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Highlights

Identified 87 miRs differentially regulated in TB patients versus healthy controls

5 miRNA signature has biomarker potential to identify active TB patients

miRNA modified with successful anti-TB treatment, potential predictor of treatment success

Not all miRNA at baseline by therapy completion maybe ongoing remodelling in lungs

Identification of a plasma microRNA profile in untreated pulmonary tuberculosis patients that is modulated by anti-mycobacterial therapy

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Running title: Changes in miRNAs during TB therapy

Keywords: Tuberculosis, Biomarker, plasma miRNA, anti-mycobacterial therapy, disease progression

Abstract

Objective: microRNA expression profiles are of interest as a biomarker of tuberculosis (TB). How anti-TB therapy effects miRNA profiles is unknown and was examined.

Methods: We identified 87 plasma miRNAs that were significantly modified in an exploratory group of 19 Chinese pulmonary TB (PTB) patients compared to 14 healthy controls. We selected 10 of these miRNAs for analysis in a cohort of 100 PTB patients prior to, and at one, two and six months during treatment.

Results: Five miRNAs were differentially expressed in PTB patients compared to controls at diagnosis; miRs -29a and -99b were up-regulated, whilst miRs -21, -146a and -652 were down-regulated. A combination of 5 miRNA distinguished TB from healthy controls with a sensitivity of 94%, a specificity of 88%, and an AUC of 0.976. Within one month of treatment, significant changes in miRs -29a, -99b, -26a and 146a levels occurred in successfully treated patients, although not all miRNAs returned to baseline by treatment completion.

Conclusion A 5-miRNA signature shows potential for development as a novel biomarker for TB disease with potential to predict response to treatment. The failure of all miRNA to return to baseline levels may reflect ongoing remodelling in the lung parenchyma that continues after completion of anti-TB therapy.

Introduction

Tuberculosis (TB) continues to be a major global health problem with over 10 million new cases of active TB and 1.8 million deaths from TB each year(1). The WHO has set the ambitious goal of reducing TB deaths by 95% and the incidence of new TB cases by 90% by 2035 (2), but this will not be achieved without the development of new interventions, including new therapeutics to combat drug-resistant TB and shorten the length of therapy and a more effective vaccine. New biomarkers that could help identify individuals with active disease or predict the response to treatment would also aid efforts to control TB disease (3). Diagnosing active TB can be challenging. Sputum can be difficult to collect from children, the elderly and HIV co-infected patients. Facilities for sputum culture are often not available. Most commonly TB is diagnosed based on symptoms, and X-ray where available and WHO estimates that over 3 million individuals who develop TB every year go undiagnosed or unreported (4). Therefore, a simple and reliable biomarker that correlates with the presence of active TB would be useful to prevent delay in diagnosis. Furthermore, biomarkers that correlate with the response to anti-microbial therapy may allow early recognition of treatment failure and possible drug resistance and be used as surrogate endpoints in late phase drug and vaccine studies, by greatly reducing the prolonged follow-up period currently required (5, 6).

microRNAs (miRNAs) show great promise as both diagnostic and prognostic biomarkers(7). miRNAs influence nearly all aspects of cell physiology, including cell proliferation, differentiation and apoptosis (8). *Mycobacterium tuberculosis* infection of macrophages modifies the expression of multiple miRNAs, although the functions of most of these miRNAs are currently unknown. Some, such as miR-155 and miR-146, have been demonstrated to exert multiple effects on the inflammatory response to *M. tuberculosis*

infection, both *in vivo* and *in vitro* (9, 10). Studies have shown changes in blood miRNA levels in small numbers of patients with active TB that have biomarker potential (11-13).

Patterns of expression of plasma miRNAs have proven to be reliable as a prognostic indicator for some malignancies and infectious diseases (14, 15). The current study has examined the potential of an miRNA expression profile in the plasma to serve as a biomarker in patients with active pulmonary TB and to predict the response to therapy.

Methods

Study area and participants

This study was undertaken in Ningxia Hui Autonomous region (NHAR) in north-western China where Han make up the majority ethnic group and Hui (Chinese Muslims), ~30%, the largest minority group. Participants were enrolled from five districts within NHAR. 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) between August 2013 and June 2014 and followed for at least 6 months. Written informed consent was obtained prior to enrolment in the study.

