Utility of microRNA analysis in exhaled breath condensate of sarcoidosis and mediastinal tuberculosis patients: a pilot study

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miRNA signals in EBC may not be sufficiently discriminative to differentiate sarcoidosis and mediastinal TB. Further research is required to confirm the potential role of EBC-derived miRNAs to differentiate these two granulomatous respiratory disorders. https://bit.ly/44xYo6C

Cite this article as: Pattnaik B, PB S, Bhatraju N, *et al*. Utility of microRNA analysis in exhaled breath condensate of sarcoidosis and mediastinal tuberculosis patients: a pilot study. *ERJ Open Res* 2024; 10: 00078-2024 [DOI: 10.1183/23120541.00078-2024].

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Received: 23 Jan 2024
Accepted: 2 May 2024

Abstract

Background Sarcoidosis and tuberculosis (TB) are the two most common causes of granulomatous mediastinal lymphadenopathy. These often exhibit overlapping clinical and radiological characteristics, rendering accurate diagnosis difficult. MicroRNA (miRNA) analysis is increasingly utilised as a potential biomarker for various diseases. Exhaled breath condensate (EBC) is a noninvasive technique for biomarker evaluation in different respiratory conditions. We attempted to identify differentially expressed miRNAs in the EBC of sarcoidosis and mediastinal TB patients.

Methods EBC was obtained from subjects with a definitive diagnosis of sarcoidosis and mediastinal TB. EBC was also obtained from age- and sex-matched control subjects. From EBC, miRNA isolation, cDNA preparation and qPCR array were performed. Differentially expressed miRNAs were shortlisted. Further validation was conducted in the EBC of a new subset.

Results Subjects with a definitive diagnosis of sarcoidosis (50) and TB (50), and control subjects (50) were included. qPCR array from EBC (20 subjects from each group) shortlisted eight differentially expressed miRNAs (miR-126, miR-132, miR-139-3p, miR-139-5p, miR-181c, miR-454, miR-512-3p and miR-362-5p). In the validation set (EBC of 30 subjects from each group), miR-126 and miR-132 were differentially expressed significantly. The miR-126 and miR-132 expression ratio could differentiate sarcoidosis from mediastinal TB with an AUC of 0.618 (82% specificity and 41% sensitivity).

Conclusion While EBC miRNA expression is significantly and differently altered in sarcoidosis and mediastinal TB, a simple ratiometric approach failed to provide clinically useful signatures for differentiating between the two in patients with mediastinal lymphadenopathy.

Introduction

Sarcoidosis and tuberculosis (TB) are the most frequently encountered causes of granulomatous mediastinal lymphadenopathy, especially in TB endemic settings [1, 2]. Both conditions can present with isolated mediastinal lymphadenopathy, with similar clinical and radiological features, often leading to inaccurate diagnosis [3]. Sarcoidosis is diagnosed by demonstrating non-necrotising granulomas in the affected organs and compatible clinical and radiological features. A confident diagnosis of mediastinal TB is established by showing necrosis/necrotising granulomatous inflammation in the lymph node and supportive microbiological evidence (including Mycobacteria Growth Indicator Tube culture, Acid-Fast Bacillus staining or nucleic acid amplification tests) of *Mycobacterium tuberculosis* [4]. The GeneXpert MTB/RIF assay has a high sensitivity and specificity and is endorsed by the World Health Organisation (WHO) for the detection of TB [5]. Endobronchial ultrasound-guided transbronchial needle aspiration

(EBUS-TBNA) and conventional TBNA are helpful, minimally invasive diagnostic modalities to obtain cytological specimens from enlarged mediastinal lymph nodes [6, 7]. Endobronchial biopsy (EBB) of the bronchial mucosa and transbronchial lung biopsy (TBLB) add to the diagnostic yield of bronchoscopy for the diagnosis of sarcoidosis [8]. Biomarkers like serum angiotensin-converting enzyme (ACE) levels have poor sensitivity and specificity to diagnose sarcoidosis [9].

MicroRNAs (miRNAs) are small non-coding RNAs of 22 to 25 nucleotides in length that act through an RNA-induced silencing complex to post-transcriptionally regulate mRNAs that contain complementary sequences [10, 11]. These are enclosed in small secretory membrane vesicles, move out of the cells by exocytosis and help in the shuttling of miRNA between cells [12]. Highly stable circulating miRNAs can be found in biological fluids [13].

