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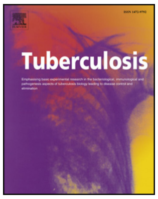

Review

Developing new TB biomarkers, are miRNA the answer?

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ABSTRACT

Efforts to reduce the global TB burden are hindered by the lack of simple, reliable non-sputum based diagnostics. To date studies investigating the biomarker potential of circulating host proteins and mRNA have not shown sufficient diagnostic utility. Recently, there has been increasing interest in circulating miRNA as a biomarker of TB disease. This review examined all published miRNA-TB biomarker studies to determine if a reproducible miRNA signature of TB disease could be elucidated. From 15 miRNA profiling studies, 894 miRNA differentially expressed between TB patients and healthy controls were identified in at least one study. Of these, 143 miRNA were validated by qPCR with 53 differentially expressed between TB patients and controls. Interestingly, only 8 of these miRNA were identified in 2 or more studies, and no consensus on a reproducible miRNA signature for identification of TB disease could be identified. TB disease is clearly associated with a wide breadth of differentially expressed miRNA. This review highlights our recent progress and the multiple factors, including environment, source of tissue, ethnicity and extent of TB disease that may influence miRNA expression. Coordinated efforts are required to validate identified targets in multiple populations to progress miRNA biomarker development.

1. Introduction

TB is primarily a respiratory disease, caused by *Mycobacterium tuberculosis*. TB remains a major global threat, responsible for over 1.6 million deaths in 2017 [1]. The World Health Organisation's (WHO) ‘End TB Strategy’ recognises that reducing the global TB burden requires the development of new tools to aid early disease diagnosis, prevention and therapy.

Accurate and early diagnosis is essential for TB control. Delay in diagnosis leads to spread of the infection within the community and hinders local and global disease control. Current TB diagnostics rely on quality sputum samples. This is difficult specifically in the elderly and young children. Expectoration creates aerosols, exposing health care workers, and promotes further disease transmission. Efforts to reliably detect *M. tuberculosis* from the circulation by culture or PCR have lacked sensitivity [2].

A hallmark of *M. tuberculosis* is its ability to enter a dormant, or latent phase within host cells, potentially for decades, known as latent TB infection (LTBI). One quarter of the world's population is estimated to have LTBI, with over 90% of those who contract the bacteria remaining asymptomatic with no active transmission [3]. Circulating antibodies and antigen specific T cell responses indicate TB infection but lack the capacity to differentiate active disease from latent infection or BCG vaccination.

2. An ideal biomarker

Biomarkers are biological indicators of a condition or disease. Host biomarkers are often used as a diagnostic tool when pathogen identification is not practical or possible. One example is the identification of type 2 diabetes based on circulating haemoglobin A1c levels [4]. A biomarker for the diagnosis of TB disease should be stable, easily measurable and specific. Circulating biomarkers are an attractive choice for diagnostic tests as sampling blood is minimally invasive, routinely performed and can be easily obtained from all individuals. Studies are also examining the utility of identifying biomarkers in saliva, pleural effusions and urine [5–8].

Suitable biomarkers must be robust enough to counter the factors which may influence biomarker expression such as age, sex, ethnicity and comorbidities. Studies have not identified a single host marker with the sensitivity or specificity required and a panel of markers or a biomarker signature is likely to be required. A robust biomarker or biomarker signature should be able to distinguish active TB disease from healthy individuals, latent infection and those with other respiratory pathological conditions.

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3. Current TB diagnostic biomarkers

Confirmed diagnosis of active TB currently relies on sputum microscopy or culture, and more recently on PCR GeneXpert®. Microscopy is relatively easy and cheap to perform, but lacks sensitivity, missing the diagnosis in over one third of patients seeking care [9]. This is in addition to the dangers of sputum microscopy as discussed above. Microscopy is also optimally effective with samples of advanced disease. Culture remains the gold standard for TB diagnosis, but due to the slow doubling time of *M. tuberculosis*, results can take up to 6 weeks [10]. Further many active TB patients, including children, cannot generate [11], or do not present with *M. tuberculosis* positive sputum [12]. GeneXpert® is a rapid PCR based diagnostic; it has the advantage of identifying *M. tuberculosis* DNA in ~2 h and will identify rifampicin resistant strains. Xpert still requires a sufficient sputum sample as well as equipment and expensive cartridges to analyse samples. Development of a new diagnostic test that can be performed in endemic settings with limited laboratory facilities, at low cost, using easily accessible non-sputum based samples such as blood, urine, or saliva, would improve TB diagnosis efficiency. Host biomarkers may be a suitable alternative for the development of this test.