Recruitment of study subjects

Patients were recruited through the TB Control Program, in Yinchuan, Ningxia, China and enrolled consecutively from each of the nominated enrolment centres and enrolled at the time of the diagnosis, prior to the commencement of anti-tuberculous therapy. Selection criteria 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 the basis of clinical and radiographic

findings, and sputum smear microscopy and mycobacterial culture according to National guidelines. TB patients enrolled in the study who were smear and culture negative, were diagnosed based on typical clinical symptoms and characteristic features of active PTB on chest radiographs. The lack of *M. tuberculosis* culture is not unusual in high-burden countries and serves to highlight the urgent need for the development of new diagnostic tools. Chest radiographs and chest-computerised tomography (where available) were reviewed independently by both a radiologist and thoracic physician.

TB patients received therapy according to national guidelines that follow WHO DOTS guidelines. Blood was collected at A, prior to the commencement of therapy; B, C and D, following one, two and six months of standard Category One regimen including two months of Isoniazid, Rifampicin, Ethambutol and Pyrazinamide followed by four months Isoniazid and Rifampicin. Patients were deemed cured if after standard therapy they achieved sputum culture conversion and/or (in the case of sputum-smear negative/culture negative TB) radiological clearance or significant radiological improvement.

TB participants were asked to peer-nominate control subjects of the same ethnic group. Healthy controls with no history of TB, and of a similar age, gender and ethnicity were screened via interview and chest radiography to ensure they did not have active TB disease.

Procedures

A venepuncture protocol was designed and strictly followed to avoid any potential factors that may affect miRNA levels. Ten mL of venous blood was drawn from the cubital fossa from each patient at each collection into EDTA (BD Biosciences) tube. Blood was processed within 2 hours of collection. Blood was separated by centrifugation at 800 g for 10 minutes at room temperature. The plasma (supernatant) was removed leaving approximately 5 mm of

plasma to prevent disturbance of the cellular layer and stored at -80°C. Samples collected from China were transported below -60°C to Australia and stored at -80°C. Prior to RNA isolation, samples were examined for haemolysis using a haemolysis chart and samples with > 100 mg/dL of haemoglobin were discarded.

RNA in 500 µl of plasma was extracted using miRCURY™ RNA Isolation Kit (Exiqon, Vedbaek, Denmark) as per the manufacturer's instructions. RNA quality was assessed using miRCURY^{TM} QC PCR panels, as per the manufacturers instructions. This measured the efficiency of amplification of low expressed miRNA and consistency of cDNA synthesis, qPCR efficiency and level of haemolysis. Samples that failed this QC were excluded from further analysis. qPCR reactions were prepared using the miRCURY LNA^{TM} Universal RT miRNA PCR Assay (Exiqon). cDNA was amplified to incorporate SYBR Green qPCR using miRNA-specific forward and reverse primers containing locked nucleic acids (LNAs) (16).

RNA for the exploratory study was loaded onto Plasma Focus miRNA PCR Panels (Exiqon) and the expression of 175 miRNAs examined as per the manufacturer's instructions using a LightCycler®480 (Roche Life Sciences, NSW, Australia). RNA for the confirmation set was loaded onto custom-made qRT-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 referenced to miR-93 which we had previously established to be a reliable miRNA to normalise miRNA in plasma samples (17). 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 using a LightCycler$^{®}$480.

Statistical analysis

The relative expression of each target miRNA was calculated according to the difference in quantification cycle (Cq) values between the target miRNA and the reference miRNA miR-93 using the $2^{-\Delta\Delta CT}$. Groups were tested for normality by the Kolmogorov-Smirnov test and Student's *t*-test and descriptive statistics, linear and logistic regression performed, and receiver operating characteristic (ROC) graphs generated with SPSS Predictive Analytic Software V.21 (IBM Corp, Chicago, Illinois) and GraphPad Prism 7 (San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to determine significance of differences in miRNA expression. Differences, $p<0.05$ were considered significant.

