cells during apoptosis. It has recently been reported that endothelial cell-derived apoptotic bodies are formed during atherosclerosis and these were found to contain miR-126. Furthermore, incubation of these apoptotic bodies with human umbilical vein cells resulted in the transfer of miR-126 into recipient cells and production of anti-inflammatory chemokines (Zernecke et al., 2009).

1.6.6.2 miRNAs bound to protein complexes

It is thought that up to 90% of plasma miRNA exist in non-membrane-bound form (Arroyo et al., 2011). A large proportion of non-vesicular miRNAs have been shown to be bound to Ago2. Ago2 is thought to be the key intracellular effector protein of

the miRNA-mediated RISC (Hutvagner et al., 2008). It remains unclear however as to how the miRNA complex is exported by the cell, it has been hypothesised that Ago2-miRNA complexes are released following apoptosis of the cell and that the Ago2-miRNA complex remains intact in the extracellular environment due to its high binding affinity (Arroyo et al., 2011). A recent study by Laffont et al., interestingly has found that Ago-miRNA-223 complexes were carried in platelet-derived microparticles where they modulate gene expression both locally and at distant sites (Laffont et al., 2013).

1.6.6.3 miRNAs associated with lipoproteins

High-density lipoprotein (HDL) was classically known for its ability to transport excess cellular cholesterol to the liver for excretion. However, it has recently become apparent that HDLs can transport and deliver miRNAs to target cells. The exact mechanism, by which they do this remains unknown. Vickers et al. found that injecting HDL into mice retrieved distinct miRNA profiles from both normal and atherogenic murine-models (Vickers et al., 2011). Vickers and colleagues also collected HLD from patients with hypercholesterolaemia and from patients with normal cholesterol levels and used these to treat cultured hepatocytes. HDL-miRNA from patients with hypercholesterolaemia induced different gene expression than that observed from HDL-treated hepatocytes from healthy controls. These data provide evidence that HDL can both transport miRNA to target cells and alter gene expression.

1.7 miRNA expression profiling

With the recent advances in miRNA research there needs to be reproducible and reliable miRNA quantification measures. There is currently little consensus regarding the best methodology to use for miRNA quantification. However, quantitative real time PCR (qPCR) is considered to be the gold standard for the accurate quantification of miRNA expression. There are several commercially available kits which employ distinct approaches to prime the miRNA for reverse transcription and then amplify the cDNA. The methods employed in this body of work are discussed below in Section 1.7.4.

Generally, cells from blood, tissue, urine or even saliva are isolated and RNA extracted and quantitated and the amount of each miRNA measured. The miRNA measured is then normalised with the aim of minimising data bias caused by external factors such as technical variation, the method used to purify the RNA, the amount of sample processed, and the quality of the sample (Peltier et al., 2008). Data is analysed in light of the available clinical or pathological information, appropriate statistics techniques are applied and the outcomes examined.

1.7.1 Sample considerations

Sample preparation and RNA extraction methods can have a substantial impact on the quantification of miRNA expression. This is especially pertinent for samples that are prone to miRNA degradation (W.-X. Wang et al., 2008; Ibberson et al., 2009; Podolska et al., 2011). Wang and colleagues performed RNA isolation on a single brain sample using eight different isolation methods with miRNA expression

measured on microarray and cross-referenced to Northern blots. Markedly different results were seen between samples obtained using different RNA isolation techniques and also between microarray and Northern blot results (W.-X. Wang et al., 2008).

It is possible to extract miRNA from a wide variety of specimens, including sputum, whole blood, plasma, serum, cell lines, formalin-fixed tissues and other bodily fluids (Accerbi et al., 2010; Weber et al., 2010). miRNA extraction is similar to RNA extraction, however the protocols are sometimes modified to capture, and sometimes enhance, small RNA isolation (Accerbi et al., 2010).

Tissues and cell lines generally deliver the greatest yield of miRNA. miRNAs were shown to be stable and remained intact when samples were formalin-fixed with comparable levels of miRNA isolated from both fresh and formalin-fixed samples (Doleshal et al., 2008). This is quite advantageous in the clinical setting, as often the only tissue sample available is formalin-fixed.

When assessing blood-based miRNA expression, rigorous quality control of plasma/serum samples needs to occur to limit factors that can erroneously alter miRNA expression. Extra consideration needs to be given in relation to the possible effects of endogenous RNase activity present in the blood, as well as the presence and effect of red blood cells, especially if haemolysed (Mitchell et al., 2008). Kirschner et al. measured miRNA from haemolysed and non-haemolysed plasma from the same donor collected at the same time (Kirschner et al., 2011). From the six donor plasma samples, miRNA was measured by microarray and later verified by

real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Significant variation in miRNA expression was noted between the haemolysed and non-haemolysed samples from the same donor. Another consideration is the time taken to process the blood sample after collection, centrifugation techniques and storage. Cheng et al. measured miRNA expression in plasma from healthy individuals subjecting the same donor's plasma to different centrifugation speeds. miRNA expression was then determined using qRT-PCR (Cheng et al., 2013). The authors observed quite marked variation in miRNA expression, sometimes more than one thousand infold differences in which were due solely to the methods of processing. This marked discrepancy was thought to be due to platelet contamination. Importantly, the authors also noted, that a second centrifugation, largely eliminated this contamination.

Measuring miRNAs present in biofluids such as plasma and serum shows great promise as a minimally invasive biomarker, however accurate quantification of miRNA expression requires rigorous quality control to limit technical variation which can easily affect miRNA levels resulting in biases that do not actually reflect the biological state of the sample.

1.7.2 Quantification and quality assessment of miRNA.

In order to maintain consistency in yield and reproducibility of results, it is important that extracted RNA is both quantitatively and qualitatively assessed. As many of the profiling methods use total RNA, specific methods to measure miRNA levels are unnecessary. However it is standard practice to measure total RNA and RNA integrity using instruments such as the Bioanalyzer 2100 (Agilent Technologies,

Santa Clara, CA) and Nanodrop Spectophotometer (Thermo Scientific, Wilmington DE). In some instances, especially when dealing with plasma samples, a synthetic spike-in can be used (such as miRNAs from *C. elegans*) The spike-in is added to the samples before RNA extraction. The spike-in is then measured in the purified RNA and acts as an endogenous normaliser to control for such factors as variation in RNA extraction and/or inhibitors of reverse transcription or PCR (Kroh et al., 2010).

There are numerous methodologies employed to quantitatively measure miRNA including northern blotting with radiolabelled probes (Válóczi et al., 2004), quantitative PCR-based amplification of precursor or mature miRNAs (Caifu Chen et al., 2005), cloning (Lau et al., 2001), serial analysis of gene expression (SAGE) (J. Lu et al., 2005; Jay et al., 2007), bead-based profiling methods (J. Lu et al., 2005; Jay et al., 2007), single molecule detection (Neely et al., 2006), oligonucleotide microarrays, (Barad et al., 2004; Cummins et al., 2006) and digital multiplex (G. K. Geiss et al., 2008; Kulkarni, 2011) also immunohistochemistry. The merits and limitations of each method are listed in Table 1.1.

Despite the many ways to quantify miRNA, there are three methods that are commonly used to measure miRNA expression: qRT-PCR; microarrays; and high-throughput sequencing. Each of these techniques has different strengths and weaknesses and they are often used at different stages of the same study with arrays or high-throughput technologies used in the discovery stage, and qRT-PCR utilised to validate findings.

Table 1-1. Methods of profiling miRNA.

Method	Merit	Limitation	Reference
Northern blot	Highly specific; gold standard.	Low throughput; low sensitivity.	(Válóczi et al., 2004)
qRT-PCR	Rapid detection; low cost; superior detection of low abundant miRNAs.	Closed-ended*.	(Caifu Chen et al., 2005)
Cloning	Discovery of new miRNAs.	Time consuming; expensive.	(Lau et al., 2001; Cummins et al., 2006)
SAGE	Discovery of new miRNAs;	Expensive.	(J. Lu et al., 2005; Jay et al., 2007)
Bead-based	High throughput. High speed; low cost; superior hybridization.	Closed-ended*.	(J. Lu et al., 2005; Jay et al., 2007)
Single molecule detection	Rapid detection.	Closed-ended*; expensive.	(Neely et al., 2006)
Microarrays	Low cost; High throughput.	Closed-ended*, lower specificity.	(Barad et al., 2004; Cummins et al., 2006; F. Sato et al., 2009)
Digital multiplex	Robust, high throughput	Expensive, time consuming.	(G. K. Geiss et al., 2008; Kulkarni, 2011)

qRT-PCR, quantitative real-time PCR; SAGE, serial analysis of gene expression.
*Closed-ended methods assay a predetermined set of miRNAs

1.7.3 Microarray measurement of miRNA

With the advent of genomics and the technology to generate large quantities of genomic data, there has been an exponential rise in the study of gene structure and function and consequently vastly improved understanding of gene regulation. The profiling of miRNAs is a relatively new field and special factors need to be considered, such as their small size, low abundance and tissue-state specificities.

There are a number of different microarray platforms that have been used to profile miRNAs. These methods differ in RNA labelling and probe design strategies. miRNA microarrays, in contrast to standard mRNA arrays, must take into account the differences between mature miRNA and miRNA precursors. Moreover, microarrays need to be able to differentiate between miRNAs that differ by as little as a single nucleotide (Shingara et al., 2005). In addition to this, miRNAs often have different melting temperatures for annealing reactions. All of these issues are specific to miRNA and present challenges to both sample labelling and probe development.

1.7.3.1 Probe design

The ideal probe should have both high affinity and high specificity that complement the sense and antisense stands of the miRNA. They also need to deal with varying melting temperatures of each miRNA, which vary between 45°C and 74°C (van Rooij, 2011). Endogenous and exogenous (spike-in controls) positive and negative control probes are also required. These help to normalise and provide reference points for quality control and facilitate quantitative comparison of different microarray platforms.

1.7.3.2 Application of miRNA arrays

miRNA microarrays allow the detection and quantification of several hundred miRNAs in the same sample at once thus requiring only small quantities of RNA. This is particularly important in the clinical setting when using blood-based biomarkers where typically the RNA yield is low. One of the major drawbacks of this technology is the inability to compare data from different array platforms. Other limitations include lower specificity and lower dynamic range compared to qRT-PCR or sequencing (F. Sato et al., 2009). The technology is also closed-ended meaning there are no avenues to discover new miRNAs.

1.7.4 Quantitative real-time PCR

qRT-PCR is the method of choice for the accurate quantification of miRNA expression. There are several quantitative platforms currently available but essentially they all rely on the conversion of RNA to cDNA and subsequent quantification by comparison to a standard gene or sample (in this case healthy/non-infected controls). Each platform employs a distinct approach to prime the miRNA for reverse transcription and then to amplify the cDNA. For this research project we utilised three different platforms: TaqMan miRNA Assay (Life Technology, Victoria, Australia); SYBR Green (GenCopoeia, Rockville, MD); and miRCURY LNA^{TM} Universal RT miRNA PCR Assay (Exiqon, Vedbaek, Denmark).

The TaqMan assay uses a miRNA-specific stem-loop reverse transcription primer to generate cDNA for subsequent qPCR amplification (Redshaw et al., 2013). There are advantages of utilising this technology. Firstly it is able to discriminate between similar miRNAs, thus increasing its specificity. Secondly it prevents the

hybridization of its RT primer to miRNA precursors as well as genomic DNA, again increasing its specificity. Finally, the stem-loop extends the 3' end of the miRNA by RT. This results in a longer RT product which is more amenable to qRT-PCR, resulting in greater sensitivity (Fu et al., 2011).

The SYBR Green-based method uses tagged and anchored olig-dT primers for reverse transcription of polyadenylated small RNAs into mature RNAs, followed by SYBR Green-based detection of reversed transcribed miRNA (Z. Zhu et al., 2013). The All-In-One qPCR reduces experimental time by providing a universal reaction that can be used with almost all primers and most real-time PCR instruments. However, this platform is less specific than the TaqMan assay.

The miRCURY LNA^{TM} Universal RT (Exiqon) involves poly(A) tailing of mature miRNAs. This is followed by the use of a poly(T) reverse transcription primer containing a 3' anchor and 5' universal tag leading to the generation of cDNA. The cDNA is then amplified by SYBR Green qPCR using miRNA-specific forward and reverse primers containing locked nucleic acids (LNAs) (Redshaw et al., 2013). LNAs are a class of nucleic acid analogues in which the ribose ring is fixed or locked by a methylene bridge connecting the 2'-oxygen and the 4'-carbon atom (Figure 1.5). The use of miRNA-specific forward and reverse primers offers an advantage in terms of the increased specificity of amplification (Vester et al., 2004).

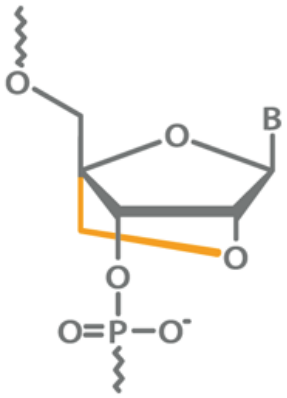

Figure 1-5. The Structure of LNA™. The ribose ring is connected by a methylene bridge (orange) between the 2'-O and 4'-C atoms thus "locking" the ribose ring in the ideal confirmation for Watson-Crick binding. When incorporated into a DNA or RNA oligonucleotide LNA™ makes the pairing with a complimentary nucleotide strand more rapid and increases the stability of the resulting duplex. Figure courtesy of Exiqon (http://www.exiqon.com/lna-technology).

1.7.5 High throughput measurement

Next generation sequencing (NGS) is a popular, widely adopted approach for profiling miRNAs. It is highly sensitive and has the ability to combine multiple biological samples in one sequencing reaction, which means that it is becoming more cost-effective. It has the advantage of being able to identify novel miRNAs as it is not reliant on previously known sequences (Vigneault et al., 2012). Studies utilising NGS have shown it to be highly sensitive, reproducible and quantitative (Burgos et al., 2013; Murakami et al., 2014; Müller et al., 2014). It also has the advantage of being able to very accurately distinguish between miRNAs of similar sequence.

1.8 miRNAs in other inflammatory lung conditions

At the commencement of this research project, little was known or understood about the role miRNAs play in *M. tuberculosis* infection. Many reports at that time

described altered expression of miRNAs in cancer samples compared with normal tissue including sarcomas (Kang et al., 2011), prostate cancer (Farooqi et al., 2012), breast cancer, colon cancer (Boni et al., 2011) and other diseases such as diabetes (Foley et al., 2012) and heart disease (Feng et al., 2011).

Interaction between *M. tuberculosis* and the host results in infection and the subsequent host inflammatory response. Chronic obstructive pulmonary disease (COPD) and asthma, although not infectious diseases, both evoke an acute and subsequent chronic inflammatory response. Both diseases affect the lungs and at the start of this research project, there were a number of studies examining the roles of miRNAs in these diseases. Listed in Table 1.2 are the miRNAs that have been found to be regulated in asthma and COPD. These findings supported the notion that miRNAs had an important role to play in the pathogenesis of both conditions.

Given the increasing amount of interest in miRNA research coupled with numerous studies highlighting their important roles in disease states such as malignancy and lung pathologies, we set out to discover the miRNAs that are modulated in *M. tuberculosis* infection.

Table 1-2. List of miRNAs associated with other inflammatory lung conditions.

miRNA	Tissue/Cell Type	Function	Species	Condition	Reference
miR-221	Serum	Pro-inflammatory	Human	Asthma	(Qin et al., 2012)
miR-221 & -485-3p	Serum	Pro-inflammatory	Human	Asthma	(F. Liu et al., 2012)
miR-126	Cells	Airway modelling	Murine	Asthma	(Collison et al., 2011)
miR-192	Peripheral blood	Airway remodelling	Human	Asthma	(Yamamoto et al., 2012)
miR-146a	BAL	Allergic inflammation	Murine	Asthma	(Garbacki et al., 2011)
mir-126	Respiratory epithelium	Anti-inflammatory	Murine	Asthma	(Mattes et al., 2009)
miR-1	Respiratory endothelium	Anti-inflammatory	Murine	Asthma	(Takyar et al., 2013)
miR-487b & -181	Respiratory epithelium	Pro-inflammatory	Human	Asthma	(Coulter, 2008)
let-7	Primary cultured T cells	Regulation of IL-13	Murine	Asthma	(Ansari et al., 2003)
miR-146a	Sputum	Airway inflammation and tissue remodelling	Human	COPD	(T. Sato et al., 2010)
miR-199a-5p	Respiratory epithelium	HIF-1α leading to prolongation of lifespan of neutrophil	Human	COPD	(L. Zhang et al., 2008)
miR-452	Human alveolar macrophages	Increasing levels MMP-12 which degrades elastin	Human	COPD	(Baffa et al., 2009)

BAL, bronchoalveolar lavage; IL-13, interleukin-13; HIF-1α – hypoxia-induced factor; MMP, matrix metalloproteinase; COPD, chronic obstructive pulmonary disease.

1.9 miRNAs in tuberculosis

Upon their discovery, miRNA research predominately focussed on their expression in malignant disease. It wasn't until very recently that the role of miRNA in other pathology became of interest. A seminal paper by Guo et al. published in 2010, was the first work that studied the possible targets of human miRNAs to *M. tuberculosis* mRNA (Guo et al., 2010). Utilising miRanda software 26 genes were identified as possible targets for miRNA from host lungs and macrophages (Ueda et al., 2010). The published works relating to *M, tuberculosis* miRNA research that occurred either prior or during my candidature is presented below. A summary of these findings is presented in Table 1.3.

1.9.1 Cell-based studies

In 2011, Lui and colleagues published the first study profiling miRNA expression in peripheral blood mononuclear cell (PBMC)s from active TB patients and their matched healthy controls (Liu et al., 2011). Using microarray technology the authors found 30 miRNAs were significantly regulated in active TB, 28 miRNAs up-regulated and two miRNAs down-regulated. Functional analysis of miR-144, which was found to be up-regulated in TB, showed that it inhibited TNF and IFN-γ and T cell proliferation.

Also in 2011, Wang et al. examined miRNA expression from PBMCs isolated from patients with active TB, LTBI and healthy controls (Wang et al., 2011). Utilising microarray technology, 17 differentially expressed miRNAs were observed between

the patients with active disease and those with LTBI. The majority (12 out of 17) were up-regulated in patients with active TB.

The following year, in 2012, Walter et al. showed that THP-1 macrophages infected with *M. tuberculosis* modulated miRNA expression (Walter et al., 2012). miRNA expression was measured by qRT-PCR with 12 miRNAs noted to be significantly regulated including miR-155 and -146a.

1.9.2 Serum-based studies

It wasn't until 2011 that the first paper profiling circulating miRNA in patients with active pulmonary TB was published (Fu et al., 2011). 75 patients with active pulmonary TB were recruited for the study together with 52 healthy age- and sex-matched individuals. A total of 1,223 miRNAs were screened using microarray technology, with 92 miRNAs with significant regulation detected, 59 up-regulated, and 33 down-regulated. The authors found that up-regulation of miR-29 could discriminate TB patients from healthy controls with reasonable sensitivity and specificity (83% and 80%, respectively).

The following year, Qi and colleagues used TaqMan Low-Density Arrays followed by qRT-PCR to examine miRNA expression profiles from the serum of 30 patients with active TB. Compared to healthy controls, the serum from patients with TB had 90 up-regulated and seven down-regulated miRNAs. When utilising a multiple logistic regression, a combination of three miRNAs (miR-361-5p, -889 and -576-3p) achieved an area under the curve (AUC) of 0.863.

Abd-EL-Fattah et al. in 2013 examined miRNA expression in serum from patients with TB, lung cancer, and pneumonia compared with matched healthy controls (Abd-El-Fattah et al., 2013). In the TB arm, there were 29 patients enrolled with 37 healthy controls. Utilising microarray profiling followed by qRT-PCR verification the authors observed that for the TB subjects, up-regulation of miR-197 was increased in patients with TB compared with normal controls. In the same year, Zhang and colleagues, used Solexa sequencing data that showed 91 serum miRNAs were differentially expressed in pulmonary TB patients, compared to healthy controls (Zhang et al., 2013). Using the combination of six serum miRNAs (miR-378, -483-5p, -22, -29c, -101 and -320b) in a logistic regression analysis revealed a 95% sensitivity and 91.8% specificity of a TB diagnosis.

1.9.3 miRNA in other tissues

In 2012, Yi et al. profiled miRNA expression from human saliva from patients with pulmonary TB comparing their expression to healthy controls (Yi et al., 2012). Using microarrays, the authors found 95 miRNAs that were differentially expressed in human saliva, with miR-19b, -3179 and -147 being the most significant.

The following year, Spinelli and colleagues examined the expression of six chosen miRNAs (miR-424, -365, -144, -421, -223 and -146a) from PBMCs obtained from pleural fluid as well as peripheral blood from newly diagnosed TB patients (Spinelli et al., 2013). These miRNAs were chosen based on studies published previously and as mentioned above. Interestingly, miRNA signatures from each compartment were different with a strong down-regulation of miR -223, -144 and -421 in the pleural

fluid while miR-424 was elevated in PBMCs from the peripheral blood. The down-regulation of miR-146a was common to both tissues.

As highlighted above, there is a great need for the development of new biomarkers to not only assist in the diagnosis of *M. tuberculosis* but also to monitor response to treatment and as an avenue to evaluate new vaccines and assist in new drug therapy development. miRNAs had shown promise to act as a biomarker in other diseases.

Table 1-3. miRNAs modulated in tuberculosis.

Tissue/Cell Type	miRNA	Modulation	Reference
Blood (PBMC)	miR-144	up-regulated	(Liu et al. 2011)
Blood (PBMC)	miR-424 & miR-365	up-regulated	(Wang et al. 2011)
THP-1 macrophages	miR-155 and miR-146a	up-regulated	(Walter et al. 2012)
Blood (serum)	miR-93 and miR-29	up-regulated	(Fu et al. 2011)
Blood (serum)	miR-3125	down-regulated	(Fu et al. 2011)
Blood (serum)	miR-361-5p, miR-889 and miR-576-3p	up-regulated	(Qi et al. 2012)
Blood (serum)	miR-197	up-regulated	(Abd-El-Fattah et al. 2013)
Blood (serum)	miR-378, miR-483-5p, miR-22 and miR-29c, -101 and -320b	up-regulated	(Zhang et al. 2013)
Blood (serum)		down-regulated	(Zhang et al. 2013)
Sputum	miR-19b	down-regulated	(Yi et al. 2012)
Sputum	miR-3179 and miR-147	up-regulated	(Yi et al. 2012)
Sputum	miR-29a	up-regulated	(Fu et al. 2011)
Blood (PBMC and PFMC)	miR-146a	down-regulated	(Spinelli et al. 2013)

PBMC, peripheral blood mononuclear cell; PFMC, pleural fluid mononuclear cell.

1.10 Aims of this study

Despite the availability of cheap and effective therapy, TB remains a global health problem. Efforts to control TB infection have been hampered by the spread and co-infection with HIV and the rise in prevalence of drug-resistant *M. tuberculosis*. New biomarkers are required to improve diagnostics, monitor response to therapy and develop new drugs and vaccines. miRNA has been shown to be a promising biomarker in other diseases such as malignancy. Therefore the aims of this study are as follows:

1. To identify miRNAs that are regulated in *M. tuberculosis* disease.
2. To determine if the expression of miRNAs can be utilised as a biomarker that:
    a. Assists in the diagnosis of TB
    b. Monitors response to TB therapy and can rapidly identify non-responders who may therefore be suffering from drug resistant TB.
3. To gain a better understanding of the role that miRNAs play in TB pathogenesis.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

The chemicals and reagents used in this study were obtained from Sigma Aldrich (NSW, Australia) unless otherwise stated.

2.2 Sample preparation

2.2.1 Isolation of primary human monocytes from buffy coat and differentiation of monocyte-derived macrophages

Sixty mLs of buffy coat from the Blood Bank or 60 mLs of blood from healthy donors, was equally divided into six, 50 mL conical tubes (Life Sciences). Blood was diluted 1/3 with phosphate buffered saline (PBS) and underlaid with 15 mLs of Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden), centrifuged for 30 minutes at 800 g at room temperature (RT) with no brake. Following centrifugation, the monocyte layer was collected, and washed twice with PBS and centrifuged for 10 minutes at 800 g. The final pellet was re-suspended in 10 mLs of media and the cells counted.

The cells were centrifuged at 2,350 g for 10 minutes and the pellet was re-suspended in MACS buffer (saline + 0.5% foetal bovine serum [FBS], 2 mM ethylene-diamine-tetra-acetic acid [EDTA]) per 10^7 cells. 10 µl of CD 14^+micro beads/10^7 (Miltenyi Biotec, NSW, Australia) was added and incubated for 15 minutes at 4°C, washed and

centrifuged at 550 g for 5 minutes at 4°C. The pellet was then re-suspended in MACS buffer with 1 mL MACS buffer per 5 x 10^8 cells.

The LS Column (Miltenyi Biotec) was prepared by rinsing with 3 mL of MACS buffer. 500 µl of cell suspension was added to the LS column and run through. It was then washed thrice with 3 mL of MACS buffer. Positively selected cells, of which greater than 90% were $CD14^+$, were eluted by removing the column from the magnetic field and applying plunger force.

Monocytes were counted and re-suspended in Dulbecco's modified eagle medium (DMEM) (Life Technologies) and differentiated into macrophages for 6 days with 10 ng/ µl granulocyte macrophage colony stimulating factor (GM-CSF) (1000 ng/mL).

2.2.2 Isolation of plasma from human blood

A venepuncture protocol was designed and was strictly followed so as to avoid any potential factors that may affect miRNA levels. 10 mL of venous blood was drawn from the *cubital fossa* from each patient at each collection. Blood was collected using the Vaccutainer Venepuncture System (BD Biosciences, NSW, Australia) with a 22 gauge needle into EDTA (BD Biosciences) tube,

The blood was separated by centrifugation at 800 g for 10 minutes at room temperature. The plasma (supernatant) was separated from the cellular layer by pipetting and approximately 5 mL of plasma was sacrificed to prevent disturbance of the cellular layer.

The plasma was stored at -80°C in 1 mL aliquots. Samples collected from China were transported below -60°C to Australia where they were stored at -80°C until further required.

Prior to RNA isolation, samples were examined for haemolysis using a haemolysis chart (Figure 2.1) and samples with > 100 mg/dL of haemoglobin were discarded.

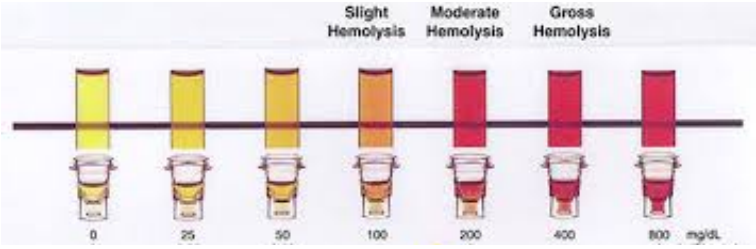

Figure 2-1. Haemolysis chart. Used to visually determine the presence of haemolysis after plasma separation. Plasma samples > 100 mg/dL were discarded.

2.3 Molecular methodology

2.3.1 RNA extraction (miRNAeasy [Qiagen, Limburg, Netherlands])

Samples in TRIzol® (Invitrogen) were thawed at room temperate. 140 µl chloroform was added and the samples were shaken vigorously for 15 seconds and incubated for 2-3 minutes, then centrifuged for 15 minutes at 12 000 g at 4°C. The upper aqueous phase was transferred to a new 1.5 mL collection tube and 1.5 times the volume of 100% ethanol was added. Up to 700 µl of sample was transferred into RNeasy Mini column and centrifuged at 8000 g for 15 seconds at RT. This step was repeated for the remainder of the sample. 700 µl Buffer RWT® was added to RNeasy Mini column and this was then centrifuged for 15 seconds at 8000 g. The flow-through was discarded. 500 µl of Buffer RPE® (mild washing buffer) was added to the column

and centrifuged at 8000 g for 2 minutes and flow through discarded. 20 µl of RNase-free water was then added to the column in a new collection tube and centrifuged for 1 minute at 8000 g. RNA concentration was then determined by NanoDrop 2000 Spectrophotometer (Thermo Scientific) and stored at -80°C until use.

2.3.2 RNA extraction (TRIzol® or TRIzol LS®)

For plasma samples, TRIzol LS® was used with the same method as below with 500 ul of plasma extracted with 1 mL TRIzol LS®.

Samples were thawed at room temperature and 0.2 mLs of chloroform per 1 mL TRIzol® or was added to the sample. The sample was shaken vigorously for 15 seconds incubated at RT for 2-3 minutes, then centrifuged at 12000 g for 15 minutes at 2-8°C. The upper aqueous phase was transferred to a 1.5 mL collecting tube and 1 µl glycogen was added. RNA was then precipitated by the addition of isopropyl alcohol at 0.5 mL of isopropyl per 1 mL TRIzol® at room temperature for 10 minutes then centrifuged at 12000 g for 10 minutes at 2-8°C. The pellet was washed with 1 mL 75% ethanol. The sample was vortexed for 10 seconds and then centrifuged for 5 minutes at 7500 g at 2-8°C. The pellet was air dried for 10 minutes and re-suspended in 10 mLs of deionized, diethylpyrocarbonate (DEPC)-treated water (Thermo Scientific).

1 µl of Sodium acetate was added with 40 µl 100% ethanol to each sample and incubated at -20°C for 12 hours, then centrifuged for 30 minutes at 14000 g at 4°C. The supernatant was removed and the pellet washed with 500 µl of 75% ethanol then

centrifuged at 14000 g for 5 minutes at 4°C. The supernatant was removed and the pellet was re-suspended in 10 µl of DEPC-treated water. RNA concentration was determined by NanoDrop 2000 Spectrophotometer (Thermo Scientific).

2.3.3 RNA extraction (miRCURY™ RNA Isolation Kits [Exiqon])

To prepare the reagents, 80 µl of >99% RNA grade ethanol was added to the supplied wash solutions. RNA and cDNA spike-ins were each re-suspended with 80 µl nuclease-free water and kept on ice for 20 minutes.

60 µl of lysis solution was added to each 200 µl of plasma together with 1 µl of spike-in and then vortexed for 5 seconds and incubated for 3 minutes at RT. The spike-ins utilised were UniSp2, UniSp4, UniSp5, UniSp6 and a synthetic version of *C. elegans*, cel-miR-39-3p. The spike-ins resemble miRNAs in structure but lack close sequence similarities to known miRNAs. Spike-ins act as a quality control for the RNA isolation and the cDNA synthesis.

20 µl of protein precipitant was added, vortexed for 5 seconds, incubated for 1 minute at RT and then centrifuged for 3 minutes at 11,000 g. The supernatant was then transferred to a new collection tube and 270 µl of isopropanol was added and the sample was loaded onto the column. 100 µl of Wash Solution 1 was added and then centrifuged for 30 seconds at 11,000 g with the flow through discarded. 700 µl of Wash Solution 2 was added, centrifuged for 30 seconds at 11,000 g with flow through discarded. 250 µl of Wash Solution 2 was added to the column, centrifuged for 2 minutes at 11,000 g. 50 µl of RNAase free water was added to the column, incubated

for 1 minute and then centrifuged at 11,000 g for 1 minute. The purified RNA sample were either used immediately or stored at -80°C.

2.4 RNA quality control

2.4.1 Nanospectrophotometer

RNA samples were run on NanoDrop 2000 Spectrophotometer (Thermo Scientific) to determine RNA concentrations (as measured in ng/ µl) and organic solvent contamination (260/230 ratio). The machine was set to a factor of 40, lid factor 10 and with a background "on" in order to measure the extracted RNA. Before running the samples the machine was "blanked" with the same nuclease free water (Invitrogen) that the sample had been eluted into. A 260:280 ratio > 1.7 and a 260:230 ratio >1.1 were required in order to pass quality control.

2.4.2 Bioanalyzer

Prior to running samples on microarray, all RNA was quality checked on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Two parameters of RNA quality were assessed; RNA integrity number (RIN) and RNA quality 28S:18S. The bioanalyzer measures the time the sample RNA takes to travel through the gel-filled microfluidic channels of an RNA Nano LabChip® and compares this to a RNA sample with pre-determined amounts of different sized RNA strands (known as the ladder). A RIN > 8 was considered to be of good integrity with a 28S:18S ≈ 2.

2.4.3 Ethanol precipitation

If the RNA had a 260:230 ratio ≤ 1:1 then it underwent ethanol precipitation with the aim of removing inorganic solvent contamination. 3 molar Sodium acetate was added to the sample at a volume equal to one-tenth of the sample amount. Two-times the sample volume of RNA-grade 100% ethanol was added, together with 1 µl of glycogen (20 mg/ µl) (Riche, Basel, Switzerland) as a carrier. The solution was vortexed and stored at -20°C overnight. The following day the sample was centrifuged at 4°C at maximum speed for 30 minutes. The supernatant was carefully removed. 500 µl of 80% RNA-grade ethanol was added and centrifuged at maximum speed at 4°C for 10 minutes. The supernatant was carefully removed and the samples were allowed to air-dry. The RNA was re-suspended in 10 µl of RNase free water and then measured by nanospectrophotometer.

2.4.4 qRT-PCR of miRNA (TaqMan [Life Technologies])

2.4.4.1 cDNA Synthesis

The RT master mix was prepared by scaling the volumes as listed below:

Component	**Master mix volume per 15-ul reaction**
100mM dNTPS (with dTTP)	0.15 µl
MultiScibe™ Reverse Transcriptase, 50 U/ µl	1.00 µl
10XReverse Transcription Buffer	1.50 µl
RNase Inhibitor, 20 U/ µl	0.19 µl
Nuclease-free water	4.16 µl

Total volume	**7.00 µl**

Each 15-µl RT reaction, consisting of 7 µl master mix, 3 µl of 5XRT primer and 5 µl RNA sample, was vortexed and then centrifuged at 12 000 g for 5 seconds. It was then incubated on ice for 5 minutes before being loaded into the thermal cycler with the following parameters set:

Time	**Temperature**
30 minutes	16°C
30 minutes	42°C
5 minutes	85°C
∞	4°C

2.4.4.2 qPCR amplification

Samples were analysed on Fast Optical 96-Well Plate (Life Technologies) in the following quantities.

Reagent	**Volume**
Taqman™ Small RNA Assay	1.00 µl
Product from RT reaction	1.33 µl
TaqMan™ Universal PCR Master Mix II (2X)	10.00 µl
Nuclease free water	7.67 µl
Total Volume	**20.00 µl**

qRT-PCR was performed on a 7900HT Fast Real-Time PCR System (Life Technologies) machine with the following parameters set.

Run mode: Standard

Sample Volume: 20 µl

Thermal Cycling Conditions

Step	Optimal AmpErase® UNG activity	Enzyme Activation	PCR	
	Step 1	Step 2	Cycle (40 cycles)	
			Denature	Anneal
Temperature	50°C	95°C	95°C	60°C
Time	2 minutes	10 minutes	15 seconds	60 seconds

2.4.5 qRT-PCR of miRNA (GenCopoeia™ [Rockville, MD])

2.4.5.1 cDNA synthesis

The RT master mix was prepared by scaling the volumes as listed below with 100 ng of total RNA used per 25 µl sample.

Component	Master mix volume per 25 ul reaction*	Quantity
Total RNA		100 ng
2.5 U/ µl Poly A Polymerase	1 µl	
RTase Mix	1 µl	
5X PAP/RT Buffer	1 µl	
Spike-in-control	1 µl	
RNase-/DNase free water	To final 25 µl	

Each 25-µl RT was vortexed and then centrifuged at 12 000 g for 5 seconds. It was then incubated on ice for 5 minutes before being loaded into the thermal cycler with the following parameters set:

Time	Temperature
60 minutes	37°C
5 minutes	85°C
∞	4°C

The resultant reverse transcription reaction product was diluted 50 times with sterile RNase-/DNase free water.

2.4.5.2 qPCR amplification

Samples were analysed on Fast Optical 96-Well Plate (Applied Biosystems) in the following quantities.

Reagent	Volume
2X All-in-One qPCR Mix	10 µl
All-in-One miRNA qPCR (2 µM)	2 µl
Universal Adaptor PCR Primer (2 µM)	2 µl
First-strand cDNA (diluted 1:5)	2 µl
RNase-/DNase free water	4 µl
Total Volume	**20 µl**

qRT-PCR was performed on a 7900HT Fast Real-Time PCR System (Life Technologies) machine with the following parameters set.

Run mode: Standard

Sample Volume: 20 µl

Step	Initial denaturation	PCR	
		Cycle (40 cycles)	
		Denature	Anneal
Temperature	95 °C	95°C	T_m -2°C
Time	10 minutes	10 seconds	20 seconds

Thermal Cycling Conditions

T_m, melting temperature.

2.4.6 miRNA array (Affymetrix® GeneChip®, Santa Clara, CA)

Total RNA containing low molecular weight RNA was labelled using the Flashtag RNA labelling kit (Genisphere, Hatfield, PA). For each sample, 150 ng total RNA was subjected a tailing reaction (2.5 mM MnCl2, ATP, Poly A Polymerase - incubation for 15 minutes at 37^0C) followed by ligation of the biotinylated signal molecule to the target RNA sample (16 Flash Tag ligation mix biotin, T4 DNA ligase - incubation for 30 minutes at room temperature) and adding of stop solution.

Each sample was hybridized to a GeneChip miRNA Array (Affymetrix) at 48°C and 60 g for 16 hours then washed and stained on Fluidics Station 450 (Fluidics script FS450_0003) and finally scanned on a GeneChip Scanner 3000 7G (Affymetrix). The image data were analysed with the miRNA QC Tool software for quality control.

Two versions of the microarray chips were used: Version 2.0 and Version 3.0. Four samples (from two donors: two infected and two uninfected) were run on a Version 2.0 miRNA microarray chip (Affymetrix) with 1105 human miRNAs and eight samples (from four donors: four infected and four uninfected) were run on a Version 3.0 miRNA microRNA chip (Affymetrix) with 1693 human miRNAs.

2.4.7 miRCURY™ Universal RT miRNA PCR (Exiqon)

All methods outlined below were utilised for the study detailed in Chapter Four. All materials were supplied by Exiqon, unless otherwise stated.

Protocol was followed according to the instruction manual and all the internal quality control criteria were met. In brief,

2.4.7.1 cDNA Synthesis

Each sample was prepared as followed:

Reagent	**Volume**
5x Reaction buffer	4.00 µl
Nuclease-free water	9.00 µl
Enzyme mix	2.00 µl
Synthetic RNA spike-in	1.00 µl
Template total RNA	4.00 µl
Total Volume	**20.00 µl**

Samples were incubated for 60 minutes at 42°C followed by heat inactivation of the reverse transcription for 5 minutes at 4°C. Samples were either used immediately or stored at -20°C.

2.4.7.2 Plasma Focus miRNA PCR Panels

The plasma focus panels (miRCURY LNA™ Universal RT microRNA PCR Serum/Plasma Focus microRNA PCR Panel, 96 well, V1.RO, Exiqon, Denmark) were comprised of 2 plates; plate 1 and 2. In total 175 different locked nucleic acids (LNA) primer sets for real time amplification of human miRNAs which are known to be expressed in humans in both healthy and diseased states were examined. These panels were utilised for the Pilot Study in Chapter Four.

For the validation study in Chapter Four, a total of ten miRNAs were chosen for further evaluation on custom-made miRNA PCR Panels (Exiqon), with miR-93 chosen as reference miRNA.

LNA are a class of nucleic acid analogues that contain one or more nucleotide building blocks in which a methylene bridge fixes the ribose moiety conformation (Vester et al., 2004) (Figure 1.5). The LNA oligonucleotides have extremely high hybridisation affinity toward their complementary single-stranded RNA and complementary single- or double-stranded DNA (Kauppinen et al., 2006).

Seven microRNA LNA™ primer sets present on both plates were designated candidate reference genes based on the literature. Included in this was hsa-miRNA-93 which we have shown to be a stable reference miRNA in the study population.

Each plate also contains an inter-plate calibrator in triplicate and a control primer set for detection of the synthetic spike-in (UniSP6).

2.4.7.3 Quality Control Measures

RNA quality was assessed using miRCURY™ QC PCR panels consisting of 96 well plates containing dried down LNA™ primer sets for each 10 µl set of reactions. The 96 well plate was able to assess 8 samples with the layout of the plate as shown below.

RNA quality was assessed on each plasma sample using miRCURY™ microRNA QC PCR Panel (Exiqon). The protocol was followed according to the manufactures' instructions. Briefly, 4 µl of cDNA from RT reaction was mixed with 198 µl of nuclease free water (reach a 50x dilution). 60 µl of SYBR Green® Master mix was combined with 60 µl of the diluted cDNA and gently mixed. The PCR panel plates were spun gently prior to the removal of the plate seal. 10 µl Mastermix:cDNA mix was added to each well. The plate was sealed with optical seal as recommended by the instrument manufacturer.

Sample quality was tested against the following;

1. RNA Purification – UniSp2,4 and 5 show consistent values across data set (<2-3Cq) and ΔCq= 5-7 between spike-ins. The presence of UniSP5 signal indicates efficient purification of low expressed miRNA.
2. cDNA Synthesis- UniSP6 and/or cel-39-3p show consistent values across the sample set with low variance (<1-2 Cq).
3. qPCR evaluation- UniSp3 shows consistent values across data-set (<2 Cp across the data-set).
4. Evaluation of haemolysis – if ΔCq (miR-23a – miR-451) is lower than 7, it is unlikely that the sample is haemolysed

2.4.7.4 Quantitative real-time PCR

All qRT-PCR experiments were carried out on a LightCycler®480 (Roche, Life Sciences, NSW, Australia). The PCR reactions were initiated with 10 min incubation at 95°C followed by 45 cycles of 95°C for 10 sec and 60°C for 1 minute. Melting curve analysis was performed at the end of amplification to verify the amplification.

2.5 Cytokines assays

2.5.1 Cytometric bead array

Cytokines, interleukin (IL)-1β, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, tumour necrosis factor (TNF), and interferon (IFN)-gamma inducible protein (IP)-10, were quantified simultaneously using a human chemokine cytometric bead array (CBA) kit (BD Biosciences). These assay kits provided a mixture of six micro-bead populations with distinct fluorescent intensities (FI)-3 and were pre-coated with

capture antibodies specific for each cytokine. 1 µl of supernatant collected from *M. tubculosis* infected and uninfected macrophages at 24 hours post infection, or the provided standard cytokines, were added to the pre-mixed microbeads in Falcon tubes (BD Biosciences). After the addition of 10 µl of a mixture of conjugated antibodies against the chemokines, the mixture was incubated for 2 hours, in the dark at RT. The mixture was washed and centrifuged at 800 g for 5 minutes, and the pellet was re-suspended in 300 µl of wash buffer. The LSR II flow cytometer (BD Biosciences) was calibrated with set-up beads and 3000 events were acquired for each sample. Individual cytokine concentrations were indicated by their Fl-2 and were computed using the standard reference curve of CELLQUEST and CBA software (BD Biosciences).

To address intra-assay performance of the CBA chemokine assay, 10 replicates samples of three different levels of the chemokine standards were tested in a single assay. Inter-assay reproducibility was assessed using two replicate samples of three different levels of the human standards. Normal ranges for cytokine levels were established using supernatants from uninfected macrophages. Each biological sample had three replicates.

CHAPTER THREE

This chapter has been adapted from an accepted publication as referenced by:

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Authors Contributions:

Conception and Design: SB, BS and BC

Acquisition, Analysis and Interpretation: SB, BC, ME, YY, MP, GG, XW, WB, BS

Drafting the manuscript for important intellectual content: SB, BC, ME, YY, MP, GG, XW, WB, BS

3 IDENTIFICATION OF A SUITABLE REFERENCE MIRNA

3.1 Introduction

Tuberculosis (TB) is an infectious disease caused by the bacillus, *M. tuberculosis* that normally affects the lungs. It is estimated that one third of the worlds population is infected with *M. tuberculosis*. Of those infected however, only about 10% will ever develop active disease (Lawn et al., 2011) whereas the rest remain in a latent state. It is estimated that TB results in the death of about 1.5 million individuals annually (World Health Organization, 2013).

Initial diagnosis of TB is made most commonly by sputum smear microscopy. Mycobacterial culture remains the gold standard for TB confirmation and resistance testing, with rapid molecular tests such as genXpert® (Xpert), also employed where available (Boehme et al., 2010). However, lengthy delays in diagnosis are an on-going problem in TB management. Therapy for TB disease requires a minimum of six months on multiple antibiotics. Monitoring a patient's response to infection to identify non-responders, which may be indicative of poor compliance or drug resistance, is another important requirement for managing TB. Currently, conversion to sputum negative culture at two months is the gold standard marker of successful treatment, but this means it is often three months or more into therapy before non-responders are identified, during which time they remain infectious. New biomarkers that aid diagnosis and identify treatment non-responders early, quickly and cheaply would greatly assist in reducing the burden of TB infection (Lawn et al., 2011).

