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Diagnostics

MicroRNA *hsa-miR-29a-3p* is a plasma biomarker for the differential diagnosis and monitoring of tuberculosis

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ABSTRACT

The diagnosis of tuberculosis (TB) continues to pose substantial public health problems. The quest for diagnostic biomarkers for TB is therefore primordial. This study aimed to evaluate the diagnostic and anti-TB treatment monitoring potentials of some selected miRNAs. Quantitative real time polymerase chain reaction and Receiver operating characteristics were used to estimate the ability of miRNAs to discriminate between healthy controls (HEC), latent (LTB) and active TB (ATB). The study showed that: *hsa-miR-29a-3p*, *hsa-miR-155-5p* and *hsa-miR-361-5p* were significantly upregulated in ATB compared to HEC while *hsa-miR-29a-3p*, and *hsa-miR-361-5p* were also significantly up-regulated in ATB compared to LTB (all $P \leq 0.05$). *MiR-29a-3p* showed a good (81.37%) distinguishing performance in discriminating ATB from HEC and a good (84.35%) diagnostic performance in discriminating ATB from LTB. The performance of *miR-29a-3p* present in the blood in discriminating active TB from latent TB and healthy controls indicates it may be a useful biomarker for diagnosis of TB. Because this miRNA is found in blood (plasma) which is easy to collect compared to sputum it could be used in pediatric and extra-pulmonary TB cases.

1. Introduction

Tuberculosis (TB) is a very contagious infectious disease, caused by various strains of mycobacteria, usually Mycobacterium tuberculosis (MTB) in humans [1]. Tuberculosis typically attacks the lungs (pulmonary TB), but can also infect other parts of the body (extra-pulmonary TB). Most infections are asymptomatic and latent, but about 10% eventually progress to active disease which, if left untreated, kills more than 50% of those infected. Tuberculosis is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS [2]. Roughly one-third of the world's population has been infected with *Mycobacterium tuberculosis*. Efforts to control it are hampered by difficulties with diagnosis, prevention and treatment [3].

The diagnosis of TB still remains a global problem with diagnostic delays and misdiagnosis increasing the rate of morbidity and mortality. National tuberculosis programs in disease endemic countries continue to rely largely on direct smear microscopy, solid culture, chest radiography and tuberculin skin test (TST) [4]. Infants of less than six years of age are unable to produce sputum for microscopy and culture which

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are currently considered as the confirmatory tests for TB. In this age group, the tuberculin skin test (TST) is used but this test is not specific for MTB. Diagnosis by culture is considered the gold standard for TB but it is time consuming and not very suitable for extra-pulmonary TB [5]. The diagnosis of smear-negative and extra-pulmonary tuberculosis continues to pose substantial clinical challenges with pediatric TB not left out because of the difficulty of sampling sputum. The quantiFERON-TB test was developed which could help out with the extra-pulmonary cases but has a limit that it cannot distinguish between latent and active TB and also cannot be used to monitor anti-TB treatment. There is need for more efficient diagnostic methods with alternative specimens like blood, feces and urine which can be collected from all age groups. One of the biomarkers being evaluated for diagnosis of TB in blood are microRNAs (miRNAs) [6,7].

MiRNAs are short fragments of about 18–22 nucleotides which regulate gene expression by binding to the 3′-UTR of messenger RNAs (mRNAs) while temporally inhibiting their translation or inducing their degradation [8]. In this study we describe the expression profile of eight selected miRNAs in a central-west African population where the prevalence of HIV is high (4.3%). For the first time we assessed the ability of eight plasma miRNAs in distinguishing active TB from latent TB and also their ability to serve as biomarkers for monitoring anti-TB therapy.

2. Materials and methods

2.1. Human subjects

A total of 162 subjects were categorized into three main groups; active TB (ATB), latent TB (LTB) and healthy controls (HEC) were enrolled between September 2013 and May 2015. An ethical clearance was obtained from the national ethical research committee in Cameroon (N° 2013/05/242/CNERSH/SP) as well as an authorization issued by the Ministry of public health (N°D30-685AAR/MINSANTE/SG/DROS/CRC/CEA1/AB). Questionnaires and informed consent forms were administered before sampling. Subjects were enrolled at the Jamot hospital in Yaounde, Cameroon and blood samples collected were transported in ice coolers (temperatures were monitored) to the Chantal Biya International Reference Center (CBIRC), Yaounde for further analyses and storage (−30 °C). ATB was confirmed by observation of clinical signs and symptoms by a medical specialist, microscopy and/or culture. Those with TB (ATB and LTB) were further classified according to their HIV status. ATB were either at the initiation of anti-TB therapy, 2 months of therapy or at completion of therapy (6 months) (see Table 1). The QuantiFERON-TB Gold In-Tube test (Qiagen, Chadstone, Victoria, Australia, Cat. N° 0594-0201) was added to the analyses to distinguish between latent TB and healthy controls. This test was conducted on health Care personnels who showed no signs/symptoms of TB, apparently in good health and had no past history of TB infection. From this group, those with positive quantiFERON test were grouped as latent TB infected while those with negative quantiFERON test results were considered healthy controls (Table 1). The HIV test was carried out in conformity with the National algorithm for the diagnosis of HIV while the T-lymphocytes quantification ($CD4^+$ count) was done using Becton Dickinson (BD) Facs calibur cytometry (BD Biosciences).