4. miRNA as biomarkers

It is well established in many disease settings, that changes in host microRNA (miRNA) expression, have diagnostic potential. Clinical trials are currently underway assessing the biomarker potential of miRNA panels to diagnose different cancers [13,14], with the hope this technology could also be used for diagnosing infectious diseases.

miRNA are short, non-coding RNA molecules of approximately 22 nucleotides in length. miRNA are relatively stable, usually found within extracellular vesicles or bound to Argonaut proteins [15,16]. This stability is retained throughout freeze thawing and extended storage, indicating that miRNA may be excellent markers of clinical disease [17,18].

To date over 2000 human miRNA have been identified by *in silico* analysis though many of these remain to be experimentally confirmed [19]. miRNA modulate post-transcriptional gene regulation. miRNA can bind to mRNA with incomplete complementarity, meaning that the seed region of an individual miR may have multiple potential binding targets and modulate the function of multiple genes [20]. While the identity and functions of individual miRNA are still being elucidated, it is estimated that miRNA may regulate up to 60% of the protein-encoding genome [21].

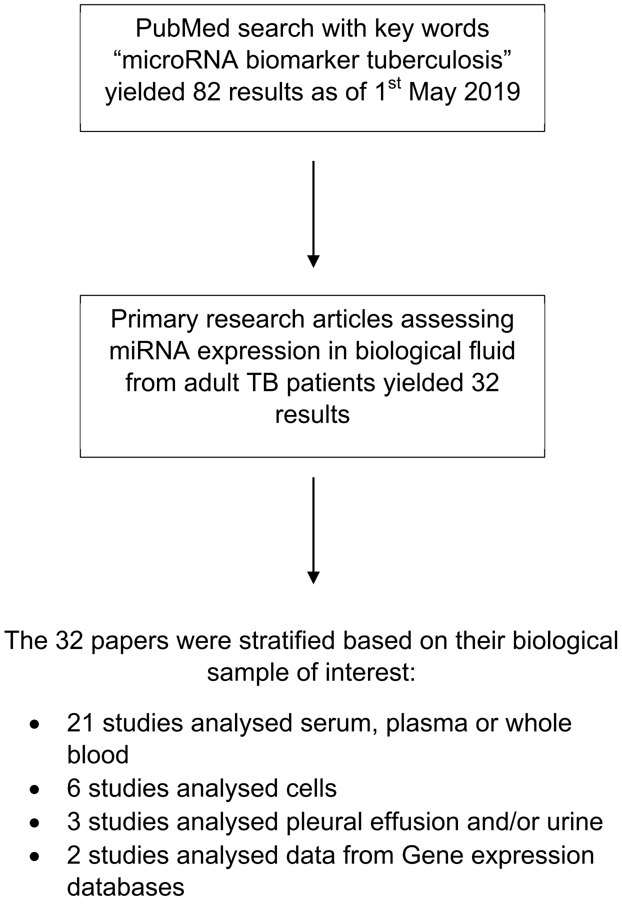
4.1. Current knowledge about miRNA as TB biomarkers

While TB disease can modulate the expression of multiple miRNA, the role of singular miRNA in TB pathogenesis is not well established. Publications to date have reported marked variability in the expression profile of individual miRNA. Whether this is due to the small sample size, the method of testing, variation in miRNA levels based on time of sample collection, extent of disease, age, sex, ethnicity, other comorbidities or environmental factors remains to be clarified. This review has focused on studies assessing miRNA expression within the circulation of active TB patients in comparison to healthy controls.