MicroRNAs (miRNAs) are single-stranded RNA molecules of 21-23 nucleotides in length that play an important role in post-translational regulation of gene expression through targeting messenger RNA (mRNA) (Mause et al., 2010). They circulate in a stable, cell-free form and are increased in levels in many disease processes such as malignancy, schizophrenia, heart failure and sepsis (He et al., 2004; Oak et al., 2011; Mastronardi et al., 2012). Their use as a biomarker to aid diagnosis and predict response to therapy is of growing interest. They may also represent a novel therapeutic target to modify the host response (Osaki et al., 2008; Cho, 2011; Ochs et al., 2014).

Normalisation of miRNA levels is critically important to enable correction of inter-sample variation and differing reaction efficiencies. Commonly RNU6B (U6) has been used in many cell and tissue studies, but other studies have shown that U6 is not stably expressed in sera (Benz et al., 2013). To date there are no published studies that systematically evaluate reference miRNAs for normalizing plasma-derived miRNAs in the setting of TB (Ng et al., 2009; F. Ji et al., 2011) or in different ethnic and geographical groups to determine if these factors influence miRNA expression

3.1.1 Strategies to normalise miRNA data

There are three recognised strategies to normalise data. The study performed here utilised two of these: GeNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004). The other recognised method is Bestkeeper (Pfaffl et al., 2004).

GeNorm measures the geometric, as opposed to the arithmetic, mean of carefully selected miRNAs. It utilises a pairwise comparison to rank candidate miRNAs by summed individual variation and in a step-wise fashion discards the most variant gene before repeating the analysis. Every potential reference miRNA is assessed and given an *M* score. miRNAs with the lowest *M* scores are the most stably expressed and are deemed the suitable reference miRNA (Vandesompele et al., 2002). GeNorm works on the principle that the expression ratios of reference miRNAs are consistent in all samples regardless of the experimental condition or cell type. It is therefore recommended to perform an independent study of variance; in this case we performed a Levene's test, an inferential statistic that is used to assess variability between two different sub-samples, with a p-value less than 0.05 considered to demonstrate significant variation between samples.

Normfinder utilises a mathematical model to directly measure the expression variation of miRNAs between samples and conditions of interest. The application of the model is able to estimate the intra- and the inter-group expression variation and calculate a "stability value" for each candidate miRNA assessed. This model also relies on the careful selection of candidate miRNAs and also assumes minimal variation between miRNA levels within the groups studied (Andersen et al., 2004).

Bestkeeper is another commonly used method for selecting a suitable reference miRNA. It is available as a prepared Excel spreadsheet file obtained at (http://www.gene-quantification.de/bestkeeper.html). It calculates the geometric average for the entire dataset and uses that as a global normalisation factor, or "bestkeeper" (Pfaffl et al., 2004). Bestkeeper assumes that as the number of miRNAs

measured increases variations resulting from genuine biological changes in miRNA expression make a progressively lower overall trend, than global variations in cDNA concentrations that arise from sample preparation and handling. Thereby, making pairwise comparisons between the expression data from miRNAs of individuals and the global normalisation factor, one can identify those miRNAs with the least variation.

Both geNorm and Bestkeeper results are highly sensitive to the dataset presented; meaning that removal of a single high-scoring candidate reference miRNA will lower the correlation of similar high-scoring candidates. This will result in lower ranked candidates being given a higher relative pairwise correlation, and thus re-shuffling the order of rankings. The benefit of this is that these methods are more tolerant of imperfect datasets stemming from variable mRNA quality or cDNA synthesis (Hildyard et al., 2014).

Normfinder, on the other hand, avoids direct miRNA-miRNA comparison, and instead focusses more on each miRNA individually, rather than giving group-wide averages. This technique, however, makes the assumption that the dataset averages do not exhibit significant intergroup variation (Hildyard et al., 2014).

By using the two varied techniques in this study, we aimed to minimise the limitations of either method whilst benefiting from each of their strengths. Furthermore, we chose 12 miRNAs to study as potential candidate reference miRNAs, as generally these techniques benefit from larger datasets. This then adds greater confidence when

a particular miRNA is suggested as a suitable reference miRNA, particularly if the same miRNA is selected by each method.

3.1.2 Other factors which may influence miRNA expression

Recent studies have supported the notion that miRNA expression is tissue and disease specific, and that normalisation strategies, which are highly important to control for external-related variation, need to be considered for each group and disease being studied (Peltier et al., 2008; H. T. Zhu et al., 2012; Song et al., 2012; Chen et al., 2013; Zheng et al., 2013; Tang et al., 2015). What is not clear is whether other factors, such as ethnicity and/or geographical location, also have an effect on miRNA expression. There have been limited studies in the literature that have reported significant variation in miRNA expression that seemed to be related to either geographical area or ethnicity (X. Chang et al., 2014; Rawlings-Goss et al., 2014; Wang et al., 2014). Given the unique opportunity of being able to study miRNA profiles in subjects from two very different geographical locations, these being Australia and north-west China, we also sought to examine the miRNA expression between these two different geographical cohorts.

3.1.3 Aims

The aims of this study were to:

1. Identify suitable and stable reference miRNAs for the normalisation of miRNA levels in patients with active TB, in comparison with individuals with latent tuberculosis infection (LTBI) and healthy controls.

2. To examine if geographical or ethnic differences influence miRNA levels in TB patients in Australia and the Ningxia Hui Autonomous Region (NHAR) of north-west China (J. J. Liu et al., 2005).

3.2 Methods

3.2.1 Ethics statement

This study was undertaken with the approval of the Ethics Review Committees at St Vincent's Hospital, Sydney (Protocol No X11-0141 and HREC/11/RPAH/201), The University of Sydney (Protocol No 2012/1076) and The Ningxia Medical University, China (approval date 6/6/2013). All participants provided written informed consent. A copy of the patient information sheet, patient questionnaire and consent form, and ethics approval documentation are included in Appendix B.

3.2.2 Australian cohort

We invited suitable consecutive consenting patients presenting to the TB clinic at St Vincent's Hospital to participate in the study (Table 3.1). Enrolment for the study ceased once the required enrolment quota was reached. Blood was collected from 12 patients with culture-proven pulmonary tuberculosis, 12 with LTBI (based on a Tuberculin skin test induration of greater than or equal to 15 mm, a lack of symptoms and a normal chest radiograph), and 12 healthy controls with no exposure to TB. 10 mL of peripheral blood was collected into EDTA tubes and processed within three hours.

3.2.3 The Peoples Republic of China (PRC) cohort

Blood was collected from 12 patients with either culture-proven TB or TB diagnosed on clinical and radiological grounds, and 12 matched healthy controls. 10 mL of peripheral blood was collected into ETDA tubes and processed within three hours. All patients recruited from China were born in PRC and classified themselves of Hui ethnicity (Table 3.1).

3.2.4 Isolation of plasma from human samples

Ten millilitres of blood was collected from each donor and plasma separated as explained in Section 2.2.2 of the Materials and Methods.

3.2.5 RNA extraction and quality control

Total RNA, including small RNAs, was extracted using TRIzol LS® (Life Technologies) as described in Section 2.3.2 (Materials and Methods). RNA concentration and integrity (RIN) was measured using a nano-spectrophotometer (Thermoscientific, Wilmington, DE, USA).

3.2.6 qRT-PCR

Reverse transcription was carried out using All-in-One^{TM} miRNA First-Strand cDNA Synthesis kit (GeneCopoeia) as described in Section 2.4.5 (Materials and Methods). The following miRNAs were measured: miR-192, -22, -221, -26, -451, -16, -103, -191, -423, -425, *let-7*, and U6.

3.2.7 Data analysis

Data was analysed using SDS Relative Quantification Software Version 2.3 (Life Technologies). Endogenous controls must be consistently expressed, therefore only the miRNAs detected in all 60 samples were selected for further analysis using Normfinder and geNorm software packages (GenEx Version 5, Multi D, Göteborg, Sweden). Normfinder and geNorm reference gene validation software was used to identify the most stably expressed miRNAs.

3.2.8 Statistical analysis

The difference in miRNA expression was analysed by Mann Whitney *U* test for comparison of two groups, and Wilcoxon rank-sum test for multi-group comparisons; p-values below 0.05 were considered significant.

Table 3-1. Baseline characteristic of subjects by country of birth and disease state.

Cohort	**Australian**	**Australian**	**Australian**	**PRC**	**PRC**
Clinical Pathological Variable	TB n=12	LTBI n=12	Healthy Control n=12	TB n=12	Healthy Control n=12
Median age, years (Range)	36 (20-60)	34 (18-59)	44 (27-76)	45 (16-76)	39 (16-68)
Male (%)	4 (33.3)	6 (50)	6 (50)	11 (92%)	7 (58)
Place of Birth*	South Asia (n=2) East Asia (n=6) South Eastern Asia (n=3) Oceania (n=1)	South East Asia (n=3) Southern Africa (n=1) East Africa (n=1) Eastern Europe (n=1) Oceania (n=6)	Oceania (n=10) East Asia (n=1) Western Europe (n=1)	PRC**	PRC**

* Regions as classified by WHO, **All PRC subjects of Hui ethnicity.
TB, tuberculosis; LTBI, latent tuberculosis infection.

3.3 Results

The clinical characteristics of study participants are listed in Table 3.1. The Australian cohort patients with TB were mainly born in Asia (91%), with one third being male. Those with LTBI were born in a variety of regions, with 50% from the Oceania region. In the Australian healthy control group the vast majority were born in Australia (83%). All subjects in the PRC cohort were born in PRC and identified themselves as Hui in ethnicity; and were typically male (92%), with an average age of 42 years. The extent of the TB disease as classified radiologically was generally less advanced in the Australian group compared with the PRC cohort, suggestive of later presentation and/or diagnosis and possible concurrent chronic lung disease in the PRC cohort.

The quantitation cycle (Cq) values for the 13 candidate miRNAs were measured for all 60 individuals, with Cq thresholds ranging from 13 to 39 (Table 3.2). U6 was poorly expressed with Cq values above the threshold of detection and was therefore excluded from further analysis. The expression levels of some miRNAs varied markedly between the two populations, independent of disease status (Figure 3.1 and Table 3.2). Two miRNAs (miR-192 and *let-7*) were highly variable between the cohorts with differences of seven to nine cycles (128-512 fold) of average values between the cohorts, while the level of expression of four other miRNAs (miR-26, -16, -423, -425) were within one cycle between the PRC and Australian cohorts (Figure 3.1 and Table 3.2). By stratifying groups based on location we found

statistically significant differences in many of miRNAs examined according to cohort and not disease state.

Table 3-2. Expression levels of candidate reference miRNAs in the Australian and PRC cohort, independent of disease status.

miRNA	Cq Mean (Range) Australian cohort n=24	Cq Mean (Range) PRC cohort n=36	Cq mean Variability between Cohorts
miR-192	22.14 (15.87-33.31)	31.46 (29.33-34.84)	9.31
miR-22	29.59 (23.81-35.59)	32.62 (28.23-36.08)	3.03
miR-221	26.79 (23.41-32.95)	31.42 (25.77-38.31)	4.64
miR-26	25.90 (18.18-32.68)	28.41 (22.42-36.34)	2.50
miR-451	21.56 (13.66-33.21)	22.37 (15.73-34.82)	0.81
miR-93	25.64 (20.82-29.13)	27.93 (25.53-30.35)	2.29
let 7	25.38 (17.77-34.41)	32.45 (29.32-34.79)	7.06
miR-16	22.69 (14.07-34.70)	23.13 (15.67-33.86)	0.45
miR-103	30.20 (21.40-37.81	28.58 (25.70-31.07)	-1.62
miR-191	33.36 (28.96-39.57)	30.20 (27.66-32.35)	-3.16
miR-423	31.53 (25.25-38.50)	30.72 (27.58-34.43)	-0.81
miR-425	29.76 (24.26-34.71)	29.92 (27.41-32.19)	0.15

Values are given as mean and range in brackets of the quantification cycle (Cq) values of candidate reference genes by cohort.
miR, microRNA.

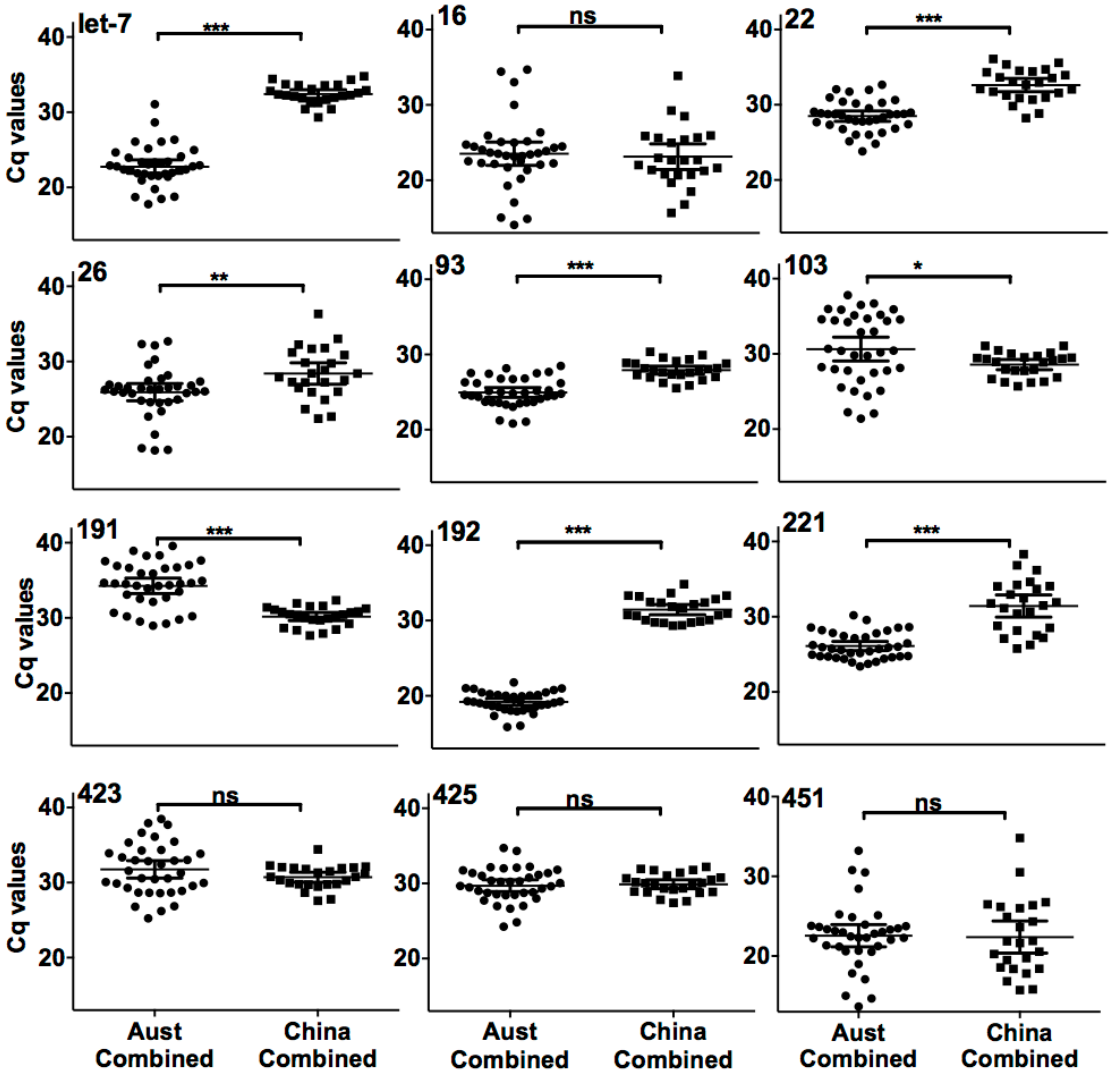


Figure 3-1. Expression levels of candidate reference miRNAs by cohort. Expression levels of the 12 candidate reference miRNAs in the combined Australian cohort (n= 36) and the PRC cohort (n=24) were determined by qPCR. Values are given as the quantification cycle (Cq mean of duplicate sample). Significant differences were calculated by Wilcoxon rank-sum test. *<0.05, ** <0.01, *** <0.001, ns= not significant. AUST, Australian cohort; CHINA, People Republic of China cohort.

3.3.1 Determination of reference miRNA expression stability using geNorm and Normfinder.

GeNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004) software were used to examine the stability of candidate reference miRNAs from the plasma of all groups from both cohorts. Both geNorm and Normfinder assume equal variance of the candidate miRNAs between the populations. Therefore Levene's test of variance was carried out on all miRNAs from the respective cohorts to determine if there was significant variance between the groups (Larson, 2008). miRNAs with p-values below 0.05 were considered significantly different, and were excluded from further analysis. In the PRC cohort four miRNAs were excluded (miR-16, -26, -221 and -451), while none were excluded from the Australian cohort, and overall when all samples were examined collectively four miRNAs were excluded from subsequent analysis (miR-26, -192, -221 and -451).

For Normfinder the maximum expression of each candidate reference miRNA (as given by lowest Cq value) was used as a control and set to one. Relative expression levels were then calculated from Cq values using the formula: $2(-\Delta\Delta C(T))$, in which ΔCq is equal to the corresponding Cq value minus the minimum Cq value. For geNorm analysis, data files containing the mean Cq values as determined by SDS Relative Quantification Software were imported directly into GenEx software for analysis. GeNorm generates an *M* value based on the average standard deviation of the ratio of the pairwise normalisation miRNAs accompanied by stepwise exclusion of the least stable miRNA. The lower the *M* value, the more stable the miRNA.

3.3.1.1 miR-93 and miR-425 are the most stable reference miRNA in the PRC cohort.

Analysis of the PRC cohort using both Normfinder and geNorm identified the expression of miR-93 and miR-425 to be the most stably expressed miRNAs (Figure 3.2).

miRNA	Normfinder		geNorm	
	Stability Value	Ranking Order	Stability Value	Ranking Order
miR-93	0.23	1	0.47	**1**
miR-425	0.23	1	0.47	**1**
miR-191	0.60	3	0.64	**3**
miR-103	0.83	4	0.77	**5**
miR-423	0.95	5	0.72	**4**
miR-192	1.40	6	1.00	**6**
Let-7	1.56	7	1.20	**7**
miR-22	3.52	8	1.83	**8**

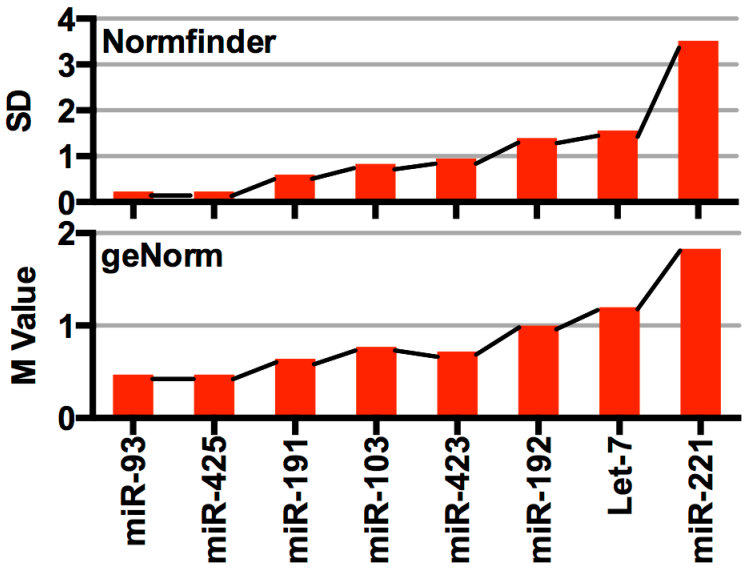


Figure 3-2. **Stability values of candidate reference genes for the PRC cohort as determined by Normfinder and geNorm.**
Expression levels of 12 miRNAs in the plasma of newly diagnosed TB patients (n=12) and healthy controls (n =12) were subject to Levene's test to determine if there was significant variance between groups. miRs -16, -26, -221 and -451 displayed significant variance between groups and were removed from subsequent analysis. The remaining eight miRNAs were ranked using Normfinder and geNorm. Greater expression stability is indicated by a lower stability value (*M*). Low standard deviation (SD) values indicate stable gene expression (Normfinder).

3.3.1.2 miRs-93, -221, -22 and let-*7 are the most stable reference miRNAs in the Australian cohort.*

geNorm and Normfinder analysis of the Australian cohort identified a panel of four miRNAs, including miR-93, as suitable reference miRNAs in this sample set. The rankings of the candidates as determined by geNorm and Normfinder are shown in Figure 3.3. The top five miRNAs were the same using both analysis tools, with similar *M* values, suggesting that either in combination or alone they are suitable normalisers for this cohort. miR-93 was found to be the most stable when utilising geNorm software, and even though it is ranked number four according to Normfinder, the stability values differed little between the top four candidates (Figure 3.3).

3.3.1.3 miR-93 and miR-22 are the most stable reference miRNAs in a combined cohort analysis.

When examining all patient groups from both cohorts geNorm and Normfinder identified miR-93 and miR-22 to be the most stable (Figure 3.4), although the stability values in the combined groups were higher than when each cohort was analysed separately. To explore this in more detail we examined miRNA variation across the cohorts.

miRNA	Normfinder		geNorm	
	Stability Value	**Ranking Order**	**Stability Value**	**Ranking Order**
miR-221	1.35	1	1.62	1
let-7	1.36	2	1.88	4
miR-22	1.37	3	1.67	3
miR-93	1.47	4	1.62	1
miR-26	1.94	5	2.05	5
miR-192	2.31	6	2.61	8
miR-451	2.76	7	2.27	6
miR-425	2.77	8	2.79	9
miR-423	2.80	9	2.99	10
miR-16	3.34	10	2.40	7
miR-191	3.52	11	3.19	11
miR-103	3.93	12	3.40	12

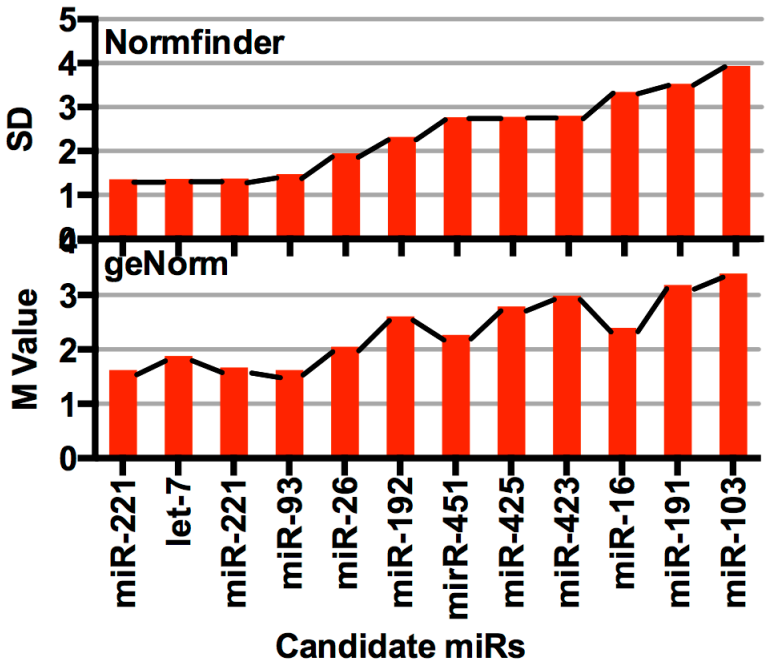


Figure 3-3. Stability values of candidate reference genes for Australian cohort as determined by Normfinder and geNorm.
Expression levels of 12 miRNAs in the plasma of newly diagnosed TB patients (n=12) and healthy and latent controls (n =24) were subject to Levene's test which determined there was no significant variance between any of the groups. The 12 miRNAs were then ranked by Normfinder and geNorm. Greater expression stability is indicated by a lower stability value (*M*) (geNorm). Low standard deviation (SD) values indicate stable gene expression (Normfinder).

miRNA	Normfinder		geNorm	
	Stability Value	Ranking Order	Stability Value	Ranking Order
miR-93	1.10	1	1.65	1
miR-22	1.76	2	1.65	1
miR-425	2.05	3	2.43	3
miR-423	2.37	4	2.86	4
miR-103	2.44	5	3.24	5
miR-191	2.93	6	3.51	6
miR-16	3.50	7	3.88	7
***let*-7**	3.55	8	4.27	8

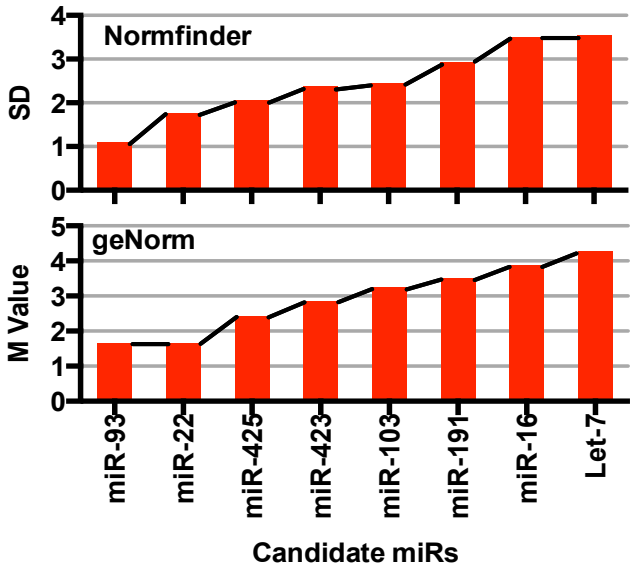


Figure 3-4. Stability values of candidate reference genes for all subjects as determined by Normfinder and geNorm.
Expression levels of 12 miRNAs in the plasma of newly diagnosed TB patients in both the Australian and PRC cohort (n=12/group) and healthy and latent controls (n =12/group) were subject to a Levene's test to determine there was significant variance between groups. miRs -221, -26, -192 and -451 displayed significant variance between groups and were removed from subsequent analysis. The remaining eight miRNAs were ranked using Normfinder and geNorm. Greater expression stability is indicated by a lower stability value (*M*). Low standard deviation (SD) values indicate stable gene expression (Normfinder)

3.3.1.4 Variation in miRNA expression may be cohort rather than disease driven.

The Cq values for the 12 miRNAs examined in the five groups were plotted by disease state and geographical location (Figure 3.5). This data suggests that there is variation in miRNA expression that is independent of disease state, but relates to geographical, or potentially ethnic differences between the cohorts. Further studies in larger cohorts will be required to confirm this. Despite these differences in miRNA expression between the populations, both geNorm and Normfinder analysis identified miR-93 as an appropriate reference miRNA to normalise miRNA levels in plasma samples from both cohorts independent of disease status.

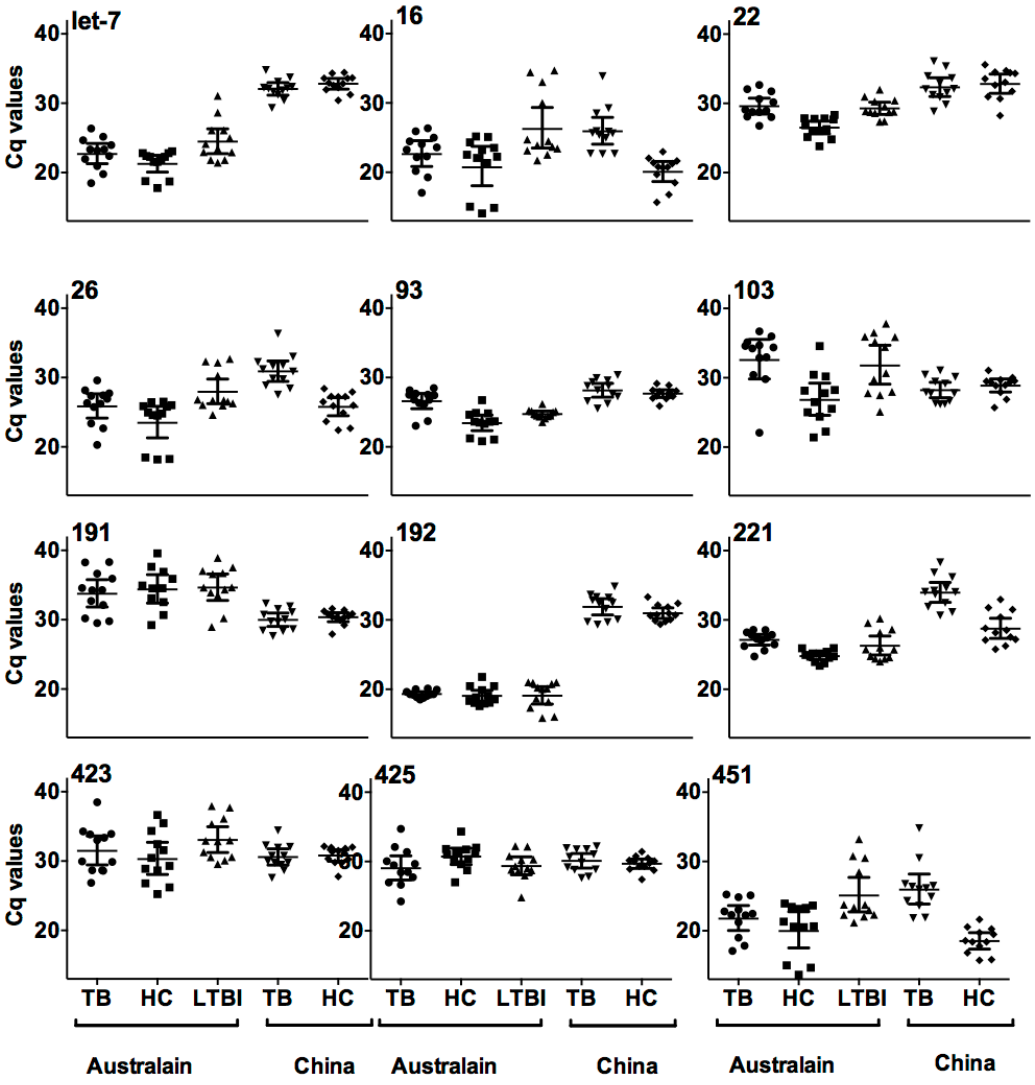


Figure 3-5. Expression levels of candidate reference miRNAs in healthy controls, latent *M. tuberculosis* infection, and active *M. tuberculosis* disease. Expression levels of the 12 candidate reference miRNAs in the plasma of TB patients, healthy controls and latently infected individuals from Australia and in TB patients and healthy controls from the PRC (n=12/group). Values are given as the cycle threshold (Cq mean of duplicate sample).

3.4 Discussion

A growing body of evidence has highlighted the critical regulatory roles that miRNA play to tightly regulate biological processes. miRNAs are commonly found in the blood, often associated with cellular vesicles, such as microparticles or exosomes (Diehl et al., 2012; Boon et al., 2013). miRNA are stable, and numerous studies have investigated the potential of plasma miRNAs to serve as biomarkers for multiple conditions, such as cancer, stroke, and infection (Suryawanshi et al., 2013; Marcucci et al., 2013; Sanchez-Espiridion et al., 2013; Sozzi et al., 2014). Normalisation of miRNA levels in samples is an essential step in biomarker analysis. This study examined the suitability of 12 miRNAs to normalise miRNA levels in plasma from TB patients and controls in two geographical and ethnically diverse populations. We found significant variability in miRNA levels across these populations, independent of disease status. Despite these differences we have identified miR-93 to be a stable, plasma-based miRNA, suitable to normalise miRNA levels across diverse ethnic and geographical populations.

Many of the current miRNA normalisation strategies have been adopted from tissue studies where U6 is often used as a reference to normalise miRNA data (Liang et al., 2007). Recent studies, however, have found that U6 is not stably expressed in plasma (Chen et al., 2008; Appaiah et al., 2011). Our study confirmed these recent findings as U6 was not stably expressed in the plasma of our subjects, indeed in over 75% of our samples U6 was not detected within the 40 cycles of the qPCR assay (data not shown). For this reason U6 was excluded from further analysis as it is not suitable as a reference miRNA for plasma samples. Other studies have added non-human

miRNA from *C. elegans* to samples to control for technical variations, however it does not control for variation in the efficiency of the reverse transcription (Akbas et al., 2012).

This study examined the expression of miRNAs previously reported as suitable to normalise miRNA levels in a variety of biological tissues (Davoren et al., 2008; Peltier et al., 2008; K. H. Chang et al., 2010; H. T. Zhu et al., 2012; Song et al., 2012; Chen et al., 2013; Zheng et al., 2013; Z. Liu et al., 2014). The selection of miRNAs used to normalise is influenced not only by the tissue being examined, but also the disease condition being evaluated. miR-16 and -451 are commonly used to normalise miRNA levels in plasma samples (Song et al., 2012); however more recent studies have reported up-regulation of plasma miR-16 and -451 as part of the miRNA signature in multiple conditions, including autoimmune thyroid disease (Yamada et al., 2014), experimental sepsis (Wu et al., 2013), and gastric cancer (C. Zhu et al., 2014). Another study demonstrated that miR-16 and -451 are highly expressed in red blood cells so that any haemolysis of blood samples strongly affects their level in plasma (Kirschner et al., 2011). We found variable expression of miR-16 and -451 between individuals, such that these miRNAs did not meet the criteria for inclusion as a stable reference for changes in TB disease in either population.

It is clear from the literature that utilising different reference miRNAs can greatly affect the profiles of target miRNAs. Recently Tang and colleagues compared the expression of five miRNAs: miR- 16, -219, -221, -55 and *let-7*, in plasma from a group of patients with gastrointestinal diseases (hepatocellular carcinoma, gastric malignancy, hepatic cirrhosis and hepatitis B) to that of healthy subjects, utilising

either U6, miR-106a, -21 or the geometric mean of miR-106a and -21 as reference miRNAs (Tang et al., 2015). Not unexpectedly they found quite significant variations in the target miRNAs expression depending on which reference miRNA was used. U6 is often used to normalise miRNA data, however this study showed that when normalising with U6, the results failed to detect a statistical difference in the expression profile of miRNAs shown to regulated in gastric malignancy. The results from this study highlight the bias introduced, and erroneous results generated, when utilising non-validated reference miRNAs.

A secondary aim of our study was to determine if there was any miRNA variation that was cohort specific rather than disease based. Even though the sample size was small this study identified significant variability in miRNA expression in different cohorts. This has important implications for not only identifying suitable reference miRNAs, an essential component in accurately analysing qRT-PCR data, but also when considering utilising miRNA expression as a potential biomarker. Whilst the reason for this variation has not as yet been determined recent reports have shown the miRNA expression can be influenced by ethnic diversity (Wang et al., 2014). One possible explanation may relate to inherent genetic variation in miRNA expression. Rawlings-Goss and colleagues utilised whole genome sequencing to examine genetic variation in 1,524 miRNAs in a group of 69 unrelated otherwise healthy individuals from 14 global populations, and identified 31 miRNA variants that were population-differentiated in terms of frequency between African and non-African populations (Rawlings-Goss et al., 2014). These variations were not thought to be somatic, as un-described mutations identified in a single individual were removed from the analysis.

As has been demonstrated, there is an important need to accurately correct for non-biological variation with stable reference genes that are suitable for the population being studied, otherwise important regulated miRNAs that may have a role as biomarkers of disease, may be missed. These data demonstrate the importance of determining the suitability of a reference miRNA directly in the study population to account for variations in miRNA expression attributable to ethnic and environmental factors.

In general this study found that individuals from the PRC grouped together more closely than the Australian cohort, whose ethnic background was far more heterogeneous and included individuals born in nine different countries. In the PRC population, in part due to the tight clustering of the samples, Levene's test of variance identified four miRNAs that were differentially regulated and were therefore excluded from further analysis in that cohort. When the Australian cohort was analysed alone no miRNAs were excluded after Levene's test, but the stability values demonstrate that, when compared to the PRC cohort, there was a greater degree of variability in this sample set. geNorm analysis, which indicates the optimal number of miRNAs that should be used to normalise the samples, found that the best stability values were achieved with analysing four miRNAs for the Australian cohort, but only two miRNAs was required to achieve a similar stability value in the PRC cohort. When the two cohorts were combined a number of miRNAs were again removed after application of the Levene's test.

The baseline expression of some miRNAs, most notably miR-192 and *let* -7, differed by 128-512 fold between the Australian and PRC cohort. Most of this variation was cohort and not disease driven, suggesting that ethnic and/or environmental factors contribute to baseline miRNA levels in the blood. The increased variability seen in the Australian cohort may also reflect the increased ethnic variation in the subjects or other undefined environmental factors. However the individuals participating in this study were not screened or asked about other potentially confounding factors. Comorbidities, such as diabetes or concurrent infections, are likely to also influence miRNA expression, and further research is required to evaluate the impact of comorbidity factors on an individual's miRNA disease signature.

miR-93 has been identified as a suitable reference miRNA to normalise miRNA levels in studies of disparate diseases from gastric cancer to major depression disorders (Song et al., 2012; Z. Liu et al., 2014). Other studies in ovarian and breast cancer have found that miR-93 is differentially regulated in cancer tissue and levels may be predictive of disease state (Eichelser et al., 2013; T. Ji et al., 2014), further highlighting the need to confirm the suitability of selected miRNAs to normalise miRNA levels depending upon the tissue under investigation, the disease state and potentially the ethnicity of the subject population. In summary, miR-93 is a suitable reference miRNA for analysing miRNA levels in TB patients, however ethnic and environmental factors influence miRNA expression in addition to the effect of specific diseases and the suitability of a reference miRNA should be assessed directly in each study population.

4 PLASMA MICRORNAS AS BIOMARKER FOR TUBERCULOSIS

4.1 Introduction

Tuberculosis remains a major global health problem and is second only to HIV as the leading cause of death from a single infectious disease (World Health Organization, 2014). The host's control of the disease is dependent on both the innate and adaptive immune system (Kleinnijenhuis et al., 2011) (Ottenhoff et al., 2005). The immune response determines whether the infection is eliminated, arrested in the latent state, or progresses to active disease either as pulmonary or extra-pulmonary infection (Lawn et al., 2011). Approximately 90% of those infected will remain in the latent state, with the remaining 10% developing active disease, either at the time of infection or later in their life. It is assumed, therefore, that genetic factors, in addition to environmental factors such as malnutrition and HIV co-infection, play an important role in regulating the progression of disease (Frahm et al., 2011; Azad et al., 2012).

Improving the diagnosis of LTBI, recognising those most likely to progress to active disease, promptly diagnosing pulmonary and extrapulmonary disease, identifying those who are most likely to relapse, and identifying drug resistance, are fundamentally important and critically required in order to curtail the rate of disease and its associated morbidity and mortality. In order to achieve this, a detailed molecular and immunological understanding of the pathogen and the host's response to the *Mycobacterium* is essential.

The potential impact of developing a reliable biomarker is substantial. There are the obvious clinical benefits, such as an improved prediction of those that will require chemopreventative therapy and earlier recognition of treatment failure and drug resistance. These tests, however, may also be helpful to be use as surrogate endpoints in late phase drug and vaccine studies, as this would greatly reduce the prolonged follow-up period currently required (Nahid et al., 2011; Kim et al., 2012).

Current standard treatment for TB consists of six months of multi-drug chemotherapy. Although this is generally well tolerated, this prolonged treatment regime does have myriad potential serious side-effects, including hepatitis, optic neuritis, and drug interactions. Ensuring compliance for such a long period of time can also be problematic. Failure to complete an adequate course of therapy can lead to treatment failure, drug resistance and on-going transmission of the disease. Although durable rates of cure with three to four months of treatment are inferior to the standard six months of therapy, the identification of suitable biomarkers may be able to identify early responders for whom shorter courses would be suitable.

Diagnosing and managing children and adolescents with TB has also proven to be very challenging. Often children cannot produce sputum, making diagnosis and monitoring of the response to treatment difficult. They also often do not have what would be considered the “typical” radiographic features of TB. These problems have been amplified by the HIV epidemic, that has increased the number of children with TB and made the clinical and radiological means of diagnosing the disease even more difficult (Ansari et al., 2003; Coulter, 2008). Biomarker work in adult

populations with TB may be transferable to the paediatric group. However, specific studies in this population will need to be undertaken.

4.1.1 miRNA as a potential biomarker

The ideal biomarker must meet certain criteria in order for it to be considered worthwhile. A good biomarker needs to be highly sensitive and specific and it must be practicable, meaning that the sample must be easily obtained by the least invasive measure. It also needs to be a reliable indicator of disease before clinical symptoms appear (early detection), sensitive to changes in pathology (disease progression or therapeutic response) and readily translatable from model systems to humans. A biomarker needs to be predictable with a relatively long half-life in blood that ensures its detection in plasma. Finally, it must be robust, meaning that detection methods need to be quick, reliable, sensitive, accurate, preferably inexpensive and able to be performed at point-of-care.

Human miRNAs isolated from plasma are highly stable and remain so when subjected to prolonged periods at room temperature or freeze-thawing a number of times. It is thought that they exist in a form that is resistant to plasma RNase activity (Turchinovich et al., 2011). Blood is easily accessible. MiRNA are remarkable stable in blood and expression patterns seem to be tissue specific. Recently, circulating miRNAs have been studied as surrogate markers of diseases such as cardiovascular disease, a prognosticator for malignancy and infectious diseases (Chen et al., 2008; Li et al., 2010; Feng et al., 2011).

This project will examine the expression of specific miRNA in the plasma derived

from patients with pulmonary TB and healthy controls to determine if miRNA expression can be used as a biomarker of TB disease and as an early marker of response to treatment.

GENERAL HYPOTHESES

1. miRNA expression profiles measured in the blood are differentially expressed in patients with TB compared with healthy controls.
2. Blood miRNA levels normalise with effective treatment for TB.
3. Failure of miRNAs to normalise with treatment is a marker of treatment failure.

OVERARCHING AIM

To develop an effective biomarker that will:

a. aid in the diagnosis of TB.

b. be used as a marker to monitor treatment progress and detect treatment failure.

SPECIFIC AIMS

1. Measure miRNA expression in 100 patients with active TB disease and 100 healthy controls over the course of treatment to determine if miRNA expression differs between the two populations.
2. To determine if miRNAs normalise with effective treatment.
3. To determine if failure of normalisation of regulated miRNAs is predictive of treatment failure.

4.2 Methods

4.2.1 Recruitment of study subjects

The subjects recruited for the studies described below can be divided into two groups – those with recently diagnosed TB, who were treatment naïve, and healthy controls. All individuals were unrelated.

4.2.2 Study area

The Peoples Republic of China (PRC) is an upper middle-income country located in East Asia. It is the world's most populous country with a population of 1.35 billion with approximately 50% residing in rural areas (Bureau, 2015). It is a single party state that is governed by the communist party. The country's largest cities are Shanghai in the east (23.7 million people) and its northern capital, Bejiing (21.1 million people). Data from the World Bank estimates that nearly 99 million people in PRC still live below the poverty line (Chaudhuri, 2009).

PRC is still profoundly affected by TB and has the second largest number of TB cases, only behind India (World Health Organization, 2014). It is estimated that one million new cases of TB develop every year, accounting for 11% of global TB incidence (World Health Organization, 2014). Further, 80% of TB cases are in rural areas, especially in the poorest provinces in the north and north-western areas, including Ningxia Hui Autonomous region (NHAR) (Liu et al., 2005).

During the 1990s, the country began to address the TB problem by implementing a national TB control programme, containing key elements of the internationally recommended directly observed treatment, short-course (DOTS) strategy. Since the

implementation of the DOTS programme there has been a reported 30% reduction in TB prevalence in areas that implemented the strategy in the ten years between 1990 and 2000 (Wang et al., 2014). The estimated overall prevalence in 2010 was 108 cases per 100 000 and this compares favourably to the 1990 estimate of 210 per 100 000 (World Health Organization, 2014).

Our study was undertaken in Ningxia Hui Autonomous region (NHAR) in north-western China. It is one of five provincial level autonomous regions in PRC. It has an area of 66 400 square km and a population of 6.3 million. NHAR is bounded to the east by the Shaanxi province; to the south, west and east by the Gansu province; and to the north by Inner Mongolia Autonomous Region. NHAR is divided into 5 prefectures, located in three geographic zones (Figure 4.1). Areas included in the study included Tongxin County, Zhongwei County and Haiyuan in Central NHAR, and Yuanzhou City in South NHAR.