Results

Exploratory study

Twenty patients with newly diagnosed PTB and an average age of 46 (range 18-69) were recruited and compared to 20 healthy controls (Table 1). Plasma miRNAs were extracted and examined with the Plasma Focus miRNA PCR panel. QC analysis of the plasma, the miRNA and the miRNA plates excluded 7 samples from further analysis with 19 TB patients and 14 healthy controls included in the final analysis. Among the 175 miRNAs analysed, 87 miRNAs were identified with significantly altered expression between PTB patients and healthy controls by qRT-PCR, with 69 down-regulated and 18 up-regulated in PTB patients (Supplementary Table 1 and 2).

Ten miRNAs that were significantly differentially expressed were selected for analysis in the confirmatory group of 100 TB patients and control subjects (Figure 1 and Table 2). These were chosen based on a mean fold-change ≥ 2, the strength of expression in the assay with Cq

values < 32, and published evidence of their regulation in TB infection or macrophage function.

Confirmatory study

A total of 100 treatment naïve patients with newly diagnosed PTB, were successfully followed for six months, together with matched healthy control subjects (Table 1). Eighty-nine of the TB patients were successfully treated as defined by either sputum conversion (AFB smear negative and/or *M. tuberculosis* culture negative) and/or clinical and radiological resolution as determined independently by both a radiologist and a thoracic physician, with eleven classified as treatment failures. At the commencement of therapy five of the ten miRNA measured were differentially expressed in the TB patients (Figure 2). miRs -21, -146a and -652 were significantly down-regulated in the TB patients, while miR-99b and miR-29a were significantly up-regulated (Table 2).

To determine if any of the significantly regulated miRNAs, either alone or in combination, could successfully differentiate TB from healthy controls, receiver operator curves (ROC) were constructed (Supplementary Figure 1). These showed values for the area under the curve (AUC) of between 0.64 and 0.70 when individual miRNAs were used. A logistic regression analysis was performed to examine if combining miRNA expression levels improved the accuracy of assignment. A combination of all ten miRNAs yielded a sensitivity of 94.6% and specificity of 88.8% for the diagnosis of TB prior to therapy, with a positive predictive value (PPV) of 88% and a negative predictive value (NPV) of 95% (Figure 3, Table 3) and associated AUC of 0.978. Examining the effect of different combinations of the miRNA determined that a group of 5 miRNA (miRs -146a, -99b, -28, -221 and -29a) yielded a sensitivity of 94% and a specificity of 88% for the presence of active TB with an AUC of 0.976. Predictive values are critically dependent on the population chosen and the prevalence

of a disease. Likelihood ratios, on the contrary, are independent of disease prevalence (18). The positive (PLR) and negative (NLR) likelihood ratio for active PTB were calculated for the miRNA panels, and this yielded a PLR of 5.29 and NLR of 0.12 for the five-miRNA signature and a PLR of 8.6 and 0.06 for the complete 10 miRNA signature.

In many parts of China and indeed globally PTB is diagnosed when the sputum smear is negative and mycobacterial culture is not available. Of the patients enrolled in this study 33 were confirmed by culture or sputum at diagnosis. miRNA expression was examined in this subset of patients and compared to their matched controls. The 10-miRNA panel actually gave a 100% specificity and sensitivity, successfully separated all 33 TB patients from their respective healthy controls. The 5-miRNA-panel similarly gave yielded a sensitivity of 97% and a specificity of 94% with an AUC of 0.984 for active PTB, indicating a strong correlation with this smaller subset of culture confirmed TB patients.

TB patients and healthy controls were further stratified based on ethnicity, age and gender. There were no significant differences in miRNA levels based on gender or age (stratified as under 40 and over 40 years) between the groups (Supplementary Figure 2). The individuals recruited to this study self-identified as either Han Chinese or Hui Chinese. Analysis of plasma miRNA levels showed a difference in miR-29a expression between PTB patients from these two groups (Supplementary Figure 2), however a larger sample size would be required to validate this finding.