Novel and potential noninvasive techniques have recently been studied to assess respiratory diseases. One of the techniques involves exhaled breath condensate (EBC) collection [14], which is considered to be the representative signature of airway lining fluid [15]. The surge of interest in the study of EBC led to the formation of guidelines by a joint task force of the American Thoracic Society (ATS) and European Respiratory Society (ERS) in 2001 [16]. Previous studies have used EBC samples and blood plasma for molecular genetic analysis for early lung cancer diagnosis and showed promising results [17]. Other studies have identified various miRNAs in EBC samples collected from respiratory disease subjects [18]. A previous study from our group has reported the differential expression of exosome-enclosed miRNAs in EBC samples collected from asthma and TB subjects [19].

This pilot study aimed to assess differentially expressed miRNAs in EBC as potential biomarkers to differentiate sarcoidosis and mediastinal TB.

Study design and methods

Study population and design

This prospective study enrolled consecutive subjects with a suspected diagnosis of sarcoidosis and mediastinal TB. The EBUS-TBNA-centred approach was employed for a definitive pathological diagnosis. The study was carried out in a large tertiary care and referral facility in north India. The study was approved by the Institutional Ethical Committee (IECPG-235/23.08.2017). Informed consent was obtained from all the subjects.

Diagnosis of sarcoidosis and mediastinal TB

A definitive diagnosis of sarcoidosis was defined as consistent clinical and radiological findings, along with the demonstration of non-necrotising granulomas in tissue biopsy. A diagnosis of mediastinal TB was defined as the presence of a consistent clinico-radiological profile and microbiological evidence of *M. tuberculosis* infection, along with the presence of necrotising granulomas/necrosis in the tissue biopsy.

Age- and sex-matched subjects with no respiratory symptoms were recruited as control subjects. The sample groups were divided into two groups, including the initial development and validation groups. Both groups were divided into control subjects, sarcoidosis and mediastinal TB patients for EBC samples. In the initial development group, 20 subjects from each subgroup were included. In the validation set, 30 EBC samples were included.

EBC collection

As per recommendations, EBC samples were collected with the R-Tube (Respiratory Research, Austin, TX, USA) over a 10-min protocol [20]. As the subject breathes normally through this device, saliva is separated from the main exhalate stream in the blue “Tee” section of the mouthpiece and directed downwards with the assistance of gravity into the saliva drain. Hence there is minimal chance of salivary contamination. Further, the collected samples were centrifuged to concentrate. The samples were then stored at −80°C. Samples were collected from treatment-naïve patients.

miRNA isolation

miRNA was isolated using the miRvana small RNA extraction kit (Ambion; ThermoFisher Scientific, Waltham, MA, USA), per the manufacturer’s protocol. The isolated miRNA was quantitated with the Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific).

cDNA synthesis and qPCR

cDNA was prepared with 60 ng of RNA using the QuantiMir cDNA synthesis kit (System Biosciences, Palo Alto, CA, USA). Finally, 5 µL of cDNA was used as a template for real-time analysis by using the

miRNome real-time quantitative PCR setup (System Biosciences). The Light Cycler 480 II (Roche, Basel, Switzerland) was used for profiling. In brief, 5 µL of cDNA sample was mixed with SYBR Green I master mix (System Biosciences), and the miRNA profile was carried out in a pre-primer coated 384-well plate using the Light Cycler 480 II.

Validation of miRNome results in fresh EBC samples

The shortlisted miRNAs were further validated in new EBC samples collected from each group. miRNA was isolated using miRvana miRNA isolation kit (Ambion, ThermoFisher Scientific) from EBC samples from the 30 sarcoidosis and 30 mediastinal TB patients and 30 control subjects. cDNA followed by qPCR using KAPA FAST sybr Green (KapaBiosystems, Sigma-Aldrich, St Louis, MO, USA) was performed on those samples [21].

Data preprocessing and normalisation

A total of 905 miRNAs were measured for each sample. The Cq values >18 and ⩽35 were only included in the analysis. Only 121 miRNAs were expressed in at least 20% of the samples and were included in the calculation. The global mean normalisation method normalised the data [21, 22]. The −ΔCq values were calculated by subtracting each Cq value of the sample from the global mean of the Cq values of the same sample ($-\Delta Cq = -(Cq_{miRNA} - \text{global mean})$).

In the case of reverse transcription quantitative real-time PCR validation, the Cq values ⩽35 were included in the analysis. The Cq values were normalised using Cel-miR-39 reference control. The −ΔCq values were calculated as follows:

$$(-\Delta Cq = -(Cq_{miRNA} - Cq_{Cel-miR-39}))$$

Statistical analysis

The differences in the ΔCq values were compared using the t-test or Mann–Whitney U test followed by the Benjamini–Hochberg correction. An adjusted p-value <0.05 was used to reject the null hypothesis. The predictive power of the selected miRNAs was assessed using receiver operating characteristic (ROC) curves. All the analyses were done using R version 3.6.2.