Initially, a PubMed search with key words "microRNA biomarker tuberculosis" yielded 82 results as of 1st May 2019. Of these, 21 primary studies assessed miRNA levels in biological fluids (Fig. 1), assessing miRNA expression in whole blood, plasma or serum, in adult TB patient cohorts.

4.2. TB biomarkers in circulation

A blood based biomarker is of interest for multiple reasons. Blood is routinely collected and relatively non-invasive to sample, making it an attractive diagnostic target. In comparison, collecting pleural effusion is highly invasive and the challenges of collecting sputum have already been mentioned. Similarly, isolating specific cell types from blood requires specialised knowledge and technical equipment and is unlikely to provide a rapid and simple diagnostic for healthcare workers to administer and read.



PubMed search with key words "microRNA biomarker tuberculosis" yielded 82 results as of 1^{st} May 2019

Primary research articles assessing miRNA expression in biological fluid from adult TB patients yielded 32 results

The 32 papers were stratified based on their biological sample of interest:

- 21 studies analysed serum, plasma or whole blood
- 6 studies analysed cells
- 3 studies analysed pleural effusion and/or urine
- 2 studies analysed data from Gene expression databases

Fig. 1. Flow diagram illustrating criteria used to select papers for review.

To examine the studies assessing circulating miRNA, they were stratified depending on the treatment status of the TB patient cohorts. There is evidence that anti-TB therapy may significantly alter miRNA expression profiles in the circulation, indeed this may be useful as a marker of treatment response [22,23]. Studies assessing samples from TB patients who had not started anti-TB therapy are summarised in Table 1 while, studies not specifying treatment status are summarised in Table 2.

The sample size, participant recruitment sites, study method and main results for 19 studies were reviewed. As the study by *Honeyborne* et al., did not include healthy controls as a cohort, it was excluded from further comparison [41]. Likewise, *Duffy* et al., analysed miRNA expression in household contacts of TB cases only to assess progression to disease, thus did not show direct miRNA expression comparison between TB patients and healthy controls [42].

Of the reported studies 15 out of 19 undertook an initial profiling investigation to assess miRNA expression in their cohorts, either by microarray or sequencing. This generated a list of differentially expressed miRNA with biomarker potential to distinguish TB patients from healthy controls (Supplementary Table 1 and Supplementary data file – http://bit.ly/Saunders_CytoScape). To date 894 miRs have been reported to be significantly regulated by TB infection. Of these, only 250 were reported in at least 2 studies and only 75 in 3 or more studies. miRNA that were identified by 3 or more studies during profiling were

Table 1
Studies assessing miRNA expression in pulmonary TB (pTB) patients, who were treatment naïve at sample collection, in comparison to healthy controls (HC).