The effect of anti-tuberculous treatment on the miRNA plasma profiles of the TB patients were measured after one, two and six months of standard anti-TB therapy. After one month, significant changes in the expression levels of miRs -99b, -29a, -146a and -26 were observed

compared to the levels at the commencement of therapy (Figure 4), however only miR-99b and miR-29a returned to levels equivalent to the healthy controls by the completion of therapy (Figure 4). Of the 100 TB patients in the cohort, 89 were classified as cured at the completion of therapy. Interestingly miRNA levels in the 11 individuals who were classified as treatment failures showed a trend to be more differentially expressed as compared to the healthy controls than the plasma miRNA levels in the patients who successfully completed treatment at the time of diagnosis (Figure 5). The miRNA levels in the treatment failures did not change significantly during the first month of therapy, but approached the levels seen in the treatment success by the completion of therapy (data not shown). However, these data are from a small sample of only 11 treatment failures, and further study of a larger sample size is required to confirm this finding.

Discussion

The exploratory study demonstrated that *M. tuberculosis* disease resulted in significant changes in the levels of 47% plasma miRNAs examined demonstrating that miRNA expression is significantly modulated during active TB. The levels of 10 of these miRNA were then examined in a longitudinal confirmatory study of 100 PTB patients sampled at four time points during therapy. Of the ten selected miRNAs that were differentially expressed in the exploratory study, only six were significantly modulated in the larger cohort study, highlighting one of the potential risks of variation in miRNA levels in relatively small sample sizes. This variation may also be due to differences in baseline miRNA expression. Our understanding of baseline miRNA expression across populations is limited. In this study stratifying for age or gender did not show any significant differences in the control or TB groups, however some differences in miRNA levels were noted between the Han Chinese and Hui Chinese. Previous studies by our group and others (17, 19, 20) have also identified

differences in baseline miRNA levels. Whether these differences are due to genetic differences or factors such as environment, diet, lifestyle or other co-morbidities requires further examination in larger cohorts.

Our understanding of how *M. tuberculosis* infection modulates miRNA expression and the most appropriate miRNA to include in a biomarker panel also requires further research. We selected 10 miRNA from 87 that were differentially regulated in our study, with only six reaching significance in our confirmatory study. Other recent biomarker studies have identified additional miRNA that may have biomarker potential to aid TB diagnosis. miRs-144, -223, -29a, -155 and 424 have all been reported as providing AUC over 0.8 or sensitivity and specificity over 80% as a single miRNA biomarker aiding TB diagnosis (12, 22-25). Of these, only miRs -223 and -29a were significantly regulated in our exploratory study, with only miR-29a remaining significantly regulated in the validation study. These differences may reflect differences in ethnicity, latent TB status, and environmental or other risk factors, such as diabetes or smoking which were not evaluated in this study, but should be examined in larger future studies.

Using a miRNA panel over a single miRNA is one option to increase the sensitivity and specificity of the biomarker. This study found a five-miRNA combination demonstrated a maximum sensitivity of 94% without compromising specificity, at 88%, for the presence of active PTB in a Chinese cohort. A study by Miotto et al. (21) on TB patients from Italy, Uganda and Tanzania, identified a 15 miRNA panel that differentiated HC from PTB with an AUC of 0.9528. Seven of these miRs were also down regulated in our study, although another seven were not differentially expressed in our exploratory study. Larger studies, comparing different ethnicities, other respiratory diseases and monitoring controls at multiple

time points are required to confirm the most promising miRNAs with diagnostic potential for TB.

Another finding from this longitudinal study was the significant changes in miRNA expression over the course of therapy. Initially it was hypothesised that a positive response to anti-tuberculous therapy would be reflected by changes in the miRNA profiles back to the levels present in healthy controls. This was the case for only two of the miRNA examined, including miR-99b that returned to levels equivalent to healthy controls within a month of starting anti-tuberculosis chemotherapy and miR-29a where this took six months. In the 89 TB patients classified as treatment successes, a significant change in miRs -99a, -29 and -26a occurred within a month of commencing treatment, suggesting that changes in miRNA levels may be utilised as a measure of treatment response.