2.2. Database search for potentially overexpressed miRNAs in TB patients

The methods used for the collection and compilation of miRNA data have already been described [9]. Briefly, a systematic PubMed search done with the queries ‘miRNAs associated with tuberculosis, miRNAs related to tuberculosis, miRNAs + tuberculosis, and miRNAs as potential biomarkers for tuberculosis’, indexed 282 records. This data were sorted to retain only miRNAs upregulated in active TB. A total of 11 miRNAs were identified (Supplementary Table 1) and confirmed with three miRNA disease data bases namely; 1) PhenomiR 2.0 database (http://mips.helmholtz-muenchen.de/phenomir) which is a knowledgebase database on miRNA expression profile in diseases and other biological processes [10], 2) Human MiRNA Disease Database (HMDD) 2.0 (http://www.cuilab.cn/hmdd) which is a curated database on experiment-supported evidence for human miRNA and disease associations [11], and 3) miR2Disease database (http://www.miR2Disease.org) which is a manually curated database, aim at providing a comprehensive resource of microRNA deregulation in various human diseases [12]. Eight of the 11 identified miRNAs were then selected for further validation based on the criteria that, they were either published by more than one author or have been quantified in blood (PBMC or Serum). The sequences were accessed from miRBase Release 21 [13] and their accuracy ensured by miRBase Tracker [14].

2.3. Blood collection

Five milliliters of blood were collected into Ethylene diamine tetra-acetate (EDTA) tubes through venous puncture on the left arm. Blood samples were transported in ice coolers (temperature monitored) from the Jamot hospital to the CBIRC. Blood tubes were centrifuged at 3000 rpm for 10 min after which plasma were aliquoted into 2 ml screw-cap tubes and stored at −30 °C till needed for total RNA extraction.

2.4. Extraction of total RNA

Total RNA was extracted and purified from Plasma with the miRNeasy mini kit (Qiagen, Inc., Valencia, USA, Cat N° 217004) following the manufacturer's instructions. Briefly, 200 µl of the plasma samples were lysed by QIAzol and chloroform. The total RNA was adsorbed onto the silica-gel membranes in the RNeasy mini columns at a high chaotropic salt concentration. The resulting RNA were washed and eluted in 50 µl of RNase-free water. The quality of RNA was assessed using a nanodrop (NanoDrop One, by ThermoScientific; Verona Rd, Madison, Assembled USA) prior to cDNA synthesis.

2.5. cDNA synthesis

Total RNA were reversed transcript to cDNA by the miRCURY LNA universal RT microRNA PCR kit (Qiagen, Maryland, USA) following the manufacturer's recommendations. Before setting the reaction, the concentration of all total RNA samples were normalized to 5 ng/µl by diluting with nuclease free water. The synthesized cDNAs were then used as templates in the qRT-PCR reactions.

Table 1
Demographic and clinical characterization of study population.

Characteristics	Groups		
	Active TB (ATB)	Latent TB (LTBI)	Healthy controls
Gender (Male/Female)	(57/26)	(8/27)	(13/29)
Age(Mean ± Stdv)[range]	(33 ± 12)[16, 76]	(34 ± 11)[19, 53]	(27 ± 7)[19, 56]
HIVstatus (Positive/Negative)	(17/67)	(3/32)	(0/42)
Duration on anti-TB therapy (No anti-TB/2 months on anti-TB/6 months on anti-TB)	(62/16/6)	(35/0/0)	(42/0/0)

Table 2
Fold changes of miRNAs in different TB/HIV- groups compared to the healthy control group.

MiRNAs	Fold Change Mean ± SEM(N)			Analyses of Variance (P-values)		
	HEC	(LTB/HIV-)	(ATB/HIV-)	(HEC) X (LTB/HIV-)	(HEC) X (ATB/HIV-)	(LTB/HIV-) X (ATB/HIV-)
miR-155-3p	0	0	0			
miR 576-3p	0	0	0			
miR-889-5p	0	0	0	All P > 0.05 (ns)		
miR-29a-3p	1.00 ± 0.64 (40)	2.78 ± 2.0 (28)	2.82 ± 1.84 (50)			
miR-144-5p	1.00 ± 0.66 (32)	1.92 ± 1.3 (24)	0.62 ± 0.40 (46)			
miR-155-5p	1.00 ± 0.77(25)	2.63 ± 1.9 (15)	3.69 ± 2.67 (38)			
miR-196b-5p	1.00 ± 1.40(11)	2.68 ± 2.2 (10)	2.22 ± 1.90 (20)			
miR-361-5p	1.00 ± 0.67 (39)	1.67 ± 1.2 (26)	2.65 ± 1.68 (49)			

2.6. Quantitative real time PCR (qRT-PCR)

The miRCURY LNA SYBR Green PCR kit (Qiagen, Maryland, USA) was used while respecting the manufacturer's recommendations. Briefly, For 1 reaction in a final volume of 10 µl, the reagents were mixed in the following order; 5 µl of PCR master mix, 1 µl of primer mix and 4 µl of diluted cDNA (the cDNAs were diluted 1:80 times). A qRT-PCR master mix was prepared and 6 µl were distributed into different wells in a 96 well plate. 4 µl of the diluted cDNA samples were then added. The 5 S rRNA was used as the normal expression control. The thermal cycling conditions were set at 95 °C for 10 min, 45 cycles at 95 °C for 10 s, 60 °C for 1 min. The Ct values were read and the fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Statistical analysis

GraphPad Prism version 6.0 was used as the statistical tool for miRNA data analysis. The analysis of variance (ANOVA) with multiple comparison was used to compare the fold changes between groups (ATB, LTB and HEC). P-values less than or equal to 0.05 were considered significant in all analyses. Data was represented as Mean ± SEM (standard error on mean).

2.8. Receiver operating characteristics (ROC) curves

ROC curves were used to estimate the diagnostic performance of significantly overexpressed miRNAs. The area under the ROC curve (AUC) is a measure of the ability of a parameter to distinguish between two groups. In ROC curve analysis, AUC values lie between 50% and 100% with 50% being a bad classifier and 100% an excellent classifier. Values from 90 to 100% are generally considered excellent, 80–90% good, 70–80% fair, 60–70% poor and 50–60% bad (or failed). An AUC below 50% is said to indicate random values not capable of distinguishing between two groups. Thus, ROC curves were used to evaluate the ability of the fold changes to discriminate between the groups (ATB, LTB, HEC).