The representative data are shown as means±SD or median (interquartile range) for quantitative data. For categorical data, n (%) are described.

Results

155 subjects with a clinico-radiologically suspected diagnosis of granulomatous mediastinal lymphadenopathy were recruited from the pulmonary medicine outpatient clinic. Of these, 50 patients were diagnosed with sarcoidosis and 50 with mediastinal TB. EBC from all the subjects were stored for further analysis. The remaining patients were diagnosed with other causes of mediastinal lymph node enlargement, including reactive lymphadenitis, and excluded. Out of patients diagnosed with sarcoidosis or mediastinal TB, 20 EBC samples from each group were used for initial exploratory analysis by miRNA qPCR array. 30 EBC samples from each group were used for validation by qPCR. 50 age- and sex-matched control subjects (for both the disease groups) were also recruited. Of these, 20 EBC samples were used in the qPCR array analysis, and 30 EBC samples were used for validation. The overall patient recruitment criteria are presented in figure 1. The diagnostic algorithm for sarcoidosis and mediastinal tuberculosis is presented in figure 2.

Demographic and clinical profile of the initial development group

Control subjects (20) and sarcoidosis and mediastinal TB subjects (20 each) were recruited. The baseline characteristics are presented in table 1. All the recruited subjects were followed up for 1 year.

Differentially expressed miRNAs between the groups

131 miRNAs were identified as differentially expressed in EBC of sarcoidosis, mediastinal TB and control subjects. 44 miRNAs were upregulated in sarcoidosis compared with control subjects (figure 3a). 46 miRNAs were upregulated and six were downregulated in mediastinal TB compared with control subjects (figure 3b). 15 miRNAs were downregulated, and 20 were upregulated in patients with sarcoidosis compared with mediastinal TB (figure 3c). A paired-wise differentially expressed miRNAs list is available in supplementary tables S1, S2 and S3.

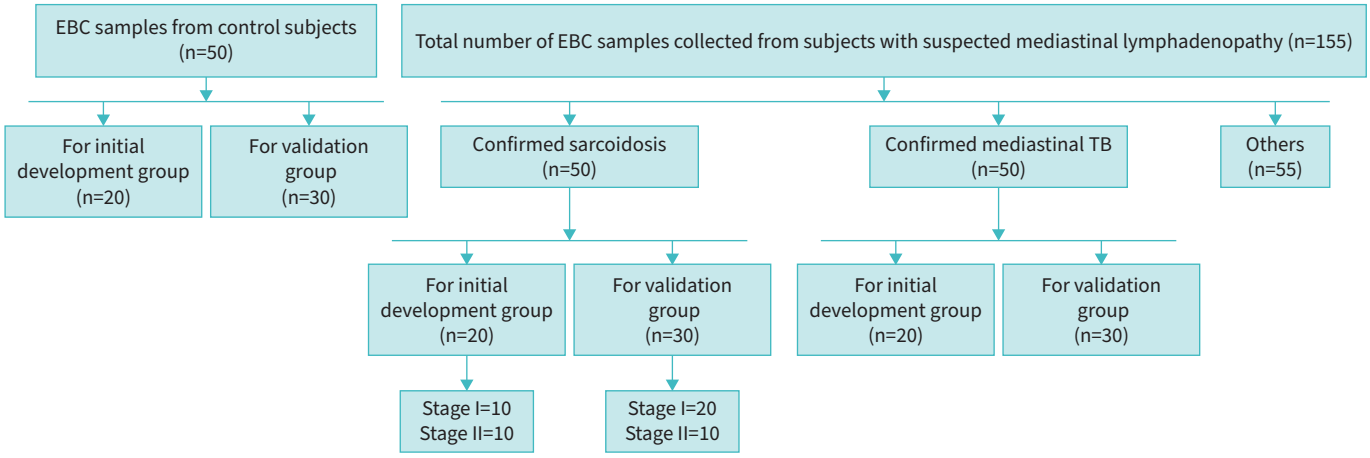

FIGURE 1 Overall patient recruitment criteria. A total of 155 suspected mediastinal lymphadenopathy patients were recruited and exhaled breath condensate (EBC) samples were collected, out of which 50 patients were confirmed with sarcoidosis and 50 patients were confirmed with mediastinal tuberculosis (TB) upon pathological examination with microbiological evidence. 55 patients were excluded as they were having reactive lymphadenitis or cancer.