Some miRNAs had not returned to the levels in healthy controls by the end of the treatment period. The reasons for this require further investigation. Possibly the persistent down-regulation of miR-146a may be a protective measure to control for the deleterious effects of an unregulated inflammatory response, and this may counteract the pro-inflammatory effects thought to be associated with down-regulation of miR-21a (26) and miR-26a (27, 28). The miRNAs may remain down-regulated because of continuing lung inflammation and parenchymal re-modelling that accompanies resolving pneumonic infection. It is important to note that the cause of the observed changes in plasma miRNAs or the target sites for these miRNAs are not known. Recently, serial PET scanning of the lungs during treatment for PTB revealed that at the completion of therapy, only 14% of patients had fully resolved lesions, 51% of patients had increased metabolic activity in the original lung lesions, and in 18% of cases new PET-positive lesions developed during therapy (29). This indicates that

inflammatory changes persist despite microbiological improvement, and this inflammation may contribute to persistent changes in some plasma miRNA levels. Other factors may also have an impact on plasma miRNA expression, such as the duration of active TB prior to therapy, intercurrent illness, diet and smoking history, and these should be addressed in a larger cohort of patients to determine whether miRNA expression patterns could be used successfully as a predictor of treatment outcome.

This study demonstrates that miRNA levels in PTB patients differ from health controls. One limitation of this study is that we did not compare the sensitivity or specificity of our biomarker panel to distinguish PTB from other respiratory diseases, such as bacterial pneumonia. This may produce similar radiological changes, particularly in the context of immune-compromised individuals (30). A serum seven-protein biosignature was able to distinguish active PTB from other chest infections and acted as a triage tool in the diagnosis of PTB in a primary care setting (31). Second, the control population in the current study were screened to exclude active TB by symptoms and chest radiography, but not for latent TB infection (LTBI). As TB is endemic in China with an estimated case detection rate of 423 per 100,000 in western China (32) it is likely that many of the healthy controls have LTBI. Both transcriptional and protein biomarkers that distinguish subjects with active TB from health controls are also negative in the vast majority of subjects with LTBI (31, 33), however future studies should determine if miRNA expression is affected by LTBI. Finally. miRNA levels were measured in the healthy control subjects once at only one time point. It would be informative to test the control subjects at multiple times to determine if plasma miRNA expression fluctuates in healthy individuals. Nevertheless, the five-miRNA signature clearly identified subjects with active TB with a sufficient PLR of 5.29 and NLR of 0.12 to suggest it is a plausible diagnostic marker worthy of further exploration.

Recent studies have shown that suppression of a single miRNA can partially regulate *M. tuberculosis* survival (34, 35). Further translational research to enhance our understanding of miRNAs as a diagnostic and prognostic tool as well as a target for host-directed therapy has the potential to improve the management of patients with TB disease.

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Table 1. **Characteristics of the TB patients and controls for exploratory and confirmation studies.**

		TB		**Healthy Controls**	
		Exploratory Set	Confirmation Set	Exploratory Set	Confirmation Set
Total Number		19	100	14	100
Gender (male/female)		10/9	58/42	8/6	57/43
Age (years, average; range)		46 (18-69)	43 (19-91)	37(18-67)	35(18-78)
Ethnicity	Han Hui	1 18	42 58	1 13	46 54
Sputum Smear Positive		5	33		
Sputum Culture Positive		5	10		
Culture DST		2	10		
Chest X-ray with active TB		19	100		
Treatment success (%)		14 (74%)	89 (89%)		

Table 2. Expression levels of 10 candidate plasma miRNAs in TB patients from the exploratory and confirmation sets from prior to the commencement of therapy.

	Exploratory Set		**Confirmation Set**	
miRNA	**Fold Change**	**p –Value***	**Fold Change**	**p –Value***
miR-21-5p	-2.28	**<0.001**	-1.83	**0.005**
miR-99b-5p	2.54	**<0.001**	1.52	**0.002**
miR-29a-5p	2.79	**0.006**	4.09	**<0.001**
miR-223-5p	2.09	**0.002**	-1.01	0.767
miR-221-3p	2.56	**0.003**	1.47	0.176
miR-146a-5p	-2.29	**0.002**	-2.66	**<0.001**
miR-26a-5p	-2.10	**0.001**	-1.26	0.192
miR-28-5p	2.93	**<0.001**	1.72	0.105
miR-133a	-2.94	**0.002**	-1.08	0.305
miR-652-3p	-1.73	**0.020**	-1.37	**<0.001**

miRNA levels for the exploratory set were measured by Plasma Focus miRNA PCR Panels (Exiqon), and for the confirmatory set by qRT-PCR, and all data normalised to levels of miR-

*p-value calculated using Student's *t*-test

Table 3. Logistic regression analyses of the combination of ten plasma miRNAs for the diagnosis of pulmonary TB in confirmation set.