Most of the region is desert, however there is a vast plain, Huang He (Yellow River) in the north that has been irrigated for agriculture for many centuries. This fertile plain presents ideal conditions for growing crops, breeding livestock and commercial fishing. This greatly benefits the northern Yellow River areas, notably Wuzhong and Zhongwei as well as its capital city, Yinchuan. In the south, however, it is very mountainous with low production levels and poor economic development (Yang et al., 2010). This disparity in the natural environmental resources has led to considerable variability in gross domestic product, with the highest levels in urban Yinchuan and surrounding counties in the north and the lowest levels in the south and central areas of NHAR.

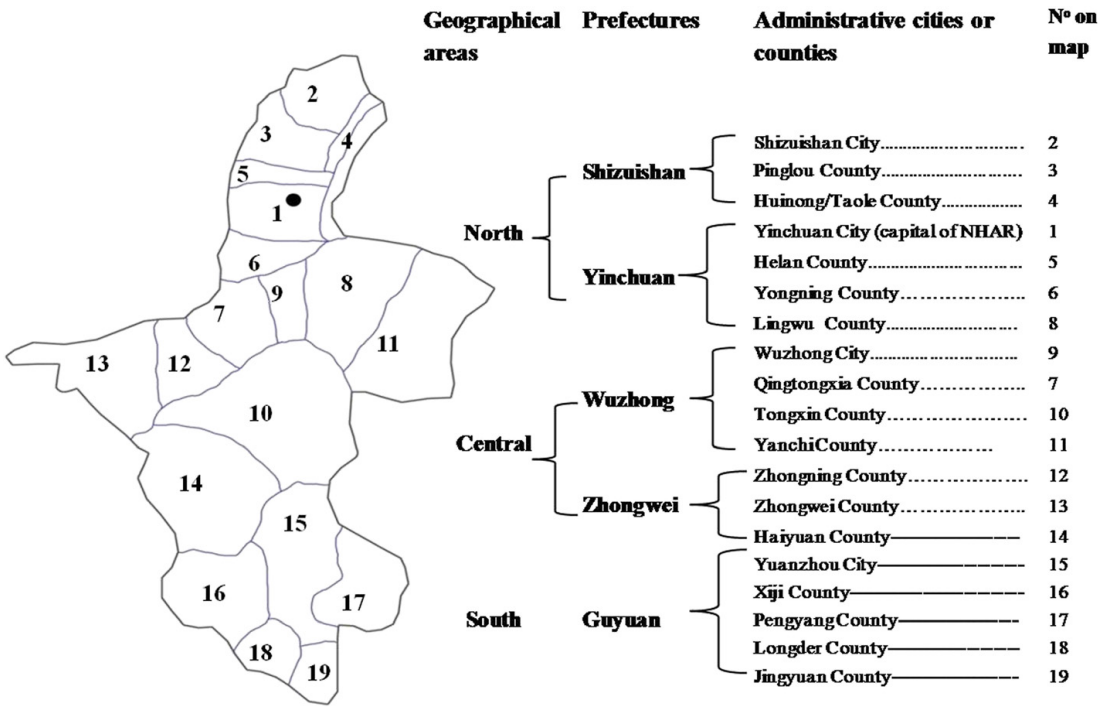


Figure 4-1. Geographical areas of Ningxia Hui Autonomous Region. Figure from Yang et al. (Yang et al., 2012).

PRC is comprised of 56 ethnic groups with nearly 92% being Han Chinese (Bureau, 2015). In NHAR, Han still make up the majority ethnic group, however, Hui (Chinese Muslims) make up about a third of the population, the largest minority group. In addition there are the Manchu ethnic group and a small number of persons from Tibet and Mongolia. Most people speak Mandarin Chinese, with some speaking Mongolian and Tibetan. The predominant religions are Buddhism and Islam (primarily among the Hui).

Hui Chinese are dispersed throughout all parts of China with one of the major concentrations within NHAR. The Hui trace back to the mid-seventh century when Arab and Persian merchants came to China to settle. The Hui have a distinct identity that differentiates them from Han Chinese, mainly in clothing, diet and religion. The

Hui are relatively poor compared with the Han and this may be secondary to historical marginality. Within NHAR, the Hui are generally concentrated in the poorer farming areas.

4.2.3 Tuberculosis in NHAR

A retrospective analysis was undertaken that examined TB cases in the NHAR from 2005-2009. The authors found TB to be hyper-endemic with an estimated 75.5 incident cases per 100 000 population in 2008. 94% of TB cases were new, with no prior history of TB in the past. The vast majority were pulmonary (97%), 2.8% were pleural and the remainder were extra-pulmonary. The rates of extra-pulmonary disease, however, are likely to be underestimated as the extra-pulmonary TB cases are often registered at general hospitals and not at TB dispensaries. About two-thirds of pulmonary cases were sputum-smear positive at the time of diagnosis with many cases considered advanced at the time of diagnosis. Incident rates were higher in men than in women. Standard short course therapy duration is six months, however 30% of patients at the time of the study were on treatment for greater than 12 months and 42% were found to have continuing treatment for longer than two years (Yang et al., 2012).

In 2004, 93% of the county and township levels in NHAR were covered by the DOTS programme. Diagnosis of TB is made on the basis of clinical examination, sputum microscopy and culture and chest radiography. Following diagnosis, patients are enrolled into the DOTS programme. Treatment for drug-susceptible TB is provided free of charge through the National Tuberculosis Programme (NTP).

4.2.4 Ethics statement

This study was undertaken with the approval of the University of Sydney Ethics Review Committee (Protocol No 2012/1076) and The Ningxia Medical University Human Ethics Committee (approval date 6/6/2013). Written informed consent was obtained prior to their enrolment in the study.

4.2.5 Study design

A multi-phase, cohort study was designed to identify plasma miRNA as surrogate markers for pulmonary TB. In NHAR, all patients suspected of having TB are referred to specific TB clinics in the provincial towns for formal diagnosis and commencement of treatment. These clinics are funded by a WHO initiative for TB control. All clinics currently implement the DOTS strategy for diagnosis and treatment, involving routine sputum tests and x-rays, as well as sputum cultures and computerised tomography (CT)-scans if additional information is required for diagnosis. As part of the programme, all medication is provided free of charge to the patient. Part of the DOTS strategy is directly observing patients as they are taking their TB medication so to ensure treatment compliance and improve outcomes. The district nurse or a community representative routinely carries out this essential part of treatment. Patients enrolled were required to attend regular follow-up clinics to determine the efficacy of treatment and to monitor their recovery.

Patients who were diagnosed with TB for the first time and had not commenced treatment were invited to take part in this study. In total, we aimed to recruit 100 cases and 100 controls in NHAR, and follow these cases through the course of their treatment. Patients were asked to peer-nominate age- and sex-matched controls. Prior

to enrolment it was emphasised to the patient that they were under no obligation to take part and that it would not affect their treatment should they refuse to participate. They were informed as to the nature and scope for the project and their role and rights as a participant prior to providing written consent.

Patients were compensated with 10 Yuan to cover the return bus fare to the clinic. Control participants were compensated with 10 Yuan to cover the return fare to the clinic. In addition, 50 Yuan was reimbursed for the time that they had to spend away from their work (loss of wages).

All study documents, including Patient Consent, Patient Questionnaire, and Ethics Clearance in both English and Chinese can be viewed in Appendix C.

4.2.6 Rationale for study design

We aimed to collect a total of four samples from the pulmonary TB patients:

1. Time Point A - at the time of diagnosis prior to or within 24 hours of commencing treatment
2. Time Point B - one month after commencement of treatment
3. Time Point C – two months after commencement of treatment
4. Time Point D – six months after commencement of treatment

One blood sample was collected from healthy subjects.

We aimed to measure the miRNA expression over the course of each TB patient's treatment. Time point A was chosen with the aim of identifying miRNAs that were significantly regulated in TB compared to healthy subjects prior to the instigation of

anti-tuberculous chemotherapy. Identifying significantly regulated miRNAs at this time point opens up the possibility of utilising the regulated miRNAs as a diagnostic biomarker.

Time point B was chosen as we sought to gain a better understanding of miRNA expression after treatment commencement. At present sputum-smear conversion to acid-fast bacillus (AFB)-negative is a surrogate used after two months of therapy to determine if treatment is working or not. This poses two major problems:

1. AFB identification and later *M. tuberculosis* culture is not achievable in all patients (in this cohort only 33 out of the 100 subjects had AFB detected in their sputum). In cases of extra-pulmonary disease it is not possible to collect a two-month sample for testing and therefore determination of treatment success is by clinical assessment only.
2. Getting results from the two-month culture often takes six to eight weeks. During this time, if the patient is not improving (often a sign of drug-resistance) there is ongoing transmission of disease to others and further amplification of drug resistance as the patient remains on inadequate therapy.

We wished to determine the nature of miRNA expression in patients on treatment and to determine if those that ultimately failed treatment could be identified early during the course of their therapy.

Time Point C marks the end of the intensive phase of a patient's therapy. After this time the patient's medications are usually reduced from the standard four-drug regime (isoniazid, rifampicin, ethambutol and pyrazinamide) to two drugs (isoniazid and rifampicin) for the remainder of their treatment (usually total of six months of

treatment). This reduction of therapy is not usually done until the results of the two-month sputum culture are known. As mentioned, this can often take six to eight weeks and is not possible for many patients as highlighted above. In some cases, where culture is not possible and drug-sensitivity profiles of the organism are not known, patients will often remain on four-drug chemotherapy for the duration of treatment. In those with fully sensitive *M. tuberculosis*, this is ultimately unnecessary. The continuation of ethambutol and pyrazinamide increases the risk of side effects which is particularly important with ethambutol as toxicity is driven by cumulative dosing (Griffith et al., 2005). Ethambutol can cause serious ocular toxicity, manifested by optic or retrobulbar neuritis, which can result in irreversible blindness (Griffith et al., 2005). Identifying a biomarker that is a point-of-care tool, which can predict early treatment failure or success, would reduce the unnecessary continuation of drugs in those who do not require them and therefore reduce potential side effects relating to those agents and again, as highlighted above, recognise those failing therapy earlier than current methods. A reliable biomarker at both one- and two-month time points may also be useful in new drug and vaccine development as it would allow treatment efficacy to be examined earlier than what is currently possible.

The final collection at six months (Time Point D) was chosen as most cases of pulmonary TB are treated for six months. We hypothesised that if a patient achieved treatment success, their miRNA profile would return to healthy control baseline.

Studying miRNAs over a time course improves the understanding of their biology and to date has not been reported in the literature.

4.2.7 Data collection

Patients diagnosed with TB between June 2013 and May 2014 were invited to participate in the study. All new cases of TB were reported to the Division of Tuberculosis Control of the Department of Public Health of NHAR. There is a provincial level electronic reporting system that was introduced in 2005 to NHAR. Data collected at the time of diagnosis included age, sex, ethnicity and domicile. Clinical information included:

- Date of diagnosis
- Clinical site/sites of disease
- Result of microbiology tests (sputum smear, culture and drug sensitivity (DST) profile)
- Chest radiography both at time of diagnosis and at completion of treatment
- Treatment regime
- Treatment outcome.

4.2.8 Data storage

Records from the clinic were collected for each patient with regard to their diagnosis, relevant medical history, culture DST results, demographic data and exposure. These data were pseudo-anonymised so that the samples were not able to be identified by the researcher. Each sample was allocated a unique index number, and only the original provider of the information, who was not directly carrying out the research outlined, had the means of identifying individuals. The electronic databases

containing the recoded patient data were locked so that only authorised individuals directly involved in the project could access the information.

4.2.9 Case management

Diagnosis of TB is made based on clinical grounds, sputum smear and culture and chest radiography. Following diagnosis, patients are commenced on DOTS which consists of short course chemotherapy comprising an intensive two-month phase of four drugs followed by four-month continuation phase of two drugs, as outlined above. This is in alignment with the current WHO guidelines for the management of treatment naïve pulmonary TB with either sputum smear/culture positivity and/or chest radiography highly suggestive of active pulmonary disease (World Health Organization, 2010b).

4.2.10 Patients and control subjects

For the pilot study, 20 newly diagnosed patients with pulmonary TB and 20 age-matched controls, were recruited. Consenting patients were enrolled consecutively from each of the nominated enrolment centres. The patients with the following criteria were included: age greater than 18; no previous history of treated or untreated TB; and, not known to be infected with HIV. Diagnosis of TB was made on clinical grounds, sputum smear results, bacterial culture and radiographic findings.

For the validation study, we enrolled 100 patients with either microbiologically proven pulmonary TB or those with suspected pulmonary TB based on clinical

suspicion coupled with chest radiograph consistent with active disease. Patients were enrolled at the time of the diagnosis and prior to the commencement of anti-tuberculous chemotherapy. The same inclusion criteria as for the pilot study was followed.

For both the pilot and validation studies, TB participants were asked to peer-nominate controls. They were asked to take an information sheet home and to invite friends/neighbours to participate in the study. The invitee should have no history of TB, be of similar age, sex and ethnicity and, if they agreed to participate, they would join the patient on his/her next clinical follow-up.

Following written formal consent, 10 mL of blood was drawn into EDTA tubes and processed as described in Section 2.2.2 of the Materials and Methods Chapter. Plasma samples were stored at -20°C at the collection centre and then, in batches, transported to Yinchuan Medical University where they were stored at -80°C.

4.2.11 Establishing the study

Prior to the commencement of the study a site visit to Ningxia was undertaken. Visits to all participating sites were carried out to ensure adequate facilities such as a suitable centrifuge and adequate storage existed for samples. During this time we met the key personnel who would be assisting in the study. An educational seminar was given to all staff that would be involved in the study, outlining the project with our aims and expected outcomes based on previous miRNA biomarker studies in other diseases. A Standard Operating Procedure was prepared (Appendix D), translated into Chinese Mandarin and discussed during the visit.

A person in NHAR was employed to oversee the running of the study. The job of the study assistant was to support and assist in the training of staff, manage the database that recorded enrolments, results of diagnostic tests such as sputum smear and radiography, and ensure the safe transport of samples from the provinces to Yinchuan Medical University. She also made available chest radiographs of patients prior to the commencement of their treatment and at the time of completion of therapy. Frequent contact was made with this person, either when visiting PRC or via email and Internet video chat (Skype) during the course of enrolment and collection of samples.

During the second visit, a training day was organised. It aimed to teach and train the staff that would ultimately be involved with consenting the patients, collecting and processing the samples and recording the necessary details of each person enrolled. One of the most important elements covered during this training workshop was the importance of obtaining written informed consent from those eligible to enrol into the study. The patient was made aware of their right to not participate or withdraw from the study and that such an action would in no way influence their treatment.

During subsequent visits the study progress was reviewed and any difficulties or problems that the team might be experiencing in relation to any part of the study was discussed and appropriately dealt with. Samples were transported back to Australia frozen with Techni Dry Ice Packs (Techni Ice, Victoria, Australia). Permission was obtained from the relevant Chinese authorities to export the samples and documentation was presented to Australian Customs to allow the importation of

plasma samples. Following arrival into Australia, samples were transported frozen and then stored at -80°C at Centenary Institute until required for further processing.

4.2.12 Isolation of plasma from human samples

Ten mL was collected from each donor and plasma collected as explained in Section 2.2.2 of the Materials and Methods.

4.2.13 RNA extraction and quality control

Total RNA was extracted using miRCURY™ RNA Isolation Kit (Exiqon) as described in Section 2.3.3 of the Materials and Methods. Quality control was undertaken for pilot study utilising the miRCURY™ QC PCR panel as described in Section 2.4.7 of the Materials and Methods.

4.2.14 qRT-PCR

Reverse transcription was carried out using miRCURY™ Universal RT cDNA Kit (Exiqon) as described in Section 2.4.7 of the Materials and Methods.

RNA for the training set was loaded onto Plasma Focus miRNA PCR Panels (Exiqon) with a list of all miRNAs measured in Appendix A. qRT-PCR was performed on LightCycler®480 (Roche Life Sciences) as described in Section 2.4.7 of the Materials and Methods.

RNA for the validation set was loaded onto custom-made qRT-PCR PCR Panels (Exiqon) with the following miRNAs measured: miR-21-5p; -99b-5p; -29a-5p; -223-5p; -221-3p; -146a-5p; -26a-5p; -28-5p; -133a; and, -652-3p with miR-93 acting as a reference miRNA. qRT-PCR was performed on LightCycler®480 (Roche) as described in Section 2.4.7.4 of the Materials and Methods.

4.2.15 Statistical analysis

miRNA expression was determined by qRT-PCR with data normalised to miR-93. miR-93 was chosen as a reference miRNA based on our findings from Chapter Three. The Cq is defined as the fractional cycle at which the fluorescence exceeds the defined threshold. The relative expression levels of each target miRNA were calculated according to the difference in Cq values between the target miRNA and miR-93 using the $2^{-\Delta\Delta CT}$. Data were logarithmically transformed to achieve a normal distribution before a Student's *t*-test was used for comparisons between two groups. P-values less than 0.05 were deemed significant.

For the validation set, miRNA expression was determined by qRT-PCR with miRNA-93 as the reference miRNA. The relative expression of each miRNA was calculated using the 2(-ΔΔC(T)) method. Further analysis was undertaken in GraphPad Prism 6 (Graphpad Software Inc., San Diego, CA, USA) and SPSS Predictive Analytic Software V.21 (IBM Corp, Chicago, Illinois). Student's *t*-tests along with descriptive statistics, linear and logistic regression were performed and receiver operating characteristic (ROC) was generated. A one-way analysis of variance (ANOVA) was performed to determine differences in miRNA expression within the TB cohort over the four collection points.

Following consultation with the Sydney University Medical School Statistician, it was thought impossible to perform a power calculation to determine an adequate sample size. Due to the nature of miRNAs with the effect of most variables, such as age, sex, ethnicity, time of day of sampling and other factors, still unknown it was deemed impossible to do an accurate power calculation in order to determine an adequate sample size. We instead examined the literature to determine sample sizes used in other miRNA work. For both the pilot and validation study, our sample size was much larger than previously published works.

4.3 Results

4.3.1 Pilot study

4.3.1.1 Patient and Control Subjects

A summary of patient characteristics is outlined in Table 4.1. In total, 20 patients with newly diagnosed TB, with an average age of aged 46 (range 18-69) were recruited between June 2013 and September 2013. Patients and their matched controls were recruited from Tongxin and Yuanzhou districts in the province of Ningxia. Most patients and their matched controls were of Hui ethnicity with just one pulmonary TB case and one healthy control of Han ethnicity. There were ten men (50%). The healthy controls had 50% men with an average age of 37/(18-67). 50% of patients enrolled had a diagnosis based on sputum smear that was positive for AFB; the remainder were diagnosed based on clinical grounds with symptomatology suggestive of pulmonary TB coupled with chest radiography in keeping with the diagnosis.

4.3.1.2 QC Panel

All 20 pulmonary TB and healthy control samples were run on QC Panel (as described in Section 2.4.7.3 of Materials and Methods) prior to proceeding to the focus panels. Following the QC analysis six samples from the healthy control group and one sample from the pulmonary TB group were eliminated due mainly to haemolysis of the sample. This meant that in total the plasma from 19 patients with newly diagnosed pulmonary TB and 14 healthy controls were analysed.

4.3.1.3 PCR Focus Panel

Among the 175 miRNAs studied, 87 with significantly altered expression between pulmonary TB patients and healthy controls by qRT-PCR were identified, of which 69 were down-regulated and 18 were up-regulated in pulmonary TB patients (Table 4.2 and Table 4.3 respectively).

Of the 87 significantly regulated miRNAs, ten miRNAs were selected to examine in the validation set. These miRNAs were chosen based on a mean fold change ≥ 1.5, a p-value < 0.01, strength of expression in the assay with Cq values < 35, and with miRNAs previously reported in the literature to be regulated in the setting of TB, other infections or known to be important in macrophage function were of particular interest. Of the ten miRNAs chosen, four were up-regulated and the remaining down-regulated in pulmonary TB (Figure 4.2 and Table 4.4).

Table 4-1. Patient characteristics for test and validation studies.

			TB		Healthy Controls	
			Test Set	Validation Set	Test Set	Validation Set
Total Number			20	100	20	100
Gender (male/female)			10/10	58/42	10/10	57/43
Age (years, average; range)			46 (18-69)	43 (19-91)	37(18-67)	35(18-78)
Ethnicity		**Han**	1	42	1	46
		Hui	19	58	19	54
Region	**Yuanzhou**	**Han**	1	17	1	9
		Hui	15	32	15	24
	Zongwei	**Han**	-	22	-	30
		Hui	-	0	-	0
	Penyang	**Han**	-	3	-	7
		Hui	-	6	-	5
	Tongxin	**Han**	0	0	0	0
		Hui	4	20	4	25
Sputum Smear Positive			5	33		
Sputum Culture Positive			5	10		
Culture DST			2	10		
Chest X-ray			20	100		
Treatment success (%)			14 (60%)	89 (89%)		

Table 4-2. Down-regulated miRNAs in plasma of pulmonary tuberculosis patients compared with healthy donors.

miRNA	Fold change	p-Value
let-7a	-2.15	0.002
let-7c	-2.02	0.006
let-7d	-2.24	0.005
let-7d*	-2.08	<0.001
let-7e	-3.08	<0.001
let-7f	-2.53	<0.001
let-7g	-1.50	0.003
let-7i	-1.42	0.045
miR-103	-2.22	0.005
miR-106b*	-2.71	0.004
miR-107	-2.11	0.007
miR-126	-1.93	0.001
miR-127-3p	-2.17	0.040
miR-128	-1.80	0.037
miR-133a	-2.94	0.002
miR-133b	-3.42	0.005
miR-139-5p	-2.62	<0.001
miR-140-5p	-2.16	0.001
miR-142-3p	-3.59	<0.001
miR-146a	-2.29	0.002
miR-148a	-1.55	0.047
miR-148b	-1.67	0.018
miR-151-3p	-2.35	0.001
miR-151-5p	-2.38	<0.001
miR-152	-2.50	<0.001
miR-15b	-1.82	<0.001
miR-17	-1.26	0.023
miR-181a	-1.84	0.004
miR-182	-2.01	0.033
miR-186	-1.42	0.003

miR-18a	-1.37	0.028
miR-18b	-1.39	0.046
miR-197	-1.91	0.013
miR-1974	-3.02	0.003
miR-199a-3p	-1.97	0.031
miR-20a*	-3.07	0.002
miR-21-5p	-2.28	<0.001
miR-223*	-1.83	0.011
miR-23a	-2.25	<0.001
miR-23b	-2.28	<0.001
miR-24	-2.48	<0.001
miR-26a	-2.10	0.001
miR-26b	-1.41	0.032
miR-27a	-1.61	0.015
miR-28-3p	-2.66	<0.001
miR-28-5p	-2.93	<0.001
miR-301a	-1.45	0.043
miR-30d	-1.58	0.002
miR-30e	-1.39	0.025
miR-30e*	-1.95	0.001
miR-320a	-1.98	<0.001
miR-320b	-1.90	0.001
miR-328	-1.78	0.016
miR-335	-1.69	0.015
miR-338-3p	-1.88	0.049
miR-361-3p	-2.68	0.019
miR-374a	-1.83	0.001
miR-374b	-2.18	0.002
miR-421	-2.04	0.005
miR-425*	-1.74	0.029
miR-484	-1.54	0.004
miR-505	-2.25	<0.001
miR-532-5p	-1.69	0.014

miR-574-3p	-1.66	0.043
miR-584	-2.59	0.001
miR-590-5p	-1.53	0.019
miR-652	-1.73	0.020
miR-766	-2.24	0.001

All data normalised to miR-93. Fold change calculated using ΔΔCT method. p-value calculated using Student's *t*-test (normal distribution assumed as data logarithmically transformed).

Table 4-3. Up-regulated miRNAs in plasma of pulmonary tuberculosis patients compared with healthy donors.

miRNA	Fold change	p-Value
miR-125a-5p	2.83	<0.001
miR-145	1.81	0.023
miR-191	2.09	0.001
miR-191-3p	2.26	0.001
miR-20a*	2.28	<0.001
miR-2110	1.78	0.037
miR-221	2.56	0.003
miR-223	2.09	0.002
miR-29a*	2.79	0.006
miR-30b	1.93	0.003
miR-30c	2.18	<0.001
miR-324-5p	2.06	0.006
miR-339-3p	2.35	0.002
miR-339-5p	2.05	0.040
miR-423-3p	2.61	0.001
miR-423-5p	1.77	0.019
miR-423*	1.69	0.040
miR-720	2.66	0.004
miR-99b	2.54	<0.001

All data normalised to miR-93. Fold change calculated using ΔΔCT method. p-value calculated using Student's *t*-test (normal distribution assumed as data logarithmically transformed).

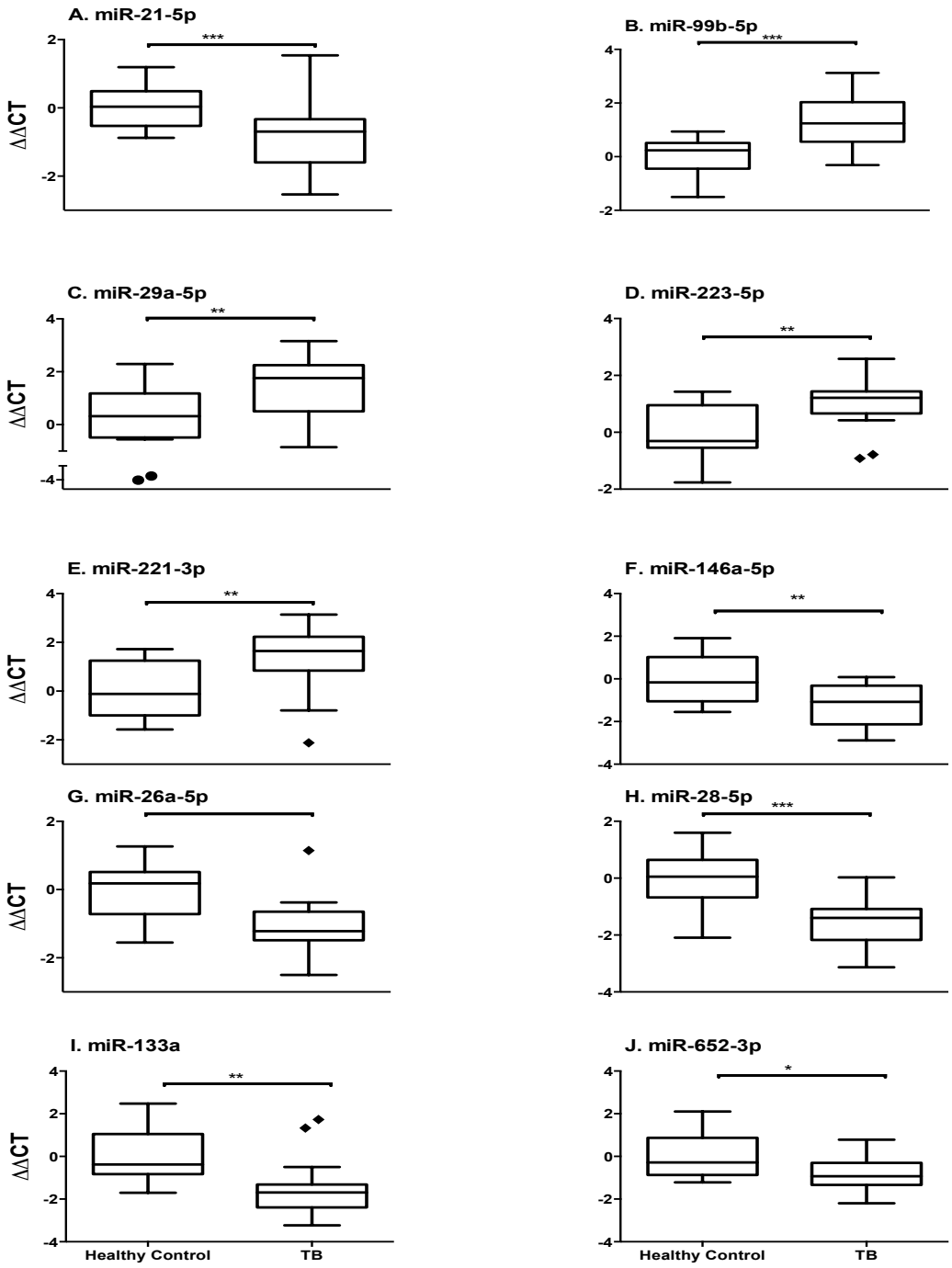


Figure 4-2. Plasma levels of miRNAs in test set of 19 TB patients and 14 healthy controls. Expression levels of miRNAs from 19 TB patients prior to commencement of treatment and 14 healthy controls were measured by using miR-93 as a reference miRNA. Significant up-regulation of miR-99b-5p, -29a-5p, 223-5p and -221-3p (B, C, D, E) and down-regulation of miR-21-5p, -146a-5p, -26a-5p, -28-5p and -652-3p was observed. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results are depicted using a boxplot with median and interquartile range within the box and +/- 1.5 interquartile range to the inner fences. Outliers are plotted individually. The differences were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001.

Table 4-4. Selected candidate miRNAs from test phase result.

miRNA	Regulation	Fold Change	p-Value	Sequence
21-5p	Down	-2.28	<0.001	UAGCUUAUCAGACUGAUGUUGA
99b-5p	Up	2.54	<0.001	CACCCGUAGAACCGACCUUGCG
29a-5p	Up	2.79	0.006	ACUGAUUUCUUUUGGUGUUCAG
223-5p	Up	2.09	0.002	CGUGUAUUUGACAAGCUGAGUU
221-3p	Up	2.56	0.003	AGCUACAUUGUCUGCUGGGUUUC
146a-5p	Down	-2.29	0.002	UGAGAACUGAAUUCCAUGGGUU
26a-5p	Down	-2.10	0.001	UUCAAGUAAUCCAGGAUAGGCU
28-5p	Down	2.93	<0.001	AAGGAGCUCACAGUCUAUUGAG
133a	Down	-2.94	0.002	UUUGGUCCCCUUCAACCAGCUG
652-3p	Down	-1.73	0.020	AAUGGCGCCACUAGGGUUGUG

4.3.2 Validation set

4.3.2.1 Patient characteristic

A total of 100 patients with newly diagnosed pulmonary TB who were treatment naïve were successfully followed for six months, together with matched healthy controls (Table 1.1). For the TB group, plasma samples were collected at four time points with plasma miRNAs measured at each point. The mean age for TB cases was 43 (19-91) and 35 (18-78) for healthy controls. 57% were male in the TB group and 59% male in the healthy control group. There were 42 (42%) Han Chinese and 58 (58%) Hui Chinese in the TB group and 46 (46%) Han Chinese and 54 (54%) Hui Chinese in healthy subjects. In the TB group 33 were smear positive for AFB (Ziehl-Neelsen staining). All sputums found to be positive for AFB were sent for culture on two slopes of Lowenstein Jensen medium per specimen. Of the 33 sent for culture, five were found to have resistance to at least one first line agent with one patient resistant to isoniazid and rifampicin (multi-drug resistant). 89 were successfully treated, with 11 classified as treatment failure based on radiological progression or end-of-treatment sputum results.

4.3.2.2 Newly diagnosed TB compared with healthy controls

We examined the ten selected miRNAs that were identified in the test study as significantly regulated in 100 patients with pulmonary TB and their matched healthy controls. miRNAs were normalised to miR-93. At Time Point A, prior to the commencement of treatment we set out to explore whether there was any statistical significant differences between miRNA expression in patients with *M. tuberculosis* infection and their matched healthy controls. Graphical representation of relative

expression of miRNA is presented in Figure 4.3. In order to explore this, we first performed a Student's *t*-test. This found that miR-21-5p, -99b-5p, -29a-5p, -146a-5p and -652-3p were specifically discordantly regulated. Specifically, TB patients were found to have significantly lower means with respect to miR-21-5p, -146a-5p and -652-3p, meaning miRNAs were down-regulated, while miR-99b and -29a-5p were significantly up-regulated with statistically significant higher mean values (Table 4.5).

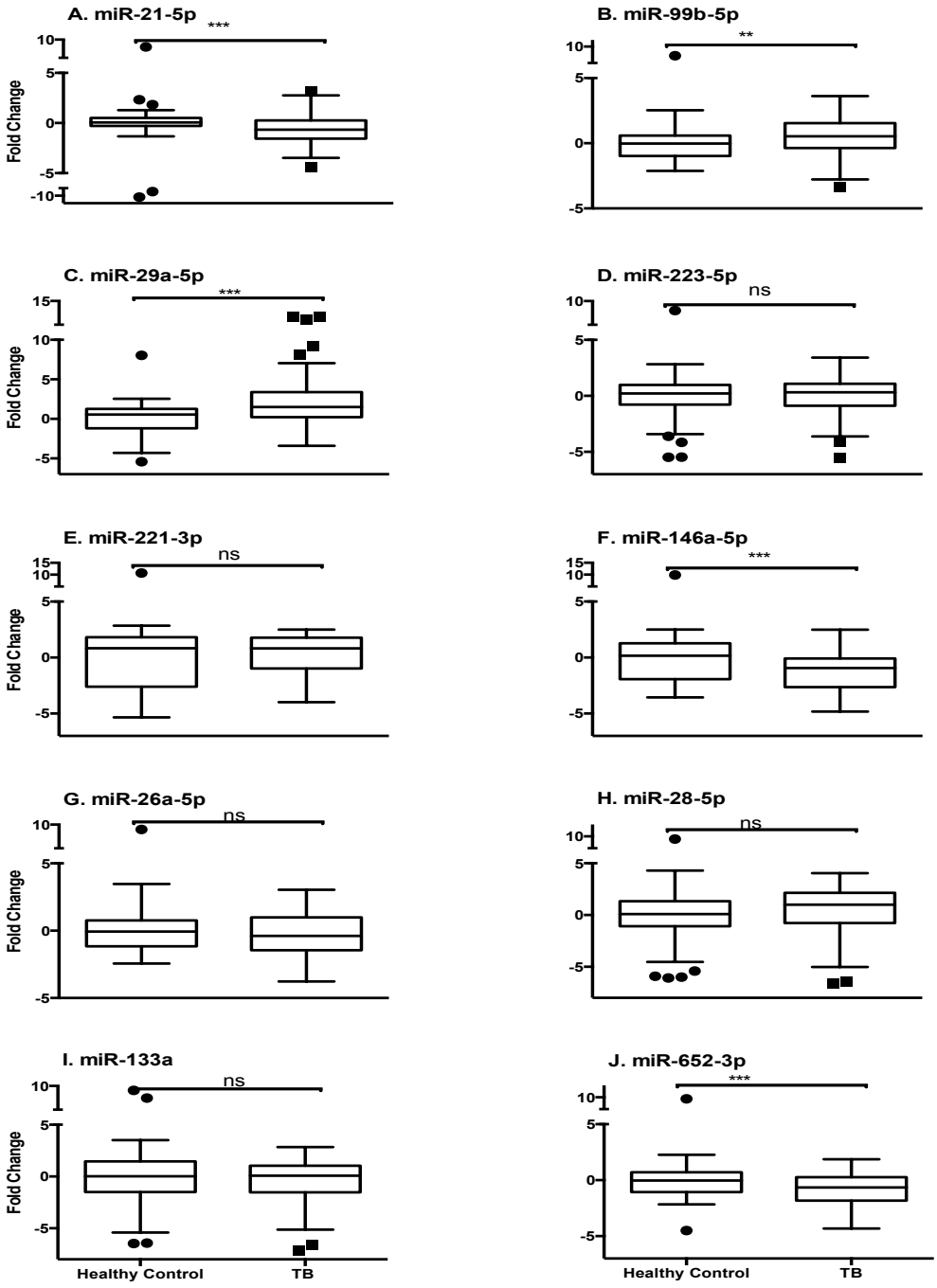


Figure 4-3. Plasma levels of miRNAs in validation set of 100 treatment naïve TB patients prior to therapy and 100 healthy controls. Expression levels of miRNAs from 100 TB patients prior to commencement of treatment and 100 healthy controls were measured by qRT-PCR using miR-93 as a reference miRNA. Significant up-regulation of miR-99b-5p and -29a-5p (B and C) and down-regulation of miR-21-5p, -146a-5p and -652-3p (A, F and J) was observed. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results are depicted using a boxplot with median and interquartile range within the box and +/- 1.5 interquartile range to the inner fences. Outliers plotted individually. The differences were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001.

Table 4-5. Expression profiles of 10 candidate miRNA on qRT-PCR in validation set from TB patients prior to the commencement of therapy.

miRNA	Fold Change	p –Value*
miR-21-5p	-1.83	**0.005**
miR-99b-5p	1.52	**0.002**
miR-29a-5p	4.09	**<0.001**
miR-223-5p	-1.01	0.767
miR-221-3p	1.47	0.176
miR-146a-5p	-2.66	**<0.001**
miR-26a-5p	-1.26	0.192
miR-28-5p	1.72	0.105
miR-133a	-1.08	0.305
miR-652-3p	-1.37	**<0.001**

To determine if any of the significantly regulated miRNAs could successfully differentiate TB from healthy controls, a ROC was constructed for the five miRNAs that were significantly regulated (Figure 4.4). miR-146a had the highest ROC of 0.69 ($p < 0.001$, 95% CI 0.62-0.76) with the others as follows: hsa-miR-29a, 0.70 ($p < 0.001$, 95% CI 0.63-0.77); hsa-miR-99b-5p, 0.64 ($p = 0.001$, 95% CI 0.56-0.71); hsa-miR-21-5p, 0.70 ($p = 0.001$, 95% CI 0.62-0.77); and, hsa-miR-652-3p 0.64 ($p = <0.001$, 95% CI 0.56-0.71)

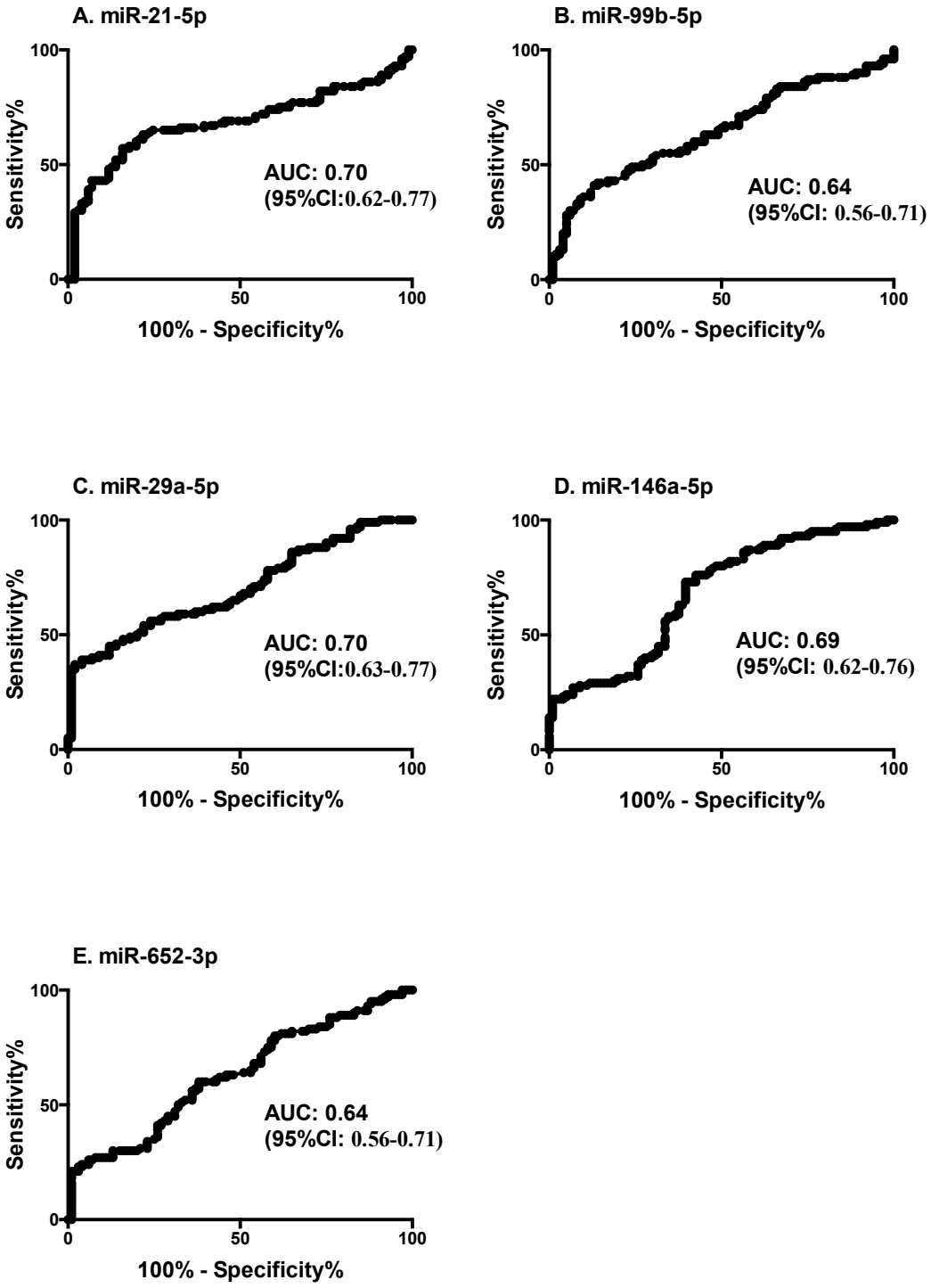


Figure 4-4. Receiver operating characteristic curves for the ability of the five individually significantly regulated miRNAs to differentiate the TB patients from the control group at Time Point A. (A), miR-21-5p, (B) miR-99b-5p, (C) miR-29a-5p, (D) miR-146a-5p and (E) miR-652-3p. AUC: area under the curve.

A good biomarker needs to be both sensitive and specific. A logistic regression analysis was performed in order to determine if measuring miRNA expression utilising a number of miRNAs would improve the accuracy. The data consisted of all healthy subjects (n=100) and all TB subjects at Time Point A (n=100) (prior to the commencement of therapy). The following regression equation was built: Logit (p) = −3.648 − 0.997*(hsa-miR-21-5p) − 2.022*(hsa-miR-99) − 1.013*(hsa-miR-29a-5p) − 0.350*(hsa-miR-223-5p) − 2.092*(hsa-miR-221-3p) + 3.791*(hsa-146a-5p) + 1.214*(hsa-miR-26a-5p) − 0.836*(hsa-miR-28-5p) + 0.314*(hsa-miR-133a) + 1.004*(hsa-miR-652-3p) (Table 4.6).

When a logistic regression is calculated, the exponential function of the regression co-efficient is the odds ratio associated with a one-unit increase in the exposure. Each unit increased (in this case miRNA expression) is associated with the odds of being in the healthy control group as compared with the TB group. Examining Table 4.6, we see that for every unit increase in miRNA expression in miR-29a-5p there is 0.363 (95% CI 0.21-0.63) chance of being in the healthy control group or inversely a 2.75 times chance of being in the TB group. Conversely, as miR-146a-5p was shown to be down-regulated in the setting of TB disease, increase expression of miR-146a is associated with a 44 times increase in the likelihood of being in the healthy control group.

Using all ten miRNAs in combination, the logistic regression equation yielded a sensitivity of 94.6%, specificity of 88.8% with a positive predictive value (PPV) of 88% and a negative predictive value (NPV) of 95%. Predictive values are critically dependent on the population chosen and the prevalence of a disease. These tests tend

to perform less well when the prevalence of disease is low. Predictive values can generally not be transferred to another setting or population. Likelihood ratios, on the contrary, are independent of disease prevalence (Attia, 2003). The positive likelihood ratio was calculated to be 8.44 meaning that, regulation of these miRNAs is associated with a nearly 8 ½ fold increase in the chance of having TB. The negative likelihood ratio is 0.06 meaning that if these miRNAs are not regulated there is a large and often conclusive decrease in the likelihood of disease (Table 4.7).

Table 4-6. Logistic regression analyses of a combination of ten plasma miRNAs for the diagnosis of pulmonary TB in validation set.