Study (PMID)	Patient Cohorts	Method(s)	Significant Results (TB patients/HCs)	Participant Recruitment Site
miRNA biomarkers in Whole blood				
[24] Maertzdorf et al., 2012 (22547807)	8 pTB 8 HC	- *Agilent human 8 × 15-k miRNA microarray chips (measuring* 795 miRs – *719 human miRs)*	*Array (8 pTB and 8 HC)* 111 miRs upregulated 193 miRs downregulated	Berlin, Germany
[25] Latorre et al., 2015 (25657026)	17 pTB 16 HC	- *SurePrint G3 8 × 60 K miRNA microarray (measuring ~2000 miRs)* - *qPCR (9 miRs validated)*	*Array (9 pTB and 6 HC)* 8 miRs upregulated 13 miRs downregulated *qPCR (17 pTB and 16 HC)* 2 miRs upregulated 1 miR downregulated	Barcelona, Spain
miRNA biomarkers in Serum and plasma				
[26] Abd-El-Fattah et al., 2013 (23559272)	29 pTB 37 HC	- *qPCR (4 miRs measured)*	*qPCR (29 pTB and 37 HC)* 1 miR upregulated:	Cairo, Egypt
[27] Zhang et al., 2013 (24349033)	128 pTB 108 HC	- *Solexa Sequencing (*233 miRs *found in TB sample and* 253 miRs *found in HC sample)* - *qPCR (*15 miRs *validated)*	*Sequencing (20 pTB pooled and 20 HC pooled – 2 groups)* 10 miRs upregulated 5 miRs downregulated *qPCR (108 pTB and 88 HC)* 4 miRs upregulated 2 miRs downregulated	Zhejiang, China
[28] Xu et al., 2015 (25936536)	11 pTB 10 HC	- *TaqMan miRNA Array (measuring* 754 miRs*)*	*TLDA – (11 pTB and 10 HC)* 10 miRs upregulated 25 miRs downregulated	Shanghai, China
[29] Zhang et al., 2015 (26309574)	90 pTB 31 HC	- *qPCR (*1 miR *measured)*	*qPCR (90 pTB and 31 HC)* 1 miR downregulated	Xinxiang, China
[30] Wang et al., 2016 (26884721)	60 pTB 60 HC	- *Solexa Sequencing* - *qPCR (3 miRs validated)*	*Sequencing (10 drug-sensitive pTB and 10 HC)* 20 miRs upregulated 11 miRs downregulated *qPCR (60 pTB and 60 HC)* 1 miR upregulated	Shaoxing and Zhejiang, China
[23] Wagh et al., 2017 (28061948)	30 pTB 30 HC	- *qPCR (4 miRs measured)*	*qPCR (30 pTB and 30 HC)* 1 miR upregulated 1 miR downregulated	Navi Mumbai, India
[22] Barry et al., 2018 (29746939)	100 pTB 100 HC	- *Plasma Focus miRNA PCR Panels (Exiqon) (measuring* 175 miRs*)* - *qPCR (*10 miRs *validated)*	*Array (19 pTB and 14 HC)* 18 miRs up-regulated 69 miRs down-regulated *qPCR (100 pTB and 100 HC)* 2 miRs up-regulated 3 miRs down-regulated	Ningxia Hui Autonomous Region, China
[31] Wang et al., 2018 (29523324)	53 pTB 53 HC	- *Solexa Sequencing* - *qPCR (4 miRs validated)*	*Sequencing (10 pTB pooled and 10 HC pooled – 2 groups per cohort)* 66 miRs upregulated 34 miRs downregulated *qPCR (53 pTB and 53 HC)* 3 miRs upregulated	Zhejiang, Jiaxing and Shaoxing in China
[32] Ndzi et al., 2019 (30711160)	83 pTB 42 HC	- *qPRC (8 miRs measured)*	*qPCR (83pTB and 42 HC)* No significant results	Yaounde, Cameroon
[33] Hu et al., 2019 (30745169)	103 pTB 91 HC	- *Affymetrix Genechip Array (measuring* 2578 miRs*)* - *qPCR (11 miRs validated)*	*Array (7 pTB and 8 HC)* 98 miRs upregulated 4 miRs downregulated *qPCR (62 pTB and 91 HC)* 6 miRs upregulated	Chengdu, China
[34] Tu et al., 2019 (30898038)	108 pTB 86 HC	- *Solexa Sequencing* - *qPCR (19 miRs validated)*	*Sequencing (10 pTB and 10 HC)* 93 miRs upregulated 88 miRs downregulated *qPCR (60 pTB and 41 HC)* 4 miRs upregulated	Zhejiang, China

compiled into a network map, illustrating the relationships between studies and miRNA that were up or down regulated in TB patients compared to controls (Supplementary Fig. 1).

Following profiling, 12 of the 15 studies undertook qPCR validation of selected targets. Interestingly, most of these studies reported that only a fraction of the miRs selected remained significantly regulated in the larger validation studies (Tables 1 and 2). Indeed from a total of 143 miRs analysed by qPCR (Supplementary Table 2), only 53 were reported to be significantly regulated in TB patients compared to controls and only 8 miRNA (miR- 20b, 21–5p, 22–3p, 26a, 29a-5p, 29c-3p, 155 and 378a-3p) were identified in 2 or more studies (Fig. 2 and Supplementary Table 3).