3. Results

3.1. Demographic and clinical characterization

A total of one hundred and sixty-two subjects were enrolled among which 84 had active TB (ATB), 35 latent TB infection (LTB) and 43 were healthy controls. Twenty of those with TB (ATB and LTB) were co-infected with HIV. The CD4 count in the sample population ranged-from 46 cells/mm^3 to 2605 cells/mm^3 with a Mean ± Stdv of 811 ± 548 cells/mm^3 and median of 746 cells/mm^3. Among the 84 people with active TB (ATB), 16 were at 2 months of anti-TB treatment, 6 had completed anti-TB treatment (≥6 months of treatment) while the rest had not yet initiated anti-TB therapy. The 22 ATB subjects on anti-TB treatment were not included among those with ATB in any of the analyses. Those who completed anti-treatment were all sputum-negative. Pearson correlation was used to check for possible associations between HIV-status, gender, age-group, CD4$^+$ count and TB infection. We found correlations between HIV-status, gender and TB infection with respective p-values of 0.0303 and 0.0035. HIV$^+$ subjects (94% of HIV + cases) and men (80% of men) were more likely to be infected with TB than HIV$^-$ subjects (69% of HIV$^-$) and women (62% of women). No correlation was found between age-group, CD4$^+$ count and TB infection. The distribution of these parameters is summarized in Table 1.

3.2. Retrieved miRNAs

We identified a total of eleven miRNAs upregulated in active TB (ATB) with respect to healthy controls from the online miRNA mining (Supplementary Table 1). Eight of these miRNAs namely; *hsa-miR-29a-3p*, *hsa-miR-361-5p*, *hsa-miR-155-3p*, *hsa-miR-155-5p*, *hsa-miR-196b-5p*, *hsa-miR-576-3p*, *hsa-miR-889-5p*, and *hsa-miR-144-5p* were selected for further validation. *Hsa-miR-29a-3p* and *hsa-miR-361-5p* were selected on the basis that, they were published by more than one author and the remaining six (*hsa-miR-155-3p*, *hsa-miR-155-5p*, *hsa-miR-196b-5p*, *hsa-miR-576-3p*, *hsa-miR-889-5p*, and *hsa-miR-144-5p*) were selected on the basis that they have been quantified in blood (PBMC or serum).

3.3. Plasma miRNA expression profiles in TB subjects with or without HIV co-infection

The expression levels of *hsa-miR-29a-3p*, *hsa-miR-361-5p*, *hsa-miR-155-3p*, *hsa-miR-155-5p*, *hsa-miR-196b-5p*, *hsa-miR-576-3p*, *hsa-miR-889-5p*, and *hsa-miR-144-5p* were evaluated in patients with active TB not co-infected with HIV (ATB/HIV$^-$), those with latent TB infection not co-infected with HIV (LTB/HIV$^-$) in comparison to healthy controls (Table 2). Our analysis revealed that three miRNAs; *hsa-miR-155-3p*, *hsa-miR-576-3p* and *hsa-miR-889-5p* were not expressed in any of the sub-groups. Four miRNAs: *hsa-miR-29a-3p*, *hsa-miR-361-5p*, *hsa-miR-155-5p* and *hsa-miR-196 b-5p* were upregulated in ATB/HIV$^-$ compared to HEC and upregulated in LTB/HIV$^-$ compared to HEC. Meanwhile *hsa-miR-144-5p* was upregulated in LTB/HIV$^-$ but down regulated in ATB/HIV$^-$ compared to HEC although the differences were not significant (Table 2 and Fig. 1 (a) and (b).

The plasma expression levels of the eight miRNAs were also evaluated in patients with TB (ATB and LTB) co-infected with HIV (ATB/HIV$^+$ and LTB/HIV$^+$). The expression profiles were similar to those with TB not co-infected with HIV (ATB/HIV$^-$ and LTB/HIV$^-$), although the size of some subgroups was not optimal for definitive conclusion (Table 3 and Fig. 1 (c)).

3.4. Evaluation of the combined effect of miRNAs

The combined effect of the five expressed miRNAs was assessed using backward multivariate logistic regression analysis. The dependent variable was TB status (TB-ATB/LTBI versus control). The independent variables were the five miRNAs. No associated or combined effects were found among miRNAs (All p > 0.05).