Logistic Regression Analyses			
Coefficients and standard errors			
Variable	**Coefficient**	**Std. error**	**p-value**
miR-21-5p	-0.997	0.775	0.198
miR-99b-5p	-2.022	0.600	0.001
miR-29a-5p	-1.013	0.282	<0.001
miR-223-5p	-0.350	0.303	0.248
miR-221-3p	-2.092	0.544	<0.001
miR-146a-5p	3.791	0.834	<0.001
miR-26a-5p	1.214	0.662	0.067
miR-28-5p	-0.836	0.306	0.006
miR-133a	0.314	0.209	0.133
miR-652-3p	1.004	0.709	0.157
Constant	3.648	0.627	<0.001
Logit (p) = −3.648 − 0.997*(hsa-miR-21-5p) − 2.022*(hsa-miR-99) − 1.013*(hsa-miR-29a-5p) − 0.350*(hsa-miR-223-5p) − 2.092*(hsa-miR-221-3p) + 3.791*(hsa-146a-5p) + 1.214*(hsa-miR-26a-5p) − 0.836*(hsa-miR-28-5p) + 0.314*(hsa-miR-133a) + 1.004*(hsa-miR-652-3p)			
Odds ratios* and 95% confidence intervals			
Variable	**Odds Ratio**	**95% CI**	
miR-21-5p	0.369*	0.08-1.69	
miR-99b-5p	0.132*	0.04-0.43	
miR-29a-5p	0.363*	0.21-0.63	
miR-223-5p	0.704*	0.39-1.28	
miR-221-3p	0.123*	0.04-0.36	
miR-146a-5p	44.320*	8.65-227.20	
miR-26a-5p	3.366*	0.92-12.33	
miR-28-5p	0.433*	0.24-0.79	
miR-133a	1.369*	0.91-2.06	
miR-652-3p	2.730*	0.68-10.96	

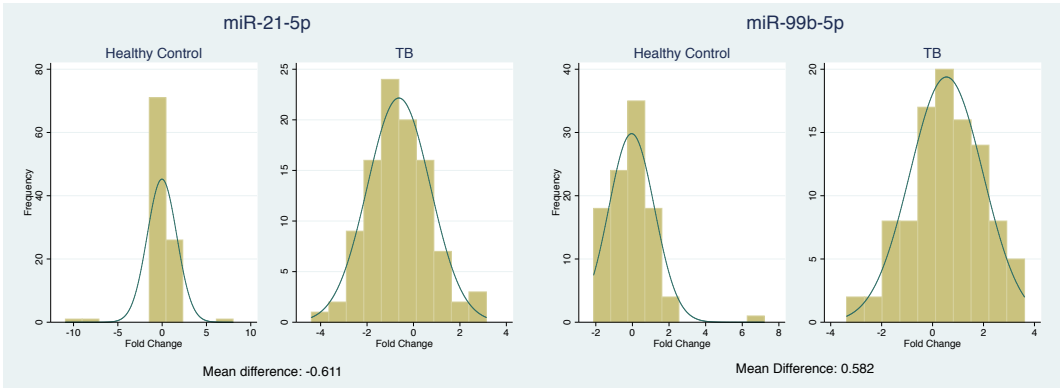
*Odds ratio (OR) can be interpreted as: each unit increase (in this case miRNA expression) is associated with an odds of being in the healthy control group category as compared with the TB group which, is increased by a factor equal to that of the OR.

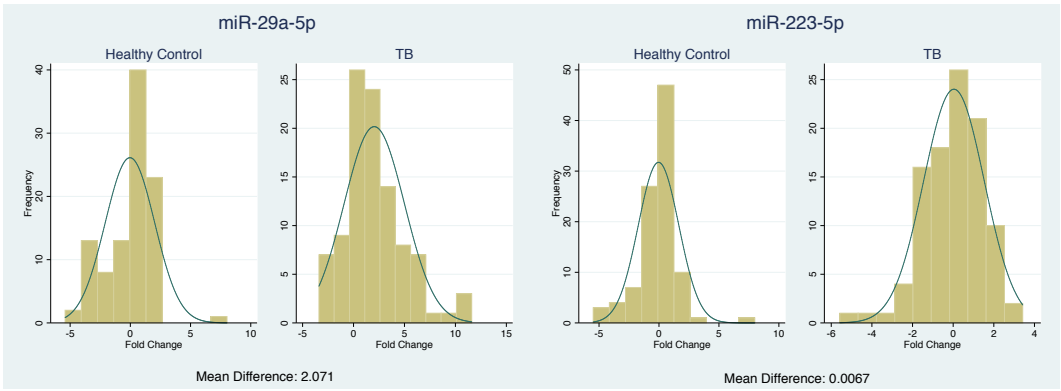
Table 4-7. Accuracy of plasma miRNAs at predicting TB prior to the commencement of treatment.

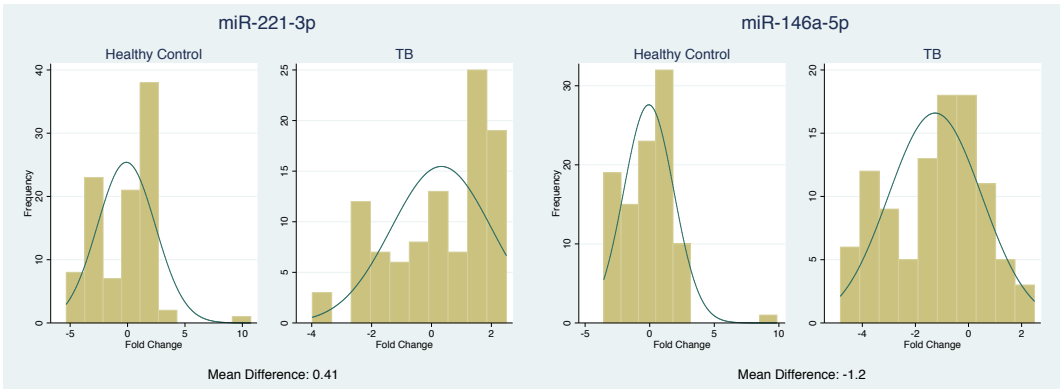
Category	Sensitivity	Specificity	PPV	NPV	PLR	NLR
TB versus Healthy Controls	94.6%	88.8%	88.0%	95.0%	8.4	0.06

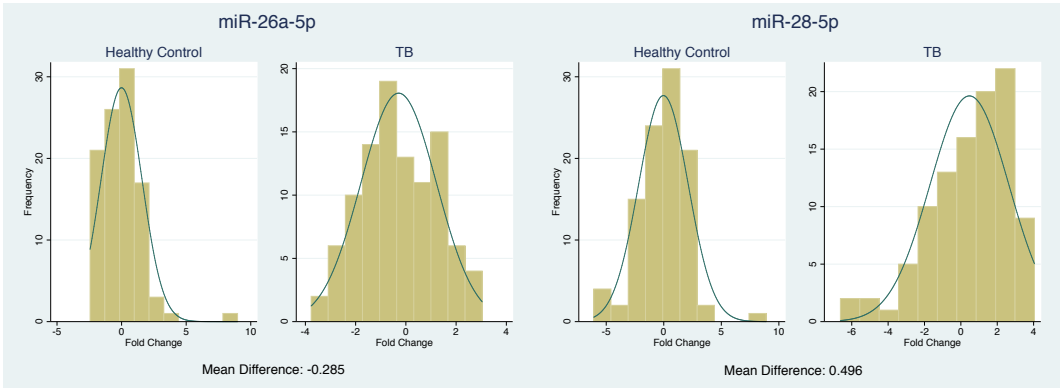
PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ration.

The logistic regression, utilising all ten miRNAs, was able to differentiate healthy individuals from TB subjects with a high degree of accuracy. Next, we wanted to determine if a cut-off that could potentially separate according to disease status was possible using a single miRNA. As shown below in a series of histograms, in Figure 4.5, this was not possible as there was an extreme degree of overlap between healthy and TB subjects with regards to these data, suggesting that these individual miRNA expression levels could not be reliably used to separate persons on this basis (Figure 4.5).


miR-21-5p
Healthy Control
TB
Frequency
Fold Change
Mean difference: -0.611
miR-99b-5p
Healthy Control
TB
Frequency
Fold Change
Mean Difference: 0.582


miR-29a-5p
Healthy Control
TB
Frequency
Fold Change
Mean Difference: 2.071
miR-223-5p
Healthy Control
TB
Frequency
Fold Change
Mean Difference: 0.0067


miR-221-3p
Healthy Control
TB
Frequency
Fold Change
Mean Difference: 0.41
miR-146a-5p
Healthy Control
TB
Frequency
Fold Change
Mean Difference: -1.2


miR-26a-5p
Healthy Control
TB
Frequency
Fold Change
Mean Difference: -0.285
miR-28-5p
Healthy Control
TB
Frequency
Fold Change
Mean Difference: 0.496

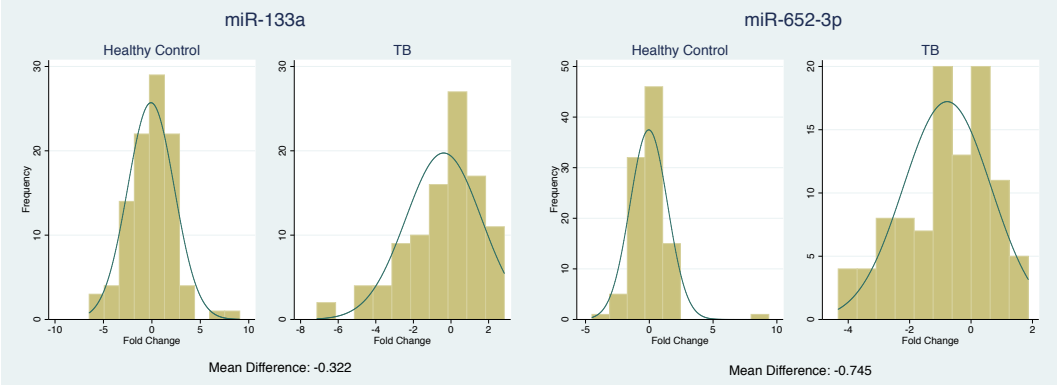


Figure 4-5. Histogram plots of miRNA expression as measured in fold change from the plasma of tuberculosis patients prior to treatment and their matched healthy controls. Expression levels of miRNAs from 100 TB patients prior to commencement of treatment and 100 healthy controls were measured by qRT-PCR using miR-93 as a reference miRNA. The mean difference reported represents the mean expression of miRNA in TB patients minus the mean expression of miRNA from healthy controls. There is considerable overlap in expression levels between TB and healthy subjects, meaning that the level of miRNA expression could not be reliably used to separate persons with TB from healthy controls.

4.3.2.3 miRNA expression after one month of TB therapy.

Next, changes in the expression of miRNA over the course of treatment for the TB patients was examined for the ten miRNAs at one month relative to levels at time point zero, before the commencement of therapy. Table 4.8 shows the fold change along with their corresponding p-value. One hypothesis being tested was that miRNAs would return to that of the baseline seen in the healthy controls with effective treatment. Examining the miRNA expression after one-month of therapy, only miR-99b fulfilled this hypothesis (Figure 4.6 B). miR-99b was significantly up-regulated in the TB subjects prior to commencement of therapy (Figure 4.3 B) and following one month of therapy, no significance was found between the TB subjects and the healthy controls (Figure 4.6 B).

There were five miRNAs that were differentially expressed after one month of therapy (Figure 4.6). miR-29a was the only miRNA to remain significantly up-

regulated. There were four down-regulated miRNAs: miR-21-5p; miR-146a-5p; -26a-5p; and, miR-652-3p (Figure 4.6 A, F G and J). Interestingly miR-26a-5p, which was found not to be significantly differentially expressed in the TB subjects compared to the healthy controls prior to the commencement of therapy, was significantly down-regulated following one month of anti-tuberculous therapy. The reason/s for this observation is unclear but may relate to a delayed inflammatory response accompanying *M. tuberculosis* infection.

Table 4-8. Expression profiles of 10 candidate miRNAs from plasma as measured by qRT-PCR in validation set from TB patients after one month of therapy.

miRNA	Fold Change*	p Value
miR-21-5p	-2.15	**<0.001**
miR-99b-5p	-1.30	0.653
miR-29a-5p	1.48	**0.031**
miR-223-5p	-1.52	0.426
miR-221-3p	1.05	0.689
miR-146a-5p	-3.04	**<0.001**
miR-26a-5p	-1.52	**0.003**
miR-28-5p	1.16	0.908
miR-133a	-1.49	0.156
miR-652-3p	-1.71	**<0.001**

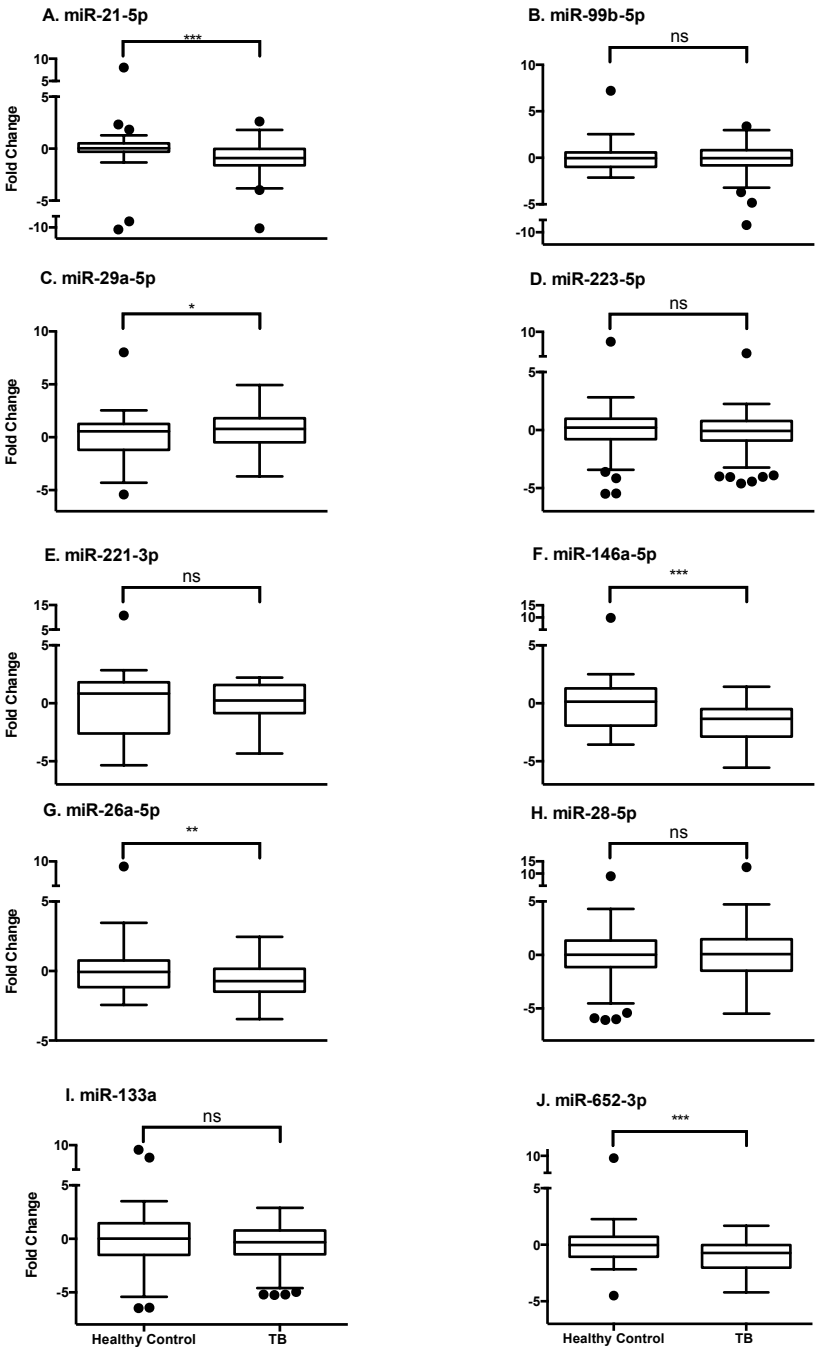


Figure 4-6. Plasma levels of miRNAs in validation set of 100 TB patients after one month of anti-tuberculous chemotherapy and 100 healthy controls. Expression levels of miRNAs from 100 TB patients after one month of treatment and 100 healthy controls were measured by qRT-PCR using miR-93 as a reference miRNA. Significant up-regulation of miR-29a-5p (C) and down-regulation of miR-21-5p, -146a-5p, -26a-5p and -652-3p (A, F, G and J) was observed. The relative expression of miRNAs was determined using $2(-\Delta\Delta C(T))$. Results are depicted using a boxplot with median and interquartile range within the box and +/- 1.5 interquartile range to the inner fences. Outliers are plotted individually. The differences were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001.

4.3.2.4 miRNA expression after two months of TB therapy

Following two months of therapy there were again five miRNAs that remained discordantly regulated. These were the same five miRNAs significantly regulated after one month of therapy (Table 4.8). The fold change along with their corresponding p-value are presented in Table 4.9. miR-29a-5p was the only miRNA to be up-regulated (Figure 4.7 C). miR-21-5p, -146a-5p, 26a-5p and -652-3p were all down-regulated following two months of therapy (Figure 4.7 A, F, G and J). miR-99b-5p expression remains not statistically different to healthy subjects (Figure 4.7 B).

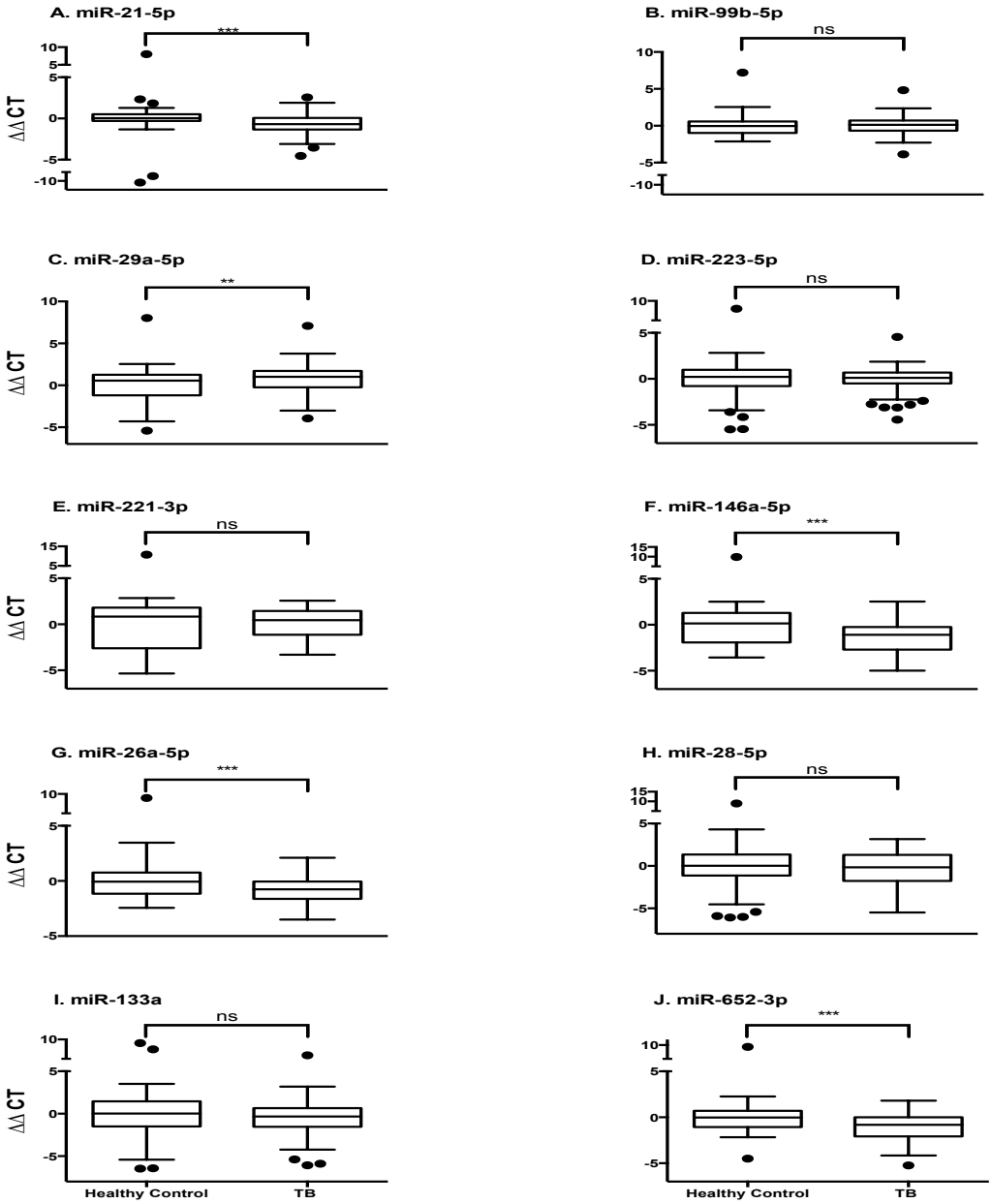


Figure 4-7. Plasma levels of miRNAs in validation set of 100 TB patients after two months of anti-tuberculous chemotherapy and 100 healthy controls. Expression levels of miRNAs from 100 TB patients after two months of treatment and 100 healthy controls were measured by qRT-PCR using miR-93 as a reference miRNA. Significant up-regulation of miR--29a-5p (C) and down-regulation of miR-21-5p, -146a-5p, -26a-5p and -652-3p (A, F, G and J) was observed. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results are depicted using a boxplot with median and interquartile range within the box and +/- 1.5 interquartile range to the inner fences. Outliers are plotted individually. The differences were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001.

Table 4-9. Expression profiles of 10 candidate miRNAs on qRT-PCR in validation set from TB patients after two months of therapy.

miRNA	Fold Change	p Value
miR-21-5p	-1.92	**0.002**
miR-99b-5p	-1.06	0.410
miR-29a-5p	1.80	**0.004**
miR-223-5p	-1.20	0.928
miR-221-3p	1.21	0.407
miR-146a-5p	-2.22	**<0.001**
miR-26a-5p	-1.33	**<0.001**
miR-28-5p	1.27	0.298
miR-133a	-1.28	0.178
miR-652-3p	-1.45	**<0.001**

4.3.2.5 miRNA expression after six months of TB therapy

Six months is the standard duration of treatment for fully sensitive pulmonary TB. Following clinical assessment, which would routinely include sputum culture for *M. tuberculosis* and chest radiography, if there are no contraindications, a patient's therapy is usually ceased. We collected plasma from all enrolled subjects following six months standard short course therapy. Fold change with the associated p value is shown in Table 4.10.

We hypothesised that miRNA levels would return to healthy control levels with treatment. Interestingly this was not the case for all regulated miRNAs. Of the six miRNAs that were regulated in the plasma of TB patients, only two returned to levels seen in the healthy control: miR-99b-5p at the one month collection time point (Figure 4.6 B); and, miR-29a-5p at the final collection point at six months (Figure 4.8 C). At the conclusion of treatment, four miRNAs remained that were

differentially modulated in the plasma of TB patients: miR-21-5p; -146a-5p; 26a-5p; and, 652-3p. (4.8 A, F, G and J). All of these miRNAs were down-regulated.

Table 4-10. Expression profiles of 10 candidate miRNAs by qRT-PCR in validation set from TB patients after six months of therapy.

miRNA	Fold Change	p Value
miR-21-5p	-2.32	**0.002**
miR-99b-5p	1.11	0.170
miR-29a-5p	1.19	0.257
miR-223-5p	-1.10	0.935
miR-221-3p	1.24	0.603
miR-146a-5p	-2.23	**<0.001**
miR-26a-5p	-1.70	**<0.001**
miR-28-5p	-1.02	0.320
miR-133a	-1.76	0.217
miR-652-3p	-1.58	**<0.001**

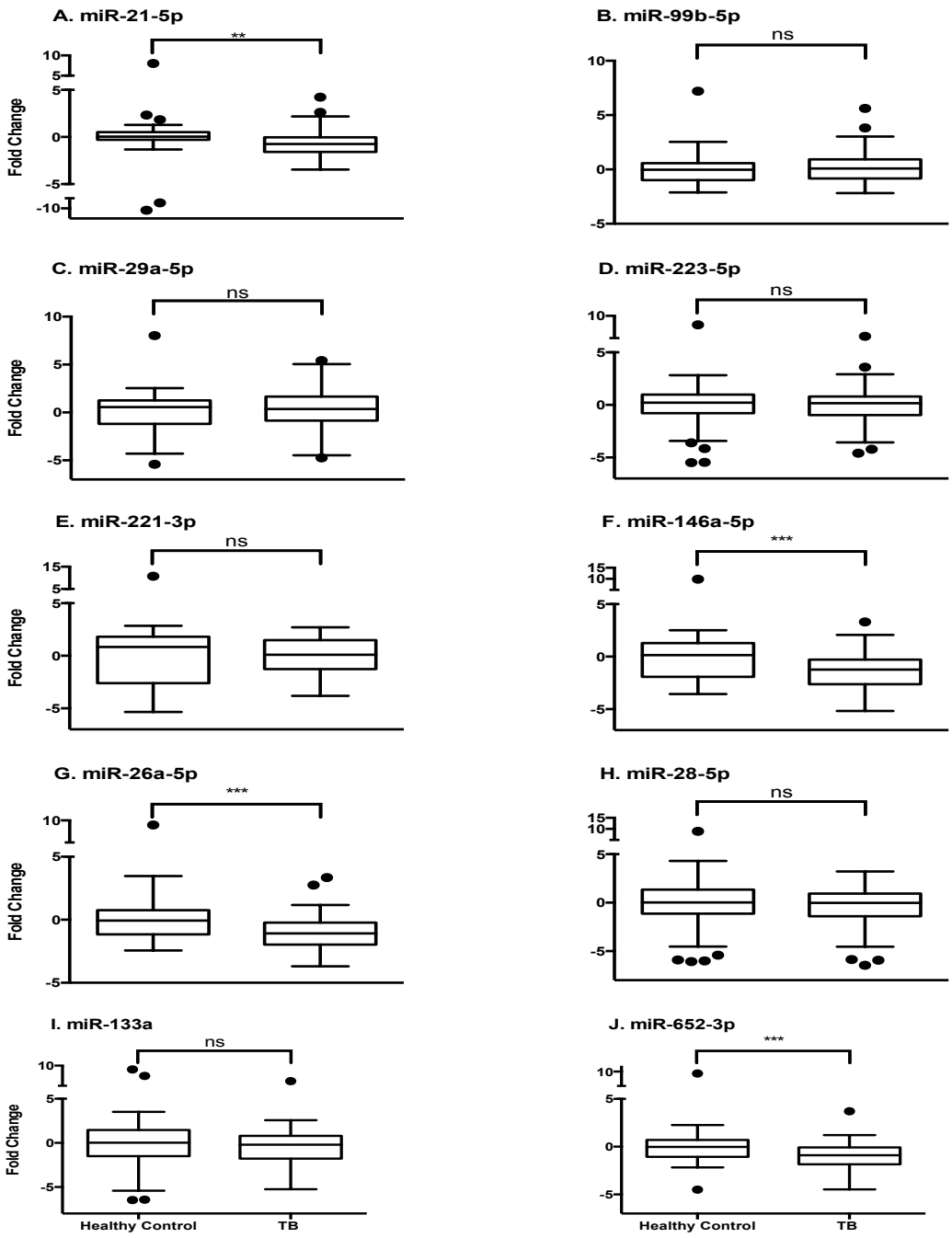


Figure 4-8. Plasma levels of miRNAs in validation set of 100 TB patients after six months of anti-tuberculous chemotherapy and 100 healthy controls. Expression levels of miRNAs from 100 TB patients after six months of treatment and 100 healthy controls were measured by qRT-PCR using miR-93 as a reference miRNA. Significant down-regulation of miR-21-5p, -146a-5p, -26a-5p and -652-3p (A, F, G and J) were observed. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results are depicted using a boxplot with median and interquartile range within the box and +/- 1.5 interquartile range to the inner fences. Outliers are plotted individually. The differences were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001.

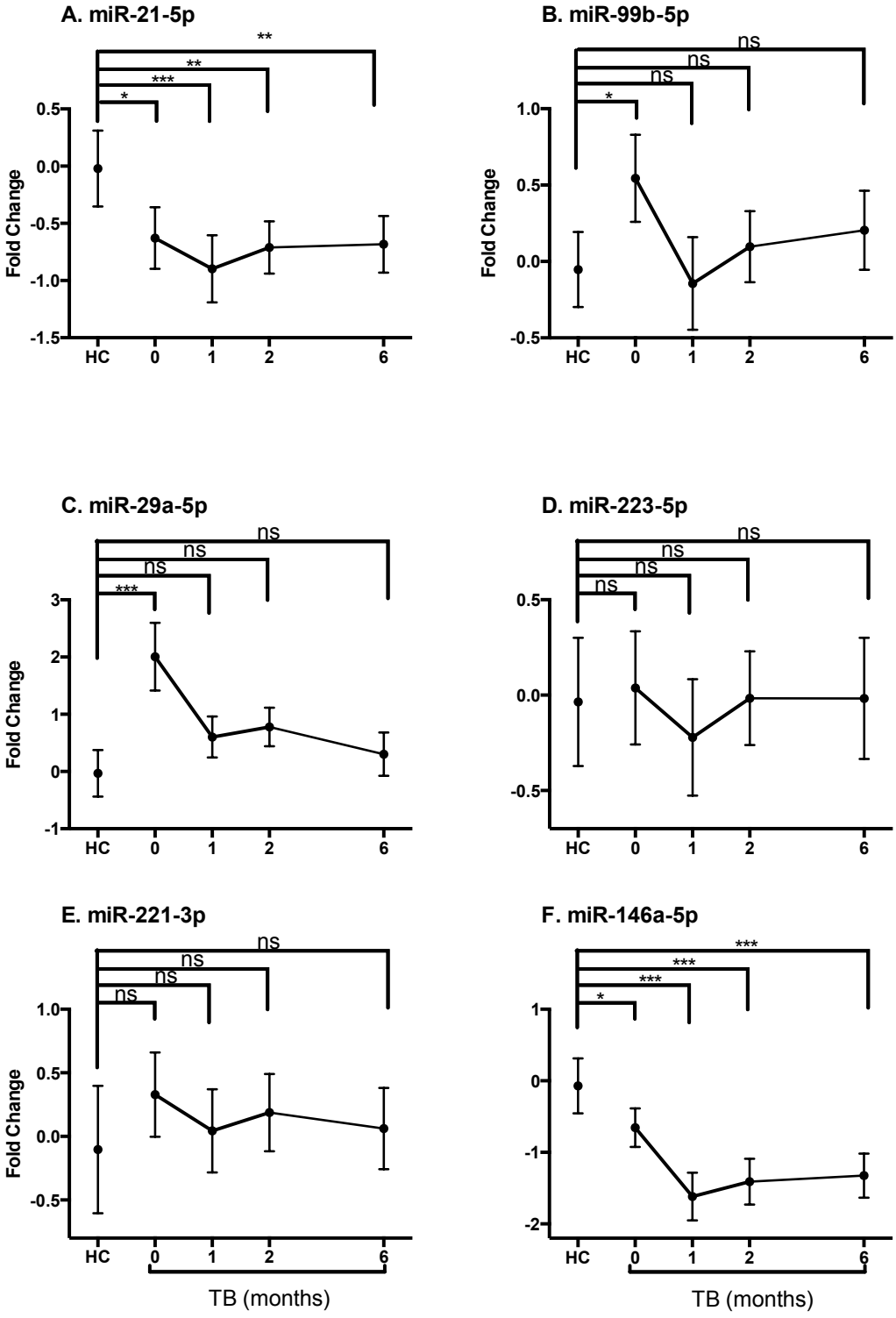
4.3.2.6 Examining the miRNAs over the six-month time course

Examining the expression levels of miRNAs over the six-month time course allows us to explore the trends in expression levels that occur with TB treatment and may help to improve current knowledge relating to the functional roles of these miRNAs. One of the hypotheses of this study was that miRNA expression would return to levels seen in healthy controls with anti-tuberculous chemotherapy. For the majority of miRNAs modulated in TB disease this was not the case. Figure 4.9 illustrates the expression of miRNAs at all collection time points and with calculated significance based on healthy control levels. Figure 4.10 documents the significance of the miRNA expression change within the TB group only.

Of the regulated miRNAs, miR-99b-5p and -29a-5p (Figure 4.9 B and C) are the only miRNAs to return to healthy control levels and they both do so after one month of therapy. The reduction in miRNA expression levels in both miRNAs is significantly less (Figure 4.10 B and C).

Some miRNAs remain persistently down-regulated and were so over the course of therapy (miR-21-5p, miR-146a-5p, 26a-5p and -652-3p) (Figure 4.9 A, F, G and J). miR-21-5p and -652-3p levels do not vary over the course of TB treatment (Figure 4.10 A and J). Whereas miR-146a-5p levels fall significantly following the initiation of therapy being the most down-regulated at one month following the commencement of therapy. The expression levels at two and six months remain low and did not vary significantly (Figure 4.10 F). miR-26a-5p expression levels in the TB subjects appears to fall throughout the course of TB treatment, however this does

not reach statistical significance until the final collection at six months (Figure 4.10 G).


A. miR-21-5p
B. miR-99b-5p
C. miR-29a-5p
D. miR-223-5p
E. miR-221-3p
F. miR-146a-5p
Fold Change
HC
0
1
2
6
TB (months)
ns
*
**
***

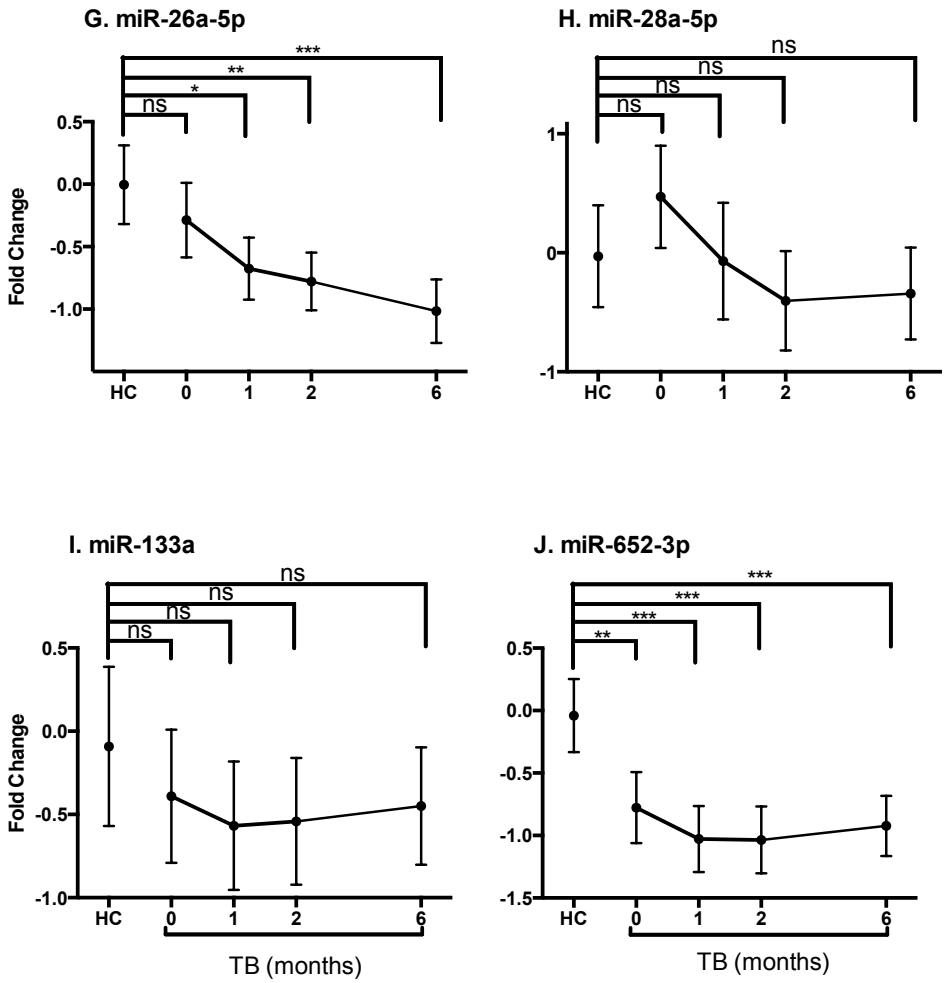
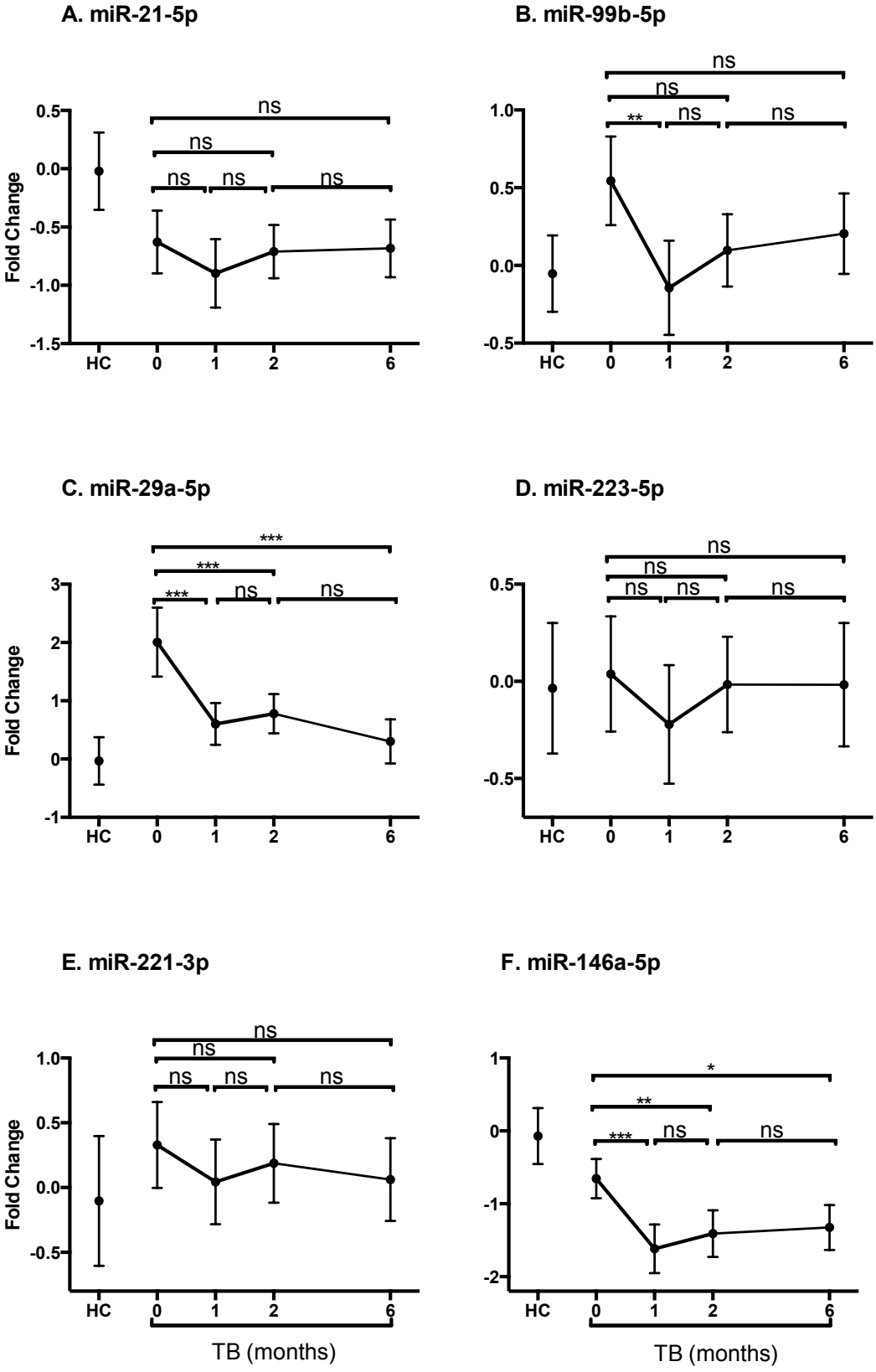


Figure 4-9. Plasma levels of miRNAs in validation set of 100 TB patients and their matched healthy controls at all collection time points. The plasma from TB subjects was collected prior to the commencement of therapy (0 months) and at one, two and six months following treatment commencement. miRNAs levels were examined by qRT-PCR with miR-93 used as a reference miRNA. miR-21-5p (A) is significantly down-regulated prior to TB treatment and remains significantly down-regulated during the course of treatment. miR-99b (B) is significantly up-regulated prior to the commencement of TB therapy and then following one month of therapy and beyond, miR-99b levels return to healthy control baseline. miR-29a-5p (C) is significantly up-regulated prior to the commencement of therapy and at one month and beyond expression levels are not significantly different to healthy controls. miR-146a-5p (F) remains significantly down-regulated throughout the six months of therapy. miR-26a-5p (G) is down-regulated which reaches statistical significance at one month measure. With time, miR-26a-5p levels reduce in TB patients despite therapy. miR-652-3p (J) is significantly down-regulated at all collection time points. miR-223, -221, -28 and -133 (D, E, H and I) are not significantly regulated in TB compared with healthy controls. The relative expression of miRNAs was determined using $2(-\Delta\Delta C(T))$. Results are depicted with mean with 95% confidence intervals. Logged data was assumed to be normally distributed. Comparisons of variables between multiple groups were done using ANOVA. In instances where p-value was < 0.05, additional two-group comparison were done by a Student's *t*-test. A p-value <0.05 deemed significant.*p <0.05, **p <0.01, ***p <0.001.


A. miR-21-5p
B. miR-99b-5p
C. miR-29a-5p
D. miR-223-5p
E. miR-221-3p
F. miR-146a-5p
Fold Change
TB (months)
HC
0
1
2
6
ns
*
**
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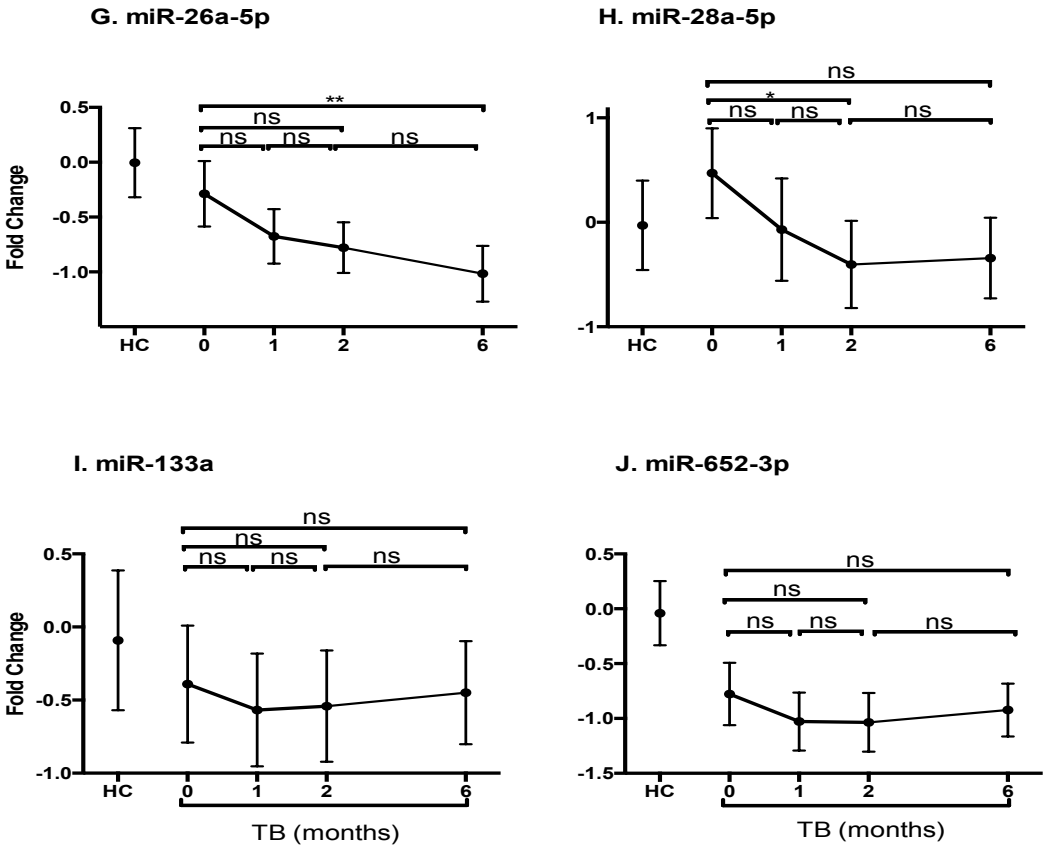


Figure 4-10. **Plasma miRNA expression levels within TB group at all collection time points.** The plasma from TB subjects was collected prior to the commencement of therapy (0 months) and at one, two and six months following treatment commencement. miRNAs levels were examined by qRT-PCR with miR-93 used as a reference miRNA. A one-way analysis of variance (ANOVA) with multiple comparisons was performed to detect significant variation in miRNA expression from the TB patients over the course of their treatment. On the far left of each graph are the healthy controls for reference. miR-21-5p (A) was significantly down-regulated in the TB patients compared with healthy controls. Over the course of therapy miR-21 remained relatively unchanged with no statistical significant variation. miR-99b-5p (B) was up-regulated in the TB patients prior to the commencement of therapy. After one month of treatment there has been a significant fall in miR-99b-5p levels, where the levels remain. miR-29a-5p (C) was also significantly up-regulated prior to the commencement of therapy. After one month of treatment plasma levels were significantly reduced. Levels of miR-146a-5p (F) were significantly reduced in the plasma of TB patients and further significantly reduced following the commencement of therapy. Levels of miR-26a-5p (G) reduced over the course of treatment, reaching significance at the six-month time point. miR-28a-5p (G) was up-regulated in the TB patients at Time Point A, although did not reach statistical significance. Following one month of therapy there was a significant fall in miR-28a-5p (H) levels in the TB subjects. miR-21-5p, -223-5p, -221-3p, -133a and -652-3-p miRNA expression did not change significantly with treatment (A, D, E,I and J). p-value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001.