Variable	Coefficient	Std error	p-value	Odds Ratio	95% CI
miR-21	0.996	0.774	0.198	2.708	0.594-12.356
miR-26a	-1.212	0.662	0.067	0.297	0.081-1.088
miR-28	0.834	0.306	0.006	2.303	1.264-4.197
miR-29a	1.011	0.283	<0.001	2.748	1.578-4.784
miR-99b	2.019	0.6	0.001	7.53	2.322-24.416
miR-133a	-0.313	0.209	0.134	0.732	0.0486-1.101
miR-146a	-3.787	0.834	<0.001	0.023	0.004-0.116
miR-221	2.092	0.544	<0.001	8.098	2.79-23.501
miR-223	0.349	0.303	0.249	1.418	0.783-2.568
miR-652	-1.003	0.709	0.157	0.367	0.091-1.471
Constant	-3.642	0.629	<0.001		

Figure Legends

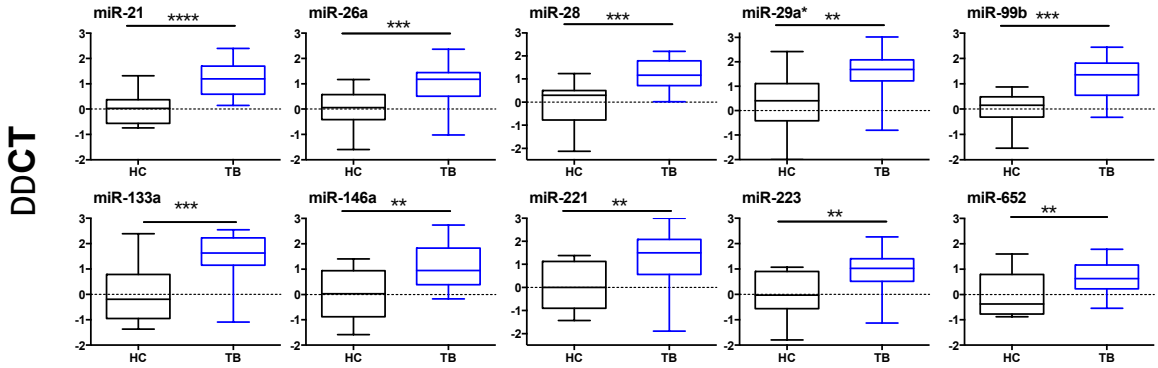

Figure 1. Plasma levels of miRNAs in exploratory set of 19 TB patients and 14 healthy controls. Expression levels of 10 miRNAs in the plasma of 19 TB patients prior to commencement of treatment and 14 healthy controls were measured by qRT-PCR. The relative expression of the miRNA as defined by $2^{-\Delta Ct}$, was normalised against miR-93. Results shown are the median and 95% central range. Outliers are plotted individually. The statistical significance of the difference between the groups for each miR was determined by Student's *t*-test. *p <0.05, **p <0.01, ***p <0.001.

