SERUM microRNAs AS BIOMARKER FOR ACTIVE AND LATENT TUBERCULOSIS INFECTION IN IMMUNOCOMPETENT AND IMMUNODEFICIENT HOSTS.

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ABSTRACT

Background: Expression patterns of microRNAs in body fluids show potential to be used as noninvasive rapid and accurate biomarkers for various diseases.The study aimed to (i) identify patterns of microRNA signatures for diagnosis of tuberculosis (TB) and (ii) assess significance of a patient's genetic background on signature composition and diagnostic performance.

Patients and Methods: The study enrolled consented participants from Europe and Africa. Circulating miRNAs were measured and compared between patients belonging to the following categories; (i) active pulmonary tuberculosis (PTB), (ii) healthy individuals (H), (iii) active pulmonary TB co-infected with HIV (PTB/HIV), (iv) latent TB infection (LTBI) and (v) other pulmonary infection (OPI). As a first step, pooled sera of 10 participants from each category and region of enrolment were measured by TaqMan low-density arrays. Secondly, the identified significant miRNA signatures were applied to56 individual sera aiming to discriminate between H and PTB patients. Next, the identified miRNA signatures were analysed for their diagnostic performances using multivariate logistic analysis, and Relevance Vector Machine (RVM). The diagnostic performance of both models was evaluated by a leave-one-out-cross-validation (LOOCV).

Results: Significant miRNA signatures that discriminated patient categories were selected from the pooled samples. After validation of these in 56 individual participants (36 from the European cohort and 20 from the African population); a signature of 15 miRNAs was observed to be significantly differently expressed between categories, and able to differentiate healthy individuals and from individuals with PTB with a diagnostic accuracy of 82% (CI 70.2-90.0) in the RVM and 77% (CI 64.2-85.9) in the logistic classification model. The analysis based on genetic background identified a signature of 10 miRNAs that was specific for the European cohort with a diagnostic accuracy of 83% (CI 68.1-92.1) in RVM, and 81% (65.0-90.3) in the logistic model. Whereas a signature of 12 miRNAs was specific to the African cohort and the diagnostic accuracy increased up to 95% (CI 76.4-99.1) and 100% (83.9-100.0) in RVM and logistic model, respectively.

Conclusion: This proof-of-concept study showed that miRNA levels were significantly higher in patient with TB than in those without TB. miRNAs are a promising diagnostic candidate for TB, therefore further prospective evaluation of this diagnostic seems warranted.

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Abbreviations

AFB Acid-Fast Bacilli
AIC Akaike Information Criteria
AIDS Acquired Immune Deficiency Syndrome
ARV Antiretroviral Therapy
AUC Area under the curve
BCG BacilleCalmette-Guerin
BRTC Bagamoyo Research and Training Centre
CCL Chemokine Ligand
CD Cluster differentiation
cDNA Complementary Desoxyribonucleic Acid
CI Confidence Interval
CFP Culture filtrate protein
CIHLMU Center for International Health Ludwig-Maximilians-Universität
CNAs Circulating nucleic acids
Ct Cycle threshold
CXR Chest x-ray
DNA Desoxyribonucleic Acid
dNTPs deoxynucleotide triphosphates
ECSFP European community's seventh framework Programme
EDCTP European& Developing Countries Clinical Trials Partnership
EIA Enzyme Immunoassay
ELISA Enzyme-Linked Immunosorbent Assay
ELISPOT Enzyme-Linked Immunosorbent Spot
EPTB Extra-pulmonary tuberculosis
ESAT-6 Early-secreted antigenic target-6
GCP Good Clinical Practice
GLP Good Laboratory Practice
H Healthy
HAART Highly active antiretroviral therapy
HIV Human Immunodeficiency Virus
HLA-G Human Leucocytes Antigens-G
IATA International Air Transport Association
ICH International conference on Harmonization
IFN- γ Interferon-γ
IFN-g Interferon gamma
IGRA Interferon-γ Release Assay
IGRAs Interferon g release assays
IL Interleukin
LDA Low Density Array
LED Light-emitting diode
LOOCV Leave-one-out-cross-validation
LTBI Latent TB Infection
MDR-TB Multiple drug resistance tuberculosis

mRNA messenger Rebonucleic Acid
miRISC miRNA-induced silencing complex
miRNA Micro ribonucleic acid
MMRP Mbeya Medical Research Programme
MODS Microscopic observation for drug susceptibility testing
MRE miRNA recognition elements
Mtb/MTB Mycobaterium tuberculosis
NAAT Nucleic acid amplification test
ND NanoDrop
NIMR National Institute for Medical Research
NK Natural killer
NPV Negative predictive value
Nt Nucleotides
OPI Other pulmonary infections
PBMC Peripheral blood mononuclear cell
PCR Polymerase Chain Reaction
POC Point-of-care
PPD Purified Protein Derivates
PPV Positive predictive value
Pri-miRNA Primary miRNA transcripts
PTB Pulmonary Tuberculosis
RIF Rifampicin
RNA Ribonucleic Acid
ROC Receiver Operating Characteristic
RT Reverse transcription
RT-qPCR Real-time quantitative Polymerase chain reaction
RVM Relevance Vector Machine
SARC Sarcoidosis
SD Standard deviation
SNPs Single nucleotide polymorphisms
TB Tuberculosis
TLDA TaqMan Low density microRNA Array card
TNF-αR Tumor Necrotic Factor -αR
TST Tuberculin Skin Test
UTR Untranslated region
WHO World Health Organization
XDR-TB Extensively drug-resistant tuberculosis
Xpert GeneXpert

1. Background and Introduction

1.1 Introduction and Problem statement

Tuberculosis (TB) is a major public health challenge globally. The World Health Organisation (WHO) estimates about 8.6 million new cases of TB each year worldwide. Although effective drugs are available and control strategies are in place for decades, TB still kills an estimated 1.3 million people every year (WHO 2013). Generally, the peak incidence rate of TB tuberculosis was recorded in 2004 worldwide and since then TB has been declining at a rate of less than 1% per year. 22 high burden countries contributed 80% of the world burden of Tuberculosis globally, and in 2009 five countries (India, China, South Africa, Nigeria, and Indonesia) rank first to fifth in terms of total numbers of incident cases. However, HIV co-infected tuberculosis cases account for 12% (1.1 million cases) in the world and most of these are from Sub-Saharan Africa and southeast Asia, as indicated in figure 1 (WHO 2010a). HIV led to a three to five fold increase in tuberculosis incidence in sub-Saharan Africa and the most affected countries are from southern and eastern part of the continent (Lawn and Churchyard 2009; WHO 2008).

Despite the fact that one third of the world population is infected with TB, only 10–12% of infections progress to active TB (Corbett et al. 2006). People with Immunosuppressed condition, HIV/AIDS are at great risk of progressing from infection to active TB(Corbett 2003).The low income countries of Africa and South –East-Asia represent the highest number of the disease(WHO 2013). Weakness in the health systems cripples TB control strategies (Corbett et al. 2006), and in some settings a high burden of HIV/AIDS and other increasingly significant epidemiological factors contributes to the tuberculosis epidemic (Dye and Williams 2010). Emergence of multidrug resistance-TB (MDR-TB) and extensively drug resistant –TB (XDR-TB) makes TB control strategies more difficult.

For decades, sputum smear microscopy has been the main technique for detecting TB and monitoring treatment response in resource-constrained countries. However, microscopy is not a sensitive test, especially in people living with HIV and children and it doesn't provide information on viability, drug susceptibility of the bacilli and cannot differentiate between *Mycobacterium tuberculosis* complex and non-tuberculosis mycobacteria (WHO 2014). Culture is the gold standard tests and also used to detect resistance, as well as monitor response to treatment for drug-resistant TB (DR-TB) though the results take weeks to obtain (WHO 2014).

In 2013, 3 million TB cases were not diagnosed or diagnosed but not reported (WHO 2014). Detection of TB clinically or without proper investigation for drug resistance can lead to ineffective treatment, fuel transmission of drug-resistant strains, additional suffering and costs for patients. Given the higher burden of undiagnosed drug resistance TB, accurate and rapid diagnosis of drug resistance TB is critical and has the potential to improve control strategies and patient management in two ways. First, it would increase the number of diagnosed and treated TB patients and interrupt transmission. Secondly, it would decrease morbidity due to earlier diagnosis and treatment (WHO 2014).

The existing diagnostic tool for active TB are less sensitive in people living with HIV/AIDS and it is clearly known that, failure or delay to detect TB in HIV co-infected individuals early is lethal. Once Acid Fast Bacilli (AFB) are detected in sputum, the diagnosis of PTB is sensitive and specific but this is not the case in people with HIV where microscopy is insensitive and results in false negative results (Parry 1993). For the smear negative, chest X-ray is not the best option because the radiographic appearance may be atypical due to other infections. This diagnostic uncertainty results in a large number of patients being treated for TB without definitive diagnosis (Mendelson 2007). It is estimated that up to 20% of all TB patients who are on treatment in sub-Saharan Africa die within a year (Harries et al. 2001). Moreover two-thirds of these deaths may happen during the first 2 months of treatments, which indicates advanced state of the diseases at the time of diagnosis. Smear negative TB in HIV-co-infected individuals constitutes a slowly progressive disease entity with limited mortality, but these patients with often have poorer treatment outcomes and higher mortality rate as compared to their counterparts with TB smear-positive (Hargreaves et al. 2001).

Latent TB infection (LTBI), which is non-infectious and does not produce symptoms of active disease, can progress to active TB disease (Dye et al. 1999). Progression can be prevented by INH preventive therapy (WHO 2014) . Currently, there is no available diagnostic tool that can differentiate old from recent infection - the latter has a higher risk to progress to active TB. Tuberculin skin test (TST) which is more than 100 years old, is the most widely used tool. Even though interferon- gamma release assays (IGRAs) was more accurate than TST, (Menzies, Pai, and Comstock 2007) as its utility unfolded, the assumed superiority of IGRAs over TST particularly for high-TB-burden turned out to be untrue (Sharma, Mohanan, and Sharma 2012).

Development of accurate, robust and rapid diagnostic tools to detect active TB at point-of-care level, diagnose latent TB infection, predict disease progression, and screen for multi- and extensively drug-resistant TB, HIV associated TB and paediatric TB are among the priority aims for the Global Plan to stop TB 2011–2015 (WHO 2010b).

It is known that circulating nucleic acids (CNAs), including micro ribonucleic acids (miRNAs), are present in serum, as well as in other body fluids, and that these may serve as potential biomarkers of physiologic and pathologic status (Kim and Nam 2006). The aim of this study was to identify a pattern of miRNAs to be used as biomarker for diagnosis of active and latent TB infection in immunocompetent and immunosuppression individuals.