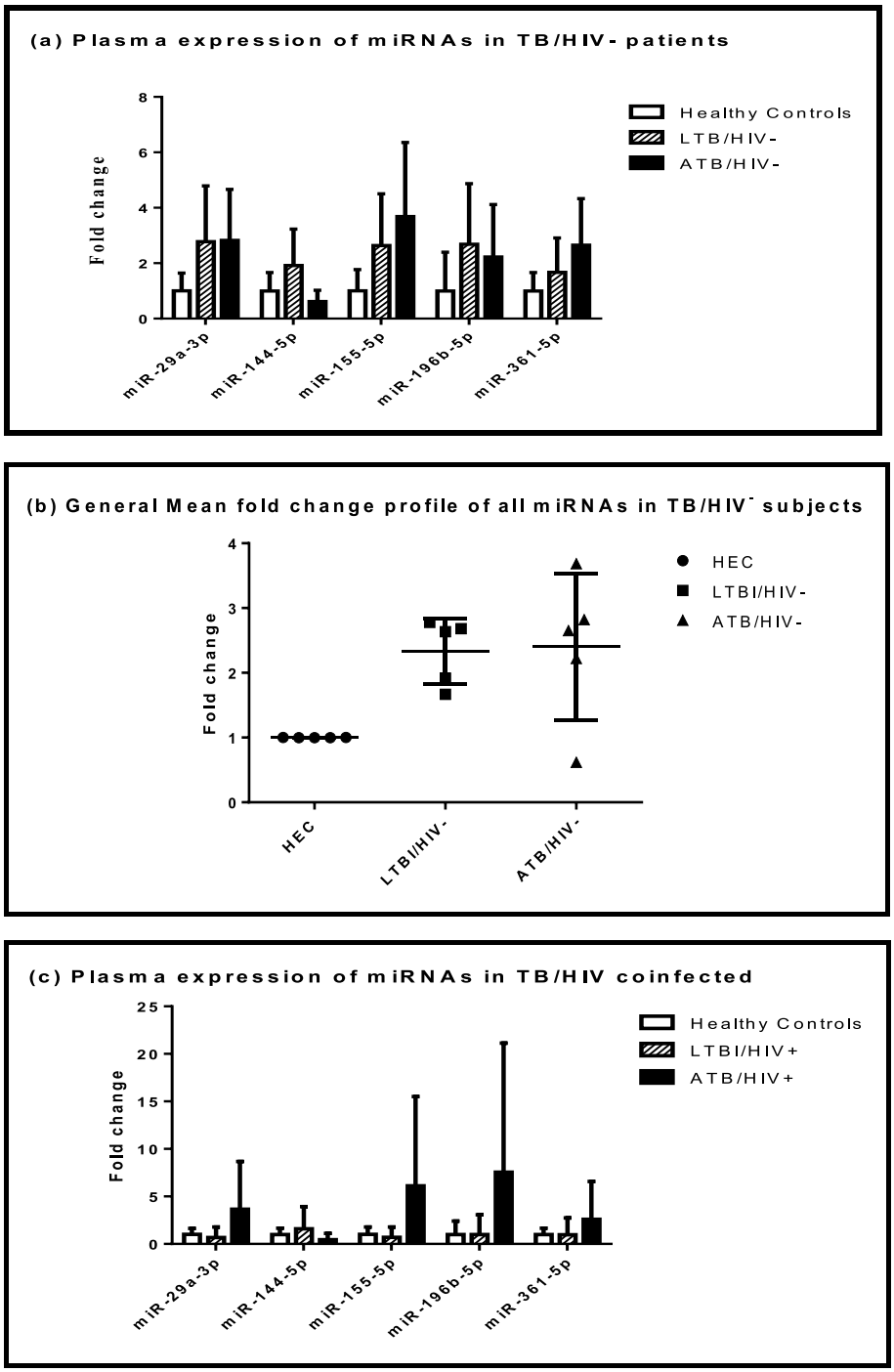

Fig. 1. Plasma expression of miRNAs (a) in TB patients not co-infected with HIV (TB+/HIV-) (b) scatter plot summary of the expression profile of all miRNAs in the TB/HIV- subjects (c) in TB patients co-infected with HIV (TB+/HIV+). Values were expressed as Mean ± SEM and were considered significant when p-values were less than or equal to 0.05 at 95 CI. (TB+/HIV- = subjects with either latent or active TB not co-infected with Human Immunodeficiency Syndrome, TB+/HIV+ = subjects with either latent or active TB co-infected with Human Immunodeficiency Syndrome, LTBI+/HIV- = subjects with latent TB not co-infected with HIV, ATB+/HIV- = subjects with active TB not co-infected with HIV, LTBI+/HIV+ = subjects with latent TB co-infected with HIV, ATB+/HIV+ = subjects with active TB co-infected with HIV).

Table 3
Fold changes of miRNAs in different TB/HIV + groups compared to the healthy control group.

MiRNAs	Fold Change Mean ± SEM(N)			Analyses of Variance (P-values)		
	HEC	(LTB/HIV+)	(ATB/HIV+)	(HEC) versus (LTB/HIV+)	(HEC) versus (ATB/HIV+)	(LTB/HIV+) versus (ATB/HIV+)
miR-155-3p	0	0	0			
miR 576-3p	0	0	0			
miR-889-5p	0	0	0	All P > 0.05 (ns)		
miR-29a-3p	1.00 ± 0.64 (40)	0.69 ± 1.1 (3)	3.65 ± 5.0 (9)			
miR-144-5p	1.00 ± 0.66 (32)	1.59 ± 2.3 (3)	0.45 ± 0.60 (9)			
miR-155-5p	1.00 ± 0.77(25)	0.69 ± 1.1 (3)	6.11 ± 9.4 (7)			
miR-196b-5p	1.00 ± 1.40(11)	0.99 ± 2.1 (2)	7.54 ± 13.6 (6)			
miR-361-5p	1.00 ± 0.67 (39)	0.98 ± 1.7 (3)	2.60 ± 3.96 (9)			

Table 4
Plasma miRNA expression levels in different TB groups in subjects with CD4 counts between 400 and 699 cells/mm^3.

MiRNAs	Fold Change compared to Healthy Control group Mean ± SEM (N)			Analysis of Variance (P-value)		
	HEC CD4 [400–700[	LTBI (CD4 [400–700[)	ATB (CD4 [400–700[)	(HEC) versus (LTB)	(HEC) versus ATB	(LTB) versus (ATB)
miR-155-3p	0	0	0	0	0	0
miR 576-3p	0	0	0	0	0	0
miR-889-5p	0	0	0	0	0	0
miR-29a-5p	1.00 ± 1.3 (8)	7.92 ± 2.17 (6)	11.78 ± 1.09 (23)	ns	****	***
miR-144-3p	1.00 ± 1.2 (7)	5.12 ± 0.97 (6)	1.20 ± 1.09 (21)	ns	ns	ns
miR-155-5p	1.00 ± 1.1 (7)	3.86 ± 1.29 (4)	6.27 ± 1.27 (20)	ns	**	ns
miR-196b-5p	1.00 ± 1.3 (2)	8.86 ± 1.42 (4)	2.77 ± 1.53 (11)	ns	ns	ns
miR-361-5p	1.00 ± 1.1 (8)	3.21 ± 2.07 (7)	10.10 ± 1.05 (25)	ns	****	***

* Significant difference ($p \leq 0.05$), ns: non significant ($P > 0.05$).