Identification of candidate miRNAs for validation

The Venn diagram in figure 4 shows that 56 of 131 miRNAs overlap between these groups. 19 were identified as unique miRNAs differentially expressed in mediastinal TB compared with control subjects. The same 19 miRNAs were found to be differentiated in mediastinal TB from sarcoidosis subjects, of which the top four upregulated (including miR-126, miR-139-3p, miR-139-5p and miR-454) and three downregulated (miR-132, miR-512-3p and miR-362-5p) miRNAs were shortlisted (based on fold change) for further validation. Likewise, 11 miRNAs were identified to differentiate sarcoidosis from control subjects. The same 11 miRNAs were differentially expressed in the EBC of sarcoidosis subjects compared with mediastinal TB subjects. The top miRNA (miR-362-5p) was selected (based on fold change) for further validation (figure 4).

Demographic and clinical profile of recruited subjects in the validation group

30 subjects from each group (control subjects, sarcoidosis and mediastinal TB) were recruited. The baseline characteristics of recruited subjects are presented in table 2. All the mediastinal TB subjects were microbiologically confirmed subjects (table 2). All the recruited patients were followed up for 1 year.

Validation of candidate miRNAs in fresh EBC samples

Out of the eight shortlisted miRNAs, miR-126 was found to be upregulated, and miR-132 was downregulated in the EBC of mediastinal TB subjects in comparison to control subjects ($p<0.05$) (figure 5a, b). However, the differential expression of these two miRNAs was not significant in sarcoidosis subjects in comparison with controls. The ROC curve was plotted based on the miRNAs expression for the diagnosis of mediastinal TB. The area under the curve (AUC) of miR-126 expression was 0.714, the sensitivity was 80% and the specificity was 56% (95% CI 0.57–0.86) (figure 5c). The AUC of miR-132 expression was 0.944, the sensitivity was 90% and the specificity was 89% (95% CI 0.89–0.99) (figure 5d). Further, the ratio was calculated based on the miRNAs expression among different groups and the miR-126/miR-132 ratio could differentiate sarcoidosis and mediastinal TB from control subjects significantly (figure 5e). The AUC of the ratio was 0.877, with sensitivity and specificity of 48% and 96% (95% CI 0.77–0.98), respectively, in the comparison of mediastinal TB and control subjects (figure 5f). The AUC of the ratio was 0.795, with sensitivity and specificity of 78% and 75% (95% CI 0.67–0.92), respectively, in the comparison of sarcoidosis and control subjects (figure 5g).The AUC of the ratio was 0.618, with sensitivity and specificity of 41% and 82% (95% CI 0.46–0.78), respectively, in the comparison of sarcoidosis and mediastinal TB (figure 5h).

Discussion

This is the first study focused on identifying the differential expression of miRNAs in EBC from patients with sarcoidosis and mediastinal TB to discriminate commonly from selectively disregulated miRNAs associated with each pathological entity. Distinct miRNA expression profiles were identified and validated, involving 131 differentially expressed miRNAs in the EBC. Of these two miRNAs, mir-126 and mir-132