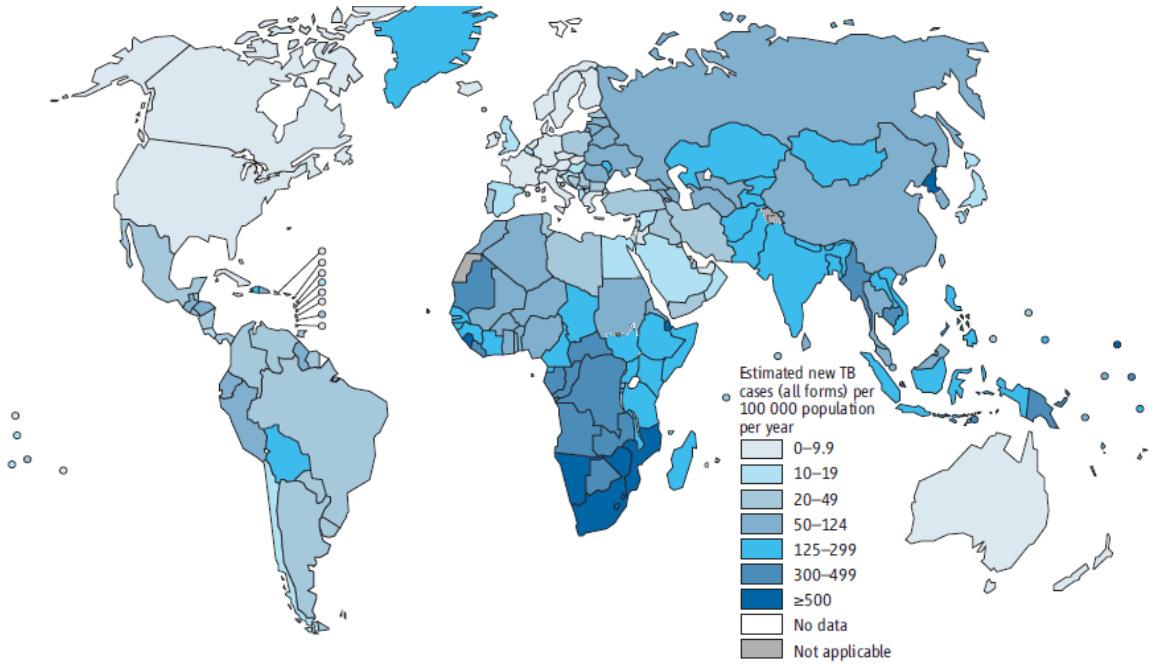


Figure 1;Tuberculosis incidence rate in 2012 (WHO 2013)

1.2 Literature review

Clinical symptoms presented by mycobacterium tuberculosis infection are non-specific which makes accurate classification of cases (latent asymptomatic infection, active pulmonary and/or extra-pulmonary disease) difficult (Amanatidou et al. 2012; Bernardo et al. 2011; McNerney and Daley 2011; Zumla and Yew 2011). Without improved diagnostic tools and effective approaches for their implementation, TB transmission cannot be interrupted and controlled in areas where HIV infection is prevalent. The good news is, opportunities for the creation of improved diagnostic tests are there (Perkins and Cunningham 2007). Developing and exploiting such tests to support TB control strategies especially in the HIV-infected populations is urgently needed. These tests should be accurate, simple to use and have a short time to result. An ideal test would also have an impact on both HIV-infected and HIV-uninfected populations (Perkins and Cunningham 2007), and supplement the existing conventional methods, microscopy and culture.

1.3 Diagnostics

Low sensitivity in detecting tuberculosis cases remains as the major hindrance in the control effort against TB (Lawn and Zumla 2011). Resource limited countries have high burden of the disease, estimated to be over 90% of the worldwide burden; the diagnosis in these countries still depends heavily on sputum smear microscopy which is insensitive especially in HIV/TB co-infection. In some cases chest radiology supplements the diagnosis (Lawn and Zumla 2011).These techniques perform poorly, and in most health care facilities in remote areas are

often or not performed when the patients visit the facilities at the first time due to the unavailability of supplies or trained personnel. The greatest diagnostic challenge is in childhood tuberculos is due to low bacillary load resulting in smear-negative samples, and in extra-pulmonary tuberculosis (EPTB) in adults. In this regards, there is an urgent need for rapid point-of-care tests that can be used at all levels of the health care facilities up to the community level (Lawn and Zumla 2011).

1.3.1 Review of currently available diagnostics

TB is endemic and the diagnosis is based on microscopic examination of sputum smear detecting acid-fast bacilli (AFB); the smear is prepared directly from the patient specimens (mostly sputum) (Foulds and O'Brien 1998; Perkins 2000). Unfortunately, the technique has several limitations. The sensitivity of this test is low in patients with EPTB or less than 10,000 bacilli per ml of sputum (Squire et al. 2005). Moreover, it requires two to three collections of sputum and laborious examination of many samples; consequently the delays of the whole process (ranging from 2 to 7 days) makes many people not return to the health facilities for their results (Squire et al. 2005).The delays or failure to diagnose the disease on time can be due to unskilled microscopist, improper functioning microscopes and inadequate reagents. Usually these challenges are commonly observed in the peripheral health facilities in developing world (Hawken et al. 2001; Martinez et al. 2005).

As the HIV-epidemic has taken root in the TB endemic countries, the performance of microscopy-based TB diagnosis worsened, because suppression of cellular immunity in TB patients co-infected with HIV results in less cavities, hence more smear-negative TB, but also more extrapulmonary TB (EPTB) (Perkins and Cunningham 2007).Culture is a more sensitive technique for detecting TB, and is defined as the gold standard. Recently, liquid culture has been approved as the reference standard for the bacteriological confirmation of TB. Its disadvantages are several weeks to yield results through slow growth of mycobacteria and requirement for advanced technical infrastructure. This technique is mostly available in the central laboratories which limit its accessibilities to the remote health care facilities where most patients are located.

Automated liquid media culture technique has been developed to supplement conventional solid media culture. ``These systems detect bacterial CO2 production or O2 consumption with radiometric, fluorescent, colorimetric, or pressure sensors that allow continuous monitoring, this obviate the need for mature colony formation, and roughly halve the time to detection, compared with Lowenstein-Jensen culture''(Gil-Setas et al. 2004; Scarparo et al. 2002) Running cost of liquid media culture machine is high due to the need for special culture vials, large and expensive incubator/readers and the need for backup culture on solid media. Thus, liquid culture is at best placed in the central laboratory level together with the drug resistance testing. Despite the sensitivity of the liquid culture there also potential disadvantages due to high risk of contamination, which commonly happens when the laboratories are not

experienced in liquid culture for TB, and lack of the possibility to examine colony morphology to discriminate between TB and non-TB mycobacteria (Perkins and Cunningham 2007).

Regardless of these challenge, there is advancement in the development of rapid diagnostic test based on molecular methods. In 2010, WHO endorsed the use of Xpert® MTB/RIF (Cepheid, Sunnyvale, CA, USA), a rapid molecular tests which can be used to simultaneously test TB and rifampicin resistance. The test has a much better sensitivity than smear microscopy and similar to solid culture (Steingart et al. 2013). Nevertheless, this test cannot be used at the peripheral health care centre but at more central levels of health systems (WHO 2013)

1.3.2 The need for more accurate and rapid diagnostics

Reduction of TB transmission depends on how quick active tuberculosis cases are being picked up and managed. Surprisingly, the detection rate remains at unacceptable low levels despite massive efforts put in place for tuberculosis control program. For example; it has been observed that only 60% of the estimated total tuberculosis caseload is detected in the WHO Africa Region, whereas the 40% remain undetected and continue to transmit *M. tuberculosis* (WHO 2011). Figure 2 illustrates that in some tuberculosis and tuberculosis/HIV-endemic countries, less than 4 of 10 cases are detected, with the huge number of HIV co-infected cases remaining undiagnosed.

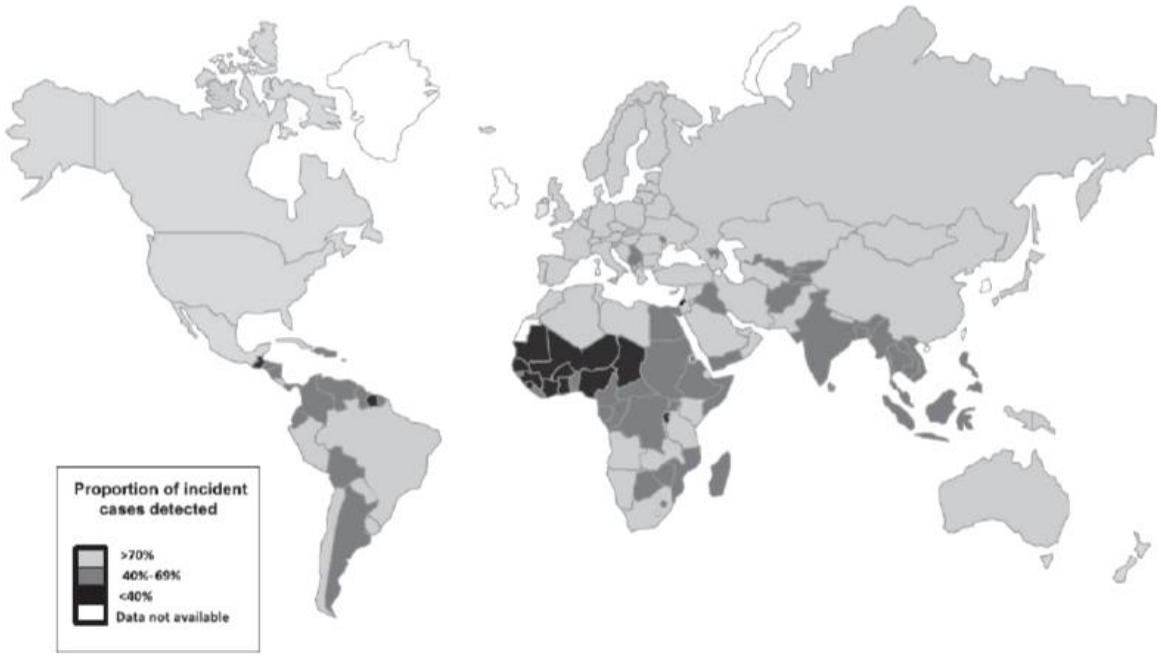


Figure 2; Estimated detection rate of global tuberculosis case (McNerney et al. 2012)

Generally, the limitations of current diagnostic methods for detecting tuberculosis or latent *M. tuberculosis* infection, especially in HIV-infected patients are still a roadblock to TB control in

resource-limited settings. Furthermore, the estimated 450,000 new MDR-TB patients each year are only detected after prolonged diagnostic delay in most cases (WHO 2013). Failure to diagnose drug resistance in time results in unsuitable treatment, poor prognosis and sometime end up in the premature death of the individual patient. It also worsens the situation by failure to interrupt circulation of resistant strains (Cuevas et al. 2012; Zumla et al. 2012).

In industrialized regions, radiography and other advanced imaging techniques, culture methods, and nucleic acid amplification tests (NAATs) are tests used to complement light and light-emitting diode (LED) microscopy for the diagnosis of active tuberculosis (Lawn and Zumla 2011). Combination of the tests during the detection of TB seems to lead to higher sensitivity which is further enhanced by the better sample collection technique like; induced sputum, or invasive techniques such as bronchoscopy lavage and tissue biopsies (WHO 2010c). Unfortunately, many of these improved technologies are not within reach of many of the world's tuberculosis cases (WHO 2010c). It is estimated that the availability of accurate widely used rapid diagnostic test for tuberculosis which will lead to initiation of correct treatment could prevent 625 000 tuberculosis deaths annually (Keeler et al. 2006).

1.3.4 Characteristics of an ideal diagnostic test

The capacity of the test to differentiate between tuberculosis disease and latent *M. tuberculosis* infection is critical.

The ideal tuberculosis test would be a point of care (POC) test capable of providing;

1. On-the-spot accurate diagnosis of tuberculosis infection, especially in problematic patient groups, e.g. HIV co-infected TB patients, patients with EPTB, and children.
2. It should be capable of detecting resistance to the first-line tuberculosis drugs to avoid treatment failure and prevent additional drug resistance (Weyer, Carai, and Nunn 2011).
3. The test should be able to detect the disease independent of sputum, rather in other sample (s) that is easier and less invasive during collection.

In addition, an ideal test should also be useful in screening all HIV-infected persons for latent *M. tuberculosis* infection simply because HIV patients with latent *M. tuberculosis* infection are at high risk of progression to active tuberculosis in the absence of preventive therapy (WHO 2010b).

There are number of tuberculosis diagnostics tests that are in different development phases or evaluation field trials. Endorsement and implementation of these tests will greatly depend on credible data on test performance, acceptance into national tuberculosis programs; affordability for both the patient and health system. The quality and durability of the diagnostic devices, as well as the access to appropriate treatment following diagnosis will also be considered. Research and development of POC tests for tuberculosis has received increasing attention in recent years. ''However, there is still lack of focused, strategic approach and insufficient integration between areas of biological discovery, test development and the establishment of well-characterized specimen repositories for initial test evaluation'' (Marais et al. 2010; McNerney and Daley 2011).