3.5. Plasma miRNA expression profiles in TB patients with CD4 counts between 400 and 699 cells/mm^3

We analysed the expression levels of the selected eight miRNAs in the sub-population with CD4 counts between 400 and 699 cells/mm^3 (Table 4). For this, analysis of variance (ANOVA) with multiple comparison of unpaired data was used. We considered this population as a normalized population. The CD4 count usually refers to the degree of the immune-competence of an individual. The assumption made was that, people with almost the same CD4 counts should have a similar immunity and therefore a similar miRNA expression profile during TB infection. Consequently a more accurate representation of the miRNA expression profile as compared to those with a wide difference in CD4 count. We used Pearson's correlation (r) to verify this assumption that CD4 count and miRNA expression may be linked but found no correlation. Despite absence of correlation between the two, our analysis revealed that: *hsa-miR-29a-3p*, *hsa-miR-155-5p* and *hsa-miR-361-5p* were significantly upregulated in ATB compared to HEC contrary to the whole population data (which showed upregulation but no significance). Also *hsa-miR-29a-3p* and *hsa-miR-361-5p* were significantly up-regulated in ATB compared to LTB (see Table 4 and Fig. 2 (a)).

3.6. Plasma miRNA expression profiles in TB patients according to anti-TB treatment duration

According to anti-TB treatment, the expression levels of *hsa-miR-29a-3p*, *hsa-miR-155-5p*, *hsa-* and *hsa-miR-361-5p* were reduced at completion of treatment compared to initiation with respective relative fold changes of 0.17, 0.26 and 0.27 (Table 5 and Fig. 2 (b)). *Hsa-miR-144-5p* was significantly up-regulated at completion of anti-TB therapy as compared to initiation of therapy (Table 5 and Fig. 2 (b)).

3.7. ROC curve analysis

The diagnostic performance of *hsa miR-29a-3p* in discriminating the active TB group from the healthy controls and the latent TB group was analysed. Our analysis showed it exhibited a good distinguishing performance in discriminating between the active TB group and the healthy controls (AUC = 81.37%, Sensitivity: 80%, Specificity: 71.43%, Positive predictive value: 0.7 and Negative predictive value: 0.8). *Has miR-29a-3p* also exhibited a good distinguishing performance between the active TB group and the latent TB group (AUC = 84.35%, Sensitivity: 80%, Specificity: 80%, Positive predictive value: 0.8 and Negative predictive value: 0.8) (Fig. 3 (a) and (b)).

MiR-155-5p exhibited a fair distinguishing performance in discriminating the active TB group from the healthy controls (AUC = 70.83%, Sensitivity: 80%, Specificity: 50%, Positive predictive value: 0.6 and Negative predictive value: 0.7) (Fig. 3 (c)).

MiR-361-5p exhibited a fair diagnostic accuracy in discriminating active TB from healthy controls (AUC = 78.29%, Sensitivity: 88%, Specificity: 57.14%, Positive predictive value: 0.8 and Negative predictive value: 0.8) and exhibited a poor performance in discriminating active TB from latent TB (AUC = 69.33%, (Sensitivity: 56%, Specificity: 83%, Positive predictive value: 0.76 and Negative predictive value: 0.65). (Fig. 3 (e) and (f)). The distribution of the fold changes of these three miRNAs in the three study groups (HEC, LTB and ATB) were also shown on scatterplots (Fig. 4).

4. Discussion

The quest for TB diagnostic biomarkers is primordial considering the difficulties faced with the currently existing diagnostic tests for TB. Developing a more efficient diagnostic test will be a great step towards the accomplishment of the sustainable development goals which is to end TB by 2030 [2]. One of the promising fields for TB diagnosis is miRNA expression profiling. Infection with TB generally leads to many changes in the host miRNA expression profile and these changes could be exploited for the diagnosis of TB.

The results of our study showed that *miR-155-3p*, *miR-576-3p*, and *miR-889-5p* were not expressed in any of the subgroups in our population. Wu et al., 2012 [15] and Qi et al., 2012 [16] had respectively showed that, *miR-155-3p* was up-regulated in PBMC while *miR-576-3p* and *miR-889-5p* were up-regulated in the serum. This difference in observation with *miR-155-3p* may be due to differences in the methods used (activated PBMC versus inactivated plasma); while the non-expression of *miR-576-3p* and *miR-889-5p* may be attributed to difference in ethnicity [17]. Plasma was chosen for this study because it is easy to collect compared to PBMC which requires further steps for separation. Plasma *miR-29a-3p*, *miR-155-5p*, *miR-361-5p*, and *miR-196b-5p* were shown in our study to be upregulated in ATB and LTB compared to HEC although the difference was not significant as was found in previous studies by Fu et al., 2011 [6]; Wu et al., 2012 [15]; Draz et al., 2014 [7]; Zhang et al., 2014 [18]. These authors respectively showed that, *miR-29a-3p* (serum), *miR-155-5p* (PBMC), *miR-361-5p* (serum), and *miR-196b-5p* (serum) were significantly up-regulated in ATB compared with HEC. *MiR-144-5p* was slightly upregulated in LTB compared to HEC but slightly downregulated in ATB with respect to HEC. Liu et al. in 2011 [19] showed that *miR-144-5p*, a miRNA mainly expressed in T-cells was significantly overexpressed in the PBMC of ATB patients compared to HEC. The expression profiles of the eight miRNAs were not very different from those of TB patients co-infected with HIV. No combined effects were found among the five expressed miRNAs.