4.3.3 Sub-group analysis

To explore whether other factors may be influencing miRNA expression we looked more closely at treatment outcome (success versus failure), ethnicity (Han versus Hui), and sex.

4.3.3.1 Treatment failure versus treatment success.

One of the secondary aims of this study was to determine if the miRNAs that we selected would be useful to predict treatment failure in early stages of a patient's course of therapy. It was interesting to observe that miRNA expression in the TB cohort remained discordantly modulated in four of the six significantly regulated miRNAs. However, not all patients that were treated were treated successfully. It was not clear what the effects of the treatment failure subjects were having on overall miRNA expression from the whole TB cohort. To explore this further we divided the TB cohort according to their outcome. Of the 100 patients treated for six months, 11 failed treatment and 89 were successfully treated. Given the small number of patients that were deemed to have failed treatment, caution will need to be taken when analysing the data, particularly from the treatment failure group.

We examined the expression of the ten miRNAs stratified according to treatment outcome below. Comparisons of variables between multiple groups were done using ANOVA as normal distribution was assumed due to logged data. In instances where the ANOVA p-value was <0.05, additional two-group comparisons were done using a Student's *t*-test. A p-value <0.05 was deemed significant. A pictorial of all miRNA expression based on treatment outcome as well as associated fold change and p-value is presented in Figure 4.11.

4.3.3.1.1 miR-21-5p

miR-21-5p (Figure 4.11 A) is down-regulated during TB disease and this was maintained when stratifying according to treatment outcome. What was apparent was that those who eventually failed treatment had greater down-regulation, particularly during the first month of treatment. At the six-month time point, the levels of miR-21-5p were similar in both the treatment success and failure groups.

4.3.3.1.2 miR-99b and -29a-5p

The levels of miR-99b and -29a-5p (Figure 4.11 B and C) are up-regulated in the treatment success group and not significantly varied in the treatment failure group. In the treatment success group there is nearly a five-fold (4.73) increase in miR-29a-5p levels in the treatment success group compared to their matched healthy controls.

4.3.3.1.3 miR-221-3p

The expression profile of miR-221-3p (Figure 4.11 E) was quite varied between the treatment success and the treatment failure individuals. Prior to treatment commencement the treatment success group was significantly up-regulated and the treatment failure group was significantly down-regulated. Following a month of therapy both groups were trending towards healthy controls with no significant differences detected in either group after the first month of therapy.

4.3.3.1.4 mir-146a-5p, -26a-5p, -133a and -652-3p

The overall trend for all these miRNAs (Figure 4.11 F, G, I and J) is that there was more significant down-regulation in the treatment failure group especially prior to

the commencement of therapy and after one month of treatment. At the two-month collection point and at the final collection point at six months, the levels in treatment failures were trending towards the treatment success individuals. With the exception of miR-26a, the levels are continuing to fall in the treatment success patients. All other miRNA levels in those that successfully completed therapy were moving towards healthy control levels of expression.

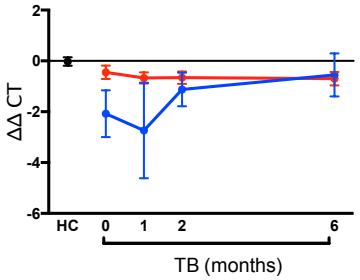
4.3.3.1.5 miR-28a-5p

When examining the expression profile of miR-28-5p (Figure 4.11 H) in the TB cohort, there was no statistically significant variability between healthy controls and TB subjects. When stratifying according to treatment outcome, we see that the expression level of miR-28-5p in the plasma was significantly down-regulated with a greater than four-fold decrease in expression levels following two-months of therapy and greater than two-fold decrease in expression levels following six-months of treatment (Figure 4.11 H).

There was neither significant variation in the expression of miR-223-5p (Figure 4.11 D) in the plasma from TB patients who ultimately failed treatment nor those that successfully completed therapy compared to healthy controls.

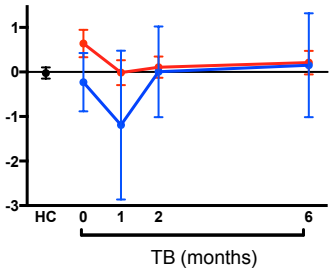
Overall we observed quite marked variation in the expression levels in those that completed treatment successfully with those that did not for most of the miRNAs examined in the plasma. Caution needs to be taken however, especially when interpreting the results from the treatment failure group, in view of the modest numbers in this group.

A. miR-21-5p



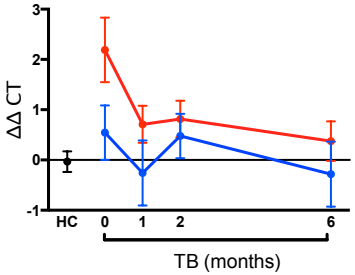
Time Point	Treatment Outcome	Fold Change	p Value
A	Success	-1.61	**0.046**
	Failure	-5.09	**<0.001**
B	Success	-1.71	**0.001**
	Failure	-14.30	**0.009**
C	Success	-1.76	**0.002**
	Failure	-4.39	**0.005**
D	Success	-2.33	**0.002**
	Failure	-2.20	0.224

B. miR-99b



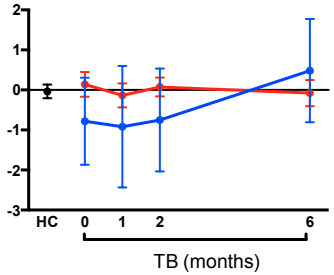
Time Point	Treatment Outcome	Fold Change	p Value
A	Success	1.63	**0.001**
	Failure	-1.13	0.588
B	Success	-1.09	0.840
	Failure	-2.31	0.163
C	Success	1.11	0.351
	Failure	-1.16	0.907
D	Success	1.11	0.149
	Failure	1.20	0.712

C. miR-29a-5p



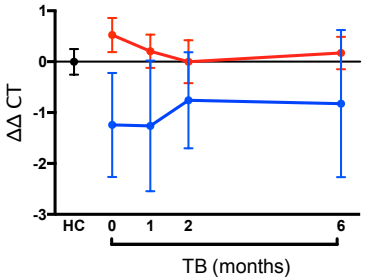
Time Point	Treatment Outcome	Fold Change	p Value
A	Success	4.73	**< 0.001**
	Failure	1.23	0.339
B	Success	1.60	**0.008**
	Failure	-1.19	0.745
C	Success	1.86	**0.002**
	Failure	1.34	0.313
D	Success	1.20	0.155
	Failure	-1.15	0.721

D. miR-223-5p



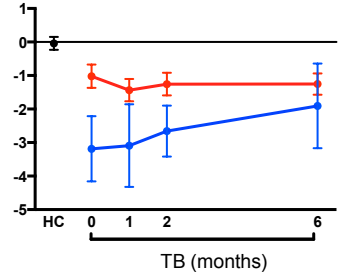
Time Point	Treatment Outcome	Fold Change	p Value
A	Success	1.07	0.448
	Failure	-1.90	0.174
B	Success	-1.46	0.659
	Failure	-2.09	0.233
C	Success	-1.09	0.594
	Failure	-2.74	0.257
D	Success	-1.16	0.854
	Failure	1.43	0.407

E. miR-221-3p

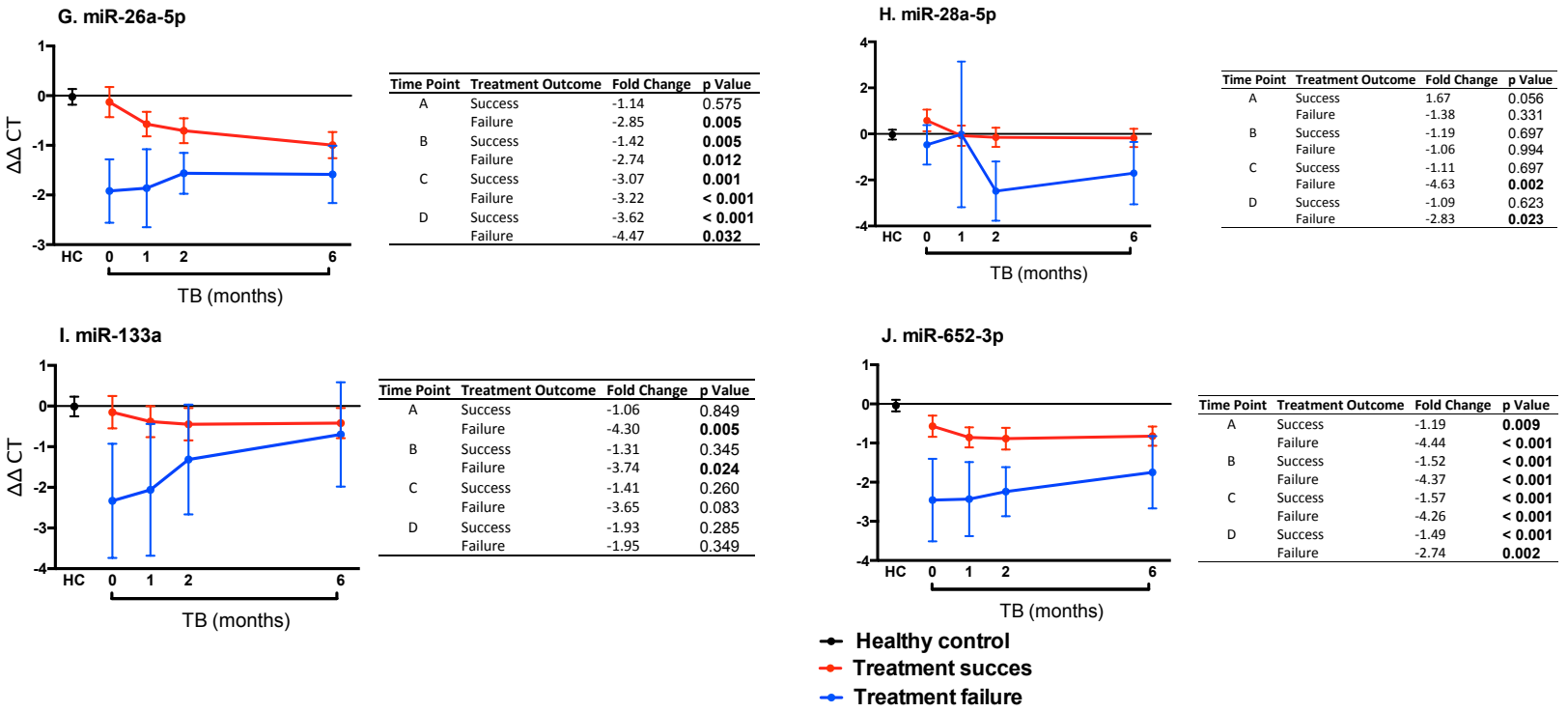


Time Point	Treatment Outcome	Fold Change	p Value
A	Success	1.71	**0.041**
	Failure	-2.33	**0.044**
B	Success	1.37	0.309
	Failure	-2.19	0.086
C	Success	1.17	0.175
	Failure	-2.57	0.199
D	Success	1.37	0.360
	Failure	-2.07	0.319

F. miR-146a-5p



Time Point	Treatment Outcome	Fold Change	p Value
A	Success	-2.30	0.066
	Failure	-8.69	**< 0.001**
B	Success	-2.70	**< 0.001**
	Failure	-8.13	**< 0.001**
C	Success	-2.42	**< 0.001**
	Failure	-7.35	**< 0.001**
D	Success	-2.43	**< 0.001**
	Failure	-3.39	**0.010**



Time Point	Treatment Outcome	Fold Change	p Value
A	Success	-1.14	0.575
	Failure	-2.85	**0.005**
B	Success	-1.42	**0.005**
	Failure	-2.74	**0.012**
C	Success	-3.07	**0.001**
	Failure	-3.22	**< 0.001**
D	Success	-3.62	**< 0.001**
	Failure	-4.47	**0.032**

Time Point	Treatment Outcome	Fold Change	p Value
A	Success	1.67	0.056
	Failure	-1.38	0.331
B	Success	-1.19	0.697
	Failure	-1.06	0.994
C	Success	-1.11	0.697
	Failure	-4.63	**0.002**
D	Success	-1.09	0.623
	Failure	-2.83	**0.023**

Time Point	Treatment Outcome	Fold Change	p Value
A	Success	-1.06	0.849
	Failure	-4.30	**0.005**
B	Success	-1.31	0.345
	Failure	-3.74	**0.024**
C	Success	-1.41	0.260
	Failure	-3.65	0.083
D	Success	-1.93	0.285
	Failure	-1.95	0.349

Time Point	Treatment Outcome	Fold Change	p Value
A	Success	-1.19	**0.009**
	Failure	-4.44	**< 0.001**
B	Success	-1.52	**< 0.001**
	Failure	-4.37	**< 0.001**
C	Success	-1.57	**< 0.001**
	Failure	-4.26	**< 0.001**
D	Success	-1.49	**< 0.001**
	Failure	-2.74	**0.002**

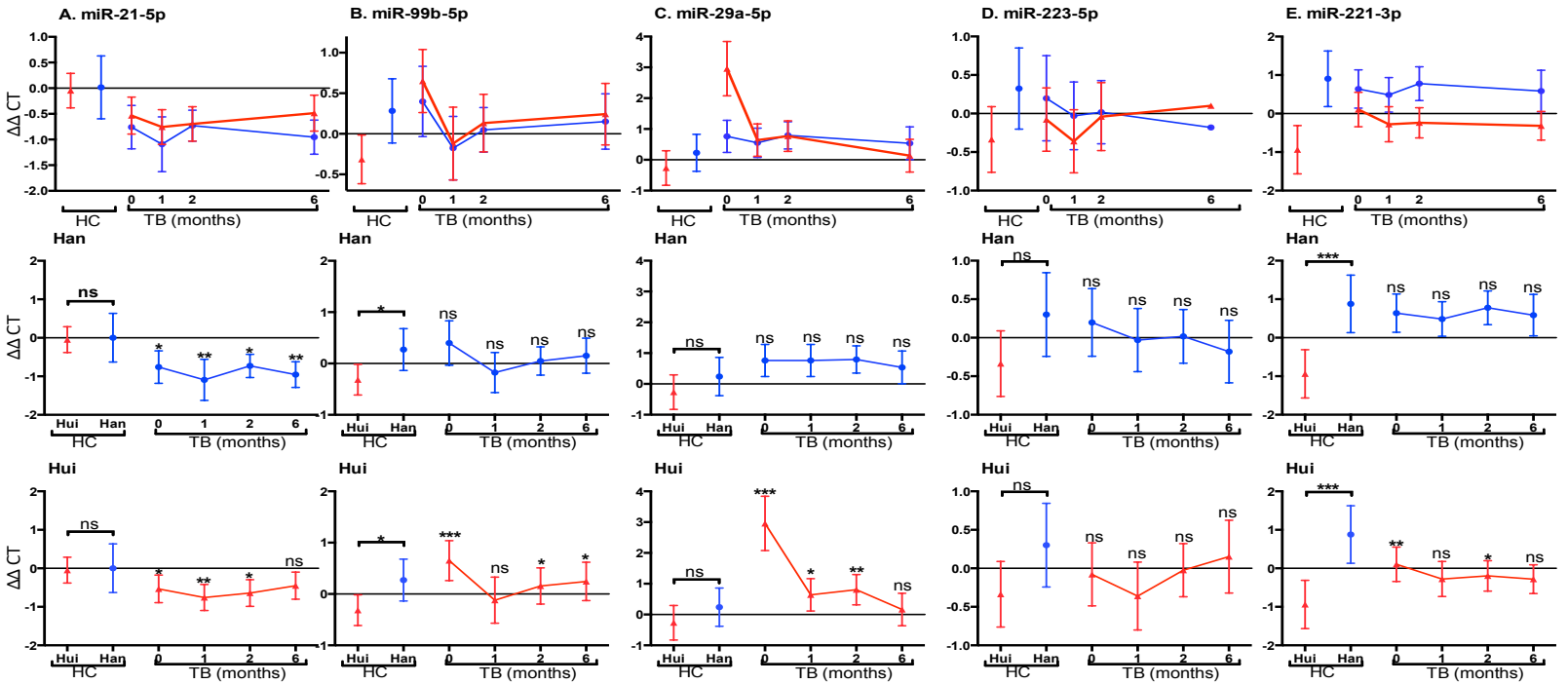
Figure 4-11. Plasma miRNA levels from validation set from 100 TB patients and 100 healthy controls as determined by treatment outcome. The plasma from TB subjects was collected at four time points (prior to treatment commencement, one, two and six months following commencement of therapy). Plasma from healthy controls was collected at one time point. miRNA expression was measured by qRT-PCR with miR-93 used as a reference miRNA. The TB group was separated according to treatment outcome following six months of therapy. Healthy controls are depicted in black, treatment success in red and treatment failures in blue. In total 89 patients were successfully treated and 11 failed TB treatment. Accompanying each figure is a table which states the fold change and p-value for each miRNA in the TB group compared with healthy controls. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results are depicted with mean with 95% confidence intervals. Logged data was assumed to be normally distributed. Comparisons of variables between multiple groups were done using ANOVA. In instances where p-value was < 0.05, additional two-group comparison were done by a Student's *t*-test. *p <0.05, **p <0.01, ***p <0.001.

4.3.3.2 Ethnicity

Previous studies have demonstrated ethnic diversity in miRNA expression (Bovell et al., 2013; Rawlings-Goss et al., 2014). We also demonstrated significant differences in miRNA expression when identifying a suitable reference miRNA (Chapter 3). This difference, however, was between Hui Chinese and an Australian cohort with many ethnic variances.

To further examine this, miRNA data from healthy controls and TB patients from Time Point A (prior to the commencement of therapy) were divided according to ethnicity. Normal distribution was assumed as all data was logged. A Student's *t*-test was performed with ethnicity as the dichotomous variable for healthy control subjects. Comparisons of variable over the time course of therapy in the TB subjects were done using ANOVA. In instances where the ANOVA p-value was <0.05, additional two-group comparisons were done using a Student's *t*-test. A p-value <0.05 was deemed significant. The results are presented in Figure 4.12. Within the healthy control cohort there were 46 that identified themselves as Han Chinese and 54 as Hui Chinese. Within the TB cohort there were 42 that identified themselves as Han and 58 as Hui Chinese.

In total, from the healthy control group there were four miRNAs which were significantly differentially expressed amongst the two ethnic groups: miR-99b-5p; -221-3p; -146a-5p; and, 652-3p (Figure 4.12 B, E, F and J). Looking at the miRNA expression level over the course of treatment course, the pattern of miRNA expression is similar, though for miR-29a-5p (Figure 4.12 C), there is a marked up-regulation in the Hui Chinese.


A. miR-21-5p
B. miR-99b-5p
C. miR-29a-5p
D. miR-223-5p
E. miR-221-3p
ΔΔ CT
HC
TB (months)
0 1 2 6
Han
Hui
ns
*
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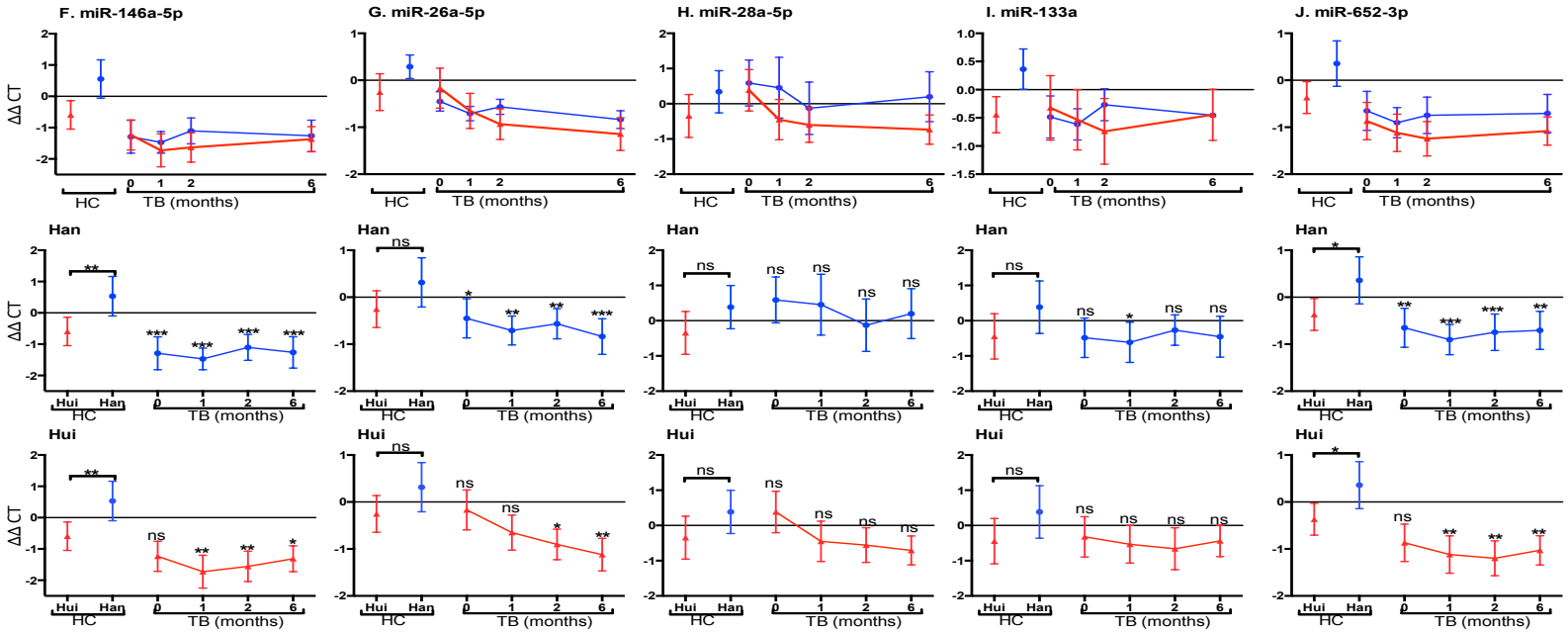

Figure 4-12. Plasma levels of miRNA from validation set divided according to ethnicity and disease state. The plasma of TB subjects was collected prior to the commencement of therapy (0 months) and at one, two and six months following treatment commencement. Plasma from matched healthy control individuals was collected at one time point. miRNA levels were examined by qRT-PCR with miR-93 used as a reference miRNA. Cohorts were separated according to ethnicity. In total there were 42 Han and 58 Hui in the TB group and 46 Han and 54 Hui in the healthy control group. Each miRNA is presented in a separate column with Han in blue and Hui depicted in red. In four of the ten miRNAs measured there was significant variation between the miRNA expression of the Han and Hui healthy subjects (B, E, F, J). The overall trend of miRNA expression in TB subjects across the four time points was similar, however the degree of modulation varied between the two ethnicities. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results are depicted with mean with 95% confidence intervals with significance calculated between healthy controls of the two ethnic groups and within the same ethnic group compared to healthy controls of the same ethnicity The differences between the healthy controls were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. Comparisons of variables between multiple groups were done using ANOVA. In instances where p-value was < 0.05, additional two-group comparison were done by a Student's *t*-test. *p <0.05, **p <0.01, ***p <0.001.

4.3.3.3 Variation in miRNA expression according to gender

We next examined if gender influenced miRNA expression. There have been no published reports in the literature regarding miRNA expression variability related to sex, however as miRNA quantification is a fairly new field we felt it important to determine if there were any significant variations. In the healthy control group there were 57 males and 43 females. In the TB cohort there were 58 males and 42 females (Table 4.1). A Student's *t*-test was performed for each cohort with gender as the dichotomous variable with the results presented in Figure 4.13. Overall there is little variability between the male and females with more obvious variation being seen according to disease state (healthy or TB). miR-221-3p and -133a (Figure 4.13 E and I) showed significant variation within the TB cohort. This will need further clarification but may be due to confounding elements such as: other co-morbidities; timing of presentation to medical facility which may be different for males and females and may translate to later presentation for a particular sex with more advanced disease; and, smoking status, much more common in Chinese males, may also have a gender effect. All of these factors are likely to contribute to miRNA expression.

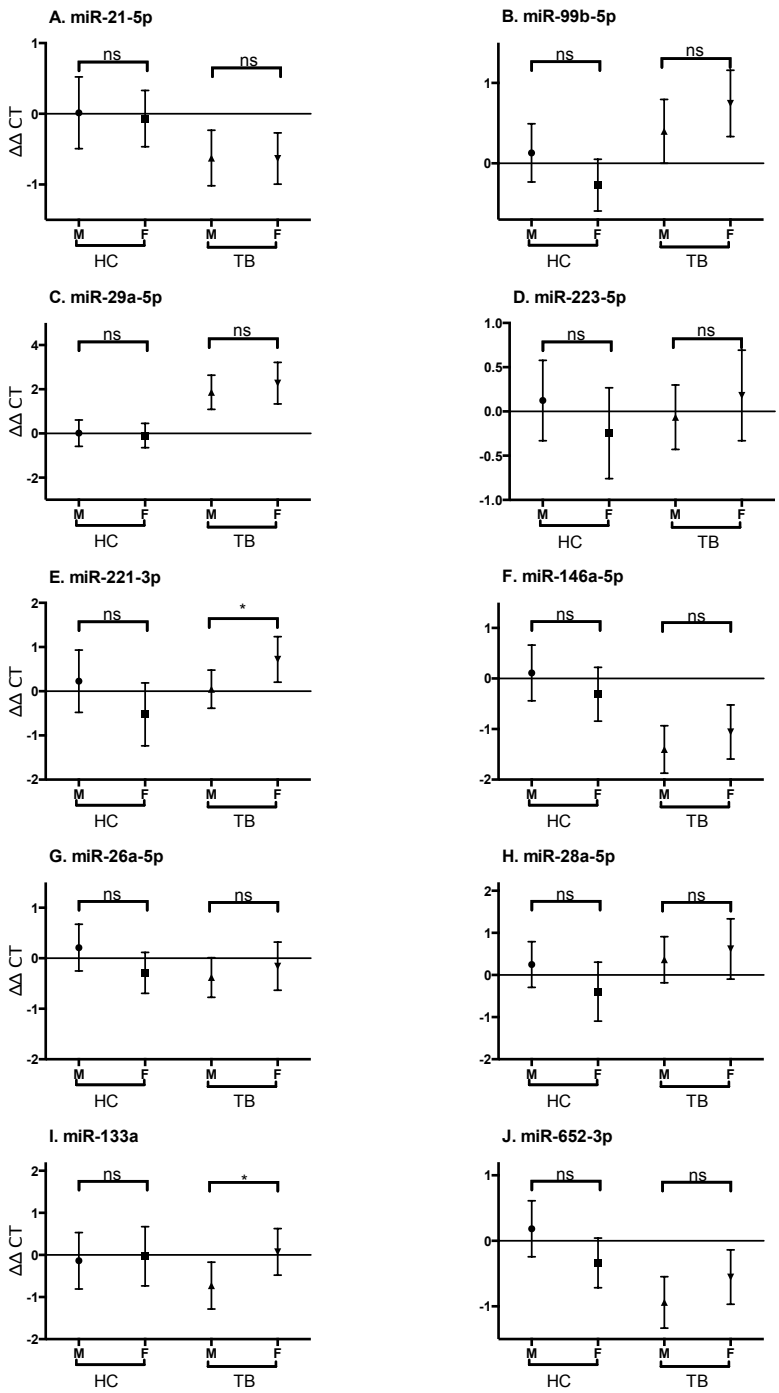

Figure 4-13. Plasma miRNA expression in healthy controls and TB subjects prior to commencement of treatment stratified according to gender. Of the 100 healthy controls there were 57 males and 43 females and in the TB cohort there were 58 males and 42 females. Expression levels of plasma miRNAs were measured by qRT-PCR using miR-93 as a reference miRNA. The relative expression of miRNAs was determined using $2(-\Delta\Delta C(T))$. Results are depicted with mean with 95% confidence intervals. The differences were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001.

4.4 Discussion

TB remains a major global health problem, causing morbidity in millions of people each year. Urgent steps are needed to curtail these alarming figures. Such steps would include the development of new biomarkers that assist in disease diagnosis and that are able to predict with accuracy early treatment failure. These factors are important so as to provide prompt and effective treatment, thus limiting on-going disease transmission. New therapies are also required to treat those with both active disease and latent infection and more efficient ways are needed to evaluate new drug and vaccine development.

With the aim of identifying diagnostic and predictive biomarkers in TB we examined the expression of 175 miRNAs in the plasma of 19 TB patients and 14 healthy controls from China by qRT-PCR. From the initial pilot study, we observed a total of 87 significantly regulated miRNAs in TB patients; 18 up-regulated and 69 down-regulated. To our knowledge, this is the first study that has detailed miRNA expression longitudinally. Subgroup analyses were performed to examine whether treatment outcome (success or failure), gender or ethnicity altered miRNA expression. Of the 100 patients enrolled into the study, 33% were diagnosed based on microbiological grounds, with the remainder diagnosed following clinical and radiological assessment.

4.4.1 miRNA as a diagnostic biomarker

A biomarker is a measurable indicator of a biological state, either normal or diseased. While a lot of research to date has focused on assessing the accuracy of a single biomarker, there is increasing interest in panels of biomarkers composed of

multiple candidates, in this case miRNAs, which when used alone are not adequately specific nor sensitive to allow clinical application, but when used in combination show good performance. Of the ten candidate miRNAs selected for further screening in this cohort of 100 patients with pulmonary TB and their matched healthy controls we found five miRNAs with significant variance. There were two significantly up-regulated (miR-29a-5p and -99b) and three significantly down-regulated (miR-21-5p, -146a-5p and -652-3p). miR-29a-5p and -21-5p individually had the best sensitivity and specificity each with a ROC of 0.70. However, by performing a multivariate logistic regression and examining the ten miRNAs collectively, the sensitivity reached nearly 95% without compromising specificity, which was close to 89%. This then achieved an 88% positive predictive value and 95% negative predictive value. The results showed that a combination of miRNAs could discriminate pulmonary TB patients from healthy controls with an excellent sensitivity and specificity.

The study of miRNAs as a potential biomarker is a relatively new field. Calculating appropriate sample sizes is difficult due to the large number of miRNAs often screened and the lack of knowledge about their various functions. Many studies that have examined miRNAs as biomarkers have used relatively small sample sizes. Wang and colleagues examined the expression of miRNA in PBMCs obtained from TB subjects and healthy controls with 29 TB subjects and 18 healthy controls enrolled into the study (Wang et al., 2011) and the study by Qi et al. that examined miRNA expression from the serum of TB patients had 30 TB subjects (Qi et al., 2012). What became apparent from this study, was that even though the pilot sample examined miRNA expression in 19 TB patients and 14 healthy controls, of the ten selected miRNAs that had both significant p values (< 0.01) and fold changes of $\geq$

1.5, only five of these miRNAs were significantly modulated in the treatment naïve TB subjects compared to healthy controls in the larger cohort study. This highlights the significant variation in miRNA expression that occurs as a result of normal human variance and demonstrates the importance of screening an adequate sample size to ensure that the findings that are made are true and representative of the target population.

4.4.2 Functional assessment of modulated miRNAs

Functions of many of the differentially expressed miRNAs are still largely unknown. Comparing findings from different studies can be problematic, even if samples are from the same tissue or cell type, due to variance in methodology including miRNA quantification techniques employed and normalisation strategies. Very few comparative studies have examined miRNA from blood samples and most miRNA studies that have examined miRNA expression in the setting of mycobacterial infection have utilised *in-vitro* models of infection, usually utilising macrophages from either patient samples or cell lines. Our study examined miRNA expression from the plasma of individuals with pulmonary TB. It is likely that the circulating miRNAs that were quantified reflect those not only secreted by macrophages but also from other cells, including platelets, dendritic and endothelial cells. Notwithstanding these limitations, we review the knowledge of the discordantly regulated miRNAs in the current literature.

miR-29 has been shown in *in-vitro* as well as in clinical studies to be up-regulated in the setting of mycobacterial infection (Fu et al., 2011; Yi et al., 2012). Our study showed a greater than four-fold increase in miR-29 levels in those with TB compared

to healthy controls prior to the commencement of therapy. A study by Zhang et al., which measured the expression of miRNA in the serum of newly diagnosed TB patients also found elevated miR-29 levels in active TB patients before commencement of therapy (Zhang et al., 2013). Our study extends these findings and shows that miR-29 levels dropped markedly by one-month post initiation of therapy. Interestingly, patients who failed therapy had a smaller increase in miR-29 expression compared to healthy controls initially but still recorded a small decline in miR-29 levels at one month.

The decline in miR-29 expression noted in our study may relate to its functional effects. While the function of miR-29 is incompletely understood, studies have shown that miR-29 suppresses the immune response by directly targeting IFN-γ (Ma et al., 2011). miR-29 targets the 3'UTR of IFN-γ mRNA as well as enhancing the association between IFN-γ and Argonaute (AGO) 2 protein to form an RNA-induced splicing complex (RISC). This has the effect of supressing IFN-γ expression post-transcriptionally (Ma et al., 2011). IFN-γ has an important role in activating the innate immune system in mycobacterial infection. Knockdown mice deficient in miR-29 show a more potent T_H1 adaptive response to intra-cellular mycobacterial infections with lungs from BCG-infected mice deficient in miR-29 showing less inflammation with less infiltrating polymorphonuclear cells than control mice (Ma et al., 2011). miR-29 has also been shown to target anti-apoptotic pathways, a mitochondrial pathway that involves myeloid cell leukaemia-1 (mcl-1) and B-cell lymphoma 2 (Bcl-2) (Xiong et al., 2009) highlighting its role in regulating apoptosis in immune cells. These functions of miR-29 may help to explain how *M. tuberculosis* is able to evade the immune system, it could be that up-regulation of

miR-29 may lead to inhibition of IFN-γ release and associated apoptosis of immune cells involved in the anti-tuberculosis response.

The underlying mechanism by which *M. tuberculosis* infection leads to increase levels of miR-29a expression in human plasma is not yet known. Questions that remain unanswered include whether *M. tuberculosis* infection can directly up-regulate miR-29a or if the host immune response generated following *M. tuberculosis* infection itself leads to increased levels of miR-29a or if it is a combination of both pathogen and host regulation that regulates this response.

While IFN-γ is absolutely essential for protective immunity to *M. tuberculosis* infection, excessive IFN-γ can lead to damaging inflammation (Lin et al., 2013). Using a transgenic mice model in which IFN-γ is overexpressed, Shelton et al. showed that increased IFN-γ, led to necrotising inflammation within muscles (Shelton et al., 1999). Similarly though, mice deficient in IFN-γ also show increased inflammation (Chen et al., 2001). This serves to highlight the pleiotrophic nature of IFN-γ whereby it can heighten innate and adaptive immune responses and at the same time promote immune homeostasis. Therefore, regulating IFN-γ levels, in *M. tuberculosis* infection, is a key host response to ensure adequate macrophage activation without inducing excessive inflammation.

This study found that miR-99b was also significantly elevated in the plasma of patients with TB. Whilst there have been no published studies reporting miR-99 in the plasma of persons with TB, Singh and colleagues noted up-regulation of miR-99 in *M. tuberculosis*-infected dendritic cells (Singh et al., 2013). By utilising

antagomirs, Singh et al. performed loss-of-function experiments and found that inhibition of miR-99b resulted in enhanced clearance of the mycobacteria from infected dendritic cells. Associated with this was significantly up-regulated pro-inflammatory cytokines such as IL-6, IL-12, IL-1β and TNF. These studies by Singh et al. then went on to demonstrate that miR-99b acts by targeting TNF mRNA.

miRNAs act to regulate mRNA (Asirvatham et al., 2009; Lu et al., 2009), Singh et al. found that miR-99b binds to 3'UTR region of TNF mRNA and destabilises the transcripts thereby reducing TNF production (Singh et al., 2013). TNF plays a key role in controlling TB through the establishment and maintenance of the granuloma as well as directing inflammatory cells to the site of infection (Lin et al., 2007). Blocking TNF biosynthesis in human macrophages allows *M. tuberculosis* to evade host immune responses. Indeed, reactivation of latent TB infection is a major risk factor for individuals taking anti-TNF therapy, highlighting the importance of TNF and its regulation, in host immunity.

This study is the first to demonstrate a significant down-regulation of miR-21 in the plasma of pulmonary TB patients compared with healthy controls. miR-21 has been shown to regulate multiple mRNAs and to be involved in progression of colorectal malignancy by reducing the expression of the tumour suppressor gene PDCD4 (Peacock et al., 2014). Previous work has reported up-regulation in miR-21 levels in human PBMCs obtained from patients with TB compared to healthy controls (Wang et al., 2011). Indeed, our work as discussed in Chapter Five, which examined miR-21 expression in *M. tuberculosis* infected macrophages, also noted significant up-regulation. It can be speculated that this apparent dichotomy reflects the exquisite

cell type-specific nature of miRNAs. miR-21 has been shown to inhibit pro-inflammatory cytokine production and at the same time enhance the anti-inflammatory cytokine, IL-10 (Sheedy et al., 2009). In *M. bovis* infection of murine dendritic cells and macrophages, miR-21 was up-regulated through various pathways including Erk and NF-κB (Wu et al., 2012). Furthermore, miR-21 was found to impair T cell responses and protective immunity and directly stimulate IL-12 and Bcl-2 mRNA resulting in increased antigen presenting cell apoptosis (Wu et al., 2012). Up-regulation of miR-21, as noted in macrophage studies, may therefore protect the host from excessive inflammatory response, whereas by down-regulating levels of miR-21 in the plasma the host may prevent the reduction in T cell responses resulting in potent anti-mycobacterial immunity.

Another of the miRNAs that was found to be markedly down regulated in the plasma of the TB patients was miR-146a, indeed this was the most down regulated miRNA measured in the plasma of TB patients during treatment. miR-146 is an early-response miRNA induced by various microbial components and pro-inflammatory mediators (Taganov et al., 2006; Fu et al., 2011). In the first published study that examined circulating miRNAs as potential biomarkers in TB, Fu et al. reported 92 miRNAs that were significantly regulated (Taganov et al., 2006; Fu et al., 2011; Hassan et al., 2012). One of the miRNAs significantly regulated was miR-146 which was down-regulated in the sera of TB patients as compared with their matched healthy controls. Similarly, our study also observed significantly down-regulated levels of miR-146 in TB patients from plasma samples. The biological function of miR-146 as a negative regulator of inflammation has been extensively studied. miR-146 has been shown to directly target the 3'UTR of TNF receptor-associated factor 6

(TRAF-6) and IL-1 receptor-associated kinase 1 (IRAK-1) and its down-regulation is thought to initiate the inflammatory response (Taganov et al., 2006; Hassan et al., 2012). Conversely, a study by Perry et al. found that while up-regulation of miR-146 resulted in increased expression of pro-inflammatory chemokine IL-8, this was independent of TRAF-6 and IRAK-1 (Perry et al., 2008), indicating there are additional mechanisms of action of miR-146 that are still to be identified. These functional studies have shown the pivotal role that miR-146 plays in regulating severe inflammation during the innate immune response such as what occurs following TB infection. Notwithstanding inflammation being an essential component of the immune response, if it remains unregulated it can lead to serious disease in the host.

In our study we observed the progressive down-regulation of miR-26a-5p. Kleinsteuber and colleagues also recently noted down-regulation of miR-26a in whole blood from children with TB compared to healthy children with LTBI. miR-26a release from T_H1 cells from the same individuals was also down-regulated (Kleinsteuber et al., 2013). Interestingly a recent study by Ni et al. examined the expression of miR-26a in primary human macrophages infected with *M. tuberculosis* and found significant up-regulation in infected cells (Ni et al., 2014). This again serves to highlight the variability of miRNA expression noted with different cell types and conditions studied (*in vivo* and *in vitro* models) compounded by the varying techniques employed to quantify their expression. Ni et al. utilised miRNA mimics to induce miR-26a expression and discovered that it acts as a negative regulator of macrophage function through IFN-γ which effectively acts to limit the immune response as well as tissue damage to lung parenchyma (Taganov et al.,

2006; Ni et al., 2014). Conversely, down-regulation, we can deduce, would have the opposite effect with increasing levels of IFN-γ inducing cytokine and chemokine release effecting a potent anti-microbial immune response.

We also observed a significant down-regulation in miR-652 prior to the commencement of anti-tuberculosis therapy. There have been no published studies that have specifically examined miR-652 in the plasma from patients with active TB. Fu et al. examined miRNA expression in $CD4^+$ T cells from individuals with latent and active TB. In this study miR-652 was significantly up-regulated in the TB group (Fu et al., 2014). Contra to these findings, a study by Roderburg et al. found that stimulating U937 cells with LPS led to significant down-regulation of miR-652 levels (Roderburg et al., 2012). The function and putative targets of miR-652 have yet to be defined, the Targetscan® predictive software identified only 8 predictive targets of miR-652 and further work is required to determine how miR-652 may be influencing immunity to *M. tuberculosis* infection.

4.4.3 miRNA timecourse

A hypothesis that was explored in this thesis related to miRNA expression over the course of treatment. We postulated that miRNA expression would return to levels not dissimilar to healthy controls with treatment. This was the first study, to our knowledge, that had examined miRNA longitudinally over the course of therapy.

As we hypothesised, miR-99b-5p which was initially significantly up-regulated returned to levels equivalent to healthy controls within a month of starting anti-tuberculosis chemotherapy. miR-29a-5p also returned to normal baseline, however

this did not occur until the six-month mark, although the levels were falling significantly following the commencement of therapy. What was interesting and somewhat unexpected was the profile of the remaining discordantly regulated miRNAs.

miR-21-5p and -652-3p were significantly down-regulated and remained so with little variation throughout the duration of therapy. miR-146a-5p and -26a-5p, which were also down-regulated in the plasma from TB patients, were even further down-regulated over the course of therapy. The reasons for these findings are unclear. Perhaps the persistent down-regulation of miR-146a-5p may be a protective measure to control for the possible deleterious effects of an unregulated inflammatory response and may counteract the effects of the pro-inflammatory effects thought to be associated with down-regulation of miR-21a-5p and -26a-5p. Another explanation may be that the miRNAs remained down-regulated due to on-going lung inflammation and parenchymal re-modelling that accompanies resolving pneumonic infection.

As the function and targets of miR-652 are yet to be explored, the reason for its persistent down-regulation are unclear.

It was hypothesised that the expression of miRNAs in individuals that failed therapy would differ both from those that succeeded and from healthy controls. To better explore this we stratified according to treatment outcome (defined as sputum positivity at six months or failure to reduce lung inflammation). By virtue of the modest sample size of 11 treatment failures, it is difficult to draw conclusions when

examining miRNA expression according to outcome, especially in the small treatment failure group. Nonetheless the data demonstrates a number of interesting trends in miRNA expression when stratifying according to treatment outcome. Overall, miRNAs that were down-regulated in TB infection tend to be more significantly down-regulated in those patients classified as treatment failure. In general, however the miRNA expression pattern that was seen during the treatment course followed a similar pattern between those that were successfully treated to those who inevitably failed therapy.

We postulated that the expression of miRNAs could act as a determinant of treatment outcome, and clinically this would be most important early in the treatment phase, as it would allow modification of therapy for those who are failing treatment. This would limit the ongoing transmission of the infection to others and would halt the propagation of drug resistance in an already failing therapy. It is not clear from the analysis of data collected from this study, if the miRNAs measured could be successfully used to delineate those that will fail treatment from those that will not. There are a number of factors that need considering: for example, of the 11 patients that were deemed to have failed therapy, we are unaware as to the reason they did not respond to the treatment prescribed. There are a number of possibilities including that the individual was infected with a drug-resistant organism significant enough to not respond to standard therapy. In those persons, we would anticipate early miRNA expression differences between TB patients. The second possibility is that the individual developed drug resistance at some stage during their treatment. This later development of drug-resistance may have arisen secondary to non-adherence of therapy, intermittent dosing of antibiotics or even malabsorption of medications.

What is also unclear is the potential role of other factors that may have impacted miRNA expression such as intercurrent illness of enrolled subjects, duration of illness prior to enrolment, and smoking history. All of these factors would need to be addressed and a larger cohort of patients enrolled in order to determine whether miRNA expression patterns could be used successfully as a predictor of treatment outcome. However, from these data there was significant differential expression of miRNAs in patients that ultimately failed treatment compared with those that were cured. We observed considerable variation in expression of miR- 21-5p, -29a-5p, -221-3p, -146a-5p, -26a-5p, -133a and-652-3p, all of which were more down-regulated in the treatment failure group. This supports the notion that miRNAs could be used in the clinical setting to determine likelihood of treatment outcome.