1.3.5 Diagnosis of latent TB disease

Latent TB disease (LTB) is the infection caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), the organism that causes TB, without any clinical symptoms of active disease. It is estimated that, one third of the world's population is infected with *M. tuberculosis* which is in the dormant state or (latent form) and most of those infected will never progress to the active form of tuberculosis (Dye et al. 1999),with an estimated lifetime risk of progression to active disease of about 10% (Corbett et al. 2003). Whereas, HIV positive persons who are infected with *M. tuberculosis* have a 5-8% annual risk and a lifetime risk of developing active tuberculosis that is three times higher compared to the immune-competent individuals (Selwyn et al. 1989) and this risk increases as immune deficiency worsens (Williams and Dye 2003).Impairing of the cell-mediated immunity in the HIV infected individuals, is the most strong known risk factor for the reactivation of latent *M. tuberculosis* infection (McShane 2005).

The ability to diagnose and use of INH preventive therapy in LTBI especially in people living with HIV depends on availability of accurate diagnostic tool to detect LTBI. The use of tuberculin skin test (TST) has been evaluated in a number of studies. Test positivity was associated with increased risk of active tuberculosis (Comstock, Livesay, and Woolpert 1974; Fine et al. 1999; Horsburgh, Jr. 2004; Menzies 1999). However, the sensitivity and specificity of TST especially in HIV infected individuals has shown some limitations (Menzies et al. 2007). Newer T cell interferon gamma release assays (IGRAs) appear to be more sensitive in detecting LTB following a recent tuberculosis exposure as compared to the TST (Lalvani and Millington 2007; Menzies et al. 2007; Pai et al. 2008). Though, data generated from different studies produced contradictory result on the predictive value of IGRAs for the risk of progression of LTBI into active TB, and just like other tests, sensitivity in HIV infected individuals remains low (Santin, Munoz, and Rigau 2012).

1.3.6 Diagnosing TB in HIV Co-infected Patients

Due to increased risk, screening for TB in all patients with HIV/AIDS has been endorsed by WHO and indicated in the guidelines although the diagnosis of TB in HIV/AIDS patients has proven to be difficult (WHO 2008).

First, *M. tuberculosis* is not the only pathogens that can cause lung infections in HIV infected individuals; rather there is a range of other microorganisms which can cause infections in the lung. Secondly, smear microscopy, the widely used technique in diagnosing pulmonary TB, has a lower sensitivity and more so in HIV infected individuals, since HIV/AIDS patients have a lower concentration of *M. tuberculosis* bacilli in their sputum. This is due to the poor formation of cavity in the lungs, or patient may not be able to produce sputum of good quality. Lastly, chest X-ray (CXR) results more often show normal findings, because TB produces less typical findings in HIV positive (Aderaye et al. 2007; Hawken et al. 1999; Zar et al. 2005). Difficulties in diagnosis of TB disease in HIV patients has resulted into more than half of TB cases being diagnosed late resulting in poor prognosis (Mendelson 2007). Limited access and low sensitivity of the diagnostic test cause substantial delay in diagnosis and patients in many resources-poor setting with a high TB burden do not receive a diagnosis for 3–6 months (Liam and Tang 1997; Madebo

and Lindtjorn 1999). This delay significantly contributes to disease transmission and severity of disease when it is finally discovered which usually end in poor prognosis of the infected patient. Although, smear-negative TB has conventionally been observed as a slower progressive disease with low mortality rate in HIV infected but patients with smear-negative disease many a times have poorer treatment outcomes and higher mortality rate than their counterparts with smear-positive TB (Friend et al. 2005; Hargreaves et al. 2001).

1.3.7 Diagnostic tests for active tuberculosis in children

In TB endemic countries, childhood tuberculosis accounts for 15%–20% of all cases (WHO 2011). The true burden of global childhood TB is not clearly known. In 2012, there were anestimated 530,000 cases and 74,000 deaths due to childhood TB (WHO 2013). The major challenges in childhood TB diagnosis are: (i) sputum recovery in young children is difficult and , it usually has low concentration of bacillary (Newton et al. 2008) and (ii) non-specificity of clinical symptoms associated with childhood TB, further makes clinical diagnosis to be very unreliable (Marais et al. 2005). Clinical scoring systems to aid diagnosis have not been validated and the diagnostic accuracy varies (Hatherill et al. 2010; Hesseling et al. 2012). Overtreatment of childhood TB is common and misdiagnosis contribute to the poor treatment outcome (Cuevas et al. 2012; Drobac et al. 2012). Furthermore, HIV infected children have an increased risk of severe and disseminating form of TB (Marais et al. 2005). To date there is no gold standard for childhood diagnosis and the existing diagnostic methods showed low sensitivity and specificity (Marais et al. 2006).Therefore, alternative, methods that will reliably detect active TB from TB negative are needed to improve clinical management.

1.4 BIOMARKERS.

1.4.1 Identifying accurate and novel biomarkers

A biomarker can be used to classify the normal physiological process against pathological changes in human or animals. Biomarkers act as an indicator for biological process, progression of pathogenesis and its response to the therapeutic intervention (Lawn and Zumla 2011; McNerney and Daley 2011; O'Grady et al. 2011; Wallis et al. 2010; Walzl et al. 2011). Monitoring a biomarker may provide useful information about the host- or pathogen in relation to the current health status, the pathogenic process and the risk of the patient to develop the disease in the future. TB- specific biomarkers could be used to categorize patients at a single time points into the following categories: active tuberculosis, latent *M. tuberculosis* infection, or no disease (McNerney and Daley 2011; Wallis et al. 2010). A biomarker as a single molecule in clinical practice is unlikely to perform better than using pattern of markers together (Walzl et al. 2011) .Biomarkers could in the future be used to detect LTBI, predict the risk of progression from LTBI to TB disease, monitor eradication programmes, and serve as a surrogate marker for cure in clinical trials following TB chemotherapy as illustrated in Figure 3.

Progress in developing and validation of TB specific biomarkers is moving at very slow pace. Further, one of the great challenge which need to be addressed during the development process is to ensure that the biomarker discovered is translated into a suitable point-of-care

test (Lawn and Zumla 2011; McNerney and Daley 2011; O'Grady et al. 2011; Wallis et al. 2010; Walzl et al. 2011). There are a number of ongoing studies which are trying to compare different gene expression profiles in patients infected with TB, LTBI as well as healthy individuals with no pre-exposure to *M. tuberculosis,* using multiplexed assays. These studies are measuring several targets with proteomics, transcriptomics, and metabolomics (Wallis et al. 2010; Walzl et al. 2011).

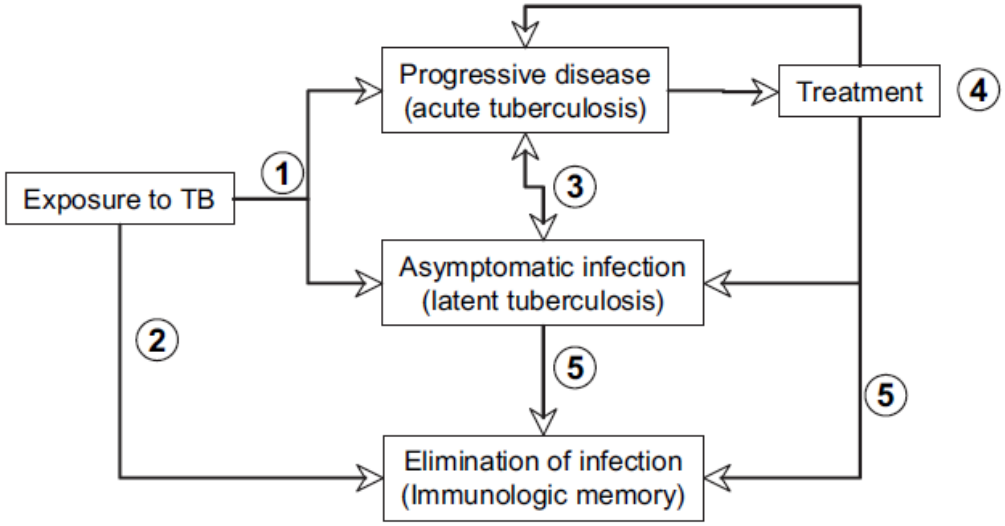


Figure 3;**The dynamics of host-pathogen interaction adapted from (Doherty, Wallis, and Zumla 2009).***This scheme indicates the different categories that are used to determine disease status*. *A double-ended arrow indicates the possible risk of the two conditions that are believed to be able to revert; while single-ended arrows indicate what is believed to be an irreversible condition in disease status. Circled numbers indicate some of the crucial questions about changes in status.*

Even though there is no breakthrough in developing new accurate, biomarkers which are specific to tuberculosis but we cannot turn the blind eye on the significant progress which has been achieved to date. Additional to diagnostic role, biomarkers have potential role in clinical care. For example, during TB drug trials, biomarkers might facilitate identification of early responders for whom shortened therapy might be appropriate (Balasubramanian et al. 1990; Hong Kong Chest Service/British Medical Research Council 1991). Also, a biomarker could detect risk of relapse which might then allow resources to be focused on those patients with high risk for poor treatment outcome. Similarly, biomarkers can indicate the risk of reactivation of latent tuberculosis infection in specific individuals, which might facilitate the targeted application of isoniazid preventive therapy in tuberculosis endemic regions (Gandhi et al. 2006). Occurrence of drug toxicity and protective immunity after vaccination can be detected by biomarkers as well; more advantages of biomarkers are detailed in Table 1.

Table 1; Predictive roles for Tuberculosis biomarkers, adapted from (Wallis et al. 2013).

Prediction of tuberculosis cure • Emergence of drug resistance • Recurrence due to relapse • Drug toxicity
Prediction of tuberculosis reactivation • Progression from primary infection to disease • Reactivation of latent infection • Eradication of latent infection
Prediction of protective immunity • Vaccine efficacy • Adjunctive immunotherapy efficacy • Recurrence due to reinfection

1.4.2 Properties of the ideal biomarker

Stable molecules which are abundantly present in the body fluids and easily recovered would make ideal biomarkers. It has been reported that circulating nucleic acids (CNAs) including miRNAs, found in the body fluids such as plasma, serum, urine, saliva and sputum (Park et al. 2009; Xie et al. 2010), could present a useful diagnostic biomarkers which is rapid, sensitive, accurate and stable for detection of different diseases (Kopreski, Benko, and Gocke 2001; Lo et al. 2007; Salani et al. 2007; Swarup and Rajeswari 2007; Wang et al. 2009).

1.4.3 miRNA - origin, processing and target selection

‘‘miRNAs are small non coding RNAs molecules regulating multiple biological processes by interfering with mRNA translation” (Hammond 2006). miRNAs are the largest gene defined as single-stranded RNAs ~22 nt in length (ranging 19–25 nt) generated from endogenous transcripts that can form local hairpin structures in silico (Ambroset al, 2003). ‘‘Primary miRNA (pri-miRNA) originate in the nucleus as a single transcript of about 1000 nucleotides long is processed by the RNase III enzyme Drosha’’(Lee et al. 2003). ‘‘The RNA binds protein DGCR8 into pre-cursor hairpin structures ~ 70-100nt long termed (pre-miRNA)”(Lee et al. 2003). . Transport to the cytoplasm is via Exportin 5 where the pre-miRNA is further processed by Dicer into a miRNA duplex consisting of the mature miRNA (Kim 2005). Transformation of mature miRNA into a miRNA-induced silencing complex (miRISC) is facilitated by Argonate protein, but importin 8 is involved in the transport to the target mRNA (Figure 4) (Weinmann et al. 2009). Single miRNA can exist in introns and exons of the host genes, while certain groups of miRNAs

are present in clusters in the genome, for example the miR-17-92 family. Each miRNA has an existing 2-8 nucleotide known as "seed region" thought to be critical for target selection (Liu 2008). Mature miRNAs use this seed region to bind selectively to miRNA recognition elements (MRE) within the 3' untranslated region (3'UTR) of target mRNAs. Different target genes may contain several MREs and therefore be regulated by numerous miRNAs.
Discovery of miRNA resulted into several theories related to the process involved in its regulation and expression. O'Connell et al describe three stages regaled to the function of miRNAs namely "(i) transcription, (ii) processing and (iii) subcellular localisation" (O'Connell et al. 2010). ''Stage (i) includes induction of miRNA expression by transcription factors in response to inflammatory stimuli and cellular stresses, stage (ii) impaired processing may be due to dicer inhibition (Wiesen and Tomasi 2009) or post-transcriptional modifications (Suzuki et al. 2009) and finally stage (iii) is where miRNA can localise to stress granules and p-bodies; a process which is poorly understood at this time.