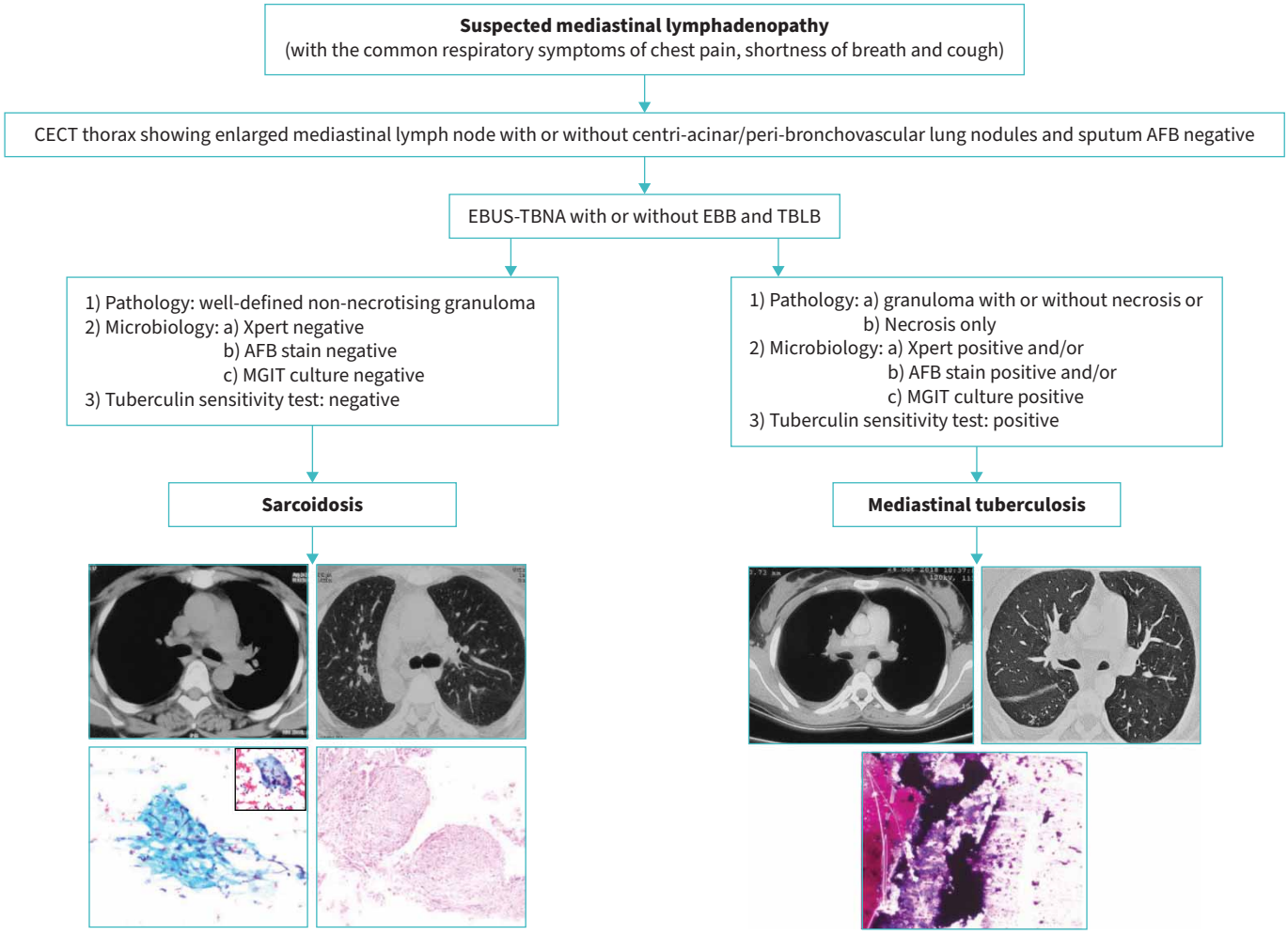

FIGURE 2 Diagnostic algorithm of mediastinal lymphadenopathy and representative cases of sarcoidosis and mediastinal tuberculosis. Representative cases from both sarcoidosis and mediastinal tuberculosis group as demonstrated in figure 1. In the case of sarcoidosis (Case A), chest computed tomography (CT) showed mediastinal lymphadenopathy with parenchymal involvement. Non-necrotising granuloma was demonstrated in endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) and transbronchial lung biopsy (TBLB) samples. An occasional multinucleated giant cell was demonstrated in the insert. In the case of mediastinal tuberculosis (Case B), chest CT showed mediastinal lymphadenopathy. Presence of necrotising granuloma demonstrated in EBUS-TBNA sample. AFB: acid-fast bacillus test; EBB: endobronchial biopsy; MGIT: Mycobacteria Growth Indicator Tube.

were identified as suitable for use as a ratio, with potentially useful discriminatory properties for mediastinal TB or sarcoidosis.

Since EBC is a noninvasive sampling method that represents the pulmonary airway niche but is variably diluted under different conditions, using two miRNAs as a ratio was considered to be a simple and robust approach. While the discrimination between mediastinal TB and sarcoidosis was suboptimal in our data, with a barely acceptable AUC this could be improved by incorporation of additional differentially expressed miRNAs *via* conventional or machine-learning based approaches. This is however likely to require larger sample sizes to prevent overfitting. We believe that this merits further exploration due to the reasons outlined below.