A sub-population with CD4 count between 400 and 699 cells/mm^3 was used to further the analyses. In this sub-population, *miR-29a-3p* and *miR-361-5p* were significantly over-expressed in ATB compared to LTB and HEC while *miR-155-5p* was significantly upregulated in ATB compared to HEC. The immune state of an individual can influence miRNA expression profile. Correlations of miRNA expression and CD4$^+$ T-cell counts have been reported [20] although we found no correlation between CD4$^+$ count and miRNA expression in our study population. The discrepancy in the results between the whole study population and the sub-population selected may be due to other immune related factors

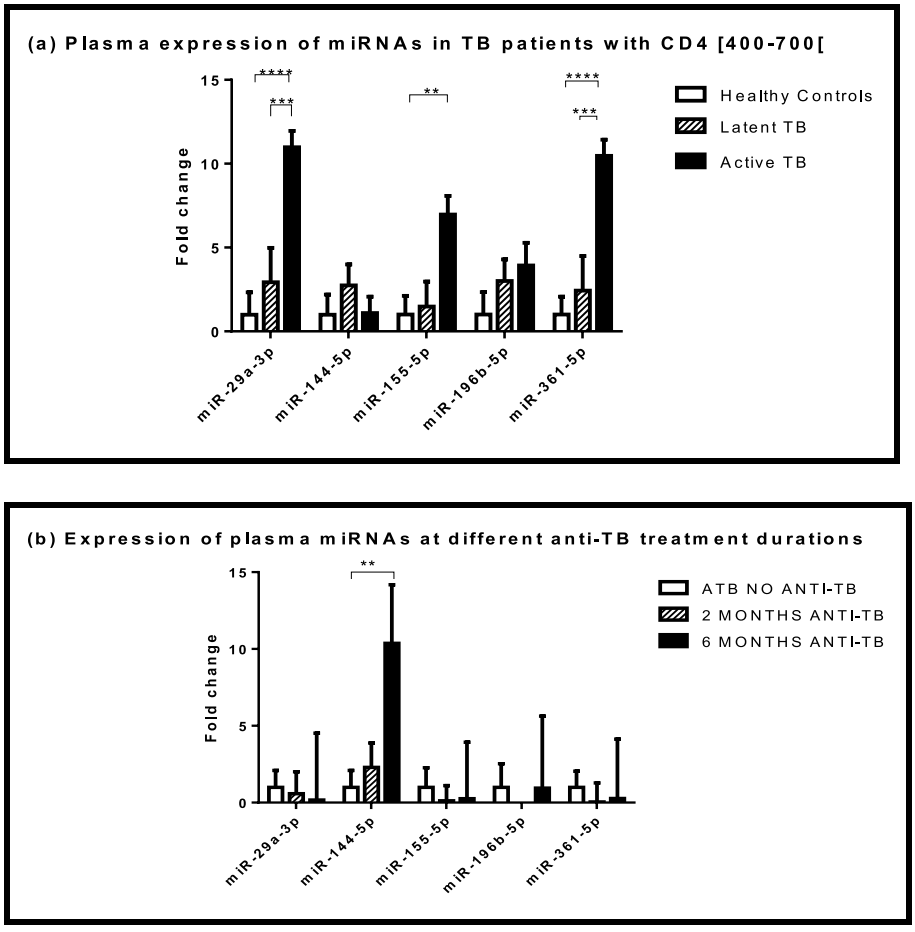

Fig. 2. Plasma expression of miRNAs (a) in TB subjects with CD4 counts between 400 and 699 cells/mm^3 (b) in active TB patients with CD4 counts between 400 and 699 cells/mm^3 at different duration of anti-TB therapy. Values expressed as Mean ± SEM and considered significant when P-value less than 0.05 at 95% CI.

other than the CD4$^+$ count. These results demonstrate the potential of *hsa-miR-29a-3p* and *hsa-miR-361-5p* not only to discriminate between ATB and HEC but also ATB from LTB. Likewise the potential of *miR-155-5p* to discriminate ATB from HEC. This finding is in agreement with that of Fu et al. (2011) [6], Wu et al. (2012) [15] and Draz et al. (2014) [7] who respectively reported that, *miR-29a-3p* (serum), *miR-155-5p* (PBMC) and *miR-361-5p* (serum) were significantly overexpressed in ATB patients compared to HEC.

The ability of *miR-29a-3p*, *miR-361-5p* and *miR-155-5p* was assessed to monitor anti-TB therapy. Their plasma levels were reduced from initiation of anti-TB therapy to completion of therapy with respective relative fold changes of 0.17, 0.26 and 0.27. This is an indication that plasma levels of these three miRNAs increase during TB infection but drop as TB is being treated. MiR-144-5p was significantly upregulated at completion of anti-TB therapy compared to initiation of treatment. The upregulation of miR-144-5p may indicate effective treatment success and disappearance of ATB from the system.

In this study, ROC curve analysis demonstrated that, *miR-29a-3p* could discriminate ATB from HEC with a good distinguishing performance (AUC = 81.37%). This observation is similar to that of Fu and colleagues, (2011) [6] who worked on a Chinese population and showed that, *miR-29a-3p* could discriminate ATB from HEC with a good diagnostic performance (AUC = 83%). On an Egyptian population, Draz and collaborators in 2014 [7] obtained an excellent distinguishing performance (AUC = 90%) of *miR-29a-3p* in discriminating TB infected patients from the healthy subjects. *MiR-155-5p* demonstrated a fair

Table 5
Plasma miRNA expression levels in subjects on anti-TB drugs with CD4 counts between 400 and 699 cells/mm^3.