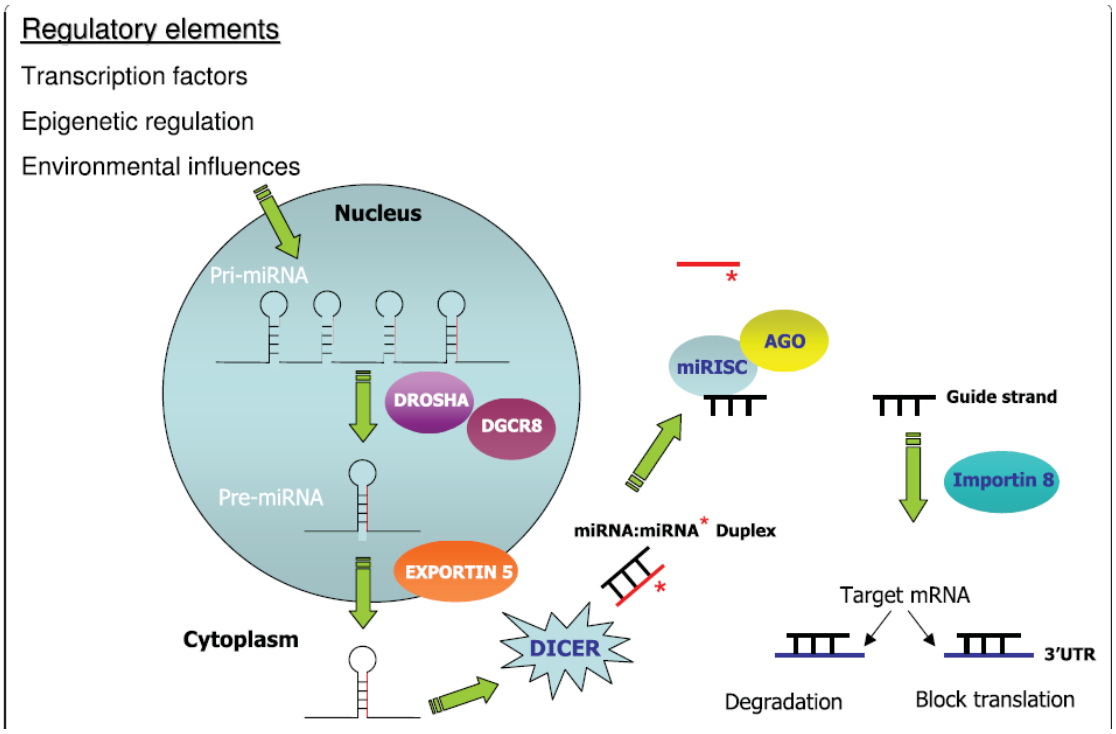


Figure 4; Representation of miRNA induction and biogenesis reproduced from (Oglesby, McElvaney, and Greene 2010).

1.4.4 Mechanism of miRNA action

Hundreds of targeted genes can be post-transcriptionally regulated with miRNAs, this process controls wide range of biological functions for instance cellular proliferation, differentiation, and apoptosis (Calin and Croce 2006). Mechanism of actions for miRNAs involves degradation of mRNA targets and translational inhibition with little or no influence on mRNA levels

(Brodersen and Voinnet 2009). For miRNAs to act on mRNAs, complementarity between the two is required (miRNAs and 3' untranslated region (UTR) of beset mRNAs). There are two mechanisms of miRNA action that are involved: (A) when miRNA is near-perfectly complementary with target mRNA, deadenylation and subsequent degradation of the target mRNA occurs (major mechanism of miRNA action); (B) when miRNA is only partially complementary to its target mRNA, translational inhibition occurs. miRNAs incorporated in the RISC (RNA-induced silencing complex) often recognize their targets — nucleotides 2–7 of miRNA (known as the 'seed region'(Brodersen and Voinnet 2009). ``Besides the complementarity between miRNA and mRNA, several other factors may influence the miRNA action such as impaired processing, methylation, gene polymorphisms, gene amplification, deletion of Dicer, translocations and others'' (Nana-Sinkam et al. 2009).

1.4.5 miRNA in pulmonary physiology and pathology

It has been documented that, miRNAs expression profile of mammalian lungs are very specific and highly conserved (Williams et al. 2007a; Williams et al. 2007b). Research on miRNA roles in pathophysiological conditions in lung compartment is limited and is mainly based on animal models. Differential miRNA expression has been observed in number of processes such as 1) lung development and homeostasis, 2) inflammation and viral infections and 3) deregulation which may contribute to several pulmonary diseases. miRNAs are also involved in the ongoing posttranscriptional regulation in the lung compartment (Tomankova, Petrek, and Kriegova 2010).

The understanding of miRNA expression patterns as potential biomarkers for diagnosis, prognosis, personalized therapy, and disease management is just starting to develop. Some miRNAs were stated to be related with some clinical outcome, such as chronic lymphocytic leukemia (Calin et al. 2005) adenocarcinoma of the lung (Takamizawa et al. 2004; Yanaihara et al. 2006), breast cancers (Iorio et al. 2005) and pancreas cancers (Bloomston et al. 2007; Roldo et al. 2006). Studies revealed that serum miRNAs correlate better to specific disease status as compared to those detected in the blood cells (Chen et al. 2008; Mitchell et al. 2008). miRNA expression levels seem to be stable and reproducible in the serum; which makes them potential markers for disease diagnosis (Agranoff et al. 2006; Jacobsen et al. 2007; Lesho et al. 2011; Maertzdorf et al. 2011; Mistry et al. 2007).

Table 2; Overview of the candidate biomarkers which emerged from studies on different genes, proteins or miRNA expression profiles.

Analysis	Candidate Biomarkers	Reference
Transcriptome	RIN3, LY6G6D, TEX264, MP68, SOCS3, KIAA2013, ASNA1, ATP5G1, NOLA3	*(Mistry et al. 2007)*
	FcγRIB	*(Maertzdorf et al. 2011)*
	127-probeset expression signature gave 100% accuracy in discriminating Healthy, PTB, LTBI and BCG vaccinated subjects	*(Lesho et al. 2011)*
	Lactoferrin, CD64, RAB33A	*(Jacobsen et al. 2007)*
Transcriptome (cell specific)	Neutrophil-driven transcript signature of IFNγand type IIFN signalling	*(Berry et al. 2010)*
Proteome	SAA, transthyretin,neopterin, CRP	*(Agranoff et al. 2006)*
Metabolome	Metabolomics patterns discriminate different mycobacterial species	*(Olivier and Loots 2012)*
Cellular miRNAs	28 miRNAs (among which miR-144*) up-regulated and 2 miRNAs downregulated in PTB	*(Liu et al. 2011)*
	17 miRNAs differentially expressed between Healthy, PTB and LTBI	*(Wang et al. 2011a)*
	*miR-155, miR-155**	*(Wu et al. 2012a)*
Circulating miRNAs	33 miRNAs up-regulated in serum from PTB Patients	*(Fu et al. 2011)*
Sputum miRNAs	95 miRNAs differentially expressed between PTB and healthy controls	*(Yi et al. 2012)*
	miR-29a	*(Fu et al. 2011)*

Yurong Fu et al. demonstrated that changes in miRNA expression can discriminate between active TB and healthy individuals. For example, circulating miR-29a could act as a biomarker for diagnosis of active pulmonary tuberculosis infection as it was reported by Ful et al (Fu et al. 2011). Wu and colleagues reported that miR-155 and miR-155* exhibited potential characteristic expression following cell stimulation with TB-specific antigen, suggesting that miRNAs can be suitable diagnostic markers (Wu et al. 2012b). Also, alterations in expression levels of miRNA could reflect and predict disease progression. For instance, some miRNAs may

control gene expression pathways that are important for the pathogenesis of TB and also involve in the transition process from latent TB disease to active TB (Wang et al. 2011a). Changes in miRNA expression levels might not only be detected in TB but also sarcoidosis (SARC), a granulomatous disease (Maertzdorf et al. 2012). The available published data suggested that miRNA could serve as a potential diagnostic marker for active TB disease.

Many non-specific markers of inflammation as single molecule have mediocre predictive value in clinical use, although a combination of the biomarkers highlighted in the table 2 above presents some potential markers that can help to determine or predict clinical cure, or risk of relapse or reactivation (Wallis et al. 2013). This study aimed at identifying patterns of miRNA signatures that can be used as a diagnostic biomarker for TB disease; which also suggest being more potent than a single marker.

We therefore started a study to investigate possible patterns of serum miRNA signatures associated with different TB statuses (PTB, EPTB, LTBI), non-pulmonary TB diseases (OPI) and multi-ethnic healthy controls, with the potential to differentiate between them. The results obtained from this study aimed to provide new knowledge which narrows the gap toward the development of POC test for TB. The focus was also on biomarkers that can discriminate the group of latent TB infected individuals, who usually act as reservoir of infection.

1.5 Purpose of the study

This study attempted to identify and describe specific patterns of miRNAs in serum, with the following aims:

I) Identification of a specific pattern of miRNAs in serum, which can be used as biomarker for active and latent TB in immunocompetent individuals.
II) Identification of a specific pattern of miRNAs in serum to be used as biomarker in with active and latent TB in immunodeficient patients: HIV
III) Identify similarities /differences of miRNAs expression based on differing genetic backgrounds in a European and African cohort.

1.6 Identification of the microRNAs

Accurate determination of miRNA expression level in a specific cell type or tissue is an essential parameter in describing the biological, pathological and clinical roles of miRNAs in healthy and diseased individuals.
Challenges in creating miRNA expression profile are:

i) mature miRNA are short (_22 nucleotides long);
ii) miRNAs are heterogeneous in their GC content, which results in a relatively large range of melting temperatures (Tm) of nucleic acid duplexes for the population of miRNAs;
iii) mature miRNAs lack a common sequence that would facilitate their selective purification Example., poly(A)
iv) the target sequence is present in the primary transcript (pri-miRNA) and the precursor (pre-miRNA), in addition to the mature miRNA (Benes and Castoldi 2010);

v) miRNAs within the same family may differ by a single nucleotide (e.g., Let-7 family)(Benes and Castoldi 2010) .

Accurate high-throughput profiling of miRNAs is a major challenge for the field. Several methodological approaches to profile mature miRNAs are available at present such as microarrays and bead-based flow cytometry, in a single experiment, but such approaches generally require significant amounts of input RNA (>1 mg) and preclude the use of very small specimen (Castoldi et al. 2006; Liu et al. 2004; Lu et al. 2005; Nelson et al. 2004; Sioud and Rosok 2004; Thomson et al. 2004)**.** In this study, total RNA was extracted from serum and an amount of RNAs as small as 2µl were used as template for reverse -transcription amplification.

2. METHODOLOGY

2.1 MATERIAL AND METHODS

Materials and methods used to evaluate miRNAs expression as a biomarker for diagnosis of TB have considerable influence on the results obtained. In this chapter, we present the method, and procedure, from enrolment, sample collection, and Processing. The results and their interpretations will be presented in the next chapter.

2.1.1 Acknowledgement

The results from the African population are the original work for this PhD. To increase the accuracy of the miRNAs as markers for diagnosis, and be able to evaluate the influence of genetic background, additional results from a European cohort were added by colleagues in Italy (Paolo Miotto and Ilaria C. Valente) and included in the final analysis as presented in this document. The statistical analysis described under section 2.3.1 to 2.3.4 was performed by (Paolo Miotto, Giovanni Sotgiu, Alessandro Ambrosi and Roberta Bosu), biostatisticians in Milan, Italy.