Overall, 131 miRNAs were differentially expressed in the EBC of two pathological groups of mediastinal lymphadenopathy (sarcoidosis and mediastinal TB) *versus* the control subjects group. Consistent with our findings, Zhao *et al.* [22] have reported 117 miRNAs identified in the EBUS-TBNA samples collected from the same disease groups. Interestingly, our top identified miRNAs, including miR-132, miR-139-3p, miR-139-5p, miR-362-5p and miR-512-3p in EBC samples, were also identified in this study. The

TABLE 1 Demographic characteristics of recruited subjects for initial development group

Parameters	Control subjects	Sarcoidosis	Mediastinal tuberculosis
Subjects, n	20	20 (SI=10, SII=10)	20
Age years, mean±SD	36.95±10.75	46.1±10.66	38.15±17.7
Male, n (%)	11 (55)	11 (55)	9 (45)
Smoker, n (%)	0	3 (15)	1 (5)
Mean node size mm, mean±SD		15.05±4.12	17.31±6.57
Serum ACE levels $\mu g \cdot L^{-1}$, median (IQR)		60 (24–191)	14.98 (15.5–80)
Comorbidities, n (%)			
Diabetes		2 (10)	1 (5)
Hypothyroidism		3 (15)	2 (10)
Hypertension		4 (20)	0
CAD		1 (5)	0
Granuloma, n (%)			
Non-necrotising		20 (100)	6 (30)
Necrotising			9 (45)
Necrosis only			5 (25)
Microbiology of mediastinal TB, n (%)			
Xpert MTB positive			14 (70)
MGIT culture positive			5 (25)
AFB stain positive			3 (15)

SI: stage I; SII: stage II; ACE: angiotensin-converting enzyme; CAD: coronary artery disease; TB: tuberculosis; MTB: *Mycobacterium tuberculosis*; MGIT: Mycobacteria Growth Indicator Tube; AFB: acid-fast bacillus.

consistency of these observations supports the potential role of EBC miRNAs in noninvasive evaluation of mediastinal lymphadenopathy.

In the sarcoidosis group, we randomly included patients from both stage I and stage II groups with pathologically confirmed sarcoidosis (presence of non-necrotising granulomas). In mediastinal TB, all the enrolled patients were pathologically and microbiologically confirmed. According to the previous reports, clinical manifestation of sarcoidosis could be a result of strong granulomatous response by mycobacteria infection [23]. Apart from this, there was presence of both necrotising (36%) and non-necrotising (43%) granuloma in the pathological specimen of mediastinal TB subjects, who all responded well to antituberculous treatment. It is thus possible that there may be molecular similarities between the two

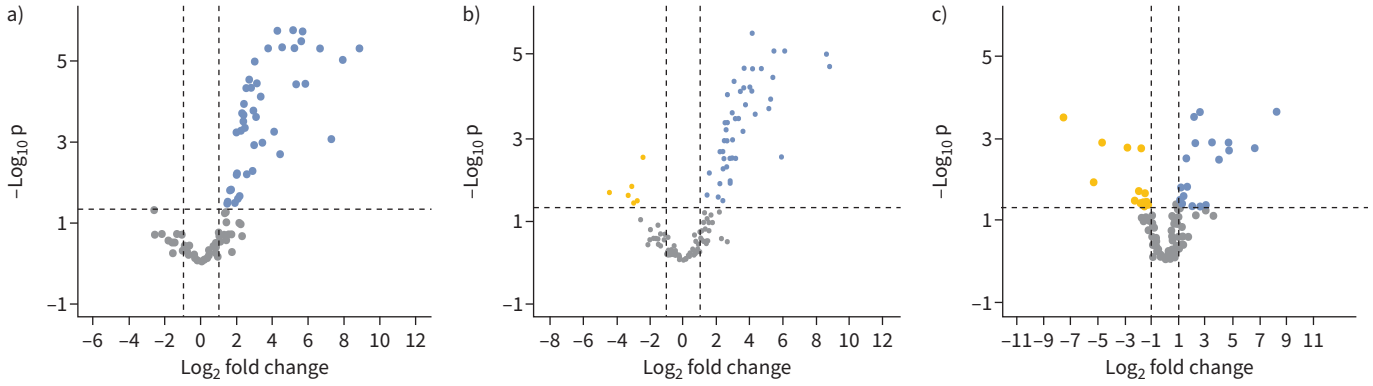

FIGURE 3 Volcano plots showing significantly altered microRNA (miRNAs) in exhaled breath condensate (EBC) samples of recruited subjects in three independent comparisons. a) In the first comparison, isolated miRNAs from EBC were compared between sarcoidosis and control subjects; 45 miRNAs were identified as downregulated in EBC samples collected from patients with sarcoidosis. b) In the second comparison, miRNAs were compared between mediastinal tuberculosis (TB) and control subjects; 46 miRNAs were found to be downregulated and 6 mRNAs were upregulated in mediastinal TB compared with control subjects. c) In the third comparison, isolated miRNAs were compared between sarcoidosis and mediastinal TB; 20 miRNAs were found to be upregulated and 15 miRNAs were downregulated in patients with sarcoidosis. The blue dots represent upregulated miRNAs, the yellow dots represent downregulated miRNAs and the grey dots represent non-significant miRNAs.