MiRNAs	Fold Change compared to No Anti-TB group Mean ± SEM (N)			Analysis of Variance (P-values)		
	NO ANTI-TB	2 MONTHS ANTI-TB	6 MONTHS ANTI-TB	(No Anti-TB) versus (2 Months)	(No Anti-TB) versus 6 Months	(2 Months) versus (6 Months)
miR-155-3p	0	0	0	0	0	0
miR 576-3p	0	0	0	0	0	0
miR-889-5p	0	0	0	0	0	0
miR-29a-5p	1.00 ± 1.1 (20)	0.58 ± 1.43 (6)	0.17 ± 4.36 (3)	ns	ns	ns
miR-144-5p	1.00 ± 1.1 (18)	2.30 ± 1.59 (6)	10.37 ± 3.82 (3)	ns	**	ns
miR-155-5p	1.00 ± 1.3 (17)	0.11 ± 0.99 (5)	0.26 ± 3.67 (3)	ns	ns	ns
miR-196b-5p	1.00 ± 1.5 (09)	/	0.94 ± 4.69 (2)	ns	ns	ns
miR-361-5p	1.00 ± 1.0 (23)	0.04 ± 1.25 (7)	0.27 ± 3.86 (3)	ns	ns	ns

* Significant difference ($p \leq 0.05$), ns: non significant ($P > 0.05$).

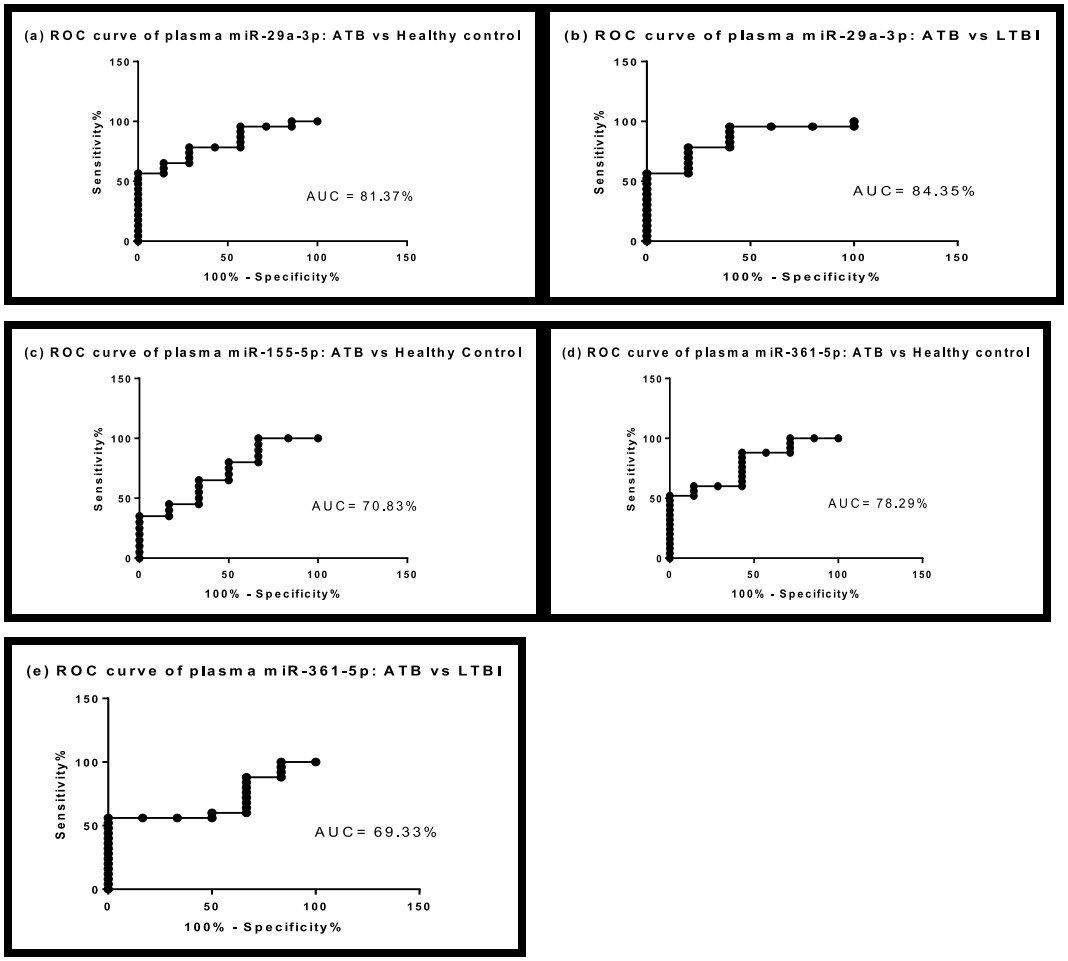

Fig. 3. ROC curves showing the diagnostic abilities of (a) miR-29a-3p to distinguish ATB from healthy controls (b) miR-29a-3p to distinguish ATB from LTBI (c) miR-155-5p to distinguish ATB from healthy controls (d) miR-361-5p to distinguish ATB from healthy controls (e) miR-361-5p to distinguish ATB from LTBI. AUC values of between 90 and 100% are considered excellent, 80–90% good, 70–80% fair, 60–70% poor, 50–60% bad classifiers. AUC below 50% indicate random values not capable of distinguishing between two groups (AUC = area under the curve, ROC = receiver operating characteristics, ATB = active tuberculosis, LTBI = latent tuberculosis infection).

diagnostic performance (AUC = 70.83%) in distinguishing ATB from HEC which was contrary to previous study by Wu et al. (2012) [15] who reported a good diagnostic performance (AUC = 89.72) for miR-155-5p. This discrepancy could be due to difference in the methods used (activated PBMC versus inactivated plasma). We observed that, *miR-361-5p* demonstrated a fair distinguishing performance (AUC = 78.29%) in discriminating active TB from healthy controls and this is similar to the results (AUC = 72%) reported by Draz et al. (2014) with this miRNA [7].