2.1.2. Study population and enrolment procedure

The study was conducted in according to the principles of the "Declaration of Helsinki" ,and followed Quality Assurance/Quality Control as indicated in protocol, study SOPs and according to the laws and regulations of the respective country; to ensure protection of the study participant and data quality. This study was nested in two studies; namely TB NEW (European cohort) and TB CHILD (African cohort), funded by ECSFP and EDTCP respectively. Inclusion and exclusion criteria of the study population are detailed below;

TB suspects:

Inclusion criteria

i. Signed written informed consent or witnessed oral consent in case of illiteracy before start of any study procedure
ii. Ages 18 years and above
iii. Persistent cough for ≥ 2 weeks and at least one of the following condition;
Haemoptysis, Chest pain, fever, night sweats, malaise, unexplained weight loss within the last 3 months, loss of appetite and contact with TB case.

Exclusion criteria

i. Tb treatment in the past year
ii. Severely ill TB suspect

Healthy individuals (Controls):

Inclusion criteria

i. Signed written informed consent or witnessed oral consent in case of illiteracy before start of any study procedure

ii. Ages 18 years and above
iii. Healthy individuals without any symptoms of active TB or any other diseases

Exclusion criteria

i. HIV infected individuals
ii. History of TB
iii. Sick with any other diseases.

Consented adult participants were enrolled from 4 sites: PTB, PTB/HIV and H were enrolled within the TB CHILD study at Ifakara Health Institute - Bagamoyo Research and Training Centre (BRTC), Pwani, Tanzania; the NIMR-Mbeya Medical Research Center (MMRC), Mbeya, Tanzania; Nsambya Hospital, Kampala, Uganda. Where at Infectious Diseases Departments and Internal Medicine Departments of Ospedale San Raffaele Fondazione- Centro San Raffaele , Milan, Italy, subjects of the categories; PTB, LTBI, H and a small population of patients with EPTB and OPI were enrolled within the TB NEW study. Sample collection from all sites was performed from September 2009 to July, 2012.

2.1.3 Study Procedures

TB suspect seeking medical help in the hospital close to the research site and meeting study criteria were enrolled. Recruitment of the patients was carried out in close collaboration with public hospital under National TB and Leprosy Programme (NTLP). After diagnosis, patients were transferred to the hospital for treatment. All results of established standard diagnostics tests were made available to assist treatment decisions. However, the results of the experimental diagnostic test did not influence clinical decisions.

All subjects included in the study underwent the following procedures:

- Symptomatic individuals.
- Asymptomatic participants were recruited and tested for latent TB infection using tuberculin skin test and/or IGRA (QuantFERON TB Gold) the potential study participants were excluded if they had a prior history of TB. In the few situations where TST and IGRA results were not available, personal and family history was collected to exclude people with potential risk of infection.
- Additional information related to the health status of all participants was collected using a questionnaire, which captured smoking, current medical problems (diabetes, transplant, silicosis, sarcoidosis, cancer), current therapies with particular focus on immunosuppressive, antiretroviral and anti-TB treatment (See questionnaire in Appendix 1).
- Peripheral venous blood was drawn from study participants, prior to the initiation of any anti-TB treatment for testing and storage.

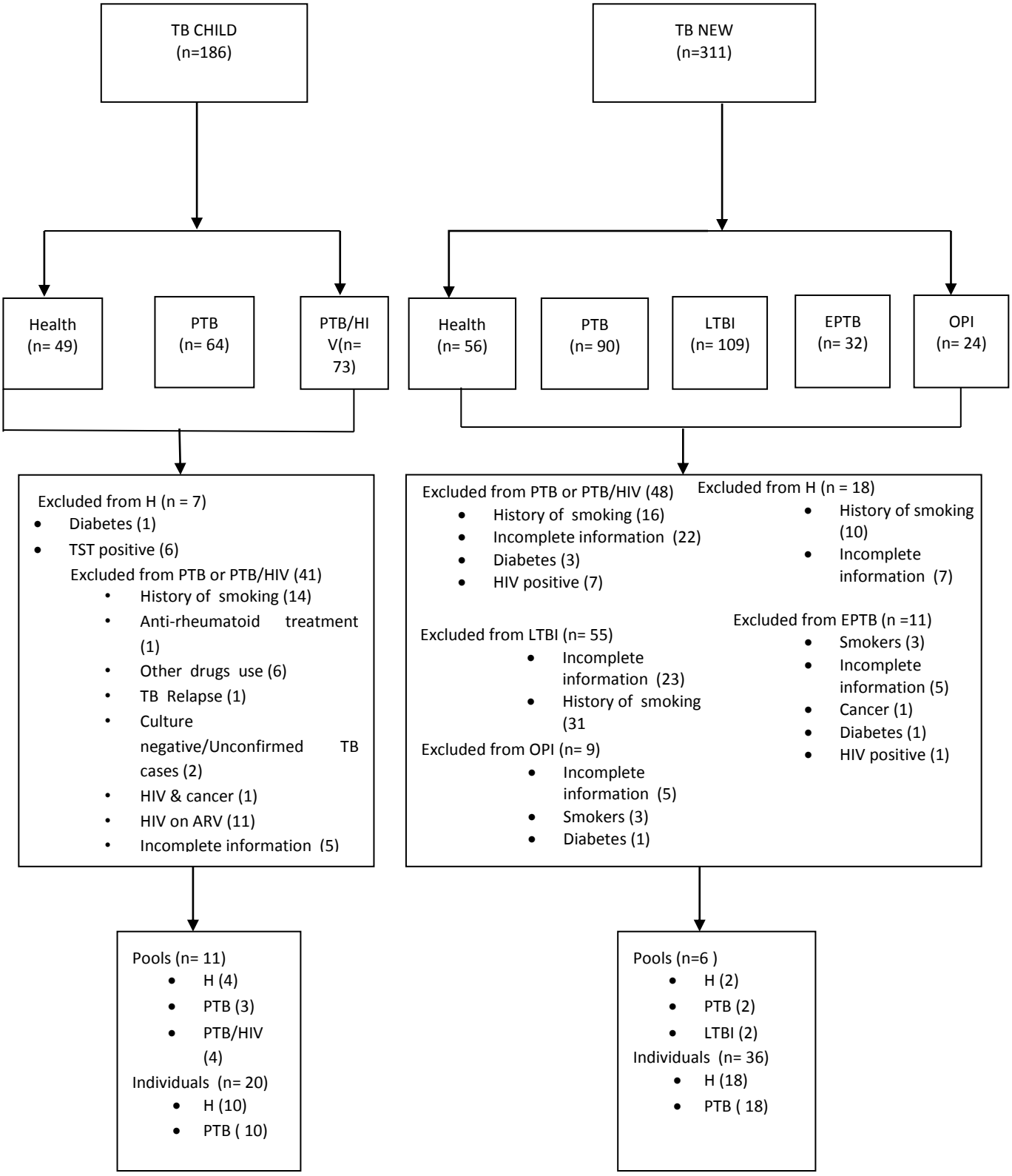
- All patients were tested for HIV, as diagnosed by the rapid diagnostics tests; Alere Determine ™ -HIV-1/2 (Alere Medical CO., Ltd, Japan) and Uni-Gold ™ HIV (Trinity Biotech Plc Bray, Ireland).

2.1.4 Selection of serum samples for analysis

Patients were classified into the following diagnostic categories based on clinical presentation, questionnaire (see Appendix 1 for details), chest radiography(X-ray), sputum smear microscopy for acid-fast bacilli, culture, Xpert MTB/RIF (GeneXpert System, Cepheid) assay (Boehme et al. 2010) as summarised in Table3. From these, serum samples for analysis were selected as detailed in flow diagram 1.

Table 3; Categorisation of patients

Patients category	Confirmatory diagnostic tests
Pulmonary active TB (PTB)	Smear microscopy positive for acid fast bacilli, solid media culture positive (Bactec™ MGIT, Becton Dickinson, Sparks, USA), GeneXpert MTB/RIF positive and HIV test negative
Pulmonary active TB co-infected with HIV (PTB/HIV)	Confirmation of PTB as above, co –infected with HIV (PTB/HIV). HIV infection confirmed by rapid diagnostic test.
Latent TB infection (LTBI)	IGRA, or TST (T- SPOT.TB, Oxford Immunuotec, Oxfordshire, UK.) tested positive with no sign or symptoms of the active disease
Extra-pulmonary TB patients (EPTB)	Positive liquid media culture from extrapulmonary specimen with localize diseases, HIV negative
Healthy individuals (H)	No disease symptoms, IGRA, or TST tested negative
Other pulmonary infectious (OPI)	Clinical presentation, with or without microbiological confirmation, IGRA and/or TST negative



Flow Diagram 1; Enrolment and criteria for sample selection

2.2 Serum preparation.

5 mL of blood were collected using BD Vacutainer (BD Falcon, BD Biosciences) for serum recovery. Serum samples were prepared according to an internal standard protocol for total RNA recovery. Blood samples were allowed to stand for 2 hours before serum was recovered. After coagulation, the blood was centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 1200 g for 7 minutes. The serum was transferred in a 15 mL Falcon tube and centrifuged (Centrifuge 5417R, Eppendorf, Germany) again at 1300 g for 10 min to remove any residual cells or debris from serum. Recovered serum was cryo-preserved at -80 °C until use. The samples were analysed in the centralized laboratory, Fondazione Centro San Raffaele, Milan, Italy where the serum from the recruitment sites was shipped under dry ice according to IATA. During total RNA extraction, serum samples were thawed on ice, and hemolysis was determined through spectrometry analysis of free hemoglobin as previously described (Kirschner et al. 2011).A sample was considered haemolysed when hemoglobin concentration was >10 mg/dL.

2.2.1. Total RNA extraction

Total RNA from serum pools or individuals was isolated using the mirVana miRNA Isolation Kit (Ambion, Austin, TX).The *mir*Vana™ miRNA Isolation Kit was designed for purification of RNA suitable for studies of both siRNA and miRNA in natural populations. The detail procedure for total RNA extraction has been descripted in *mir*Vana™ miRNA Isolation protocol (Ambion, 2008). Briefy,the kit employs an organic extraction followed by immobilization of RNA on glass-fiber filters to purify either total RNA, or RNA enriched for small species, from cells or tissue samples (Ambion, 2008).

The *mir*Vana™ miRNA isolation procedure combines the advantages of organic extraction and solid-phase extraction, while avoiding the disadvantages of both. ``High yields of ultra-pure, high quality, small RNA molecules can be prepared in about 30 min'' (Ambion, 2008). The serum samples were extracted in two aliquots with an equal volume. RNA was eluted with 70µL of the Ambion Elution Buffer solution, and approximately 60µL was recovered from each column. Total RNA quality and purity (protein or DNA contamination) were determined by measuring the absorbance at 260 nm with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, ThemoScientific). RNA concentrations ranged from 6.0 to 80ng/µL. Total RNA extracts were stored at -80 °C until use.

2.2.2 Composition of pooled and individuals samples

To minimize individual variation, serum samples from 10 subjects within the same category were pooled together and analysed for miRNAs expression. 11 pools (H (4), PTB (3) and PTB/HIV (4)) from TB CHILD study and 8 pools (H (2), PTB (2), EPTB (1), LTBI (2) and OPI (1)) from TB NEW. A serum aliquot from each selected sample, thawed on ice, and 500 µL of serum from

each selected sample were thoroughly mixed in order to get a homogeneous pooled serum. Before RNA extraction, one ml of pooled serum was aliquot in two tubes and later miRNAs analysed were performed in duplicated sample. Pooled analysis was done for the categories; PTB, LTBI and healthy individuals, from TB NEW and PTB, PTB/HIV and healthy individuals from TB CHILD patients.
The results from pooled sera were then validated by repeating analysis from individuals' sera, from 18 PTB and 18 H subjects from TB NEW, and 10 PTB and 10 H subjects from TB CHILD. 1 mL of serum from each individual selected was thawed on ice and farther analysed for miRNA expression levels as described above.

2.2.3 Retro-transciption for pooled and individuals samples

Retro-transcription was performed with TaqMan® microRNA reverse transcription components (Life Technologies), according to the manufacturer's instructions. Two independent mastermix were prepared: one containing Megaplex™ RT primers for array A and one for array B. Each reaction was prepared in duplicated and negative controls inclusive. Reverse transcription PCR reaction was carried out in a thermal cycler (Applied Biosystems 2720). Seven µL of the reaction mixtures were subjected to thermal cycling for 40 cycles of 2 min at 16°C, 1 min at 42°C, 1 sec at 50°C and 5 min at 85°C, and then held at 4°C.