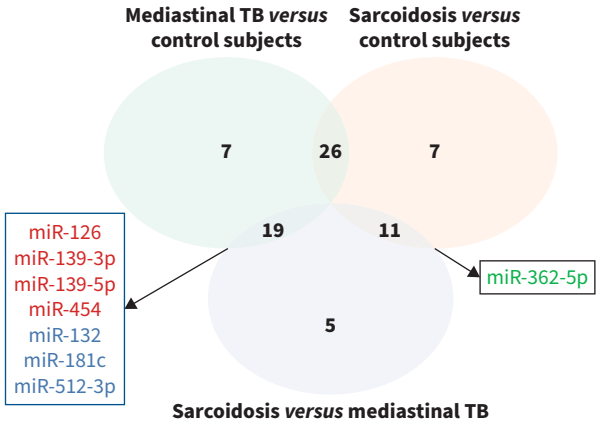

FIGURE 4 Venn diagram showing candidate microRNAs (miRNAs) identified in profiles. Identification of common miRNA signatures among three subject groups through a Venn diagram. Different colours represent the different subject groups. Seven miRNAs were differentially expressed in subjects with mediastinal tuberculosis (TB) in comparison with control subjects, which could compare with sarcoidosis subjects. Likewise, one miRNA was found to be differentially expressed in sarcoidosis in comparison with control subjects and also could compare with mediastinal TB.

groups that lead to overlaps in miRNA signatures and requires more complex approaches than a two-miRNA ratio.

The two selected miRNAs have been associated with many processes and are not specific to TB, sarcoidosis or granulomatous inflammation. miR-126 has been highly expressed in endothelial cells and has a significant role in angiogenesis [24]. It has reported roles in tumour development, metastasis and autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and cardiomyopathies [25]. Interestingly miR-126 was found to be downregulated in the peripheral blood of tuberculous meningitis patients [26]. miR-132 also plays a role in angiogenesis, neurological

TABLE 2 Demographic characteristics of recruited subjects for validation group

Parameters	Control subjects	Sarcoidosis	Mediastinal tuberculosis
Subjects, n	30	30	30
Age years, mean±SD	35.73±13.5	39.6±12.6	35±14.4
Male, n (%)	22 (73)	17 (56)	19 (63)
Smokers, n (%)	0	0	1 (3)
Mean node size mm, mean±SD		14.8±4.9	20.4±8.4
Serum ACE levels μg·L^{-1}, median (IQR)		44.18 (18.6–113)	40 (23–89)
Comorbidities, n (%)			
Diabetes		2 (6)	0
Hypertension		0	1 (3)
Radiological stage, n (%)			
Stage I		20 (66.7)	
Stage II		10 (33.3)	
Granuloma, n (%)			
Non-necrotising		30 (100)	13 (43.4)
Necrotising			11 (36.6)
Necrosis only			6 (20)
Microbiology of mediastinal TB, n (%)			
Xpert MTB positive			22 (73.3)
MGIT culture positive			5 (16.6)
AFB stain positive			6 (20)

ACE: angiotensin-converting enzyme; IQR: interquartile range; TB: tuberculosis; MTB: *Mycobacterium tuberculosis*; MGIT: Mycobacteria Growth Indicator Tube; AFB: acid-fast bacillus.