4.4.4 Ethnic variation in miRNA expression

The use of miRNA as a clinical biomarker and diagnostic tool has been actively investigated, particularly over the last decade. Little work however has focused on possible genetic variability. As described in Chapter Three, when we sought to identify suitable reference miRNAs for plasma studies in TB subjects, we observed quite significant variability between the Chinese cohort (Hui Chinese) and the Australian cohort (numerous ethnic backgrounds). This study presented a unique opportunity to explore the possibility of ethnic variation influencing miRNA expression in two distinct ethnic groups within China, the Han and the Hui Chinese.

We observed four miRNAs (miR-99b-5p, -221-3p, -146a-5p and -652-3p) that were significantly differentially expressed in the healthy control subjects based on ethnicity. We also noted differences in miRNA expression in the TB subjects, even

though the overall trend of expression (either up- or down-regulated) was similar. A recent study by Rawlings-Goss utilising whole genome sequencing also observed variation in miRNA expression among African and non-African persons in the setting of malignancy (Rawlings-Goss et al., 2014). Overall the researchers found that miRNA sequences were similar across the sampled populations, however they did observe differences in 33 variances that occurred in more than a single person, with some being population specific. This finding may help to explain the disparity observed with regards to survival rates of certain malignancies of the same stage and grade that are noted in persons of different ethnic backgrounds (Aizer et al., 2014). Although the miRNAs examined by Rawlings-Goss were different to the miRNAs studied in this body of work, ethnicity may influence susceptibility or response to therapy in TB.

Another recent study by Chang and colleagues also noted variation in miRNA expression that was associated with ethnic origin (Chang et al., 2014). The authors noted the six-fold increased likelihood of type-2 diabetes mellitus in Han Chinese compared with the Kazak Chinese. Utilising qRT-PCR, Chang et al. observed a three-fold increase in miR-375 levels in the Han Chinese. These findings further support our findings that miRNA expression levels are influenced by ethnicity.

With regards to our study, there are other factors that may have contributed to the differences in miRNA expression observed between the two ethnic groups. Overall the Hui Chinese are poorer and are more likely to work in agricultural subsistence farming, meaning that they are often living further away from urban areas thus limiting their access to medical services. Living in rural areas, away from medical

facilities compounded by poverty, means that the Hui Chinese may be more likely to seek medical attention later during the course of illness and are therefore more likely to have more advanced disease at the time of diagnosis compared to the urban dwelling and wealthier Han Chinese. More advanced TB is likely to be accompanied by a heightened inflammatory response within the lung parenchyma, which may influence miRNA expression. With regards to the healthy controls' variation in miRNA expression, this may relate to such factors as co-morbid conditions, smoking rates and general health, all of which are also likely to be affected by socioeconomic status.

Other variability, not studied here, relates to actual functional roles of miRNA. Zhang et al. discovered single nucleotide polymorphisms (SNPs) within precursor miRNAs that were associated with susceptibility to pulmonary TB in Chinese Uygur, Kazak and Southern Han populations (X. Zhang et al., 2015). SNPs of precursor miRNAs can alter miRNA processing and expression and they may also contribute to the development or the progression of the disease. Therefore, not only are the expression of miRNAs different in different ethnic groups, there also appears to be functional differences of miRNAs that in some cases are secondary to inheritable SNPs.

This study has shown significant variability between the two ethnic populations. This may be due to inheritable differences in miRNA expression. These differences may also or alternatively be secondary to environmental, socioeconomic factors or may be secondary to other factors such as co-morbid illness or disease. Future studies to further examine the role of ethnic variation are needed.

4.4.5 Next steps

The aims of this study did not extend to examine miRNA targets and ultimately determining their function. This however, is the logical next step for this project. Independent of the platform utilised or the statistical analyses performed, the end point of all global miRNA expression profiling is a list of differentially expressed miRNAs. The function of any given miRNA is determined by the genes that it ultimately targets. Having an understanding of the genes targeted by specific miRNAs is important as it provides a better understanding of the pathophysiology of the disease and also opens up the possibility of directed and targeted therapies via the manipulation of specific miRNAs. In Chapter Five, where we profile miRNA expression in *M. tuberculosis* infected macrophages in an *in vitro* environment, we will discuss the ways to explore miRNA function through molecular and computational methodologies with the aim of identifying miRNA targets.

4.4.6 Identifying and overcoming limitations of study

4.4.6.1 Effect of haemolysis on miRNA expression

In the pilot study, increased variation in miR-23a and miR-451 were used as markers of haemolysis and this method is supported by the literature (Kirschner et al., 2011). Plasma samples for the validation set were only visually inspected for haemolysis, however no further steps were taken to ensure haemolysis of the sample had not occurred. The impact of haemolysis on miRNA expression has been well documented in the literature (Kirschner et al., 2011; Blondal et al., 2013; Köberle et

al., 2013), however it has not often been considered in other studies (Mitchell et al., 2008; Blondal et al., 2013; W. Zhang et al., 2014). In addition to visual inspection, samples can be inspected by spectrophotometry, with A414 > 0.2 (absorbance peak of haemoglobin) and additional at peaks 541 and 576nm (Kirschner et al., 2011). Future studies are required to determine if haemolysis is having any impact on the miRNAs being measured.

4.4.6.2 Identifying those with LTBI

Our studies involved collecting plasma from individuals with newly diagnosed pulmonary TB and their matched healthy controls and comparing miRNA expression both prior to treatment outcome and then over the course of treatment for the TB cohort. We noted significant variation in some miRNA expression both at the time of diagnosis and also during treatment. TB is endemic in China and given the worldwide estimate that one in three has LTBI (World Health Organization, 2014), it is highly likely that many of the persons in the healthy control group had LTBI. This was not assessed as part of healthy control recruitment. It is unclear what effect LTBI has on miRNA expression. Future studies that screen all healthy controls for LTBI and then incorporate this group as a third arm of the study would be helpful to determine if miRNA expression is affected by latent infection with *M. tuberculosis.*

4.4.6.3 Measuring healthy controls subjects at more than one-time point

Limited by budgetary constraints, we screened the healthy controls at only one-time point. It would have been very informative to screen the healthy controls at the same four time points as the enrolled TB subjects. Currently it is not known what normal

fluctuation occurs in miRNA expression as part of normal and healthy variance. Certainly though, if only examining at Time Point A in the TB patients, it is clear that some of the miRNAs are significantly different in the TB population with excellent sensitivity, specificity, negative and positive predictive values, making them a plausible diagnostic marker.

4.4.6.4 Explore the role of ethnicity in miRNA expression

There appeared to be variation in miRNA expression that was related to ethnicity. As mentioned above, there are many confounding factors, most notably socioeconomic status and poverty, which may have contributed to this variability. To explore this further, a more detailed questionnaire enquiring about co-morbid illness, duration of illness prior to seeking medical attention, smoking status, as well as collecting information about socioeconomic issues, would be helpful. If taking all of these factors into consideration, a larger cohort would be required to give the study suitable power to detect if differences in miRNA expression can be used as a biomarker of active TB disease.

4.4.6.5 Gain a better understanding of the miRNA expression in treatment failure and treatment success patients

Being able to detect treatment failure early is very important, not only for the individual but for the greater community. Unless current therapy is adjusted, amplification of drug resistance is likely to occur, leading to greater morbidity and even mortality for the individual and prolongation of therapy using agents with

greater toxicity. Compounding this is on-going transmission of potential drug-resistant *M. tuberculosis* to the community.

Our results were promising, however, due to the small number of patients who were deemed to have failed treatment, it is hard to draw any conclusions. A larger study cohort would likely increase the number of patients in the treatment failure arm. Better case definition that allows stratification of treatment failures based on aetiology would also be helpful. Reasons that patients fail treatment are varied and are likely to affect miRNA expression. Such factors would include: infection with a drug-resistant organism; acquired drug-resistance (usually due to poor compliance); and, co-morbid illness such as HIV (Dooley et al., 2011; Jibrin et al., 2012).

4.4.6.6 Exploring the expression of miRNA in other lung infections

Our study consisted of two groups, those with active TB and those who were healthy. The diagnostic dilemma often with TB is being able to differentiate it from other pneumonic infections (Storla et al., 2008). Radiologically both pneumonic infections secondary to *M. tuberculosis*, or other pathogens, can look identical, with similar symptoms (McAdams et al., 1995; World Health Organization, 2014). *M. tuberculosis* is usually more insidious in nature, however, particularly in the setting of immune-compromise, TB presenting in a similar fashion to other pneumonic infections is not uncommon (Levy et al., 1987; Kunimoto et al., 2005; Nyamande et al., 2007). Being able to differentiate *M. tuberculosis* infection from other causes of pneumonic infection is very important, as the treatments are vastly different, utilising different therapies with very different duration of treatment (Kunimoto et al., 2005).

Future studies examining miRNA expression in not only TB but other pneumonic infections are required.

4.4.6.7 Examining miRNA expression in extra-pulmonary TB

Extra-pulmonary TB was not studied as part of this study. It would be interesting to profile miRNA expression in this subset of *M. tuberculosis* disease. It would be most interesting to determine if miRNA expression was different and was instead more closely aligned with the tissue/organ involved.

4.4.6.8 Discordant results between similar studies

There have been several studies that have examined extracellular circulating miRNAs from *M. tuberculosis*-infected individuals and controls, however common biomarker candidates have not been identified. There are several reasons why this may have occurred including; heterogeneous study design with inconsistent definitions of cohorts; varying profiling techniques yielding different expression profiles; lack of consistent normalizing strategies; significant inter-individual variability confounded at times by small study group sizes; and, other confounding factors which are not accounted for such as ethnicity, socioeconomic status and co-morbid conditions that may impact miRNA expression. To improve this, future biomarker studies should aim to define the criteria for disease more stringently and focus on well-defined study groups, such as LTBI with a known index case and have more standardized analysis and data evaluation.

This body of work has identified a number of miRNAs that were significantly regulated in pulmonary TB. Used in combination, these miRNAs give both an

excellent sensitivity without compromising specificity that enables differentiating those with pulmonary TB from those who are healthy with a good degree of accuracy. This is the first study, to our knowledge, that has examined miRNA expression longitudinally. Our findings were not as expected as miRNA expression did not always return to healthy baseline with treatment. This serves to highlight the current gaps in knowledge pertaining to miRNA functionality and purpose. We also noted varying miRNA expression with treatment outcome, either success or failure, and even though our cohort of treatment failures was small, it is encouraging that perhaps miRNAs could be utilised early during TB management as a biomarker to predict treatment outcome. As has been found in a few studies, we also found significant variation between the two ethnic populations, both in the healthy controls and the active TB group. Whilst some of these differences may be attributable to socio-economic differences between the groups, there may also be an inheritable difference in miRNA expression. This may translate to certain populations being more susceptible to TB and furthermore their immunological response to TB infection may be different.

5 *M. TUBERCULOSIS* INFECTION IN MACROPHAGES

5.1 Introduction

Alveolar macrophages are the first cell type from the innate immune system to encounter the bacterium and they provide the first line of defence against the invading pathogen. As *M. tuberculosis* is an intracellular pathogen, following its phagocytosis by macrophages, its ability to survive and replicate is dependent on a degree of tolerance from the host immune system (Aderem et al., 1999).

Augmentation of the host transcriptome occurs as a response to the encounter between *M. tuberculosis* and host macrophages (Ehrt et al., 2001) and is triggered by pattern recognition receptors. As a result, numerous pro-inflammatory mediators such as chemokines and cytokines are released that aid the antigen-specific acquired immune response to the pathogen. However, tight regulation of host cellular immune pathways needs to occur to prevent an excessive and inappropriate inflammatory response that causes host tissue damage (Ehrt et al., 2001).

Previous work has shown the important role that miRNAs play in modulating immune responses (Asirvatham et al., 2009; Oglesby et al., 2010). Their ability to tightly modify and calibrate key immune responses forms a critical part in the immune pathway. Recent attempts have been made to determine the effects that *M. tuberculosis* infection has on the expression of miRNA in the host (Wang et al., 2011; Fu et al., 2011). However, there are limited studies that have explored the

effect that *M. tuberculosis* infection has on host macrophages. Schnitger et al. examined the role of miRNAs in other intracellular pathogens, namely *Listeria monocytogenes* (Schnitger et al., 2011). The authors found significant regulation of miRNAs that specifically target macrophage-innate immune response at post-transcriptional levels.

There has been much effort recently to understand the role of miRNAs in innate immunity and macrophage inflammatory response. Identifying key miRNAs involved in *M. tuberculosis* infection will help gain a better understanding of its pathogenesis, and may identify miRNAs as targets for new therapies.

Here we explore miRNA expression in human macrophages infected with *M. tuberculosis* with the following hypothesis and aims.

HYPOTHESIS

M. tuberculosis infection modulates miRNA expression in infected macrophages resulting in varying expression of inflammatory cytokines.

AIMS

1. To identify key miRNAs released by macrophages during *M. tuberculosis* infection.
2. To determine if miRNA changes are reflective of inflammatory cytokine expression.

5.2 Methods

5.2.1 Human macrophages infected with *M. tuberculosis* or stimulated with LPS

Primary human monocytes were isolated from freshly collected buffy coats from six healthy voluntary blood donors (Australian Red Cross Blood Service, Sydney, Australia) as described in Section 2.2.1 of the Materials and Methods. Monocytes were plated at 1 x 10^6cells/well and differentiated into macrophages as described in Section 2.2.1 of Materials and Methods, with 18 wells in total.

On day five, 100 units of interferon-γ (Sigma-Aldrich) were added to each well. On day six, six wells per donor were infected with H37Rv *M. tuberculosis* at a multiplicity of infection (MOI) of 1, six wells were treated with 100 ng/mL lipopolysaccharide (LPS) (Sigma-Aldrich) and the remainder were uninfected/untreated controls. LPS is known to induce transcription of genes that drive pro-inflammatory regulators of the immune response (Hambleton et al., 1996) and it has been extensively used in models studying inflammation as it induces many inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Ngkelo et al., 2012). Cells and supernatant were collected from half of the total wells at six-hour post-infection time point with the remainder harvested at the 24-hour time point. Triplicate samples were assayed for colony forming units (CFU). Culture medium was removed and cells lysed with Triton X-100 lysis buffer (Sigma-Aldrich). Serial dilutions of the bacterial suspensions were plated (three replicates for each dilution) on Middlebrook 7H10/OADC agar plates. CFUs were counted after 21 days of incubation at 37°C.

5.2.2 RNA extraction and quality control

RNA was isolated using Trizol as described in Section 2.3.2 of Materials and Methods, and concentration and quality were determined by NanoDrop 2000 Spectrophotometer (Thermo Scientific) as described in Section 2.4.1 of Materials and Methods.

5.2.3 qRT-PCR

100 ng of total RNA was reverse-transcribed using All-in-One miRNA first cDNA synthesis kit (Gencopoeia) as described in Section 2.4.5 of Materials and Methods.

Based on current literature and results obtained examining the expression of miRNA from the plasma of *M. tuberculosis* patients from the Chinese cohort (Chapter Four), the expressions of seven miRNAs (miR-99b, miR-7a, miR146a, miR-21, miR-221, miR-26, and miR-29) were examined in macrophages infected with *M. tuberculosis* or stimulated with LPS and compared to uninfected/untreated macrophages. RNU6 was used to normalise the data.

5.2.4 Cytometric bead array

Cytokines, interleukin (IL)-1β, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, tumour necrosis factor (TNF)-α, and interferon-γ-inducible protein (IP)-10, were quantified simultaneously using a human chemokine CBA Kit (BD Biosciences), as described in Section 2.4.1 of Materials and Methods.

5.2.5 Data analysis

5.2.5.1 miRNA array

miRNA expression data were imported into Partek Genomics Suite (Partek, St Louis, MO) as CEL files. Version 2.0 and Version 3.0 Affymetrix chips were analysed separately. Principal-component analysis was performed to identify outliers and to evaluate whether study date or individual variation affected the result.

5.2.5.2 miRNA array validation with qRT-PCR

Data were analysed using SDS Relative Quantification Software Version 2.3 with automatic baseline (3-15 cycles) and threshold (0.2) settings for quantification cycle (Cq) determination. The threshold cycle Cq is defined as the fractional cycle number at which the fluorescence passes the fixed threshold (0.2). The mean Cq values for duplicate wells were then calculated.

5.2.5.3 qRT-PCR

qRT-PCR data were analysed using SDS Relative Quantification Software Version 2.3. For CBA, individual cytokine concentrations were indicated by their fluorescent intensities (Fl-2) and were computed using the standard reference curve of CELLQUEST and CBA software (BD Biosciences).

5.2.6 Statistical analysis

5.2.6.1 miRNA array

The differences in miRNA expression based on disease status were analysed by Student's *t*-tests for comparison of two groups and Wilcoxon rank-sum test for multi-group comparisons. P-values below 0.05 were considered significant. Differential expression analysis was performed using one-way analysis of variance (ANOVA).

5.2.6.2 qRT-PCR

The 2(-ΔΔC(T)) method was employed to analyse the relative changes in miRNA expression from qRT-PCR. The difference in miRNA expression was analysed by Student's *t*-tests for comparison of two groups in GraphPad Prism 6 (Graphpad Software Inc., San Diego, CA, USA).

5.2.6.3 CBA

The statistical significance of findings pertaining to cytokine expression measured from the supernatant of *M. tuberculosis*-infected and uninfected macrophages were determined by Wilcoxon rank-sum test using GraphPad Prism (Graphpad Software Inc.).

5.3 Results

5.3.1 miRNA array

With the aim of identifying novel miRNAs and to gain a better understanding of the macrophage inflammatory response to *M. tuberculosis* infection we initially began our work utilising miRNA microarrays. Microarray technology is a powerful high-throughput tool capable of monitoring the expression of thousands of miRNAs at

once and in parallel. It can be used to quantify differences in the abundance of, in this case, miRNA (also DNA and RNA) between two different entities (for example, one which is infected with *M. tuberculosis* and the other which is not). It has been extensively used and validated to examine expression of miRNA of normal and diseased samples, such as malignancy, and to distinguish expression signatures associated with diagnosis, prognosis and therapeutic interventions (Zhang et al., 2008; Baffa et al., 2009; Ueda et al., 2010).

Six samples of human monocyte-derived macrophages with and without infection with *M. tuberculosis* were tested on miRNA arrays. Two different versions of microarray chips (GeneChip® miRNA 2.0 and GeneChip® miRNA 3.0 [Affymetrix]) were used to measure miRNA expression. The results from the microarrays were analysed using Genomics Suite® Software (Partek). The current software does not allow microarray chips from different versions to be analysed concurrently. For this reason, analysis was undertaken separately for Version 2.0 and Version 3.0 microarray chips. In total, RNA from two donors was loaded onto Version 2.0 microarray chips and RNA from four donors was loaded onto Version 3.0 microarray chips.

There was a marked variation in the expression profile of miRNA between samples from different individuals when comparing infected versus uninfected macrophages. From principal component analysis the expression profile for miRNAs was more closely aligned for an individual, rather than for comparison of infected and non-infected miRNA expression for all donors (Figure 5.1). This is likely to be representative of normal human variance. For this reason it was decided to study the

variance of miRNA expression in each subject and then determine if there were any miRNAs commonly modulated across the donors. Differential expression analysis was performed using one-way analysis of variance (ANOVA) with infection as a variance. Gene lists were created using a cut-off of two-fold change (either two-fold increase for up-regulation or two-fold decrease for down-regulation) (Table 5.1).

Examining the results of Version 3.0 microarray chips (total of four donors), comparing miRNA expression from infected and uninfected macrophages, there were no miRNAs that had a fold change of greater than two in all four samples. However there were several miRNAs that were common to three donors. Lists of these miRNAs are given with their corresponding fold change in Table 5.1 (up-regulated miRNAs) and Table 5.2 (down-regulated miRNAs).

Examining the list of miRNAs up-regulated greater than two-fold from Version 2.0 microarray chips, there were two miRNAs (miR-1246_st and miR-125-3p_st) that were also up-regulated greater than two-fold in three of the four RNA samples infected with *M. tuberculosis* analysed on Version 3.0 microarray chips. There was one greater than two-fold down-regulated miRNA (miR-324-3p_st) from Version 2.0 microarray chips that was common to three of the four donors from Version 3.0 microarray chips.

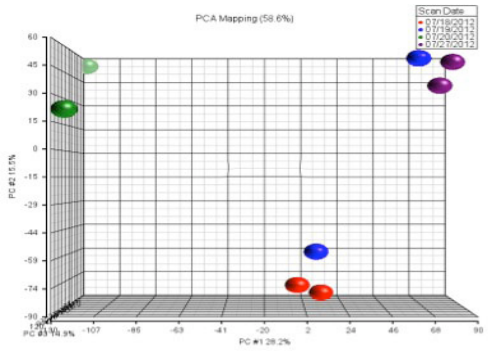

Figure 5-1. Principal component analysis (Version 3.0 microarray chips). miRNA expression was measured from PBMCs from four donors which were then either infected with *M. tuberculosis*, or uninfected controls, using microarray. Each colour corresponds to a different donor. The close proximity of both spheres of the red and purple donors suggests that there is minimal detectable difference between their miRNA expression in the infected and uninfected PBMCs. There was no clear cluster of infected and uninfected chips demonstrating quite distinct miRNA expression in each donor.

Table 5-1. Up-regulated miRNAs in human macrophages infected with *M. tuberculosis.*

miRNA	Fold Change Donor 1	Fold Change Donor 2	Fold Change Donor 3	Fold Change Donor 4
4532_st	7.58	2.43	2.50	1.47
1246_st	7.22	2.71	-1.24	25.77
125a-3p_st	2.69	3.37	1.03	3.03
193a-3p_st	2.76	2.30	0.68	5.37
3135b_st	6.69	2.43	0.81	3.00
3178_st	26.32	3.03	0.74	19.28
3197_st	61.73	5.47	1.98	44.19
4484_st	34.10	6.75	0.61	77.36
U41_x_st	6.28	1.66	7.82	2.52
3613-5p_st	16.62	-3.42	2.87	2.11
940_st	2.85	1.55	3.99	5.12

miRNA expression from monocyte-derived macrophages infected with H37Rv *M. tuberculosis* at MOI 1 compared with no infection control, analysed with Affymetrix miRNA microarray.

Table 5-2. Down-regulated miRNAs in human macrophages infected with *M. tuberculosis*.

miRNA	Fold Change Donor 1	Fold Change Donor 2	Fold Change Donor 3	Fold Change Donor 4
324-3p_st	-2.71	-1.03	-4.55	-2.85
3911_st	-19.32	1.63	-10.52	-2.11
425-star_st	-3.61	-1.56	-30.96	-2.32
4743_st	-6.71	1.17	-4.93	-2.39
574-3p_st	-3.12	-1.00	-6.24	-2.08
339_st	-1.38	-2.27	-2.10	1.20
85-26_st	-1.99	-2.25	-2.32	-2.26

miRNA from PBMC derived macrophages infected with H37Rv *M. tuberculosis* at MOI 1 compared with no infection control, analysed with Affymetrix miRNA microarray.

5.3.2 Validation

To assess the reliability of the data between the two different chip versions miRNA expression from the same donor was measured using both Version 2.0 and Version 3.0 chips. There was a large difference in miRNA expression across the majority of miRNAs measured (Figure 5.2). It was clear that some miRNAs that were up-regulated in one chip were down-regulated in the other, despite loading identical RNA from the same donor. Technical quality control was satisfactory for both microarray chips.

To assess further the reliability of the array data presented, the relative expressions of three miRNAs (miR-155, miR-146a and miR-29a) were measured. These miRNAs were selected because even though they did not feature in the arrays as being significantly modulated, previous work that we have undertaken had shown their

significant modulation with infection. Furthermore, they are frequently found in the literature to be regulated in *M. tuberculosis* infection (Fu et al., 2011; Wu et al., 2012; Kleinsteuber et al., 2013).

qRT-PCR was performed on the RNA samples using TaqMan® PCR Kit as described in Section 2.3.4 of Materials and Methods. In four of the five donors the expression of miR-146a between the array and qRT-PCR was conflicting. For example, Donor One had an approximately six-fold increase in miR-146a expression as measured by qRT-PCR, whereas the microarray reported a downregulation of miR-146 (Figure 5.3 A). Similar discordant results were observed with miR-155 and miR-29a (Figure 5.3 A, B).

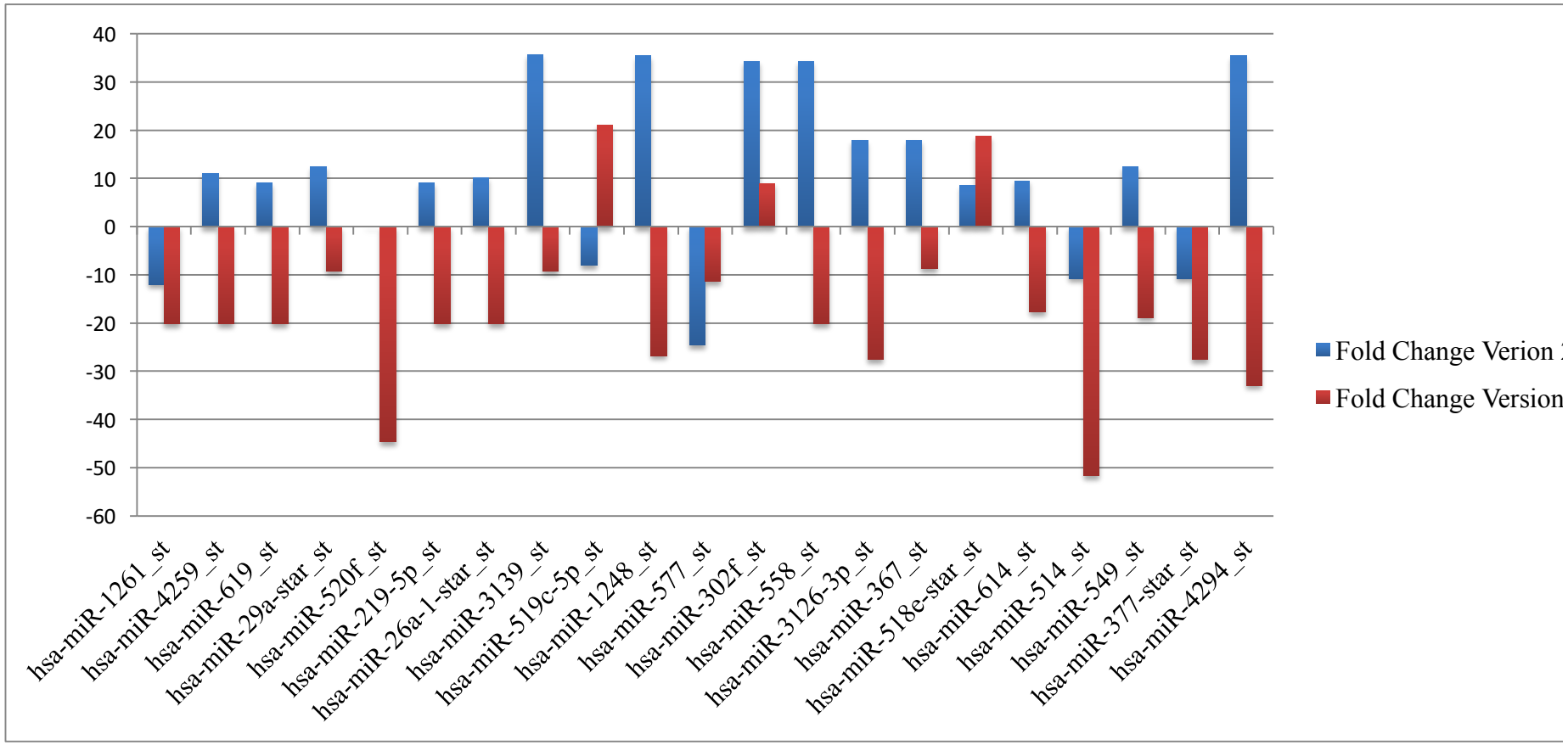

Figure 5-2. miRNA fold change from human macrophages infected with H37Rv *M. tuberculosis* measured by microarray. Two different versions of the Affymetrix microarray chips (Version 2.0 and 3.0) were used to quantify miRNA expression from identical RNA samples. There was marked variation in miRNA expression. The miRNAs shown here are a sample of the 1105 (Version 2.0) and 1693 (Version 3.0) miRNAs, which showed similar variation.

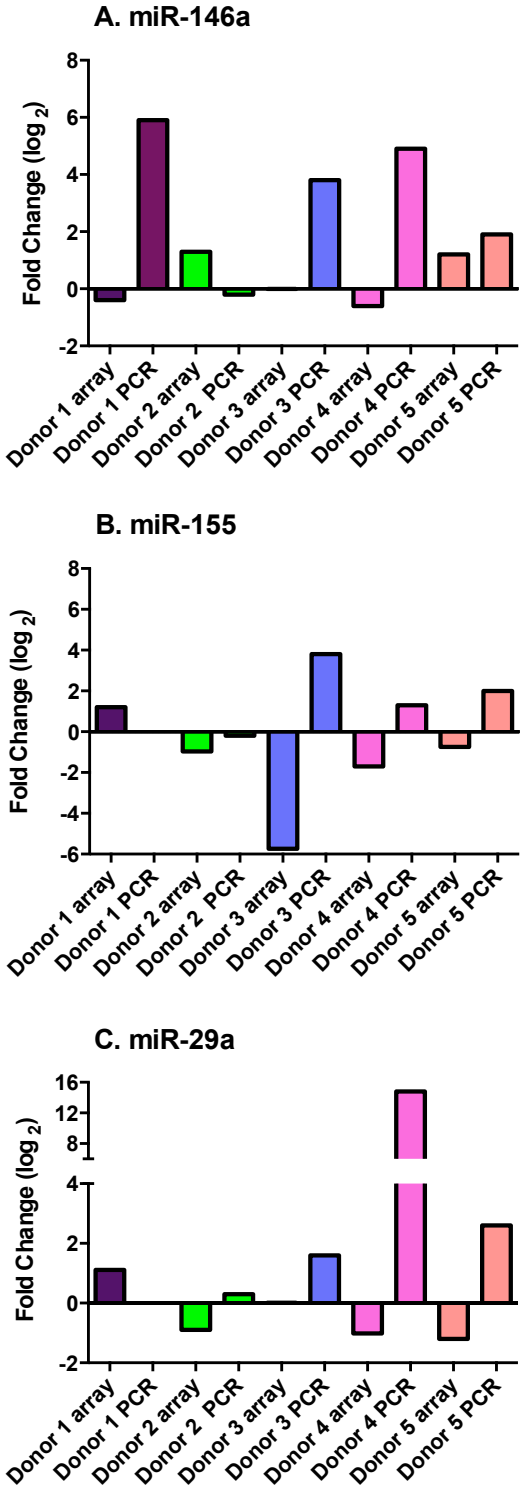

Figure 5-3. Validation of microarray results by qRT-PCR for selected miRNAs. Fold change in miRNA in *M. tuberculosis* infected samples with respect to uninfected samples for five donors as measured by microarray and qRT-PCR. There is clear difference in the expression profile in four out of five miRNA for miR-146 (A) and -155 (B) and all six donors for miR-29a (C).

Numerous studies exist in the literature, which have profiled miRNA expression utilising microarrays with concordant qRT-PCR results (O'Connell et al., 2007; Singh et al., 2013). However, we found that our microarray results could not be verified with qRT-PCR. Furthermore, identical RNA from the same donor loaded onto different microarray chips yielded different results.

Due to the unreliability of the data obtained, miRNA expression was examined by qRT-PCR as detailed below.

5.3.3 miRNA expression in *M. tuberculosis* infected macrophages

The expression of seven miRNAs in human monocyte-derived macrophages infected with *M. tuberculosis*, activated by LPS, or uninfected was examined from six donors. These miRNAs were chosen due to their significant regulation in the Chinese cohort (Chapter Four) or their reported regulation during *M. tuberculosis* infection (Wang et al., 2011; Fu et al., 2011; Yi et al., 2012). To better understand the complexity and variability of miRNA expression, we examined miRNA expression at two time points: six- and 24-hours after infection. All miRNAs were normalised to RNU6. Mean raw Cq values together with the range of all miRNAs are given in Table 5.3.

RNU6 was abundantly and consistently expressed across all samples, and there was no statistically significant variability in Cq values across the three conditions, making it a suitable reference miRNA (Table 5.3 and Figure 5.4 H). As would be expected, all Cq values for the seven miRNAs in uninfected macrophages at six- and 24-hours of culture were similarly expressed and within one cycle of each other (Table 5.3). When normalised to RNU6, there was no statistically significant

variability in the seven miRNAs between the uninfected samples at the six- and 24-hour time points (Figure 5.5).

5.3.3.1 Growth of M. tuberculosis bacterial populations

Growth of *M. tuberculosis* within the macrophages was assessed after six and 24 hours of infection. CFU assays were performed manually following a 21-day incubation. For dilutions of $1:10^{-1}$ and $1:10^{-2}$, CFUs were too numerous to count; at dilutions of $1:10^{-3}$ and $1:10^{-4}$, linear CFU counts were noted.

Table 5-3. Expression levels of miRNAs as measured by qRT-PCR.

miRNA	Cq Mean (range) UI 6h	Cq Mean (range) UI 24h	Cq Mean (range) *M. tb* 6h	Cq Mean (range) *M. tb* 24h	Cq Mean (range) LPS 6h	Cq Mean (range) LPS 24H
let-7	16.2(14.3-21.5)	15.4(13.3-19.2)	15.2(12.5-18.3)	16.2(13.1-21.8)	15.6(14.2-17.5)	15.4(13.0-17.3)
miR-146a	27.9(24.5-35.8)	26.8(23.3-32.6)	23.1(19.1-27.5)	25.1(22.0-34.0)	24.5(21.0-30.2)	25.4(22.8-29.5)
miR-21	14.0(11.7-16.3)	13.5(10.5-15.3)	12.2(9.8-13.6)	11.0(8.2-15.3)	12.7(10.1-14.7)	11.7(9.9-13.6)
miR-221	20.3(17.7-24.2)	20.3(17.0-23.9)	21.3(18.2-25.0)	24.7(20.4-31.7)	23.3(18.8-25.4)	24.1(20.9-25.7)
miR-26	16.6(14.7-21.0)	16.1(14.5-20.2)	18.5(15.8-22.0)	20.2(17.9-29.3)	18.2(15.5-22.2)	18.2(15.4-22.3)
miR-29	16.0(14.6-18.9)	16.0(14.6-18.8)	14.0(12.4-18.1)	13.7(12.3-16.5)	14.8(13.1-16.6)	14.3(12.9-16.1)
miR-99b	26.4(23.9-28.9)	26.3(23.3-29.6)	22.9(21.1-25.0)	22.2(19.9-31.9)	23.3(19.0-26.1)	26.5(24.3-28.6)
RNU6	18.0(15.7-22.0)	18.0(15.8-21.3)	18.3(15.7-21.8)	19.1(15.3-31.1)	18.1(15.5-21.6)	17.9(15.8-21.0)

Raw data presented as mean of triplicate for all 6 donors. miRNA, microRNA; Cq, Quantification of cycle; UI, Uninfected; *M. tb*, *Mycobacterium tuberculosis*; LPS, Lipopolysaccharide; 6h, 6 hours of incubation; 24h, 24 hours of incubation.

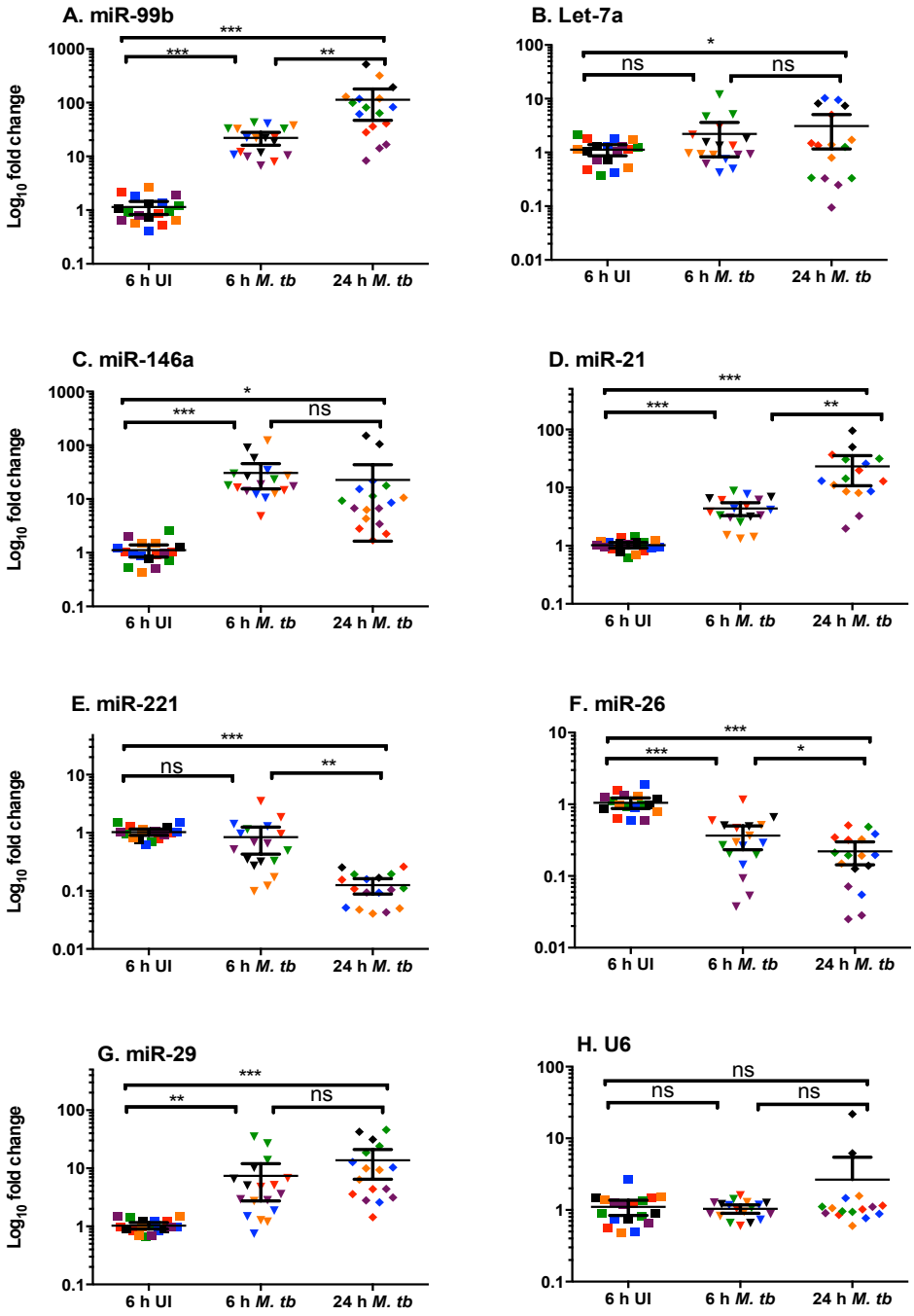

Figure 5-4. Expression levels of eight miRNAs in *M. tuberculosis* infected and uninfected macrophages. Expressions of miRNAs in monocyte-derived macrophages from six donors were measured in triplicate by qRT-PCR using RNU6B as a reference miRNA. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results from each donor are depicted in a different colour. Bars indicate mean with 95% confidence intervals. The differences were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001, ns= not significant. UI, uninfected; *M. tb*, *Mycobacterium tuberculosis*.

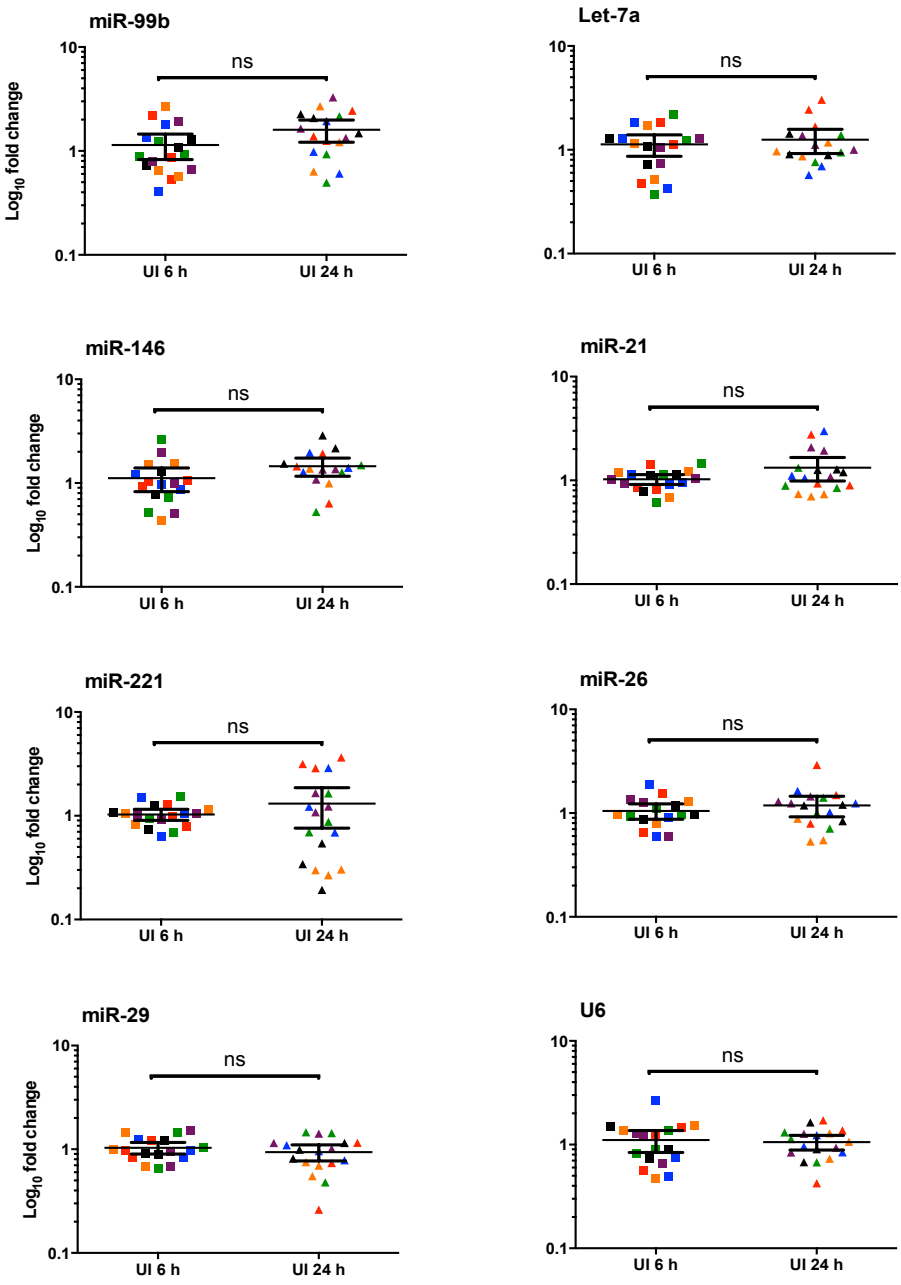

Figure 5-5. Expression levels of eight miRNAs in uninfected macrophages. Expression of miRNA in monocyte-derived macrophages from six donors was measured in triplicate by qRT-PCR using RNU6B as a reference miRNA. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results from each donor are depicted in a different colour. Bars indicate mean with 95% confidence intervals. The differences were calculated by a Student's *t*-test with a p-value <0.05 deemed significant. ns= not significant. UI, uninfected.

5.3.3.2 M. tuberculosis-infected macrophages determined by qRT-PCR

Examining the miRNA expression of *M. tuberculosis* infected macrophages and comparing it to uninfected macrophages, all of the miRNAs examined showed significant altered regulation, though at varying time points. Fold change and probability values for each miRNA examined at the varying time points are presented in Table 5.4.

All except miR-221 and *let-7* were significantly differentially regulated at six hours post *M. tuberculosis* infection (Table 5.4 and Figure 5.4 [B and E]). Of the significantly regulated miRNAs, only miR-26 was down-regulated. All of the others (miR-99b, -146a, -21, and -29a) showed significant up-regulation with miR-146 and -99b having the most significant fold change (Table 5.4 and Figure 5.4 [A and C]).