2.2.4 Pre- Amplification for pooled and individuals samples

In order to increase the sensitivity of the TLDA, a pre-amplification step was performed after the RT procedure using the TaqMan PreAmp Master Mix and the Megaplex PreAmp Primer Pools A + B. All reactions were carried out according to the protocols recommended by the manufacturer. Briefly; 25µL reaction mixture consisted of 4.5 µL of undiluted cDNA combined with 12.5 µL of TaqMan PreAmp Master Mix, 2.5 µL of Megaplex PreAmp Primers and 7.5 µL of nuclease-free water. The pre-amplification step was performed using a ABI 2720 or Bio-Rad iCycler by heating to95°C for 10 min, 55°C for 2 min, 72°C at 2 min followed by 12 cycles of 95°C for 15 sec, 60°C for 4 min and 99.9°C for 10s and then held at 4°C. The product, pre-amplified cDNA was diluted by adding 75 µL of TE buffer (0,1x, pH 8.0) and stored at -20°C.The Pre-Amp protocol is designed to maintain the relative quantities of each miRNAs as unchanged as in their original sample, and this step does not affect the results during the analysis of qPCR data.

2.2.5 Quantitative Real-time PCR reaction (qRT-PCR) for pooled and individuals samples

MiRNA profiling assays were performed using the TLDA v2.0 (Applied Biosystems, Life Technologies™, Foster City, USA) as per manufacturer instructions. Briefly, the TLDA is a 384-well microfluidic card containing dried TaqMan® primers and probes. ``Array A focuses on more highly characterized miRNAs while array B contains many of the more recently discovered miRNAs. The use of two panels (array A and array B) enables quantitation of gene expression

levels of up to 672 different miRNAs. This is accomplished by loading the cDNA product onto the array for PCR amplification and real-time analysis'' (Applied Biosystems, Life Technologies™, Foster City, USA). ``MegaPlex Pools are designed to detect and quantitate up to 380 microRNAs (miRNAs) per pool in human species because of o a set of stem-looped reverse transcription primers (MegaPlex RT Primers) that enable the simultaneous synthesis of cDNA and a set of miRNA-specific forward and reverse primers (MegaPlexPreAmp Primers) intended for use with very small quantities of starting material'' (Applied Biosystems, Life Technologies™, Foster City, USA). ``The primers enable the unbiased pre-amplification of the miRNA cDNA target by PCR prior to loading the TaqMan® MicroRNA Array'' (Applied Biosystems, Life Technologies™, Foster City, USA). ``The TaqMan® Low Density Array Human MicroRNA Panel is faster, sensitive for miRNA profiling as compared to microarrays'' (Applied Biosystems, Life Technologies™, Foster City, USA). By the use of Megaplex RT primer pools method, specific mature miRNAs are selected , quantified and easily covers range of targets (Mestdagh et al. 2008). Each serum sample was analysed in an A and B card in duplicate detection of a total of 672 miRNAs in each card which consist also of endogenous negative controls.

QRT-PCR was performed using 384-well TLDAs cards according to the manufacturer instruction. In brief, nine µL of the diluted Pre-Amp product mixture was combined with 450 µL of TaqMan Universal PCR master mix without uracil-N-glycosylase (Life Technologies), and 441 µL of nuclease-free water. Aliquots of 100 µL were pipetted into each fill port of a 384-well TLDA. The cards were centrifuged twice (12,000 rpm, 1 min, Multifuge3S-R, Heraeus, Germany) and sealed. The reaction was run on a 7900 HT Fast Real- Time PCR System or on a ViiA™ 7 Real-Time PCR System according to manufacturer's instructions (Life Technologies). Cycling conditions were as follows: 40 cycles 50^0C for 2 min, 95 ^{0}C for 10 min, 95 ^{0}C for 15 sec and 60 ^{0}C for 1 min.

For each pool, two arrays were performed: from array A 381 miRNAs and 291 miRNAs from array B were analysed. The number of miRNAs analysed on each card is less than the actual number of wells (384) because some miRNAs are analysed in duplicate: in array A card there are four wells of endogenous control *MammU6,* while on array B card: there are four wells for each of the small RNAs: *MammU6, RNU24, RNU43, RNU44, RNU48* and *RNU6B;* - rows 11, 13 and 14 of the array are duplicated in lines 12, 15 and 16 respectively. Moreover, *RNU48* and *RNU44* are also present in array A card. For pooled samples, analysis was performed in duplicate in each array (A and B) and for each individual serum samples analysis was performed in single arrays (panels A + B).

2.3 Statistical analysis

2.3.1 Data analysis and normalization.

It is important to mention that, the statistical analysis described under section 2.3.1 to 2.3.4 was performed by the biostatistician, in Milan Italy.

Data generated from this study was analysed by qRT-PCR. In comparison with other quantitative techniques, qRT- qPCR has become a powerful technique with higher sensitivity, specificity, and ability to detect signals even in the low concentration of the targets (Chen et al. 2005; Mestdagh et al. 2008). Due to the complexity of the data set, the accuracy of the results generated from qPCR, which is a high-throughput technique, will depend on appropriate data normalization methods (Dheda et al. 2005; Meyer, Pfaffl, and Ulbrich 2010). Data produced from qPCR experiments is influenced by several variables due to sample processing, stabilization, total RNA extraction and target quantification. The differences may indicate variations between sample or bulk transcriptional activity. Before data analysis begins, variations and differences caused during the experiment need to be removed by a normalization process, in order to get the true biological changes (Meyer et al. 2010; Steinhoff and Vingron 2006). On the other hand, an incorrect normalization method may produce misleading results, which may affect the subsequent analysis outcome (Bas et al. 2004; Dheda et al. 2005; Pradervand et al. 2009; Risso et al. 2009; Tricarico et al. 2002).Therefore, selection of appropriate normalization method is a critical step in the analysis of qPCR data as illustrated in figure 5.

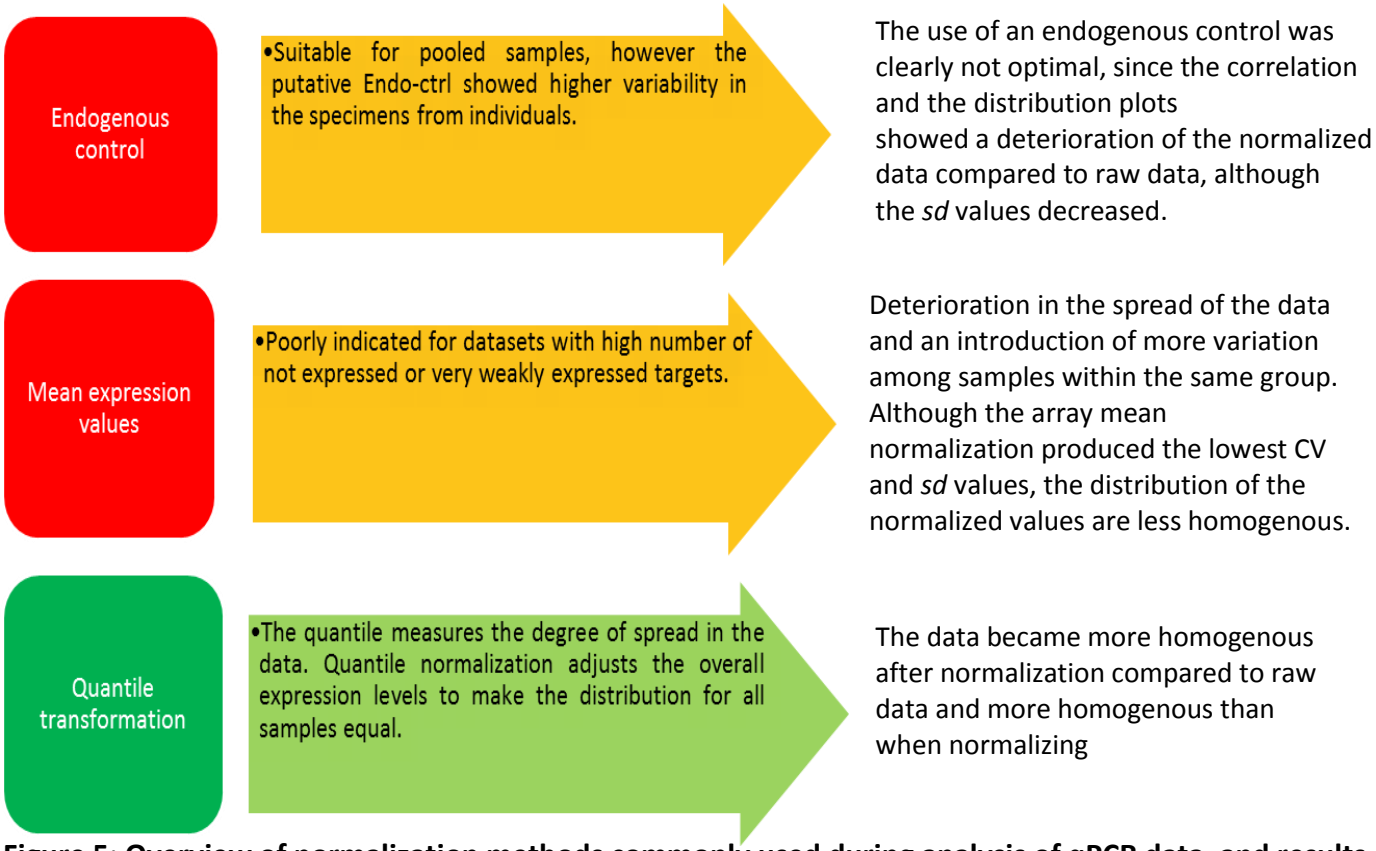


Figure 5; **Overview of normalization methods commonly used during analysis of qPCR data, and results obtained in this study**

QPCR data were collected with SDS v2.4.1, ViiA™ 7 v1.1, and RQ Manager v1.2.2 software (Applied Biosystems, Life Technologies™, Foster City, USA), (baseline criteria are: automatic; threshold: 0.20; maximum allowable Ct : 35.0). Data analysis was done by using R and Bioconductor environment (R Development Core Team, 2011, Vienna, Austria). Prior to the analysis, raw Ct values were normalized by quantile normalization, as described elsewhere (Bolstad et al. 2003; Deo, Carlsson, and Lindlof 2011). In brief, this widely used approach is based on the hypothesis that only few miRNA are differentially expressed. The advantage of using this method is that after normalization, data become homogenous with similar distribution and the correlation coefficient between observations increases as compared to the raw data.

2.3.2 Analysis of pooled samples

Analysis on the pools were done by comparing mean Ct values of miRNAs of each category with another category by fitting a constrained regression model with MM robust estimators as described by Salibian-Barrera et al. (Salibian-Barrera M 2008).The use of the robust estimator method has a great advantage in the presence of outliers. The Empirical Distribution Function of residuals was calculated and miRNAs residuals; outside the Inter quartile range were filtered and defined as significantly differently expressed miRNAs.‘‘(Residuals are the difference

between the observed value of the variable and the value suggested by the regression model)''. Data was visualized in circular format which was created using Circos software (Michael Smith Genome Sciences Center, Vancouver, Canada) (Krzywinski et al. 2009). ''Circos is a visualization tools which enable the identification and analysis of similarities and differences arising from comparisons of genome produced by sequence alignments, hybridization arrays, genome mapping, and genotyping studies'' (Krzywinski et al. 2009). ''Circos uses a circular ideogram layout to facilitate the display of relationships between pairs by the use of ribbons, which encode the position, size, and orientation of related genomic elements''(Krzywinski et al. 2009).

2.3.3 Analysis of individual sera

About 80% of miRNAs with Ct values less than 35, and detected in at least one of the patient categories were selected for farther comparison. The Two ways ANOVA statistical package was performed in the selected miRNAs from different patient categories and study cohorts. *P* values were calculated based on non-parametrical by means of permutations (Good P.I 2005). False Discovery Rate (FDR) as described by Benjamini was used; whereby miRNAs showing both (i) an adjusted p-value (*p-adj)* <0.05 on individuals and (ii) identified to be significant by pooled samples analysis were considered for miRNA signature classification (Benjamini Y 2001).