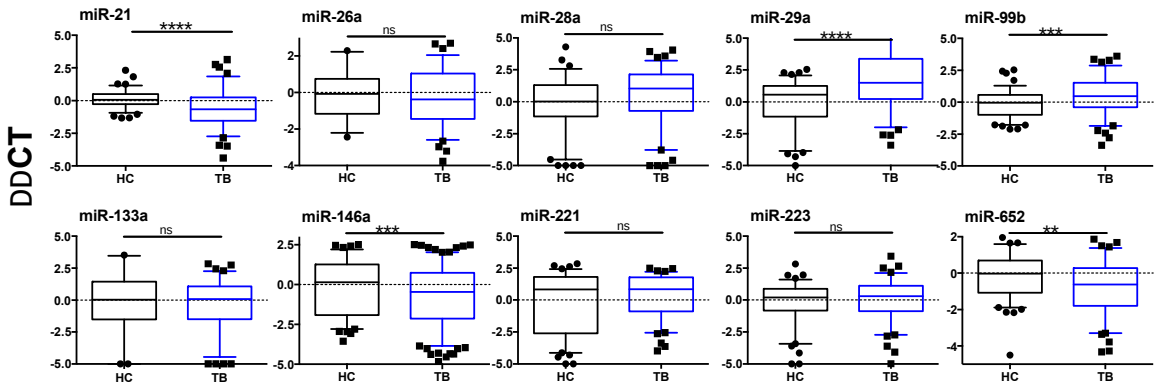

Figure 2. Plasma levels of miRNAs in confirmation set of 100 treatment naïve TB patients prior to therapy and 100 healthy controls. Expression levels of 10 miRNAs from 100 TB patients prior to commencement of treatment and 100 healthy controls were measured by qRT-PCR. The relative expression of the miRNA as defined by $2^{-\Delta Ct}$, was normalised against miR-93. Results are median and 95% central range. Outliers are plotted individually.

The statistical significance of the difference between the groups for each miR was determined by Student's *t*-test. *p <0.05, **p <0.01, ***p <0.001.

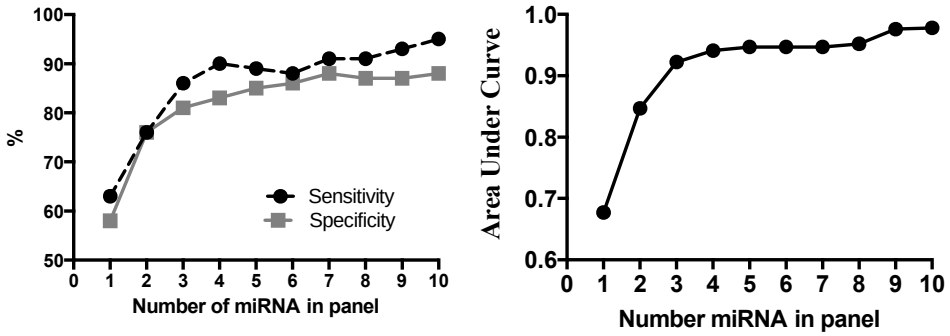

Figure 3. Increasing sensitivity and specificity of the miRNA panel to distinguish newly diagnosed TB patients from healthy controls.

The specificity and sensitivity and area under the curve for combinations of 1 to 10 miRNA measured in 100 TB patients prior to commencement of treatment compared to 100 healthy controls were generated in SPSS V.21.

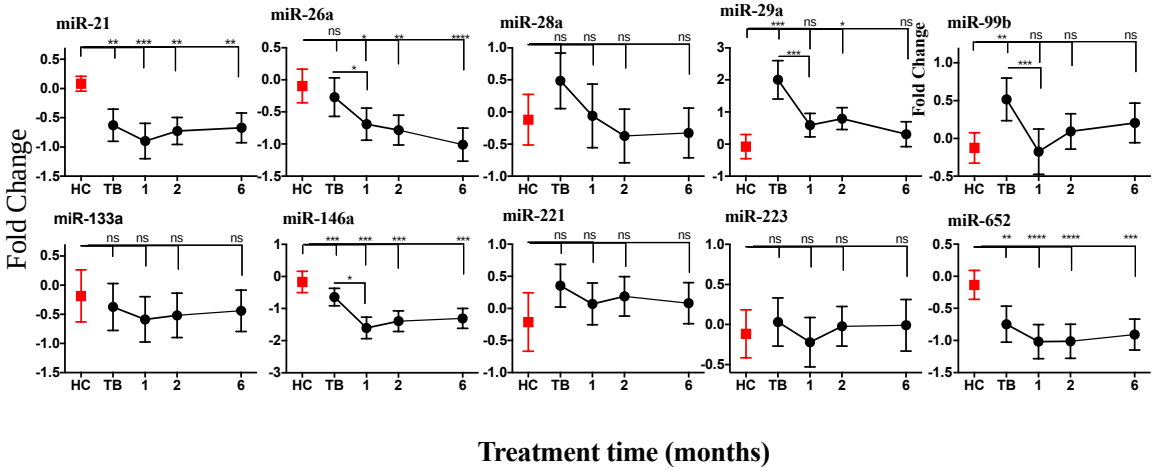

Figure 4. Plasma levels of miRNAs in confirmation set of 100 TB patients and their matched healthy controls at all collection time points. Plasma from TB subjects was collected prior to the commencement of therapy (0 months) and after one, two and six months of therapy. miRNAs levels were examined by qRT-PCR. The relative expression of the miRNA as defined by $2^{-\Delta\Delta Ct}$, was normalised against miR-93. Results are mean ± 95% confidence intervals. The statistical significance of the difference between the groups and

between time points for each miR was determined using ANOVA.*p <0.05, **p <0.01, ***p <0.001.