For the first time we reported the ability of plasma *miR-29a-3p*, and *miR-361-5p* to distinguish ATB from LTB. Other authors mentioned above worked only on ATB and HEC. We observed that, *miR-29a-3p* exhibited a good distinguishing efficiency (AUC = 84.35%) in discriminating active TB from latent TB. Meanwhile *miR-361-5p* was not good enough (AUC = 69.33%) to discriminate ATB from LTB. One of the main problems faced with currently existing TB diagnostic tests is their inability to distinguish ATB from LTB and also their inability to monitor anti-TB therapy. A previous study [7] had reported that, sera *miR-29a-3p* was significantly up-regulated in active TB compared to other bacterial infections, a report which supports the idea that, the up-regulation of sera *miR-29a-3p* could discriminate TB infection from other bacterial infections. Draz et al. (2014) [7] also reported that sera *miR-361-5p* levels in the TB infected group was not significantly different from that of other bacterial infections while Qi et al. (2012) [16] observed that *miR-361-5p* was significantly overexpressed in TB infected sera compared to other infection group mostly composed of viruses (*Bordetella pertussis*, Varicella-zoster virus, and enterovirus). These results show that *miR-361-5p* may be specific to TB when compared to viral diseases but not very specific when compared to other bacterial diseases. In the present study, it was also demonstrated that the expression profiles of the eight selected miRNAs in TB patients co-infected with HIV was similar to those of TB/HIV negative patients.

The limitation of this study includes the absence of an HIV infected/TB uninfected group which could have permitted us identify miRNAs that may serve as inhibitors or enhancers for progression to ATB in HIV patients.

5. Conclusion

Plasma *hsa miR-29a-3p* may be a useful biomarker for the diagnosis of TB as it can discriminate active TB from latent TB and healthy controls (with less or no immunosuppression). Being present in plasma is a good advantage in diagnosing pediatric and extra-pulmonary TB cases. This *miR-29a-3p* could also be used to monitor the efficacy of anti-TB treatment. Future studies are required to evaluate this biomarker on a larger population so as to set cut off value that could be applied in laboratory diagnosis.

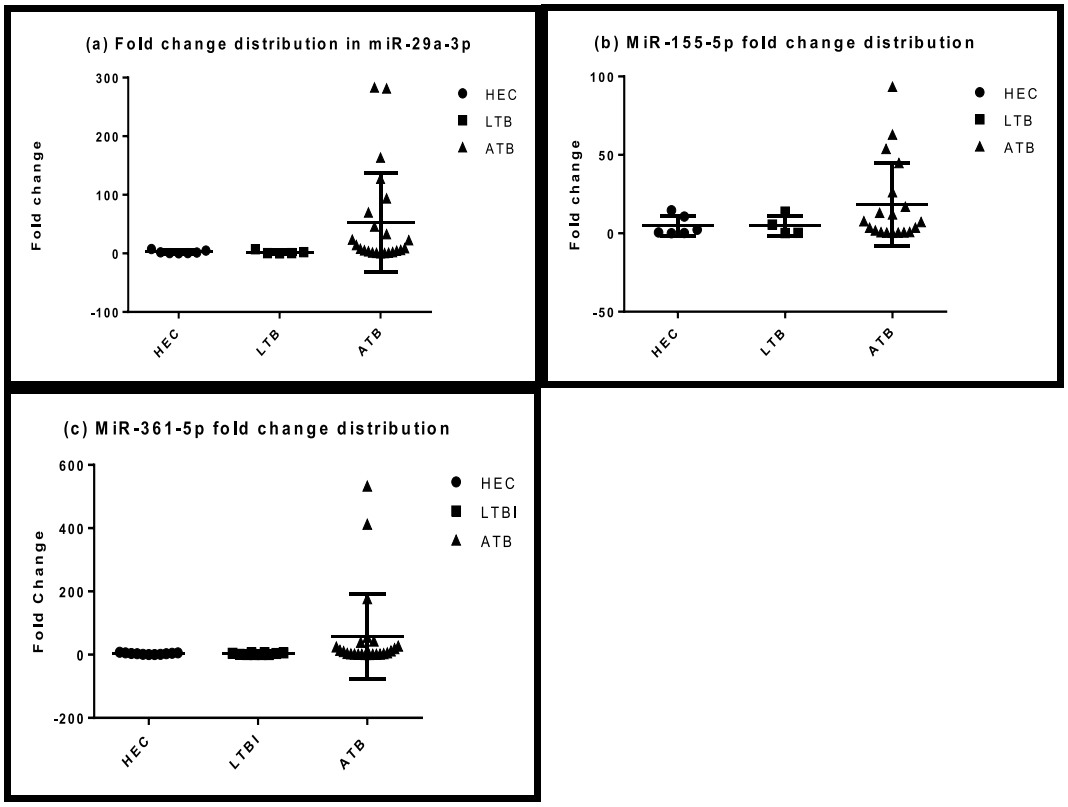

Fig. 4. Scatterplots showing the (a) MiR-29a-3p fold change distribution in HEC, LTB and ATB (b) MiR-155-5p fold change distribution in HEC, LTB and ATB (c) MiR-361-5p fold change distribution in the different study groups.

Conflicts of interest

The authors declare no conflict of interest regarding the publication of this work.

Author contribution

All authors contributed to the study conception and design, data acquisition, analysis and Interpretation, manuscript drafting, revision and approval for submission.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2018.12.001.

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