2.3.4 Performances of the signature

Capability of a single miRNA to identify health status was determined using Receiver Operating Characteristic (ROC) curve as described by Zou et al (Zou, Hall, and Shapiro 1997). Area under the curve (AUC) and the *p* values were calculated when the general performance of miRNAs signature in differentiating cases was evaluated. Akaike Information Criteria (AIC) and Relevance Vector Machine (RVM) model as multivariate logistic model were used to evaluate and associate the diagnostic performances of the miRNA signatures (Braun AC 2012). Unlike the support Vector Machine, RVM follows a Bayesian approach giving *a posteriori* probability of the class. Hence, the results from the two models become more comparable. ROC curve and associated AUC were calculated based on the logistic model.

A leave-one-out-cross-validation (LOOCV) approach was adopted for validating the performance of RVM and AIC logistic regression predictive models as indicated in figure 6. Results from the two models were presented in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy.

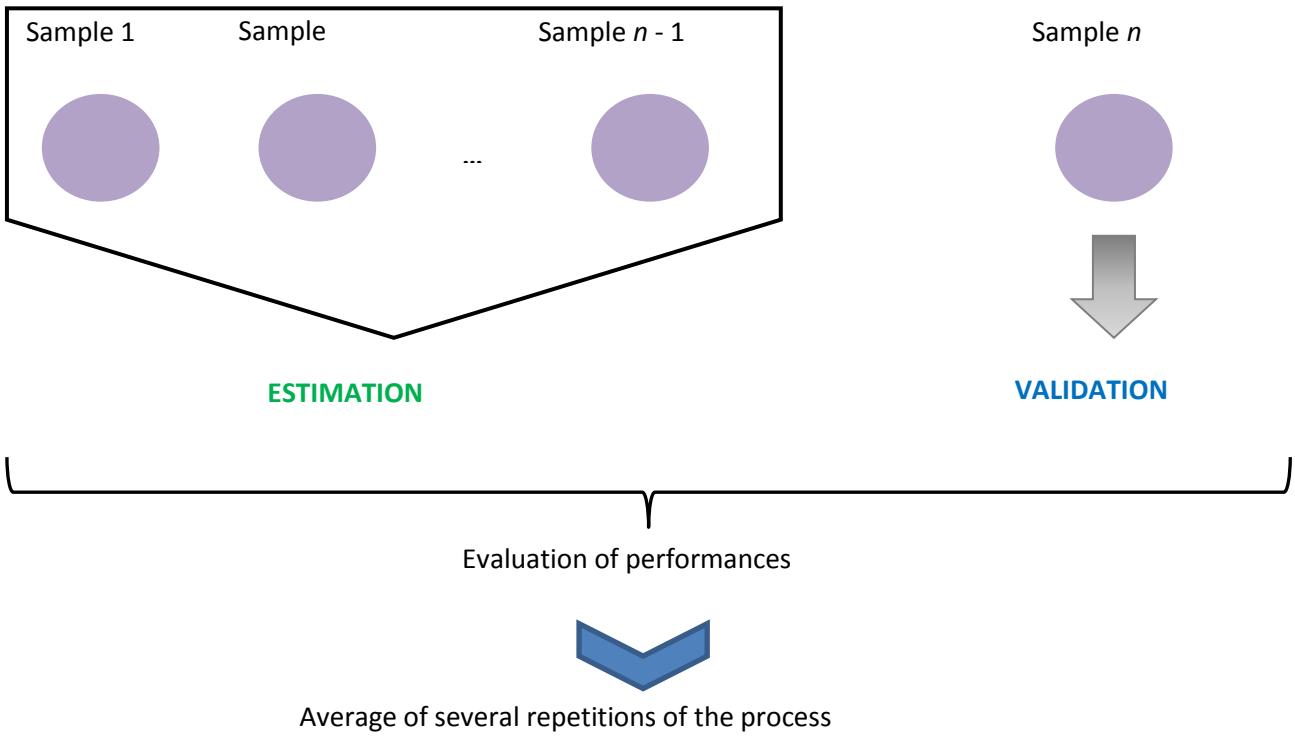


Figure 6; An overview of A leave-one-out-cross-validation (LOOCV) model.
LOOCV model a validation technique is used for assessing how the results of a statistical analysis will generalize to an independent data set. It is mainly used to estimate how accurately a predictive model would perform in practice.

3. RESULTS

3.1. Study patients

All subjects were enrolled from September 2009 to September 2012. Enrolment was done in Uganda, Tanzania and Italy. Participants are summarised with their baseline characteristics in Figure 10, with their geographic origin classified by World Health Organization (WHO) regions. The population enrolled in Africa (TB CHILD) comprised of 137 pulmonary tuberculosis patients of whom 64 patients were HIV negative, and classified into the PTB cohort, 73 were HIV positive (PTB/HIV) and 49 healthy individuals (H).

A total of 311 patients were enrolled from Italy (TB NEW) with 90 PTB patients, 56H and 109 LTBI participants. In addition, small population of 32 patients with EPTB and 24 OPI were also enrolled from Italy. Patients enrolled from Italy were all HIV negative. Detailed description of the study populations are indicated in flow diagram 1 (page 27) and figure 7. Patients with PTB/HIV had median $CD4^{+}$ cell count of 198.4 cells/mL (interquantile range: 277.1) as compared with 707.7 cells/mL (interquantile range: 813.3) of participants with PTB without HIV infection. Age differences between H and PTB patients were not statistically significant (p= 0.29).

Population	Category	Male	Female	Total	Age
TBnew	Pulmonary TB	58	32	90	40(19-78)
	Health	31	25	56	23(19-51)
	Latent TB infection	40	69	109	40(19-83)
	EPTB	16	16	32	39(19-78)
	OPI	14	10	24	61(32-85)
	Total	129	152	311	40(19-83)

Population	Category	Male	Female	Total	Age
TB CHILD	Pulmonary TB	43	21	64	36(18-73)
	Health	27	22	49	22(18-32)
	PTB/HIV	37	36	73	35(19-61)
	Total	107	79	186	53(18-73)

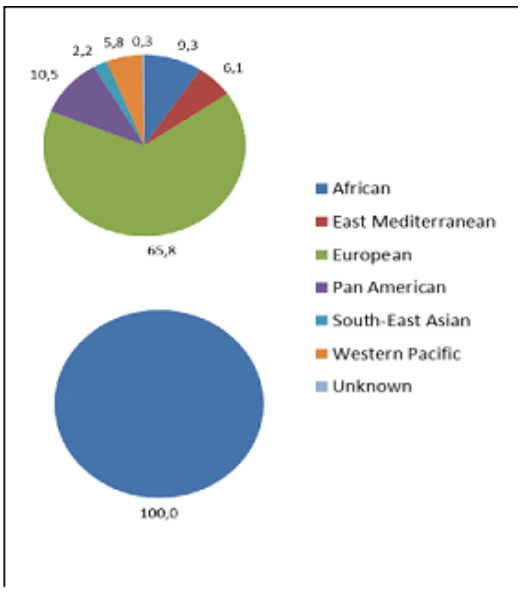


Figure 7; Demographic information of the two studies with the mean and range ages of the study participants. *The distribution of populations participated in the two studies with their countries of origin as stipulated in WHO region categorization.*

Table 4; Detail Information of constitutions of participants as allocated in each pool and individual analysis in the two study population enrolled from different geographical location.

Population	Sample type	Category	male/female ratio	Country of origin	mean age	SD
TB NEW	Pooled samples	H1	5/5	10 Europe, 0 non-Europe	19.7	0.6
		H2	5/5	9 Europe, 1 non-Europe	22.7	4.7
		LTBI1	5/5	6 Europe, 4 non-Europe	45.5	16.8
		LTBI2	5/5	4 Europe, 6 non-Europe	38.5	9.9
		PTB1	5/5	5 Europe, 5 non-Europe	33.1	12.7
		PTB2	5/5	7 Europe, 3 non-Europe	36.4	11.6
		EPTB	3/5	3 Europe, 5 non-Europe	49.9	24.1
		OPI	6/4	8 Europe, 2 non-Europe	67.0	8.7
	Individual samples	H	9/9	18 Europe, 0 non-Europe	21.8	5.8
		PTB	9/9	18 Europe, 8 non-Europe	38.5	15.4
TB CHILD	Pooled samples	H1	5/5	10 Africa	23.4	4.12
		H2	5/5	10 Africa	21.4	4.48
		H3	5/5	10 Africa	22.7	3.68
		H4	5/5	10 Africa	20.2	2.66
		PTB2	5/5	10 Africa	35.1	11
		PTB3	5/5	10 Africa	24.2	10.6
		PTB4	5/5	10 Africa	38.1	10.3
		PTB/HIV1	5/5	10 Africa	38	9.89
		PTB/HIV2	5/5	10 Africa	20.9	16.3
		PTB/HIV3	5/5	10 Africa	35.3	6.63
		PTB/HIV4	5/5	10 Africa	37.1	11.2
	Individual samples	H	5/5	10 Africa	23.7	4.1
		PTB	7/3	10 Africa	29.8	16.3

3.2 Selected samples for qRT-PCR.

Serum samples from each category were selected for analysis using the information collected in the questionnaires during the enrolment. The selection criteria varied depending on category as indicated in flow diagram 1. Basically we aimed for homogeneous categories in this proof-of-concept study, to better differentiate between targeted categories. Six pools from the TB NEW, and 11 pools from the TB CHILD were analysed. In the second step, we also validated the

signatures of miRNA from the pooled samples using individual serum by analysing 36 and 20 sera of participants from TB NEW, and the TB CHILD population, respectively (details are reported in Table 4).

3.3 Normalization of qPCR results

qPCR data was normalized by quantile normalization method. The normalized data from pooled sera, individual sera as well as from the endogenous controls (comprising the four miRNAs ath-miR159a, MammU6, RNU44, and RNU48) detected by both array A and array B, are reported in Table 5. Statistical analysis was performed to compare and determine miRNA signatures that can be used to differentiate between healthy individuals and other TB related conditions; the comparison was also done between other TB related conditions. Moreover, results from participant from TB NEW and TB CHILD were analysed separately and the detected miRNAs in groups were compared in their expression levels and presence.

Table 5; The mean and standard deviation of Ct values of endogenous control miRNAs included in the two array cards (A and B). *The table presents the C_t values detected in the two study population separately and from both populations after quantile normalization*.

	microRNA	Array A		Array B	
		Pools	Individuals	Pools	Individuals
TB NEW	ath-miR159a	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0
	MammU6	26.09 ± 2.18	24.92 ± 2.81	24.47 ± 3.97	23.99 ± 3.43
	RNU44	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0
	RNU48	33.74 ± 1.52	33.63 ± 1.77	33.24 ± 1.93	33.41 ± 1.63
TB CHILD	ath-miR159a	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0
	MammU6	24.76 ± 1.05	27.25 ± 1.66	24.93 ± 1.45	27.24 ± 2.54
	RNU44	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0
	RNU48	31.34 ± 1.45	33.54 ± 1.70	31.66 ± 1.02	33.89 ± 1.33
Both populations	ath-miR159a	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0
	MammU6	25.32 ± 1.73	25.73 ± 2.70	24.74 ± 2.76	25.15 ± 3.49
	RNU44	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0	35.0 ± 0.0
	RNU48	32.35 ± 1.89	33.60 ± 1.73	32.32 ± 1.65	33.58 ± 1.53

3.5 Serum miRNA profiles in Pooled samples.

In this study a total of 672 different miRNAs were analysed by qRT-PCR- Taqman Low density arrays from pooled sera. Results from healthy individuals were used as controls. Raw data for qRT-PCR C_t values obtained from pooled and individual samples for all 672 miRNAs analysed after quantile normalization, are too large for this thesis and can be reviewed in the online supplement (Table S3) in the already published results from this work (Miotto et al. 2013). Normalized qRT-PCR data from pooled samples showed that 277 miRNAs were undetectable in the categories H, PTB, LTBI, and PTB/HIV from both groups TB NEW and TB CHILD.
The mean C_t value for each miRNA was calculated and one-to-one comparison of miRNAs between different patient categories was carried out, with significant miRNA residuals being those outside the interquartile range after comparing two categories. Residuals of significant miRNAs from the pooled samples are shown in Table 6, with the raw data available in the supplement (table S4) in our already published results (Miotto et al. 2013).