Results indicate that miR-99, *let-7* and miR-21 (Figure 5.4 and 5.6 [A, B and D]) were up-regulated since six hours post-infection with increased expression of these miRNAs over time. Whereas miR-146a up-regulation peaked at six hours and seemed to be trending back down towards uninfected levels of expression at 24-hour time point, though the variation between the six- and 24- hour expression was not significant (Figure 5.4 and 5.6 [C]).

miR-221 and -26 had the greatest down-regulation at 24 hours post infection (Figure 5.4 [E and F]). miR-221 was found to be not significantly different at the 6-hour time point. It is clear from examining the data represented in Figure 5.4 (E) and Figure 5.6 (E) that there seems to be considerable variability between infected donors at this

six-hour time point, with some donors (as depicted in red) up-regulated at this time point and others (as depicted in orange) down-regulated. At the 24-hour time point though, all donor expression of miR-221 is significantly down-regulated. This highlights individual variability that is often seen when examining human macrophages from different donors when compared with examining cell lines where variability is minimal.

When examining the mean miRNA expression for each donor at the varying time points in *M. tuberculosis* infected and uninfected samples, the expression of some miRNAs show a consistent pattern across all six donors (Figure 5.6). miR-99b (Figure 5.6 [B]) was found to be clearly up-regulated in all six donors following six hours of infection and then was further up-regulated at 24 hours post infection. miR-146a was up-regulated early at six hours but then was seen to be reduced in five of the six donors at 24 hours, though not to the levels seen in the uninfected controls (Figure 5.6 [C]). miR-21 and -29 demonstrate up-regulation in all donors at six hours and then further up-regulation in most donors at 24 hours (Figure 5.6 [D and E]). For miR-26 all donors show down-regulation at six hours post infection with further significant down-regulation at 24 hours (Figure 5.6 [F]).

Table 5-4. miRNA expression in *M. tuberculosis* infected or LPS stimulated in human macrophages as compared with uninfected macrophages.

miR	Condition	Fold Change	p value
99b	*M. tb* 6h	2.22	0.113
	M. tb 24 h	127.23	< 0.001
	LPS 6h	17.55	< 0.001
	LPS 24 h	2.86	0.037
let-7a	*M. tb* 6h	2.22	0.113
	M. tb 24 h	3.75	0.030
	LPS 6h	1.58	0.328
	LPS 24 h	1.08	0.799
146-a	*M. tb* 6h	30.52	<0.001
	M. tb 24 h	28.44	0.037
	LPS 6h	14.43	0.017
	LPS 24 h	4.26	< 0.001
21	*M. tb* 6h	4.39	< 0.001
	M. tb 24 h	24.81	< 0.001
	LPS 6h	2.82	< 0.001
	LPS 24 h	5.64	< 0.001
221	*M. tb* 6h	0.84	0.358
	M. tb 24 h	0.13	< 0.001
	LPS 6h	0.35	< 0.001
	LPS 24 h	0.14	< 0.001
26	*M. tb* 6h	0.37	< 0.001
	M. tb 24 h	0.22	< 0.001
	LPS 6h	0.40	< 0.001
	LPS 24 h	0.31	< 0.001
29	*M. tb* 6h	7.38	0.007
	M. tb 24 h	15.04	< 0.001
	LPS 6h	3.44	< 0.001
	LPS 24 h	3.32	0.002

miR, microRNA, *M. tb, Mycobacterium tuberculosis;* LPS, lipopolysaccharide; 6h six hours; 24h, 24 hours

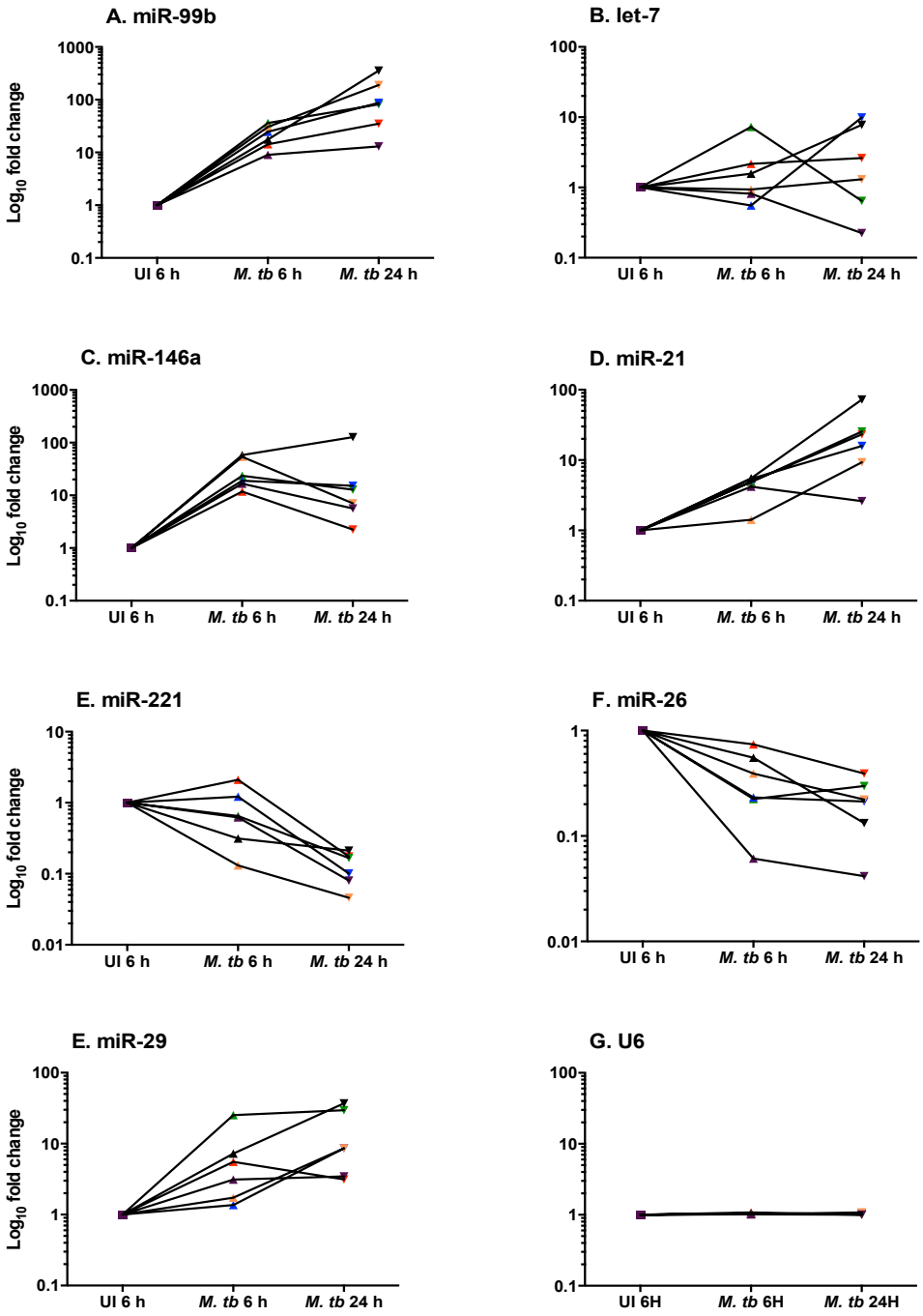

Figure 5-6. Change in miRNA expression measured at six- and 24-hours of incubation. Macrophages were inoculated with MOI 1, cultured for six or 24 hours as indicated, harvested and RNA isolated using Trizol. miRNA expression was measured by qRT-PCR in triplicate from each of the six donors. The relative expression of miRNA was determined using 2(-ΔΔC(T)) and are presented as fold change. RNU6 was used as the reference miRNA. The average of miRNA expression for each donor is presented here with individual donors represented by a different colour. UI, uninfected; *M. tb*, *Mycobacterium tuberculosis*.

5.3.3.3 Macrophages stimulated with LPS

LPS has been widely used to mimic bacterial infections, including *M. tuberculosis* infection in the *in-vitro* model (Ngkelo et al., 2012). It is well known and understood that LPS induces many cytokines and chemokines that are important in controlling *M. tuberculosis* infection and initiating the adaptive immune response (Rossol et al., 2011). It is for this reason that we chose to explore miRNA expression during LPS stimulation.

As with *M. tuberculosis* infected macrophages, there was significant regulation of miRNAs following LPS stimulation, however there were some notable differences. miR-99b was again up-regulated following six-hour LPS stimulation, however the fold change was not as marked as macrophages infected with *M. tuberculosis*, likewise macrophages stimulated with LPS were not as significantly up-regulated at the 24- hour time point (Table 5.4 and Figure 5.7 [A]).

A similar pattern of miRNA regulation was noted with miR-146a, -21, -221, -26 and -29 in both the *M. tuberculosis* and LPS models, however the degree of modulation was far less in the LPS stimulated macrophages (Table 5.4 and Figure 5.7 [C-G]). Interestingly, *let-7,* which was shown to be significantly up-regulated by more than four-fold during *M. tuberculosis* infection following 24-hour incubation, was not significantly modulated during LPS stimulation. Examining the individual donors in the LPS model reveals that *let-7* expression is more variable than was seen in the *M. tuberculosis* infected macrophages at the six-hour time point, suggesting a more variable individual response to LPS stimulation.

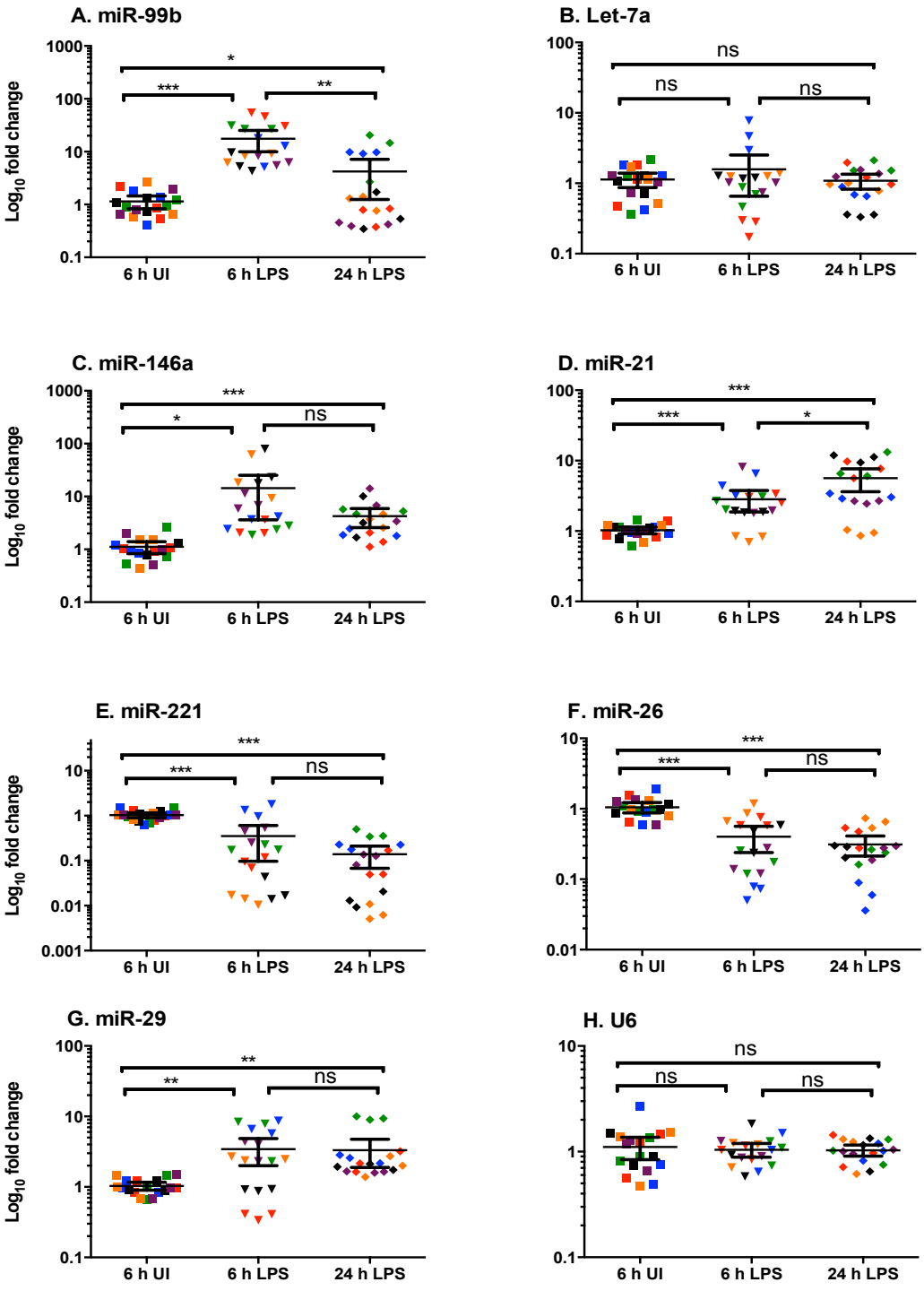

Figure 5-7. Expression levels of eight miRNAs in LPS-stimulated and uninfected macrophages. Expression of miRNA in monocyte-derived macrophages from six donors was measured in triplicate by qRT-PCR using RNU6B as a reference miRNA. The relative expression of miRNAs was determined using 2(-ΔΔC(T)). Results from each donor are depicted in a different colour. Bars indicate mean with 95% confidence intervals. The differences were calculated by a Student's *t*-test with a p value <0.05 deemed significant. *p <0.05, **p <0.01, ***p <0.001, ns= not significant. UI, uninfected; LPS, lipopolysaccharide.

5.3.3.4 Cytokine and chemokine examination in human macrophages infected with M. tuberculosis

Six key inflammatory cytokines (Figure 5.8 [A-C]) and chemokines (Figure 5.8 [D-F]): IL-1β; IL-6; TNFα; MIP-1α (CCL3); IL-8 (CXCL8); and, IP-10 (CXCL-10), were examined by cytometric bead array in supernatants obtained from *M. tuberculosis*-infected and -uninfected macrophages following 24-hour incubation for the same six donors. The mean concentrations of each cytokine in *M. tuberculosis*-infected and -uninfected macrophages are presented in Table 5.5.

Previous studies have demonstrated the crucial role that these cytokines and chemokines play in controlling *M. tuberculosis* infection (Lewis, 1990; Kaufmann, 2002; Algood et al., 2004; Mootoo et al., 2009), however there is a paucity of literature examining their levels from *M. tuberculosis*-infected macrophages.

There was a marked increase in all cytokines and chemokines levels in supernatants obtained from macrophages infected with *M. tuberculosis* across most donors (Figure 5.8). The most marked increase was IL-8 and IP-10 from the supernatant of *M. tuberculosis*-infected macrophages (Figure 5.8 [E and F]). The IFN-inducible chemokine, IP-10, was increased in the supernatant from *M. tuberculosis*-infected macrophages in all six donors (Figure 5.8 [F]). IL-8, a pro-inflammatory cytokine secreted from macrophages, plays an important role in the innate immune response. It has been found to be significantly elevated in the supernatants of macrophages and in bronchial alveolar lavage fluid from persons with pulmonary TB compared to healthy controls (Y. Zhang et al., 1995; Friedman et al., 2009). Four of the six donors

had increased levels of IL-8 during infection, however donors D and F had lower levels in the supernatants from *M. tuberculosis*-infected macrophages. Interestingly, donors D and F also demonstrated much higher levels of IP-10 in the supernatant from uninfected macrophages than all other donors. It may be that the supernatant for these donors was inadvertently contaminated or that the donor was suffering from an inflammatory illness at the time of blood donation, however, with only a small sample size and without any further medical information, it is difficult to draw any conclusions.

Levels of IL-1β (Figure 5.8 [A]), IL-6 (Figure 5.8 [B]), TNF-α (Figure 5.8 [C]), and MIP-1α (Figure 5.8 [D]) were all markedly increased in the supernatants of *M. tuberculosis* infected macrophages.

Table 5-5. Mean concentrations of cytokines in macrophages with and without *M. tuberculosis* infection.

	UI Mean (SD) (pg/mL)	*M. tb* mean (SD) (pg/mL)	p Value
IL-1β	0(0)	58 (60.5)	< 0.0001
IL-6	1893(3360.3)	11467(1560.2)	< 0.0001
TNFα	49(116.3)	5067(4948.1)	< 0.0001
MIP-1α	1396 (3284.2)	13133(3442.4)	< 0.0001
IL-8	28300(22364)	58214(42188.0)	ns
IP-10	6343(6624.3)	18044(3554.6)	< 0.0001

UI, uninfected; *M. tb*, *Mycobacterium tuberculosis*; SD, standard deviation; IL, interleukin; TNF, tumor necrosis factor; MIP, Macrophage inflammatory protein; IP, Interferon-γ-inducible protein; pg/mL, pictograms per millilitre; ns, not significant.

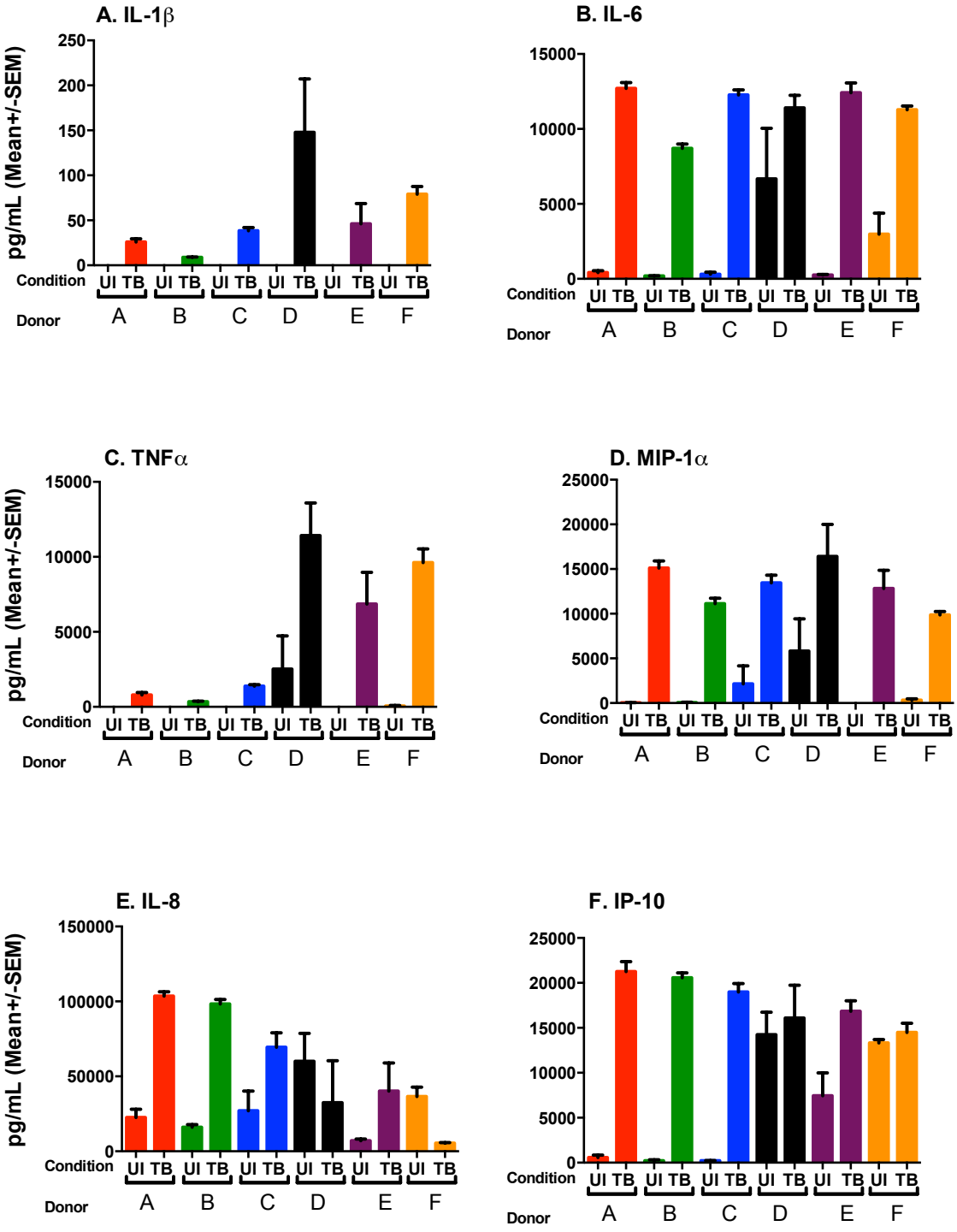

Figure 5-8. Cytometric Bead Array measuring cytokine levels from supernatants from H37Rv-infected and -uninfected macrophages collected at 24 hours post infection from six donors. Each biological sample had three replicates. Mean concentrations are given (pg/mL) with standard error. UI, uninfected; TB, H37Rv *M. tuberculosis* infection.

5.4 Discussion

miRNAs are non-coding RNAs that are emerging as key players in regulating gene expression. Our understanding of their role in the innate immune response and their ability to orchestrate cytokine and chemokine release is currently limited (O'Connell et al., 2007; Welker et al., 2007; Asirvatham et al., 2008). A greater understanding of their role in the immune response to *M. tuberculosis* infection has the potential to lead to new, targeted therapies through drug and vaccine development. Macrophages are the first immune cells to encounter *M. tuberculosis* and they are considered to play a key role in protecting the host by a means of phagocytosis and mycobacterial killing, as well as induction of the inflammatory cascade. Previous studies have shown that macrophages contribute to the immune response by modulating both cellular and humoral immunity, particularly through the synthesis and release of immunoregulatory cytokines and chemokines. There is also growing evidence that bacteria modulate immune responses by altering the expression of host miRNAs. Our study supports this concept, as it was evident that *M. tuberculosis* infection altered the expression of both miRNAs and cytokine production in human macrophages.

Initially this study measured miRNA expression using high throughput microarray technology, with the aim of identifying novel miRNAs that were regulated during *in vitro M. tuberculosis* infection. An essential component of this technology however, is to verify findings with qRT-PCR. Not only did the qRT-PCR data not correlate with the microarray data, but identical RNA from the same donor loaded onto two different microarray chips also demonstrated discordant results. It was therefore

concluded that the microarray results were unreliable for this purpose. Others have also reported problems with microarray accuracy; Kothapalli et al. examined the accuracy of several different microarray platforms and, among other things, found significant discrepancy in fold-change across the varying platforms (Kothapalli et al., 2002). Sato et al. compared repeatability and comparability across five different microarray platforms (including Affymetrix) and similarly found significantly poor inter-plate concordance, highlighting the problems of current miRNA microarray platforms (Sato et al., 2009). It has been suggested that the development of a set of commercially available standard miRNAs or samples would be helpful as it would allow comparison between different results from miRNA profiles (Cummins et al., 2006) thus overcoming some of the current difficulties encountered by this technology.

At the commencement of this study there was a paucity of published works that examined the role of miRNAs in not only mycobacterial infections but also infections in general. The emphasis of work that had been published at the time was primarily examining the role of miRNAs in malignancy. We had aimed to identify regulated miRNAs by utilising high-throughput technology. However, as this was not possible we then examined miRNAs which we had shown to be regulated in the plasma of patients with *M. tuberculosis* (Chapter Four), many of which had also been shown to be modulated in the literature.

This investigation studied the expression of seven miRNAs (miR-99b, -146a, -21, -221, -26, -29a and *let-7*) from human peripheral monocyte-derived macrophages from six donors, which were experimentally infected with *M. tuberculosis*,

stimulated with LPS, or used as uninfected baseline controls; RNU6 was utilised to normalise the qRT-PCR data. miRNA expression profiles were measured at both the six- and 24-hour time-point. This study has identified significant modulation of all seven miRNAs analysed during both *M. tuberculosis* infection and LPS stimulation of human macrophages. The pattern of expression, although not as significantly regulated, was similar in the LPS model and is concordant with most of the current literature that has examined macrophage-miRNA expression in the setting of infection or other inflammatory conditions (Taganov et al., 2006; Sheedy et al., 2009; J. Sharbati, et al., 2011a; Singh et al., 2013; Furci et al., 2013; Das et al., 2013).

When examining the expression of miR-99b, we found a marked and statistically significant up-regulation, especially in the *M. tuberculosis* model. This mirrored the results of our plasma study as described in Chapter Four. Similarly, Singh et al. found that the expression of miR-99b was highly up-regulated in murine macrophages infected with H37Rv *M. tuberculosis*. The authors also found that knockdown of miR-99b resulted in up-regulation of cytokines such as IL-1β, IL-6 and TNF (Singh et al., 2013). In our study, however we found a noticeable up-regulation of these cytokines in the setting of miR-99b up-regulation. The varying cytokine expression noted in this study may be due to the influence of other miRNAs on cytokine expression. This highlights the complexity of the innate and adaptive immune system and serves to highlight the need for a better understanding of the biological function of these miRNAs.

The miR-146 family comprises of two members: miR-146a and miR-146b. Our study of *M. tuberculosis*-infected human macrophages observed significant up-regulation of miR-146a that peaked at six hours post infection. Findings were similar in the LPS stimulated macrophages, however the degree of up-regulation was not as great.

Taganov and colleagues first reported that miR-146 modulates macrophage function and showed that it is a negative regulator of NF-κB activation. NF-κB plays a critical response in controlling host response to infection (Brasier, 2006).They also showed that in THP-1 cells, a human macrophage-like cell line, LPS stimulation resulted in the up-regulation of miR-146a. miR-146a expression peaked at around eight hours following LPS induction, after which its levels plateaued (Taganov et al., 2006). Similarly, this study found the highest expression at the earlier, six-hour time point. Human monocyte-derived macrophages from patients with *Mycobacterium avium* complex (MAC), which is less pathogenic than *M. tuberculosis*, have also been found to have increased expression of miR-146 (J. Sharbati et al., 2011). In an *in vitro* infection of macrophages with MAC, miR-146a was induced shortly after infection and showed slightly increased expression up to 48 hours post infection. These results differ from our findings, in that miR-146 reached maximum expression at the six-hour time point, however this may relate to the degree of pathogenesis of the mycobacterial species, with the less virulent MAC resulting in different miR-146 expression. Alternatively, it may be connected to the complexity and interdependent nature of miRNA expression in diseased states.

Interestingly, we observed down-regulation of plasma-derived miR-146a in patients with pulmonary TB compared to healthy controls (Chapter Four). Up-regulation of miR-146a, as observed in *M. tuberculosis*-infected macrophages, has been shown to result in a decreased inflammatory response with decreased levels of pro-inflammatory cytokines and down-regulation of TRAF6, IRAK1 and IRAK2 and NF-κB (Sharma et al., 2015). Conversely down-regulation of miR-146a has been shown to initiate an inflammatory response (Taganov et al., 2006; Hassan et al., 2012). Inflammation plays a critical role in host defence against invading microbial pathogens. The discrepancy in miR-146a expression between plasma and macrophages may be a result of the fine balance and orchestration of the innate immune system to limit the deleterious effects to host tissue of persistent inflammation. It may also represent manipulation of cellular miR-146a by *M. tuberculosis* to augment cellular inflammatory response in order to create a favourable cellular environment for the bacteria's survival.

Our study has shown that miR-21 is induced by both *M. tuberculosis* infection and LPS stimulation of macrophages. A study by Wang and colleagues examined macrophages obtained from patients with proven *M. tuberculosis* and compared the expression of several miRNAs to healthy subjects. Similar to our findings, miR-21 was also significantly up-regulated in the macrophages obtained from the TB subjects (Wang et al., 2011). It has been previously shown that LPS stimulation of macrophages induces miR-21 which indirectly results in the production of IL-10 (Sheedy et al., 2009b). Although not studied here, IL-10 has been found to be increased during *M. tuberculosis* infection and it has been proposed that IL-10 is linked with the ability of *M. tuberculosis* to evade host immune responses, possibly

by inhibiting the formation of the phagolysosome (Redford et al., 2011). Our results demonstrate that there is increased macrophage miR-21 expression over time following both infection with *M. tuberculosis* and stimulation with LPS. This aligns with findings from Sheedy and colleagues who found miR-21 expression in RAW264.7 cells, a murine leukaemic monocyte-macrophage cell type, increased over time when stimulated with LPS.

miR-21 was one of the first mammalian miRNAs identified. Despite this, there has been no published work that has examined the function of miR-21 in infection. Most work to date has examined the role of miR-21 in malignancy with more recent work examining its role in cardiac disease (Papagiannakopoulos et al., 2008; Dong et al., 2009; Patrick et al., 2010; Zheng et al., 2011). More work is needed to uncover the functional role of miR-21 in *M. tuberculosis* infection.

This study observed significant down-regulation of miR-221 in both *M. tuberculosis* infection (at 24 hours post infection) and with LPS stimulation (at six and 24 hours post incubation) which is consistent with findings from Furci and colleagues who found miR-221 was down-regulated in response to *M. tuberculosis* infection of macrophages and also down-regulated in response to less the virulent *M. bovis* (Furci et al., 2013). Stimulating THP-1 cells with LPS, Gazzar et al. observed early up-regulation in miR-221 levels, which is in opposition to this study's findings of significant down-regulation of miR-221 stimulated with LPS at the six- and 24-hour time points (Gazzar et al., 2010). The mechanism of action of miR-221 is not clear. However a study by Lu et al., which examined the effects of miR-221 on human monocytes-derived dendritic cells found that silencing miR-221, using knockdown

probes, resulted in increased apoptosis of dendritic cells (Lu et al., 2011). Down-regulation of miR-221, as was observed in this study, may be a protective effect to limit dendritic cell apoptosis and enhance the innate response to *M. tuberculosis* infection.

The area of miRNA research is in its infancy and, as such, little is known about the role and function of many miRNAs including miR-26. TargetScan® analysis of potential binding sites of miR-26 identified over 1000 conserved sites, including a number of known immune genes and kinases to name a few. This study observed significant down-regulation with both *M. tuberculosis* infection and LPS stimulation. Further studies are required to identify the key mRNAs targeted by miR-26 and to determine if down-regulation of this miRNA provides a survival advantage for the *M. tuberculosis* bacilli or is advantageous for the host response to *M. tuberculosis* infection.

miR-29a is known to control adaptive immune responses to bacterial infections by targeting IFN-γ (Ma et al., 2011). Studies demonstrated that miR-29a directly targets the 3'UTR of IFN-γ mRNA leading to suppressed IFN-γ production (Ma et al., 2011). IFN-γ plays a critical role in host defence against *M. tuberculosis*. In addition, several studies have reported that miR-29 also targets anti-apoptotic proteins such as Bcl-2 and Mcl-1 thus demonstrating another important role that miR-29 has in regulating the apoptotic pathway in immune cells (Park et al., 2008; Xiong et al., 2009).

This body of work has shown significant up-regulation of miR-29a during both *M. tuberculosis* infection and LPS stimulation of human macrophages as well as from human plasma from patients with active TB compared to no infection. In the plasma from newly diagnosed TB subjects we observed a four-fold increase in miR-29a levels compared to their matched healthy controls. A study by Das et al. also observed modest up-regulation, with a 1.86 fold change in THP-1 cells infected with H37Rv *M. tuberculosis* compared to no infection (Das et al., 2013). Similarly, Fu et al. observed up-regulated miR-29 in the setting of *M. tuberculosis* infection with significantly increased levels found in plasma, serum and saliva of human subjects with the disease compared to healthy subjects (Fu et al., 2011; Yi et al., 2012). Interestingly though, research examining miR-29 in other intracellular infections have reported significant down-regulation in murine macrophages when infected with *L. monocytogenes* (Ma et al., 2011). Overexpression of miR-29 in *M. tuberculosis* infection may explain one mechanism by which *M. tuberculosis* is able to evade macrophage digestion by inhibiting the secretion of IFN-γ, which is essential to fully activate infected macrophages.

The down-regulation of *let-7* in the setting of infection and inflammation is well documented (Rijavec et al., 2014) (Pandit et al., 2010). However, few studies to-date have examined the expression of *let-7* in *M. tuberculosis*-infected macrophages. Furci and colleagues observed a reduction in *let-7* expression from monocyte-derived macrophages infected with *M. tuberculosis* (Furci et al., 2013). Sharbati however found increased expression of *let-7e* from human macrophages infected with *M. avium*. (J. Sharbati et al., 2011) Our findings also showed an increase (three-fold) in the expression of *let-7a* in the setting of *M. tuberculosis* infection, but interestingly

no significant change following LPS stimulation. Our findings, together with those of Sharbati et al. were in contradiction to these earlier studies and require further evaluation. Other factors that could possibly explain the conflicting results that need consideration include the complex interplay and effect of other miRNA modulation in the setting of *M. tuberculosis* infection with resultant cytokine and chemokine augmentation and the effect this has on *let-7* expression.

5.4.1 Future directions

We have identified miRNAs that are modulated during *in vitro M. tuberculosis* infection of human macrophages. At the same time, we identified a number of key cytokines that are up-regulated during infection. The next step is to try to understand and determine the functions and targets of these miRNAs, and to gain a better understanding of the interrelation between miRNAs and the innate immune response. The reason for doing this is two-fold: firstly, it will allow a more detailed understanding of the pathogenesis and biology of disease; and, secondly it has the potential to represent a new class of directed therapeutics, which may yield patient benefits that are simply unattainable with current therapy.

The cytokines measured during *M. tuberculosis* infection were all significantly up-regulated. The association between miRNA and cytokine production and release is not well understood. What is apparent from the current literature is that a clear understanding of miRNA functions and their specific targets, especially in relation to infection and, more specifically, *M. tuberculosis* infection, is very limited. What is clear, however, is that miRNAs play a central role in modulating inflammatory responses and that manipulating miRNA expression has the potential to allow fine-

tuning of the inflammatory response to the advantage of the host and detriment of the pathogen. What is also clear is that each miRNA has numerous mRNA targets. In order to achieve directed manipulation of the inflammatory response an intricate understanding of miRNA networks, and how they work collectively, is required.

It remains challenging to identify the specific targets of any single miRNA. From the literature there are many conflicting reports as to the behaviour of many miRNAs in disease states. Some of this may be due to external factors such as the varying platforms used to quantify miRNA expression presenting different miRNA expression results. Certainly our study showed significant variation between miRNAs measured from the same RNA through microarrays and qRT-PCR. It is also likely to be due to the complex interactions and numerous targets that each miRNA has. One of the most interesting aspects of miRNA biology is that one miRNA can target multiple genes and a single gene can be targeted by multiple miRNA. Furthermore, there appears to be a lot of overlap with different miRNAs seemingly to serve the same purpose. However, this is likely to be a reflection of our very basic knowledge of how miRNAs function and there is likely to be combinations of complex specificity depending upon the situation. One aspect of this is that is appears that many of the functions of miRNAs are cell- or tissue-type specific (van Rooij, 2011).

The recent exponential growth in miRNA research has given rise to computational and experimental ways to functionally analyse miRNA targets. Identifying miRNA targets is key as it opens the possibility of directed miRNA-based therapeutic modalities, potentially targeting one of the receptors of a miRNA, without limiting

its other functional effects. The current methods to determine miRNA targets are discussed below.

5.4.1.1 Computational assessment of miRNAs targets

Computational framework for the functional assessment of miRNAS is one of the common methods used to determine target genes for miRNAs. Each miRNA is estimated to target approximately 100-200 genes (Friedman et al., 2009). There are several algorithms that have been developed that act to predict the mRNA targeted by each miRNA. Fundamentally, for each miRNA a list of potential target sites is determined on the 3'UTRs of human gene-coding transcripts based on a search for complementary binding sites for the sequence of the seven-nucleotide "seed" region. The miRNA "seed" is defined as positions two to eight from the 5'end of the mature miRNA. There may also be additional criteria for target prediction depending on which computer-generated algorithm is used. The major problem with computational prediction is the often high rate of false positives that are generated. Computational prediction of targets for both individual miRNAs or clusters of miRNAs can be performed using open-access platforms such as miRanda, TargetScan, Diana microT, PicTar and miRDB as listed in Table 5.6.

Table 5-6. Algorithms for computational miRNA target prediction

Algorithm	Website	Last Update	References
miRanda	www.microrna.org	Nov-10	(Enright et al., 2003; John et al., 2004)
TargetScan	www.targetscan.org	Jun-12	(Lewis et al., 2005; Friedman et al., 2009; Garcia et al., 2011)
DIANA-microT-CDS	www.microrna.gr/microT-CDS	Jul-12	(Maragkakis et al., 2009; Reczko et al., 2012; Paraskevopoulou et al., 2013)
PicTar	pictar.mdc-berlin.de	Mar-07	(Lall et al., 2006)
miR-DB	www.miRdb.org	Aug-2014	(Wong et al., 2015)

5.4.1.2 Predicting miRNA targets through experimental means

There are several tools available to selectively target miRNAs pathways. The most widely used approach is to regulate and study miRNA functions by using anti-miRs or miRNA mimics. Anti-miRs (or antagomiRs) are modified antisense oligonucleotides that can reduce the endogenous levels of miRNAs. The fundamental principle characterizing anti-miRs is that they need to bind with high affinity and be very specific for the miRNA of interest (Bartel, 2009). miRNA mimic technology, as the name suggests, mimics endogenous miRNAs thereby increasing the translational repression of the gene targets of the specific miRNAs (Z. Wang, 2011). By either blocking or enhancing miRNA expression and then studying the downstream effect, investigators can determine the specific miRNA targets and their functional effects.

Another approach used has been the development of genetic knockout mice. Knockout mice that lack key miRNA processing factors such as Dicer and Drosha are embryonically lethal (C. Y. Park et al., 2010). This highlights the crucial role that miRNAs play in early embryonic development. miRNA-mRNA interactions can be

studied using this model with observations from knock-out mice models showing in some cases profound physiological effects. However, in other cases studies have shown some miRNA functions overlap, resulting in redundancy with several related miRNAs targeting similar related proteins to exert their biological functions (Bartel, 2009). One example of this knock-out model is with the well-studied miR-155. miR-155 knockout mice (miR-155 $^{-/-}$) have been found to be defective in both B- and T-cell immunity. More recently it was shown that dendritic cells from miR-155 $^{-/-}$ mice were less apoptotic than those from wild-type mice leading them to be more efficient at antigen presentation (Rodriguez et al., 2007; O'Connell et al., 2010; Changming Lu et al., 2011). More efficient antigen presentation would favour the host response to *M. tuberculosis* infection as antigen presentation plays an important role in activating the adaptive immune response against *M. tuberculosis* (Sia et al., 2015).

The other loss-of-function method for validating targeted genes is a method known as "sponging". The "sponge" mRNA contains multiple target sites that are complementary to the miRNA of interest. Binding of the sponge mRNA to miRNA acts to inhibit the activity of a family of miRNA sharing common gene targets (Ebert and Sharp, 2010a; 2010b; Tay et al., 2015). Although no publish work exists that has examined this technology in infection, miRNA sponge has been used to examine the functionality of oncogenic miRNAs. Ma and colleagues examined miR-9 and its ability to regulate metastatic properties of breast malignancy (L. Ma et al., 2010). Inhibiting miR-9 by using a miRNA sponge in malignant cells was found to inhibit metastasis formation (L. Ma et al., 2010). These findings are important as they have the possibility of improving clinical management by the addition of directly targeted therapies against malignant disease and in the future infectious diseases such as TB.

miRNAs have numerous mRNA targets. For this reason, it may be advantageous not to target the miRNA itself but one or more of its predicted mRNA targets. The benefit of this has recently been shown by Gamble et al. where the authors were able to block one target of miR-27b, a vascular endothelial-specific cadherin, and by doing so demonstrate an essential role for miR-27a in angiogenesis in the setting of ischaemia (Young et al., 2013). Such technology not only improves our understanding of miRNA function but can also translate into new clinical application through targeted therapeutics.

Innate immunity employs a multi-layered response to control *M. tuberculosis* infection. In recent years the recognition of miRNAs and their regulatory role has uncovered another layer to the orchestrated response to infection and also other inflammatory conditions including malignancy. Alveolar macrophages are the first cell types to encounter *M. tuberculosis* and are critical in the initial innate response to this infection. This study has observed specific induction of miRNAs following *M. tuberculosis* infection of human macrophages. Concurrently, we observed modulation of known critical inflammatory cytokines and chemokines during infection. Depending on whether a miRNA functions in a pathogenic or compensatory manner, miRNA antagomiRs, or mimics, could be used as very targeted and specific therapies. In addition, a better understanding of disease processes can be achieved by defining in more detail molecular mechanisms of disease processes. Deciphering the complex network and interplay of miRNA activities and interactions, particularly with regards to cytokine and chemokine

expression, is of great significance and clearly warrants further studies especially given their potential clinical applications.

6 FINAL DISCUSSION

Tuberculosis remains one the world's deadliest communicable diseases. In 2013, an estimated nine million people developed TB that resulted in 1.5 million deaths. Given the estimated two billion people currently infected with latent *M. tuberculosis*, there is a large reservoir of potential TB disease. In recent years, due to the development of drug-resistance, TB control efforts have taken on an increased urgency. The current approaches to preventing, diagnosing and treating TB are inadequate. Developing new biomarkers that are able to predict reactivation in the latently infected individuals, assist in the diagnosis of TB, identifying non-responders to therapy, and act as a surrogate endpoint in drug and vaccine development, will advance steps towards better TB control. The results presented in this thesis serve as a foundation for further developments in the area of miRNA biomarkers.

miRNAs are small non-coding RNAs that are remarkably stable in cell-free form and measurable from samples that are easily obtainable such as plasma. This makes them an ideal biomarker, as their use is feasible in all settings including resource-poor areas, where the majority of TB is found. These data have revealed evidence for the potential role of specific miRNAs to discriminate TB disease from healthy individuals. In addition, there is a growing body of scientific evidence implicating the central role of miRNAs in modulating the molecular mechanism underlying the pathogenesis of TB. Therefore an intricate understanding of the functions and targets

of miRNAs is imperative as it opens the possibility of manipulating miRNA expression as a new directed treatment strategy.

miRNAs were first discovered in 1993 by researches screening for genes instrumental in the development of *C. elegans* (R. C. Lee et al., 1993). It was not until 2002 when Calin et al. reported the association between miRNAs and chronic lymphocytic leukaemia, that it became clear that there was a direct link between miRNA expression and malignancy (Calin et al., 2002). Since this landmark discovery, many researchers have identified abnormal miRNA expression in the setting of other malignancies, and more recently, other pathologies including inflammatory lung conditions such as asthma and chronic obstructive pulmonary disease as well as infectious diseases like tuberculosis (Pottelberge et al., 2011; C. Wang et al., 2011; Graff et al., 2012; F. Liu et al., 2012; Solberg et al., 2012; Miotto et al., 2013). Earlier studies focussed on a small numbers of miRNAs, this was mainly due to limited technologies available to enable high throughput analysis and/or the vast cost of using such high throughput technology. The number of miRNAs identified in human species continues to grow along with technologies that allow simultaneous profiling of many thousands of miRNAs at a more realistic cost. These factors mean that the number of miRNAs implicated in human disease will continue to increase substantially in the coming years.

While single prognostic biomarkers may be useful, it is likely that a combination of validated biomarkers will provide more reliable information and enable better stratification of patients. Combining several miRNAs, together with other

inflammatory mediators, such as cytokines, may serve this purpose. Identifying biomarker signatures in the setting of malignancy have already been shown in the literature to be a good diagnostic tool and predictor of treatment outcome and survival. The potential for miRNAs in current diagnostics began with a study comparing miRNA expression using miRNA microarray in 205 primary malignant tumours 131 metastatic tumours from 22 different tumour origins (Rosenfeld et al., 2008). A binary decision tree algorithm was developed that would help distinguish cell of origin in cancers with poor differentiation. Poorly differentiated malignancies are notoriously hard to trace to the cell of origin using current diagnostics. From this study, it was found that by utilising 48 miRNAs, cell of origin was able to be determined in nearly 90% of cases. Based on this study, and later similar studies, there now is an available panel using 64 miRNAs (miRview-mets2) manufactured by Rosetta Genomics available for clinical use (Mueller et al., 2011; Varadhachary et al., 2011). In addition to this panel, Rosetta Genomics also commercially produces miRNA signature panels to help distinguish the different types of lung cancers using eight miRNAs and to separate renal cancers into its four primary types (benign oncocytoma, renal cell carcinoma, papillary renal carcinoma and chromophobe renal carcinoma). All panels rely on binary tree classification as in the original paper by Rosenfeld et al. (Rosenfeld et al., 2008).