There are a myriad of factors that may influence miRNA expression profiles in individuals. Differences may be due to ethnic or environmental factors, microbiome profiles, concurrent helminth infections, HIV status, extent of TB disease, age, sex or comorbidities such as diabetes. Further studies examining miRNA expression across the spectrum of potential cofactors are clearly required to elucidate how these factors influence expression profiles. What is evident so far is that a miRNA signature that could be considered a front runner for future

Table 2
Studies assessing miRNA expression in pulmonary TB (pTB) patients, whose treatment status is not reported, in comparison to healthy controls (HC).

Study (PMID)	Patient Cohorts	Method(s)	Significant Results (TB patients/HCs)	Participant Recruitment Site
miRNA biomarkers in Whole blood – **No studies**				
miRNA biomarkers in Serum and plasma				
[35] Fu et al., 2011 (21998423)	75 pTB 52 HC	- *miRCURY LNA array (Exiqon) (measuring* 1223 *miRs)* - *qPCR (*3 *miRs validated)*	*Array (75 pTB pooled and 52 HC pooled – 2 groups)* 59 miRs upregulated 33 miRs downregulated *qPCR (30 pTB and 30 HC)* 2 miRs upregulated 1 miR downregulated	Weifang, China
[36] Qi et al., 2012 (23272999)	30 pTB 65 HC	- *TLDA*[a] *(measuring* 667 *miRs)* - *qPCR (*10 *miRs validated)*	*TLDA (20 pTB pooled and 20 HC pooled – 2 groups)* 90 miRs upregulated 7 miRs downregulated *qPCR (30 pTB and 65 HC)* 7 miRs upregulated	Jiangsu Province, China
[37] Miotto et al., 2013 (24278252)	154 pTB 105 HC	- *TLDA*[a] *(measuring* 671 *miRs)*	*TLDA (28 pTB and 28 HC)* 13 miRs upregulated 3 miRs downregulated	Milano, Italy Pwani, Tanzania Kampala, Uganda
[38] Zhang et al., 2014 (24586438)	15 pTB 46 HC	- *Solexa Sequencing (*904 *miRs found)* - *qPCR (*4 *miRs validated)*	*Sequencing (15 pTB pooled and 46 HC pooled – 2 groups)* 44 miRs upregulated 65 miRs downregulated *qPCR (10 pTB pooled and 10 HC pooled – 2 groups)* 2 miRs upregulated	Beijing, China
[39] Xin et al., 2016 (27450007)	10 pTB 11 HC	- *Microarray (measuring 1887 human miRs)* - *qPCR (*11 *miRs validated)*	*Array (10 pTB and 11 HC)* 48 miRs upregulated 18 miRs downregulated *qPCR (10 pTB and 11 HC)* 2 miRs upregulated	Zhengzhou, China
[40] Cui et al., 2017 (28910318)	64 non-cavitary pTB 64 HC	- *Solexa Sequencing (*692 *miRs found)* - *qPCR (*27 *miRs validated)*	*Sequencing (25 pTB pooled and 31 HC pooled – 2 groups)* 17 miRs upregulated 10 miRs downregulated *qPCR (64 pTB and 64 HC)* 3 miRs upregulated 4 miRs downregulated	Harbin and Heilongjiang Province, China

[a] TLDA = TaqMan low density array.