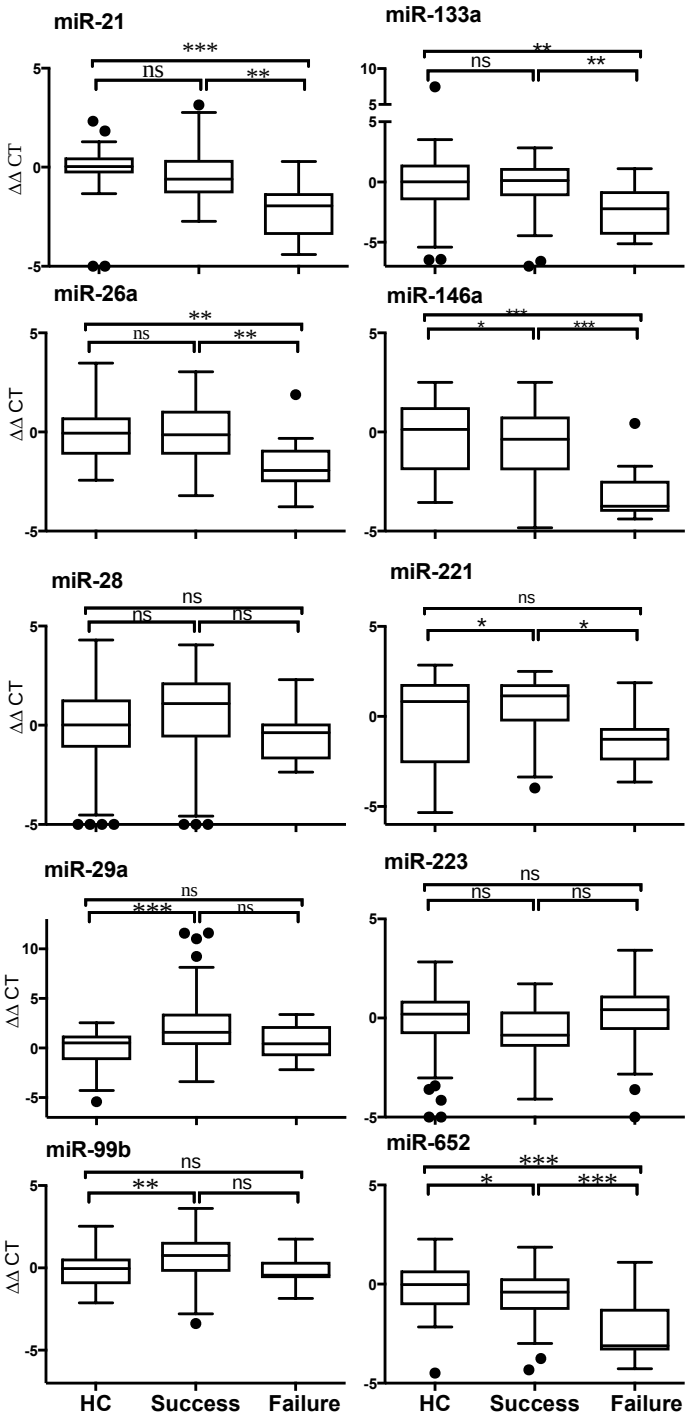

Figure 5. Plasma levels of miRNAs in patients who were classified as treatment failures compared with healthy controls and treatment successes. Plasma from TB subjects was collected prior to the commencement of therapy (0 months) and after one month of therapy

was stratified based on treatment success (n=89, treatment failure n=11). Results are median + 95% confidence intervals. The statistical significance of the difference between the groups and between time points for each miRNA was determined using ANOVA. *p <0.05, **p <0.01, ***p <0.001.