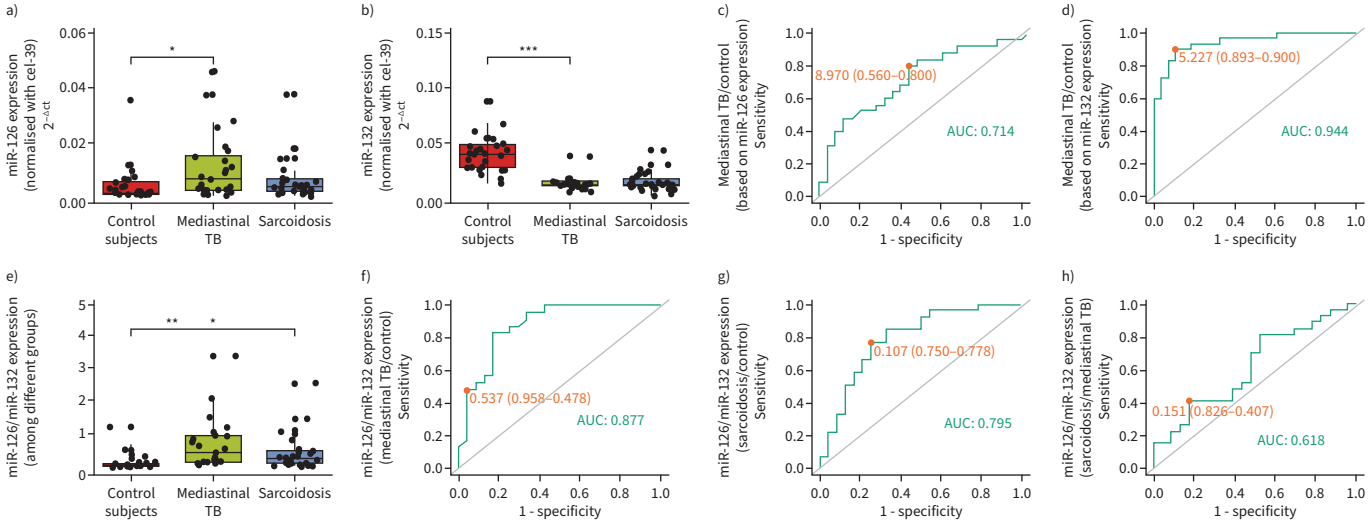

FIGURE 5 Validation of identified microRNAs (miRNAs) identified in exhaled breath condensate (EBC) samples collected from fresh subjects. Differentially expressed miRNAs were further confirmed by quantitative real-time PCR in fresh EBC samples of patients with sarcoidosis and mediastinal tuberculosis (TB) along with control subjects. Normalisation was performed with *cel-39*. a) The expression of miR-126 was significantly upregulated and b) the miR-132 expression was significantly downregulated in the EBC of mediastinal TB in comparison with control subjects. The data are presented as the mean±SEM. Significance was established with a p-value of <0.05. c) Receiver operating characteristic (ROC) curve of miR-126 in the prediction of mediastinal TB. d) ROC curve of miR-132 in the prediction of mediastinal TB. e) The ratio was obtained from miR-126 and miR-132 expression in different groups. f) ROC curves of selected miRNA ratios in the differential diagnosis of mediastinal TB. g) ROC curves of selected miRNA ratios in the differential diagnosis of sarcoidosis. h) ROC curves of selected miRNA ratios in the differential diagnosis of sarcoidosis from mediastinal TB. AUC: area under the curve.

development and inflammation [27]. Previously, miR-132 was elevated in *M. tuberculosis*-infected human macrophage cells [28].

The main disadvantage of employing EBC in miRNAs research is its unpredictable replication capabilities. Furthermore, EBC-derived miRNA levels may be close to or below the detection limit of the applicable technology. Thus, miRNA expression with enough sensitivity and specificity is required to adequately assess biomarkers in EBC. There is currently no defined procedure for assessing EBC dilution. miRNA concentrations in EBC may be influenced by various other confounding factors [29]. Aside from this, miRNA concentrations in EBC may be influenced by a variety of other confounding factors.

Conclusion

In this study, we attempted to perform miRNAs analysis in exhaled breath samples to discriminate sarcoidosis from mediastinal TB. Despite significant and reproducible differences in miRNA expression, we failed to discriminate these two diseases using a two-miRNA ratio based approach. Larger studies where more complex classifiers can be developed and tested are required for TB–sarcoidosis differentiation especially in TB endemic settings.

Provenance: Submitted article, peer reviewed.

Acknowledgements: The authors would like to thank all the subjects who have participated in this study. The authors would also like to thank Divyanjali Rai, Seetu Kashyap, Umashankar Verma, Mr. Rizwan and Neeraj Kumar for their technical assistance. The authors would like thank all the bronchoscopy staff for their technical assistance.

Ethics statement: The study was approved by the Institutional Ethical Committee (IECPG-235/23.08.2017).

Author contributions: B. Pattnaik, K. Madan, A. Agrawal and R. Guleria had full access to all of the data in the study, take responsibility for the integrity of the data and the accuracy of the data analysis, and wrote the manuscript. S. PB, N. Bhatraju and S. Mittal contributed substantially to the study design, data collection, data analysis and interpretation. S. Arava, D. Jain, B. Nayak, P. Tiwari, V. Hadda and A. Mohan contributed to the study design and interpretation.

Conflict of interest: The authors have no conflicts of interest.

Support statement: This work was supported by grants from the Indian Council of Medical Research (ICMR), India (I-1061), Council of Scientific and Industrial Research, India (MLP-1803), a Welcome Trust India Alliance Senior Fellowship (to A. Agrawal) and an ICMR Senior Research Fellowship (to B. Pattnaik). Funding information for this article has been deposited with the Crossref Funder Registry.

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