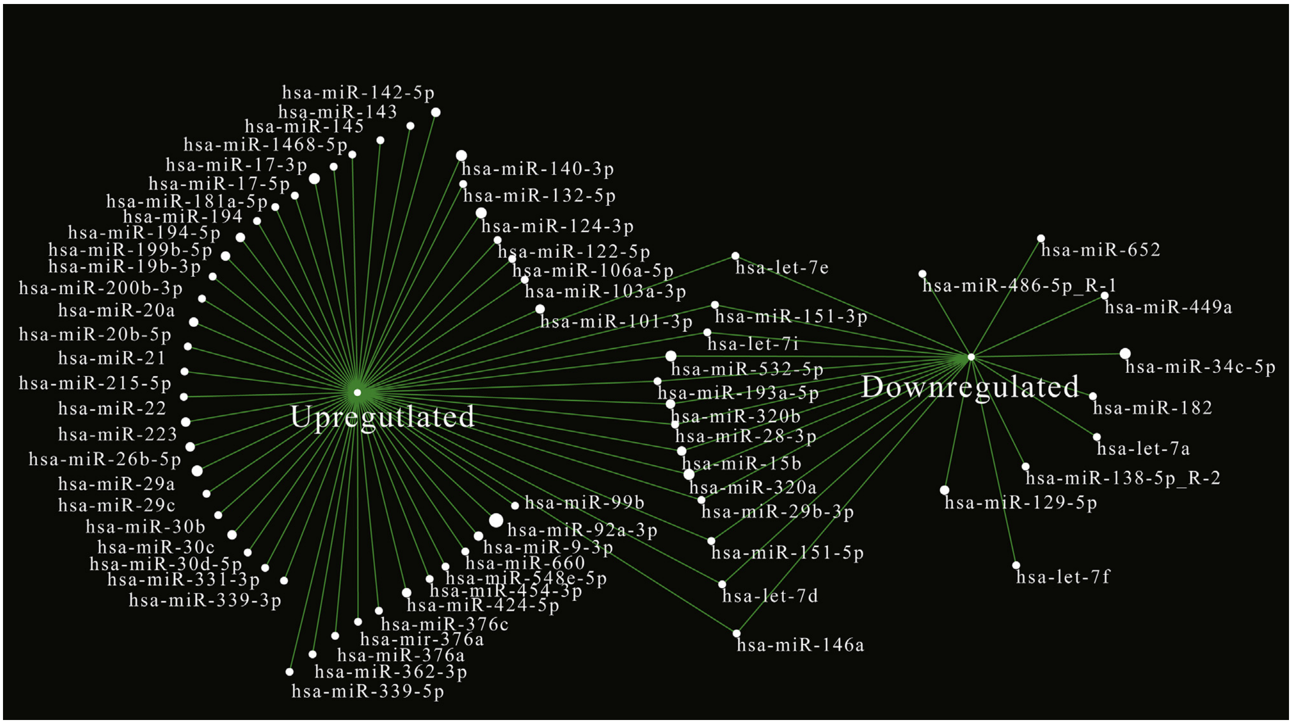


Fig. 2. Network map of miRNA that were significantly regulated in TB patients compared to controls, assessed by qPCR. The larger the dot, the more studies that identified this miR. Only miR-21 showed an overlap in up and down regulation between different studies.

diagnostic studies has not yet been clearly identified. While it may well exist within the reported studies, additional studies comparing the same miRNA profiles across different populations will be required to clarify this issue. From the data available it is evident that one miRNA is not sufficient to differentiate TB patients from healthy controls. Rather a miRNA signature is likely to be required for diagnosis, and this may have to be tailored to the local population.

4.3. Drawbacks for miRNA as TB biomarkers

The inconsistency between studies' miRNA expression results, often using similar methods and patient recruitment sites, is an issue for the advancement of a miRNA biomarker panel for identification of TB disease. Some of this variability may be attributed to the age of patient cohorts, differences in participant's ethnic and environmental background or stage of disease progression. One consideration is that different strains of *M. tuberculosis* may induce a different miRNA circulating signature. One *in vitro* study assessing miRNA expression in THP-1 cells infected with two different clinical isolates of *M. tuberculosis* identified 14 differentially regulated miRs [43]. How the different clinical *M. tuberculosis* strains influence systemic miRNA expression profiles needs to be further elucidated. *Wang* et al., assessed miRNA in the serum of drug sensitive (n = 60) and MDR TB patients (n = 42), and found differences in the expression of miR-4433–5p and 424–5p [30]. Future studies will need to assess if miRNA profiles are influenced by different *M. tuberculosis* strains.

Ideally, a miRNA biomarker profile needs to be robust enough to overcome these factors. While new miRNA biomarker candidates for TB diagnosis are plentiful, validation and clinical application of these markers beyond discovery is severely lacking. Without consistency of analytical and statistical methods, adequate sample sizes and potentially storage of samples, new biomarkers for TB disease will remain elusive.