In our work presented here, we have been able to demonstrate that combining ten miRNAs and examining their expression profile in those with TB disease compared with healthy subjects delivered both excellent sensitivity, specificity and predictive values far greater than comparing a single miRNA in disease versus healthy state. The next step would be to combine this panel of miRNAs with other known TB

inflammatory makers, to determine if this further improves the diagnostic accuracy. A number of serum proteins have been implicated as possible biomarkers for diagnosis of active TB or for predicting the response to therapy. Cytokine profiles have been associated with TB disease and treatment response (Azzurri et al., 2005; Djoba Siawaya et al., 2009; Kabeer et al., 2011; Hong et al., 2012). In particular a number of studies have demonstrated higher levels of IP-10 (CXCL-10) in newly diagnosed TB patients compared to healthy controls with levels declining with anti-tuberculosis chemotherapy (Goletti et al., 2010; Wergeland et al., 2015). Interestingly only one study by Azzurri et al. found that patients who showed a sustained decrease in IP-10 after two months of therapy went on to have successful cure, while no changes in IP-10 levels were observed in patients who failed treatment (Azzurri et al., 2005). Along with protein profiles, transcriptional responses in monocytes and in peripheral blood have shown distinct gene expression profiles associated with active TB (Berry et al., 2010; Chanyi Lu et al., 2011; Lesho et al., 2011). Again type-1 IFN-related genes, including IP-10 were up-regulated in active disease compared to latent *M. tuberculosis* infection or uninfected healthy controls. Indeed a study by Berry et al., (Berry et al., 2010) found that expression profiles correlated with the radiological extent of disease and were able to discriminate between TB and other infectious pathogens. Other studies have found that these profiles change rapidly following effective treatment, even within the first two weeks (Bloom et al., 2012). It would be interesting to determine how the protein and mRNA profiles compared with the miRNA profile during TB and to determine if it was possible to identify a combined profile that had sufficient sensitivity and specificity to distinguish active TB disease from other infections or from latent TB and to predict outcome to therapy. Predicting responses to therapy has been made more

difficult with the increase in drug-resistant TB. Drug-sensitivity testing to determine resistance patterns in TB patients has its limitations: it requires reliable and reputable laboratories together with well trained and skilled staff to perform testing that is complex with results that can be delayed for six-to-eight weeks. During this time the patient is receiving inadequate therapy with the real risk of further resistance amplification and the on-going transmission of drug-resistant TB. It is for these reasons that new ways to diagnose and predict treatment outcomes are imperative in order to combat TB.

Currently there are numerous studies underway using serum, plasma, whole blood, PBMCs and other bodily fluids to identify biomarkers that better predict outcomes of *M. tuberculosis* infection, and enable faster evaluation of new therapeutics and vaccines (Jacobsen et al., 2007; Wallis et al., 2010; Chanyi Lu et al., 2011; Phillips et al., 2012; Hong et al., 2012). There are however a number of challenges with developing new biomarkers. Biomarkers of treatment response, cure and relapse have been reported in the literature. These studies however invariably involved relatively small cohorts in geographically restricted areas. The variability not only in sampling material and technique but heterogeneity between individuals that are both intrinsic and also related to extrinsic factors such as living standards and socioeconomic status mean that extrapolating these results to the wider community has so far proved difficult.

The possible bias introduced by using small sample sizes was highlighted by our own study. The ten regulated miRNAs chosen from the test set of 19 TB patients and 14

healthy subjects, were all significantly regulated with fold changes greater than 1.5. However in the larger validation set only five of the miRNAs were significantly regulated prior to the commencement of therapy. Many of the published data relating to miRNAs have reported findings based on sample sizes similar to our test set. Before any conclusions can be drawn from such studies, larger cohorts will need to be examined.

As miRNA research is only in its infancy, little is known about intrinsic or environmental factors that may or may not influence their expression. Sub-group analyses from both Chapter Three and Four suggested that miRNA expression was possibly influenced by ethnicity and/or geographical factors. Other factors, that were not examined here nor have been reported in the literature, may also influence miRNA expression such as the timing of sample collection (there may be a diurnal difference in miRNA expression similar to what is seen with hormones such as cortisol), smoking history or even dietary factors. As a single miRNA can target multiple mRNA coupled with an overlap in function, other comorbid conditions are likely to influence miRNA expression. When considering a diagnosis of TB, it is often hard to discriminate TB from other inflammatory lung conditions such as other bacterial pneumonias. Accurate diagnosis is important as treatment is vastly different. Other inflammatory lung conditions may have similarity in their regulation of miRNA expression. In order to identify a unique miRNA expression profile specific to TB it is imperative that future studies enrol patients not only with TB but with other respiratory pathologies.

Co-infection with HIV and TB is a major problem, particularly in the African continent and studies are required to understand how co-infection modifies the expression of miRNAs and potentially other biomarkers in these individuals to determine if the same biomarker panel can be utilised to aid diagnosis of TB in individuals with or without concurrent HIV co-infection. Miotto and colleagues examined miRNA expression from the sera of patients with active TB and compared it to those with active TB whom were co-infected with HIV and found differential miRNA expression across the two groups (Miotto et al., 2013). This suggests that specific miRNA bio-signatures will need to be utilised in those with co-infection. Few studies have examined those with HIV and TB as a cohort, and more work in the future will be needed in this area, as TB still remains the major cause of death in those with HIV (World Health Organization, 2014). A diagnostic miRNA panel for HIV-TB co-infection is imperative as historically TB in those with HIV infection has been difficult to diagnose due to their often atypical presentation and paucibacillary disease, making culture identification of *M. tuberculosis* more difficult (Sreeramareddy et al., 2009).

Another major comorbidity that increases an individual's risk of developing active TB disease is diabetes mellitus. Diabetes triples the risk of developing TB and adversely affects disease presentation and treatment outcomes (Lönnroth et al., 2014). As the prevalence of diabetes increases, it is likely that it will surpass HIV as the most significant risk factor for developing active disease (L. S. Geiss et al., 2014). How diabetes effects the expression of miRNA during TB infection is largely unknown but is another compounding factor that needs to be addressed in devising

new biomarkers.

As shown in the study reported here the size of the cohort measured is clearly an issue in confirming the reliability of the results. Other potentially confounding factors include the type of sample measured, when it is collected, the control cohorts, and the clinical data and length of follow up necessary to determine relapse rates. One way to address these possible confounding factors is to develop large and centralised bio-banks that allow collection and storage of bio-specimens, such as cells, plasma and sputum, with associated clinical data from large cohorts of patients that have had adequate follow-up so as to determine clinical outcome (cure, relapse or recurrent disease). International collaborative studies with the development of bio-banks will allow standardized scientific techniques to be applied to stored clinical samples with adequate clinical data to allow stratification of subjects. Historically, one of the main factors impeding such collaboration was inadequate funding, however of late, a large number of bodies have increased investment into biomarker studies. Already the Tuberculosis Trials Consortium in collaboration with AIDS Clinical Trials Group have established a biobank for the collection and storage of sputum, serum, urine, PBMCs, host mRNA and DNA from patients with TB which will be used to evaluate candidate biomarkers of TB treatment response. Similar bio-banks could be established to examine miRNA as a diagnostic and prognostic biomarker.

In order to work towards TB elimination and curtail the morbidity and mortality associated with the disease new therapeutics are urgently required. miRNA

manipulation has already shown promise as a new targeted therapeutic in malignancy. By overexpression of *let-7* in lung cancer cell lines, it was shown that *let-7* can repress resistance to radiation therapy both *in vitro* and *in vivo* (Weidhaas et al., 2007). Manipulation of miRNAs as a new therapeutic agent in TB has not yet been reported, but may be a new and exciting mechanism for treating *M. tuberculosis* infection. It may be possible to boost immunity to *M. tuberculosis* by delivering new therapeutics that target either specific miRNA or their binding sites and boost the hosts immune response. One benefit of these new therapies is that they could be used in conjunction with existing antibiotic therapies and may help to limit the duration of treatment, or lower the development of drug resistance. Critical to the development of miRNA-specific therapeutics is an understanding of the functions of miRNA and the relevant signalling pathways in *M. tuberculosis* infection and disease. An intricate understanding of miRNA function will enable the development of novel therapeutics that target directly pathologically essential miRNAs and/or their target genes. One of the first steps towards achieving this is *in vitro* studies of a largely purified population of cells as this facilitates a greater understanding of precise function though at the expense of analysing the system as a whole. It is for this reason that this body of work studied miRNA expression in macrophages, the key innate immune cell in *M. tuberculosis* infection. Results have provided an insight into host-mycobacteria interactions. To further this understanding, genetic manipulation of miRNA is the next step, as this will enable the study of phenotypic changes in culture or within the organism that occurs with gain or loss of function. By observing these changes a greater understanding of the functional relevance of miRNA can be achieved. An exquisite understanding of miRNA:mRNA is essential to allow manipulation as a mode of new and innovative targeted therapy.

This body of work has examined the role of miRNAs as a diagnostic tool with the aim of being able to diagnose TB quickly with a high degree of accuracy and as a predictor of treatment outcome. This is the first study to examine miRNA expression longitudinally and has improved current understanding of miRNA expression in the setting of TB disease both at the time of diagnosis and also over the course of therapy. Like many other published work, this study focussed on comparing miRNA expression in *M. tuberculosis* disease states to healthy controls/no infection. In clinical practice however, one of the major difficulties in diagnosing TB is to differentiate patients with TB disease from those with other respiratory pathologies. Future work, which examines miRNA expression across a myriad of lung pathologies, is critical to enable miRNAs to be a clinically relevant biomarker.

The development of new biomarkers to diagnose and better manage TB is urgently required. This study has aimed to achieve this through the use of plasma miRNAs. The appeal of using miRNAs as a clinical biomarker is high, given their stability in the extracellular environment and their ease of measure in plasma and serum, which are readily obtainable, even in developing countries where TB is often endemic. The potential of manipulating miRNA expression as a targeted new therapy in TB is only beginning to be recognised. Further translational research that allows for a better understanding of miRNA as a diagnostic and prognostic tool as well as a targeted treatment approach has the potential to revolutionise the management of patients with this disease.

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APPENDIX A. MIRNA PLASMA FOCUS PANELS

	1	2	3	4	5	6	7	8	9	10	11	12
A	hsa-miR-20a	hsa-miR-19b	hsa-miR-126	hsa-miR-24	hsa-miR-223	hsa-miR-15a	hsa-miR-21	hsa-miR-150	hsa-miR-23a	UniSp3 IPC	hsa-miR-320a	hsa-miR-451
B	hsa-miR-106a	hsa-miR-142-3p	hsa-miR-486-5p	hsa-miR-320b	hsa-let-7g	hsa-miR-27a	hsa-miR-15b	hsa-miR-101	hsa-miR-26a	UniSp3 IPC	hsa-miR-221	hsa-miR-16
C	hsa-miR-185	hsa-miR-107	hsa-miR-222	hsa-miR-342-3p	hsa-miR-1974	hsa-miR-23b	hsa-miR-423-3p	hsa-miR-30b	hsa-let-7f	UniSp3 IPC	hsa-miR-181a	hsa-miR-103
D	hsa-miR-144	hsa-miR-30c	hsa-miR-145	hsa-miR-26b	hsa-miR-197	hsa-miR-192	hsa-miR-151-3p	hsa-miR-27b	hsa-miR-186	UniSp6 CP	hsa-let-7i	hsa-miR-425
E	hsa-miR-139-5p	hsa-miR-424	hsa-miR-17	hsa-miR-143	hsa-miR-199a-3p	hsa-miR-29c	hsa-miR-146a	hsa-miR-29a	hsa-miR-99a	hsa-miR-152	hsa-miR-148a	hsa-miR-423-5p
F	hsa-miR-19a	hsa-miR-30d	hsa-miR-22	hsa-miR-720	hsa-miR-125b	hsa-miR-378	hsa-miR-125a-5p	hsa-miR-148b	hsa-miR-484	hsa-miR-25	hsa-miR-99b	hsa-miR-93
G	hsa-miR-18b	hsa-miR-652	hsa-miR-331-3p	hsa-miR-151-5p	hsa-miR-18a	hsa-let-7d	hsa-let-7d*	hsa-miR-34a	hsa-let-7b	hsa-miR-335	hsa-miR-590-5p	hsa-miR-191
H	hsa-miR-375	hsa-miR-374b	hsa-miR-215	hsa-miR-10b	hsa-miR-140-3p	hsa-miR-130a	hsa-miR-338-3p	hsa-miR-324-5p	hsa-miR-33a	hsa-miR-195	hsa-miR-365	Blank (H20)

Plasma Focus miRNA PCR Panel, Plate 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	hsa-miR-497	hsa-miR-532-3p	hsa-miR-142-5p	hsa-miR-210	hsa-let-7a	hsa-miR-30e	hsa-miR-140-5p	hsa-miR-328	UniSp3 IPC	hsa-miR-16-2*	hsa-miR-339-5p	hsa-miR-451
B	hsa-miR-144*	hsa-miR-339-3p	hsa-miR-584	hsa-let-7e	hsa-miR-205	hsa-miR-28-3p	hsa-miR-155	hsa-miR-374a	UniSp3 IPC	hsa-miR-92a	hsa-miR-28-5p	hsa-miR-16
C	hsa-miR-20b	hsa-miR-502-3p	hsa-miR-93	hsa-miR-30a	hsa-miR-29b	hsa-miR-505	hsa-miR-223*	hsa-miR-532-5p	UniSp3 IPC	hsa-miR-106b*	hsa-miR-15b*	hsa-miR-103
D	hsa-let-7c	hsa-miR-122	hsa-miR-766	hsa-miR-10a	hsa-miR-301a	hsa-miR-346	hsa-miR-425*	hsa-let-7b*	UniSp6 CP	hsa-miR-128	hsa-miR-32	hsa-miR-425
E	hsa-miR-194	hsa-miR-18a*	hsa-miR-106b	hsa-miR-136	hsa-miR-501-3p	hsa-miR-154	hsa-miR-127-3p	hsa-miR-30e*	hsa-miR-326	hsa-miR-133a	hsa-miR-495	hsa-miR-423-5p
F	hsa-miR-1	hsa-miR-199a-5p	hsa-miR-20a*	hsa-miR-660	hsa-miR-376a	hsa-miR-193b	hsa-miR-409-3p	hsa-miR-543	hsa-miR-132	hsa-miR-363	hsa-miR-629	hsa-miR-93
G	hsa-miR-182	hsa-miR-421	hsa-miR-22*	hsa-miR-92b	hsa-miR-29a*	hsa-miR-500a	hsa-miR-103-2*	hsa-miR-2110	hsa-miR-574-3p	hsa-miR-29b-2*	hsa-let-7i*	hsa-miR-191
H	hsa-miR-382	hsa-miR-95	hsa-miR-361-3p	hsa-miR-133b	hsa-miR-324-3p	hsa-miR-200c	hsa-miR-605	hsa-miR-485-3p	hsa-miR-885-5p	hsa-miR-297	hsa-miR-551b	Blank (H20)

Plasma Focus miRNA PCR Panel, Plate 2.

APPENDIX B STUDY DOCUMENTS CHAPTER THREE

ADDRESS FOR ALL CORRESPONDENCE
RESEARCH DEVELOPMENT OFFICE
ROYAL PRINCE ALFRED HOSPITAL
CAMPERDOWN NSW 2050

TELEPHONE: (02) 9515 6766
FACSIMILE: (02) 9515 7176
EMAIL: lesley.townsend@email.cs.nsw.gov.au
REFERENCE: X11-0141 & HREC/11/RPAH/201

NSW GOVERNMENT Health
Sydney Local Health Network

3 June 2011

Professor W Britton
Department of Medicine & Clinical Immunology
Building 93
Royal Prince Alfred Hospital

Dear Professor Britton,

Re: Protocol No X11-0141 & HREC/11/RPAH/201 - "Biomarkers for tuberculosis"

The Executive of the Ethics Review Committee, at its meeting of 26 May 2011, considered Dr S Barry's correspondence of 25 May 2011 and subsequently her correspondence of 3 June 2011. In accordance with the decision made by the Ethics Review Committee, at its meeting of 11 May 2011, ethical approval is granted.

The proposal meets the requirements of the *National Statement on Ethical Conduct in Human Research.*

This approval includes the following:

- Information for Participants – Longitudinal Study (Master Version 2, 25 May 2011)
- Participant Consent Form (Master Version 1, 29 April 2011)
- Information for Participants – Biomarkers Study (Master Version 2, 25 May 2011)
- Participant Consent Form (Master Version 1, 29 April 2011)
- Questionnaire (Master Version 2, 3 June 2011)

Sydney Local Health Network
ABN 17 520 269 052
Level 11 KGV Building
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REFERENCE: X11-0141 & HREC/11/RPAH/201
5.0/9.41/NOV11

NSW GOVERNMENT Health
Sydney
Local Health District

17 November 2011

Professor W Britton
C/- Dr S Barry
Department of Medicine & Clinical Immunology
Building 93, Centenary Institute
Royal Prince Alfred Hospital

Dear Professor Britton,

Re: Protocol No. X11-0141 & HREC/11/RPAH/201 – "Biomarkers for tuberculosis"

Thank you, on behalf of the Ethics Review Committee, for Dr S Barry's correspondence of 4 November 2011.

The inclusion of the St Vincent's Hospital as a site for the above study, with Professor A Glanville as the Principal Investigator, is noted with thanks.

Yours sincerely,

Lesley Townsend

Lesley Townsend
Executive Officer
Ethics Review Committee (RPAH Zone)

HERC\EXECOR\11-11

General Correspondence
PO Box M30
Missenden Road, NSW, 2050
Email: slhn.esu@sswahs.nsw.gov.au
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CAMPERDOWN, NSW, 2050
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St Vincent's Hospital

TUBERCULOSIS BIOMARKERS

QUESTIONNAIRE FOR PATIENTS WITH TUBERCULOSIS TO BE COMPLETED BY ATTENDING PHYSICIAN

1. Name .....
2. Study Number .....

St Vincent's Hospital

TUBERCULOSIS BIOMARKERS

QUESTIONNAIRE FOR PATIENTS WITH TUBERCULOSIS TO BE COMPLETED BY ATTENDING PHYSICIAN

1. Study Number .....
2. Gender .....
3. Date of birth .....
4. Country of birth .....
5. Year of arrival in Australia .....
6. Date of Diagnosis .....

7. Establishment of laboratory diagnosis (please tick the most appropriate box or boxes if positive)

Smear	□	sputum	□	lymph node	□	other	□	*please specify* .....
Culture	□	sputum	□	lymph node	□	other	□	*please specify* .....
PCR	□	sputum	□	lymph node	□	other	□	*please specify* .....
Histology	□	sputum	□	lymph node	□	other	□	*please specify* .....

8. Clinical Manifestation
   Pulmonary □ *Please specify*.....
   Extra-pulmonary □ *Please specify*.....

9. (Intended) treatment
   Standard □
   Other □ *Please specify*.....

10. Duration of treatment (thus far) .....

11. Coexistent immunosuppressive disease (e.g. HIV)
    No □
    Yes □ *Please specify*.....
    Unknown □

12. List of current medications
    .....
    .....
    .....
    .....
    .....

13. Other comments

St Vincent's Hospital

Biomarkers in Tuberculosis

PARTICIPANT CONSENT FORM

I, ... *[name]*

of

.....*[address]*

have read and understood the Information for Participants on the abovenamed research study

and have discussed the study with .....

I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.

I understand that my participation in this study will allow the researchers and others, as described in the Information for Participants, to have access to my medical record, and I agree to this.

I freely choose to participate in this study and understand that I can withdraw at any time.

I also understand that the research study is strictly confidential.

I hereby agree to participate in this research study.

NAME: .....

SIGNATURE: .....

DATE: .....

NAME OF WITNESS: .....

SIGNATURE OF WITNESS: .....

MASTER Participant Consent Form, Version 1, 29/04/2011
St Vincent's Hospital Information for Participants, Version 1, 29/06/2012

St Vincent's Hospital

Biomarkers for Tuberculosis
INFORMATION FOR PARTICIPANTS

Introduction

You are invited to take part in a research study that will monitor the response to treatment for tuberculosis. Tuberculosis is a major health problem worldwide. In Australia there are still 1200 patients diagnosed each year with tuberculosis. Tuberculosis infects cells called monocytes. These infected monocytes release special particles, called microparticles into the blood. Special white blood cells called lymphocytes fight the tuberculosis infection. We plan to measure these microparticles to see whether their levels change with treatment. We also wish to look more closely at the lymphocytes during infection and compare this with people without tuberculosis.

The study is being conducted within this institution by:
1. Professor Warwick Britton
   Professor of Medicine. Department of Medicine and Clinical Immunology.
   Head of Mycobacterial Research Program, Centenary Institute.
2. Dr Bernadette Saunders
   Head "Host Responses to Tuberculosis" group, Centenary Institute

   In collaboration with:
1. Dr Simone Barry
   Respiratory and Sleep Physician, St Vincent's Hospital

Study Procedures

If you agree to participate in this study, you will be asked to sign the Participant Consent Form. You will then be asked to undergo the following procedures:

- You will then be asked to provide a 20 mL sample of your blood. The samples will be taken from a vein in your arm.

In addition, the researchers would like to have access to your medical record to obtain information relevant to this study.

Risks

Blood collection involves some discomfort at the site from which the blood is taken, There is also a risk of some minor bruising at the site, which may last one to two days.

Benefits

While we intend that this research study furthers medical knowledge and may improve treatment of Tuberculosis in the future, it will not be of direct benefit to you.

Costs

Participation in this study will not cost you anything, nor will you be paid.

Voluntary Participation

Participation in this study is entirely voluntary. You do not have to take part in it. If you do take part, you can withdraw at any time without having to give a reason. Whatever your decision, please be assured that it will not affect your medical treatment or your relationship with the staff who are caring for you.

Confidentiality

All the information collected from you for the study will be treated confidentially, and only the researchers named above will have access to it. The study results may be presented at a conference or in a scientific publication, but individual participants will not be identifiable in such a presentation.

Further Information

When you have read this information, Dr Simone Barry will discuss it with you further and answer any questions you may have. If you would like to know more at any stage, please feel free to contact her on 95656518

This information sheet is for you to keep.

Ethics Approval and Complaints

This study has been approved by the Ethics Review Committee (RPAH Zone) of the Sydney Local Health Network. Any person with concerns or complaints about the conduct of this study should contact the Executive Officer on 02 9515 6766 and quote protocol number X11-0141.

APPENDIX C: STUDY DOCUMENTATION CHAPTER FOUR

Ningxia Medical University

— To Whom It May Concern

This is to certify that the project entitled 'Biomarkers in Tuberculosis Study' was reviewed by Ningxia Medical University Human Ethic Committee on June 6th, 2013. The aims of the project are to investigate the specific biomarkers that may indicator the disease progression/regression and chemotherapeutic efficiency in active tuberculosis (TB) cases by use case control study. The investigation will invite 200 TB patients who have been newly diagnosed with tuberculosis with sputum TB positive and 200 healthy controls to take part in the project study. The participants will undergo the interview by questionnaire for those infectious risk factors of tuberculosis infection and transmission. Patients will be asked to provide 10 ml blood in EDTA tube at four time points (before treatment, post treatment one week, one month and six month), while health controls will be asked to provide 10 ml blood in EDTA tube at one time point. The patient sputum and chest x-ray will only be required according to TB clinics or hospitals' routine conveniences. The study outcomes will provide new insights and strategies for human TB treatment strategy. The project will last for a period of 9-months.

We note that the investigators will use standard operation procedures (SOP) with safe equipment and techniques for blood and sputum sample collection. The ethical clearance has been obtained from Ningxia Medical University and Ningxia Infectious Disease Hospital for the project implement. In addition, the committee advised the investigators that they will be asked to obtain consent letters from all participants to be involved in this study before the project commences.

Yours Sincerely

Director

Li zhengzhi 李正直

Ningxia Medical University, People's Republic of China

6 June, 2013

PARTICIPANT CONSENT FORM

..... [Print Name] Give consent to my participation in the research project

TITLE: **Biomarkers in Tuberculosis Study**

In giving my consent I acknowledge that:

1. The procedures required for the project and the time involved (including any inconvenience, risk, discomfort or duration, and of their implications) have been explained to me, and any questions I have about the project have been answered to my satisfaction.
2. I have read the participant information statement and have been given the opportunity to discuss the information and my involvement in the project with the researcher/s.
3. I understand that I can withdraw from the study at any time, without affecting my relationship with the researcher(s) of the National Tuberculosis Program now or in the future.
4. I understand that my involvement is strictly confidential and no information about me will be used in any way that reveals my identity.
5. I understand that being in the study is completely voluntary. I am not under any obligation to consent.

Signed:

Name:

Date:

If you want feedback about the study, write your address or email and dress here:

.....

知 情 同 意 书

您将被邀请参加由自治区第四人民医院和澳大利亚昆士兰医学医学研究所、悉尼大学结核病研究所合作主持的抗结核药物在体内的药代动力学研究项目。是为了解抗结核药物在体内的生物转化过程而进行的一项研究，它将历时 9 个月。这份知情同意书能帮助您决定是否参加本研究。在您同意加入本研究之前，请认真阅读以下内容，如有不理解的问题，请咨询研究者。

一、 题目：抗结核药物的药代动力学研究

二、 目的：了解抗结核药物在生物体内吸收、分布、代谢和排泄规律。

三、 试验过程

在获得您的知情同意之后，经过符合性筛查，若符合入选标准，您将作为我们的研究对象参与研究，具体安排如下：

1、若您是活动性肺结核患者，自愿参加本次研究，请在第一次就诊时配合门诊大夫抽血化验，保留您的痰标本进行痰涂片和痰培养的检测，同时拍摄 X 光片以便辅助诊断。

2、请您在治疗 1 月末、2 月末及 6 月末应复查时，按时就诊抽血化验，保留痰标本进行痰涂片和痰培养的检测，医生会告知您的痰标本化验结果。

3、若陪同您的密切接触者（也可以是家属）也同意参加此次研究，作为实验对照，仅在第一次就诊时抽血化验，1、2 和 6 月末无需抽血化验。

四、 风险

您参加本次研究没有风险存在，只需按时抽血化验，保留痰标本进行检测，拍摄 X-光片辅助诊断。我们将按计划给您用药并对您的

身体进行密切观察。

五、保密性

所有显示您姓名的记录将得到保密，您的姓名不会出现在申办者的表格中，也不会出现在任何出版物中。

六、 自愿者陈述

我已阅读以上知情同意内容（或医生已经给我解释了相关内容），并就不明的问题向医生询问，没有任何疑议。本人同意并自愿加入本次结核病抗结核药物药代动力学研究项目。

参加者签名：_____

日　　期：_____

如果你同意参加本项调查，请写下你的联系方式 或信件邮寄地址：

电子邮箱：

电话

地址：

邮编：

Patient Questionnaire

Date of Interview |__|__|__|__| / |__|__| / |__|__| (yyyy/mm/dd)

Location of clinic:

Allocated Participant ID:

Respondent for interview: |__|
(1) Self; (2) Parent; (3) Child; (4) Brother/sister; (5) Spouse; (6) Other

GENERAL INFORMATION

Sex |__| M/F

Age at date of interview:

Ethnicity:

Education |__| (1) Illiterate (2) Semi-literate (3) Primary school
(4) Junior School (5) Senior School (6) College

Years of education: |__|__|

Occupation: |__| (1) Farmer (2) Businessman (3) Teacher
(4) Student (5) Pre-School (6) Other

TUBERCULOSIS HISTORY

1) Have you ever had been vaccinated for tuberculosis? Y/N/D
If YES, do you have a BCG vaccination scar? Y/N/D

2) Have you ever been diagnosed with tuberculosis? Y/N/D

THE UNIVERSITY OF SYDNEY

Mycobacterial Research Program
Centenary Institute
Central Clinical School
Sydney Medical School

Bernadette Saunders 博士
分支杆菌研究小组

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Web: www.centenary.org.au

结核杆菌的生物标志物研究

参与结核病治疗项目信息调查表

(1) 我们的研究目的

你被邀请来参加这项研究，旨在检测结核治疗的反应

(2) 谁来负责这项科研项目

这项研究是由中国宁夏第四人民医院（宁夏传染病医院）王晓林副院长和悉尼大学的 Bernadette Saunders, Warwick Britton 博士， Magda Ellis 博士 and Simone Barry博士合作进行。

(3) 研究内容

如果你同意参加该项研究，那么你将被要求签署知情同意书，之后做以下事情：就是分8次采集静脉血，每次10 mL

a. 治疗前采血一次
b. 治疗后一周采血一次
c. 治疗后1月、2月、3月、4月、5月、6月各采集一次。

(4) 我们将占用你多少时间

每次访谈仅占用你几分钟时间，同时采集静脉血，填写简短的问卷调查。

(5) 可以退出研究吗

因为，参与该项研究完全是自愿的，任何时候都可以离开，不负任何责任，也不会影响你与治疗医生之间的关系。 无论你做什么样的决定，都不会影响你的医学治疗及与相关人员的关系。

(6) 别的人会知道结果吗

研究的方方面面，包括结果都将严格保密，只有研究人员才会接近参与者的信息。

研究报告将会发表，但是个人信息不会出现其中。

(7) 这项研究对我们有什么益处

这项研究我们是想获得有关该病的更多的医学方面的知识，我们不能保证你能从中获得什么好处。

(8) 关于这项研究我能告诉其他人吗？

没问题，可以。

(9) What if I require further information about the study or my involvement in it?

阅读该调查表时间，_____ 如果你想要知道更多信息，在你方便时我们会随时为您解答。

(10) 如果我有什么抱怨或者任何担忧怎么办？

有关该项研究的任何的抱怨或担忧你可以联系项目管理，人类伦理管理办公室:，悉尼大学，电话： +61 2 8627 8176; 传真：+61 2 8627 8177 or 电邮：ro.humanethics@sydney.edu.au (Email).

这篇信息请保留

Patient Questionnaire /病人调查表

Date of Interview（访问时间） |__|__|__|__| / |__|__| / |__|__|
(yyyy/mm/dd)

Location of clinic（诊所地点）:

Allocated Participant ID（病人病例号）:

Respondent for interview（回答者）: |__|

(1) Self（本人）; (2) Parent（父母）; (3) Child（孩子）;

(4) Brother/sister（兄弟姐妹）; (5) Spouse（配偶）; (6) Other（其他）;

GENERAL INFORMATION（基本信息）

Sex（性别） |__| M/F （男/女）

Age at date of interview（访问时年龄）:

Ethnicity（民族）:

Education（教育程度） |__| (1) Illiterate（文盲） (2) Semi-literate （半文盲）

(3) Primary school（小学）(4) Junior School（初中） (5) Senior School（高中）

(6) College （大学）

Years of education（受教育年限）: |__|__|

Occupation（职业）: |__| (1) Farmer（农民） (2) Businessman（商人）

(3) Teacher（教师）(4) Student（学生） (5) Pre-School（学龄前）

(6) Other（其他）

TUBERCULOSIS HISTORY（患结核结核病历史）

1) Have you ever had been vaccinated for tuberculosis? （是否接种过结核疫苗）

Y/N/D

If YES（如果是）, do you have a BCG vaccination scar?（有 BCG 接种痕吗）

Y/N/D

2) Have you ever been diagnosed with tuberculosis? （曾经诊断过结核吗）

Y/N/D

Commonwealth of Australia

STATUTORY DECLARATION

Statutory Declarations Act 1959

1 *Insert the name, address and occupation of person making the declaration*

I,[1] Beatrice Nagaria, ~~H~~ Centenary Institute, medical practitioner

make the following declaration under the *Statutory Declarations Act 1959:*

2 *Set out matter declared to in numbered paragraphs*

[2] It is a true and accurate copy of the English version. (Participants Consent form)

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

3 *Signature of person making the declaration*

[3]

4 *Place*
5 *Day*
6 *Month and year*

Declared at[4] Centenary Institute on[5] 13 Sept 2013 of[6]

Before me,

7 *Signature of person before whom the declaration is made (see over)*

[7] Beatrice Nagaria

Beatrice Nagaria

8 *Full name, qualification and address of person before whom the declaration is made (in printed letters)*

[8]

Full name = Beatrice Djayanti Nagaria

Qualification = M.B.B.S

Note 1 A person who intentionally makes a false statement in a statutory declaration is guilty of an offence, the punishment for which is imprisonment for a term of 4 years — see section 11 of the *Statutory Declarations Act 1959*.

Note 2 Chapter 2 of the *Criminal Code* applies to all offences against the *Statutory Declarations Act 1959* — see section 5A of the *Statutory Declarations Act 1959*.

APPENDIX D: STANDARD OPERATING PROCEDURE FOR CHAPTER FOUR STUDY

Standard Operating Procedures (SOP)
For miRNAs in Tuberculosis

Index of SOP

Details of study

1. **Title**: **MicroRNAs in Tuberculosis**
2. **Duration: 9 months** (from June 2013-February 2014)
3. **Management: Ningxia Infectious Diseases Hospital**
4. **Co-operating organizations**
   (a) In China
   Ningxia Infectious Diseased Hospital and Ningxia Medical University

   (b) In Australia
   University of Sydney, Australia
   The Centenary Institute, University of Sydney

Foreword

Tuberculosis (TB) remains a major health problem worldwide, with two million deaths from TB each year. The management of TB requires multi-drug chemotherapy for at least six months. Drug-resistant strains of TB are increasing. Currently, diagnosis of TB is dependent on culturing the organism (usually from sputum), which can take up to six to eight weeks. Treatment success is a sputum sample that is culture negative after two months of treatment. However, these culture results also take six to eight weeks, thus delaying the time taken to identify patients not responding to treatment. Identifying new biomarkers, through this research, may lead to an efficient way of monitoring response to treatment, and increase the early identification of non-responders who may be harboring drug-resistant organisms.

MicroRNA (miRNA) are small non-coding RNA sequences that affect the translation of about 30% of human genes. This project aims to determine whether levels of specific miRNAs in patient blood can be used as a biomarker for the presence of active TB disease, and furthermore whether changes in miRNA levels can indicate early treatment failure.

miRNA levels will be measured in the TB population, at the time of diagnosis, after one, two and six months of treatment month of treatment and compared to levels in age and sex matched healthy controls. miRNA levels will be measured at these four time points to determine what effect treatment has on miRNA expression.

We hypothesise that the miRNA profiles of patients who are poor responders to treatment (often a surrogate marker of drug resistance) will differs from those who are responding well to treatment.

SUMMARY of MIRNA STUDY

Tuberculosis Patients enrolled into the study will have their blood collected at 4 time points

Prior to the commencement (or within 24 hours of commencing TB treatment)

After 1 month of treatment

After 2 month of treatment

After 6 months of treatment

All patients will be paid 10 Yuan to cover their return trip to the clinic on visits 1,3,and 4

Patients returning to visit 2 (unscheduled visit) will be given 10 Yuan and an additional 50 Yuan to cover lost wages. In total for visit 2 they will receive 60 Yuan.

Enrolled Controls will have their blood collected on ONE occastion only

All controls will be given 10 Yuan to cover their return fare to the clinic. In addition 50 Yuan will be reimbursed for the time they had to spend away from their work (lost wages).

4

Flowchart 1 – For enrolling patients with pulmonary TB

STEP 1: Does the patient meet the criteria for the study?

Explain the study to the patient: we are testing for factors that are altered during TB infection

- The study will only involve blood tests and brief questions.
- The X-ray and blood tests are free of charge.
- The patient will receive a reimbursement for participating in the study.
- Give the patient the Information Form
- Show the patient the Consent Form

Ask the patient if they agree to participate in the study.

If the patient is at least 18 years old and agrees to participate in the study then they can join the study.

STEP 2: Enroll in the study

- The patient must sign the consent form and agree to have the blood tests.
- Allocate a Study Identification Number to the patient from the Study Workbook. This number must go on all forms written for this patient.
- Enrol the patient on the Study Workbook
- Complete specimen request forms:
  - On the study Blood test form complete name, number and date.
  - On the Microbiology request form: Write patient's name, study identification number, date of test on the form.

STEP 3: Obtain chest X-ray

Each subject needs a good quality chest X-ray (to confirm whether there is pulmonary TB).

Does the patient have a good CXR from the Department of Radiology?

(a) If patient already has a chest X-ray from the current admission / review: Record the radiology number of the film on the Register Book.

STEP 4: Collect blood for tests

Take the patient to Pathology Department. The staff there will collect 10 mL EDTA tube for the miRNA test

STEP 5: Complete enrolment

- Check that all forms are complete
- Complete the checklist on the Study Workbook.

STEP 6: Give patient reimbursement

After the patient has given their blood and completed their chest X-ray, give them a reimbursement.

Flowchart 2 – Enrolling healthy subjects (never had TB)

STEP 1: Does the subject meet the criteria for the study?

Explain the study to the subject: we are testing for risk factors for tuberculosis.

- The study will only involve a blood test and brief questions.
- The blood tests are free of charge.
- The subject will receive a reimbursement for participating in the study.
- Give the subject the Information Form
- Show the subject the Consent Form

Ask the patient if they agree to participate in the study.

If the patient is at least 18 years old and agrees to participate in the study then they can join the study.

STEP 2: Enroll in the study

- Allocate the subject a study number form the Study Register Book
- Complete the Registration Book with the subject.
- Get the patient to complete the study questionnaire
- Complete a Study Blood test request form:
  - Complete the subject's name, study identification number and date.

STEP 4: Send subject to Pathology Department for blood test

The subject will have a blood test at the Department of Pathology.

- Give the subject the Blood test request form
- Take the subject to the Department of Pathology for their blood test.
- Notify the staff at the Department of Pathology that this test is for the miRNA research study. Staff will take blood in a 10 mL EDTA blood tube.

STEP 5: Complete enrolment

- Complete the check-list on the Study Register Book to make sure the subject has had all of their tests. Do this before giving the subject a gift.

STEP 6: Give the reimbursement to the subject

- After the patient has had their blood test and completed their questionnaire, give them the reimbursement

8

Flowchart 3 – Laboratory processing protocol at the Department of Microbiology

STEP 1: Record the results of culture: For TB patients only

- When the results of cultures become available (or after 8 weeks if a negative culture)
  - write it in the STUDY LABORATORY BOOK

Assistance with the study

- For technical assistance with plasma extraction techniques email Dr Simone Barry at s.barry@centenary.org.au

10

Population included

(a) Tuberculosis cases
All new patients at the Outpatient Clinic with newly diagnosed tuberculosis should be considered for this study. There need to be a total of 100 patients recruited. The inclusion and exclusion criteria are written above.

(b) Control cases
Cases enrolled into the study will be asked to bring along a friend to their next visit and these people will act as controls.

IV. Guide to interviewing subjects

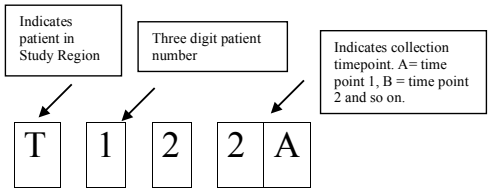
Patients with tuberculosis should be recruited at the time the diagnosis is made (such as according to smear status).

Interviews will take about 10 minutes. Follow the flow-charts above.

Training for this study will emphasise the techniques to perform a good interview.

Numbering for tuberculosis patients

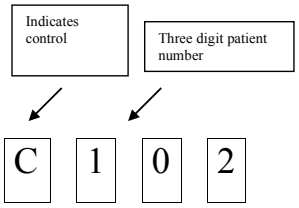
An individual study code will be allocated to each index case, which includes a letter (T for a tuberculosis patient), followed by the patient number (3 digits, 001 to 500). For example for a patient with tuberculosis:



Numbering will begin at 0 0 1, and patients will have consecutive numbers according to their time and date of enrolment in the study.

(b) Numbering for control subjects

Control subjects will be numbered with a letter (C to indicate control), followed by a three-digit number (3 digits, 001 to 500). For example for a contact of the above patient may have the following number.



Numbering will begin at C 0 0 1, and controls will have consecutive numbers according to their time and date of enrolment in the study. There will be a list for new cases and contacts. Staff enrolling patients in this study will use these forms to record details of all study subjects at the time of enrolment.

1.2. Culture

- Culture for TB will be performed on all specimens

Withdrawal from study

If a subject decides to withdraw from the study, then they can complete the Withdrawal of consent form. The study staff should place this form in the Study Folder, with other consent forms. If the patient withdraws from the study then their specimens will not be analysed any further.

Appendices

Appendix 1: Forms for MiRNA Study

Description	Details
Patient information sheet (PIS)	For consent of a patient with tuberculosis
Control Information Sheet (CIS)	For consent of normal controls (no TB)
Consent form	Consent form for patients and healthy normals to sign, if they agree to participate in the study

Appendix 2: Timetable for study

The timetable for the overall study will follow the following schedule.

April 2013	**June 2013-Feb 2014**	**Feb 2014**	**Jan-July 2014**
• Recruitment of Research staff • Translate study protocol into Chinese • Staff Training	• Begin recruitment of cases and controls • Visit by Dr Saunders and Dr Barry • Analysis of Samples	• Completion of Recruitment • Transfer of all remaining samples to Australia	• Analysis of samples

Appendix 3: List of equipment needed

(a) Equipment needed by staff performing enrolment

- Subject information forms
- Consent forms
- Study register books
- Request forms for sputum culture
- Request forms for radiology
- Study Folder (for placing completed forms)

(b) Equipment needed in Department of Haematology

- Blood collection tubes (10 mL EDTA)

(c) Equipment needed in Department of Microbiology

- Study FOLDER for placing completed request forms

Ensure tube is labeled with the study number, date and collection time

The time of phlebotomy, date and study number should be recorded in Study Workbook

2.1 Plasma Preparation

2.1.1 Equipment List

1 x EDTA for plasma (lavender lid)
Filter pipette tips
Transfer pipette
2ml eppendorf tubes
1.5 mL screw top tubes

2.1.2 Requirements

The sample should be processed as soon as possible after blood taking (within 2 hours of the blood draw).

The time elapsed between the taking of the blood and sample processing must be recorded for the sample in the Study Workbook

2.1.3 Instructions for removal of BD Hemogard closure

Grasp the blood tube with one hand, placing the thumb under the closure. With the other hand, twist the closure while simultaneously pushing up with the thumb of the other hand, only until the tube stopper is loosened.

Move the thumb away before lifting the closure. Caution: Do not use the thumb to push closure off tubes. If the tube contains blood, an exposure hazard exists.

Lift closure off tube.

2.1.3 Procedure

Keep the sample at room temperature (18-22^{0}C) throughout processing

The sample needs to be processed in sterile conditions using aseptic technique

Upon arrival at the lab, centrifuge the blood in the EDTA tube (lavender lid) at 1500 g for 15 minutes at room temperature.

This causes separation of the sample into 3 distinct phases: the upper layer of plasma (contains clotting factors), the narrow middle layer is the "buffy coat" (white blood cells), and the bottom layer is the red blood cells.

Appendix 4: Sample Collection and Processing Procedure

1.1 Sample Collection – summary

Samples to be obtained:
1 x 10 ml EDTA tube for miRNA analysis

1.2 Materials

Blood Collection Kit
- 1 x 21G butterfly needle
- 1 x Vacutainer needle holder
- 1 x gloves
- 1 x Torniquet
- Alcohol Wipes
- Cotton wool
- Small Plasters
- 1 x Sharps bin for used needle

Sample Tubes
- 1 x 10 ml EDTA tube (BD Vacutainer EDTA tube, lavender lid, Catalogue # 367525)

1.3 Obtaining Samples

1.3.1 Sample tubes should be labeled with study number

1.3.2 Record details in the Study Registry Book:
- *Study number
- *Date and time that the samples were taken

EQUIPMENT

The equipment used throughout the study for sample collection and storage should be the same as outlined in this protocol.
Any significant variation in equipment must be recorded in the Study Registry Book(G5/G6)

1.4 Protocol for taking blood

1.4.1 Label tubes with study number, date and collection time

1.4.2 Aim to completely fill the EDTA tube. EDTA tubes contain a certain amount of anticoagulant that needs to be mixed in an exact proportion to the blood

1.5 Venipuncture

When the tube is full and blood flow ceases, remove it from the holder.

Invert the EDTA (lavender lid) at least 10 times to reach a proper mix of additive and blood. Do not shake

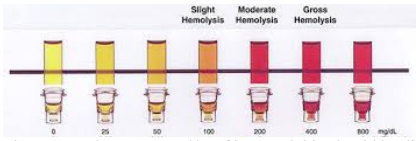
Safely discard the used needle holder and syringe into the sharps bin.

←Plasma – upper layer

←

Figure 1. This is what the tube should look like after centrifugation.

Review each sample for evidence of haemolysis



Plasma samples > 100 mg/dL of haemoglobin should be discarded.

Aliquot the plasma into 2 ml eppendorf tubes using filtered pipette.

Spin the plasma for 3 minutes at maximum speed in the microcentrifuge (~14,000 g) to pellet platelets and residual red blood cells.

Remove the top 80% and divide into 4- 1 ml screw capped tubes. Label each tube with date, study number and visit number (i.e visit 1, 2 or 3)

Sample Storage

3.1 For immediate storage, samples need to be labeled with subject number, date of sample collection, visit number (i.e visit 1, 2 or 3) and assigned box numbers to aid location in the freezer.

3.2 Protocol for sample storage

3.2.1 Plasma

Frozen at -80^{0}C

21