5. Quantification of circulating miRNA

Microarray, sequencing and qPCR methods are generally utilised to quantify miRNA expression levels in biological samples. Many studies applying microarray analysis for biomarker discovery and qPCR for validation, found discordant results between the two methodologies (Tables 1 and 2). Differences in sample preparation and methodology should also be considered when comparing results between studies. 6 of these 15 studies pooled multiple patient's samples prior to RNA extraction, meaning any individual with a strongly unique miRNA profile may have skewed the resulting output. *Latorre* et al., found that validation of miRNA by qPCR gave discordant expression levels to their microarray results, which they attributed to the array detection thresholds [25]. If a simple point-of-care test for *M.tb* diagnosis is to be developed using miRNA, it is likely to require some type of amplification step to produce sufficient sample for reliable detection.

Additionally, the choice of reference genes for miRNA expression normalisation between biological samples, remains controversional. Our previous study, *Barry* et al., tested the suitability of 12 miRs that had been used in other studies to normalise miRNA in plasma or serum [44]. miR-93 was identified as the most suitable miRNA across two different ethnic populations, irrespective of disease status. Though another publication has reported miR-93 to have biomarker potential in distinguishing TB patients from controls [27]. Other studies have suggested miR-16 levels can be used as an endogenous control [45]. Others used RNU6 as a reference gene though more recent data has shown that RNU6 is not expressed in serum but in cells and its presence in serum or plasma samples is indicative of haemolysis of the samples [46]. Haemolysis contamination of serum or plasma samples may impact the expression of some circulating miRNA, particularly those associated with erythrocytes [47]. As haemolysis in samples may create inter-study variability in miRNA expression, it has been suggested that miRs associated with erythrocytes, such as miR-144 and miR-451, should be measured to determine the level of haemolysis in samples [48]. Further research is required to identify the most appropriate method to standardise miRNA levels in samples and account for any impact of haemolysis.

6. The potential of miRNA as a marker of treatment response

There is growing interest in developing new diagnostics to not only identify TB disease but also to monitor the response to therapy, to quickly identify patients who are not responding to treatment (often a sign of drug resistance, poor compliance, or incompatibility with medications). Recent clinical trials aimed at shortening therapy showed unacceptably higher recurrence rates in the 4 month versus 6 month treatment regimens [49–51]. If you reframe these findings, most patients required only 4 months of anti-TB therapy, or potentially less, to be able to overcome TB disease. If we could identify treatment markers that indicated patients who were responding to therapy and only required shorter course treatments for cure, we could save time, medical resources, reduce the potential side effects of long treatment times and hopefully increase patient compliance and cure rates. This would leave more resources to investigate and treat patients who may require additional therapy.

In these studies, patients would be their own controls, meaning changes in miR levels could be related directly to the individual. Our recent study identified that several miRs (miR-99b and miR-29a) changed significantly after the first month of therapy, and may be markers of treatment response [22]. Similarly, *Wagh* et al., showed that miR-16 and 155 returned to control levels in the serum of TB patients following 6 months treatment [23]. Whether these changes were apparent earlier during treatment remains to be determined.

7. Future directions for host biomarkers for TB

Development of novel point of care technologies for miRNA detection in a field setting as well as additional research to identify the optimal miRNA panel for the diagnosis of active TB disease and to monitor response to therapy are needed. The WHO has released target product profiles identifying the minimum criteria for non-sputum-based diagnostic tests for TB as having a minimum 90% sensitivity and 70% specificity. A recent review by Warsinske concluded that gene expression profiles have the diagnostic capacity to fulfil this criteria [52]. Further research and validation of miRNA profiles as TB diagnostic biomarkers will be required if miRNA diagnostic signatures are to be developed.

The introduction of bead-based fluorescent technology for measuring miRNA from small sample volumes has potential for developing a new TB diagnostic test [53]. Further investigation of the effect of comorbidities on circulating miRNA expression in TB disease and the influence of ethnicity and environmental factors will all be required to progress development of a miRNA biomarker signature for TB diagnosis.

New diagnostics are essential to achieve the WHO ‘End TB strategy’. A miRNA biomarker remains an attractive option towards achieving these goals.

Conflicts of interest

The authors report no conflicts of interest with regards to this publication.

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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.2019.101860.

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