3.6 Difference in expression from the two geographic distributions.

We assessed potential differences in miRNA expression between European and African populations, with results summarized in Table 6. The residuals of the significant signature of miRNAs expressed are shown in already published results, supplement Table S5 (Miotto et al. 2013). The distribution of the residuals between categories showed little variation; 168 miRNAs were observed to be significantly differently expressed between the H and 105 between the PTB patients from the two study populations.

In the initial analysis of pooled samples, miRNAs observed from patients with extra pulmonary TB (EPTB) and other respiratory tract infections (OPI) collected from the European cohort were also observed in other categories. The 1st and 3rd quantile tails of miRNA residuals shows the distribution obtained by comparing the two different categories, summarized in Figure 8. The two quantiles considered, contain signature of miRNAs that are considered to be significantly different between the two compared categories. Based on the distribution of the residuals, from qualitative analysis a signature consisting of about 120 to 172 miRNAs could differentiate between categories that were considered in the two studies. For instance, signature consisting of 134 miRNAs observed to be significant differentiates between LTBI and PTB, 150 discriminate H and PTB (TB NEW), whereas 134 miRNAs could discriminate between PTB and PTB/HIV.

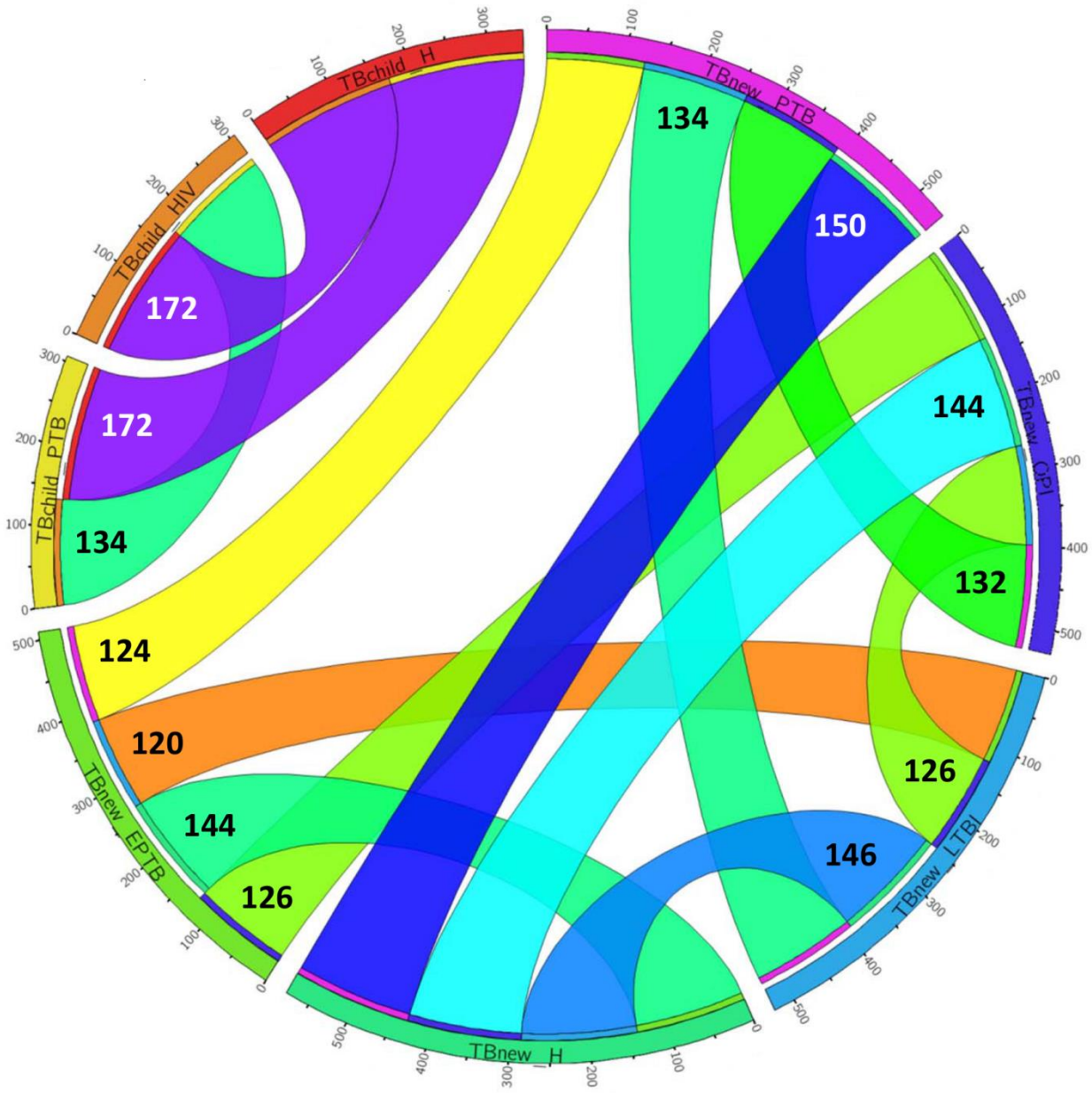


Figure 8; **miRNA signatures significantly differentiating between categories in the pooled samples**.

Two study population; African (TBCHILD) and European (TB NEW) and disease categories are reported on the circular visualization made by circos. Numbers of miRNAs residuals significantly different between categories are given in the coloured ribbons linking the two categories. The miRNAs were used to compared different categories and filtered in those defined categories : H vs PTB, H vs LTBI, H vs EPTB, H vs PTB/HIV, OPI vs PTB, OPI vs LTBI, OPI vs EPTB; Active TB: H vs PTB, H vs EPTB, H vs PTB/HIV, OPI vs PTB, OPI vs EPTB, LTBI vs PTB, LTBI vs EPTB; Symptoms: H vs PTB, H vs OPI, H vs PTB/HIV, H vs EPTB, LTBI vs PTB, LTBI vs EPTB, LTBI vs OPI; Pulmonary disease (any): H vs PTB, H vs OPI, H vs PTB/HIV, LTBI vs PTB, LTBI vs OPI, EPTB vs PTB, EPTB vs OPI.

Signatures of miRNAs observed to be of clinically significant from pooled samples were filtered. From this, we identified potential miRNAs which could be specific for Latent TB disease, active TB, pulmonary disease and any other disease statuses as shown in Figure 9.

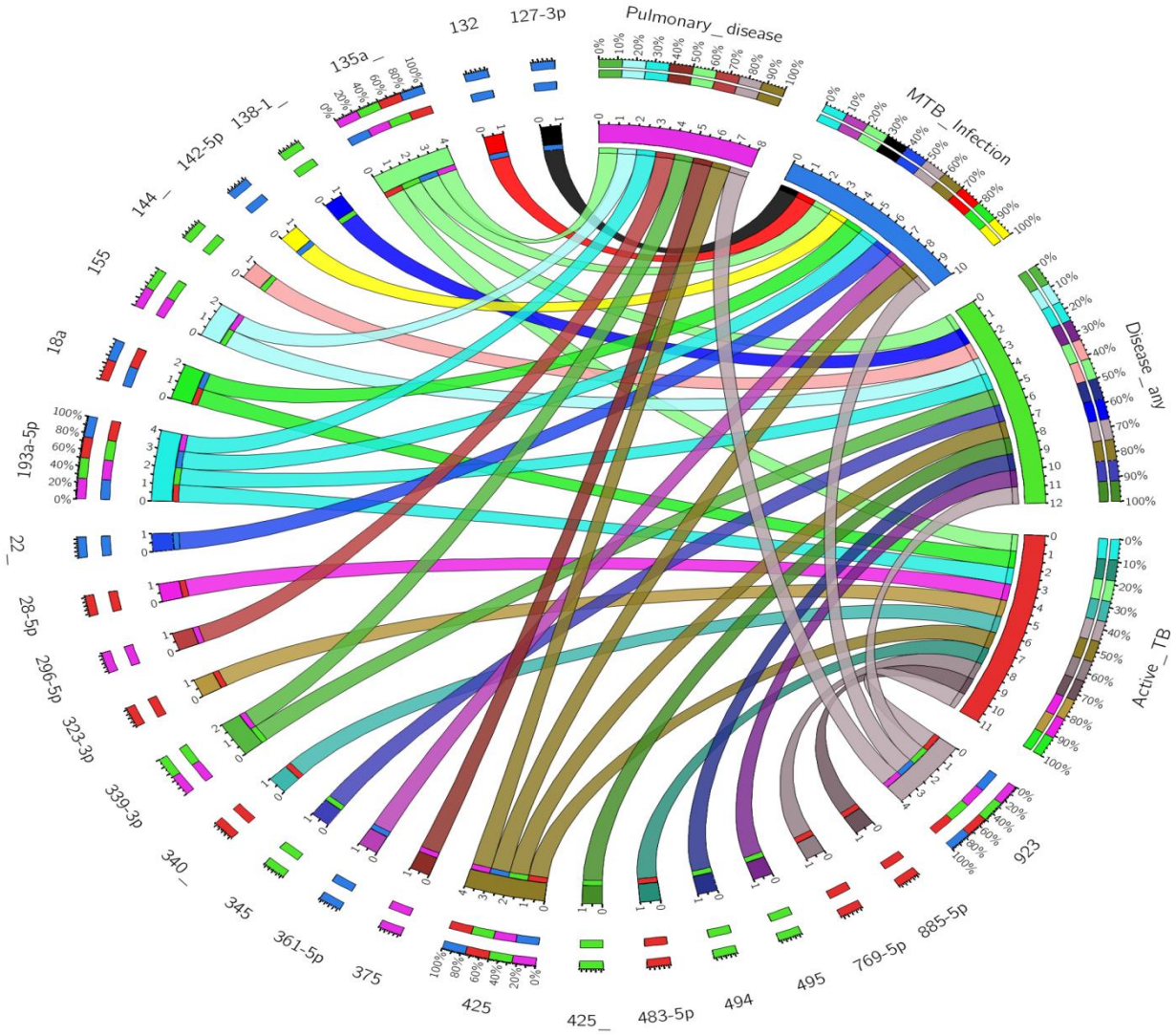


Figure 9; **Signature of miRNAs able to differentiate between different clinical categories from the analysed pooled samples.** *Clinical status and miRNAs are presented on the circumferences whereas the coloured ribbon links one disease category to specific miRNAs.*

3.7 Serum miRNA profiles in individual samples.

To verify the pattern of identified relevant miRNA from the pooled samples, we performed a validation study using individual samples from H and PTB cases. Serum miRNA profiles from 18

H and 18 PTB from *European* group as well as 10 Hand 10 PTB from the *African* group were analysed using qRT-PCR.

672 miRNAs were analysed and out of those, 126 miRNAs with Ct < 35 were detected in 80% of the subjects in at least one of the analysed categories whereas, 71 miRNAs with P<0.05 were detected. We observed significant differences in miRNA expression between the two geographical locations, European cohort and African population (35 miRNAs; 49.3%), between H and PTB (29 miRNAs, 40.8%) and differences between the two populations and the clinical status (7 miRNAs ,9.9%). Additionally, 20 miRNAs were significantly different between H and PTB in both populations (let-7e, miR-10b, miR-127-5p, miR-146a, miR-148a, miR-16, miR-185, miR-192, miR-193a-5p, miR-25, miR-365, miR-451, miR-518d-3p, miR-532-5p, miR-590-5p, miR-660, miR-885-5p, miR-223*, miR-30a, miR-30e) when false recovery rate FDR, p-adj) was calculated.

3.8 Comparison of miRNAS from Pooled samples against individual samples.

Validation analysis aimed at identifying expressed miRNAs with significant differences between H and PTB categories. From a signature consisting of 20 miRNAs with significant p-adj in individuals' samples (table 7), 16 (80%) had already been identified as significant miRNAs in the pooled samples. Out of those; nine miRNAs had significantly differentiated H and PTB in both study populations in the pooled samples; whereby four had been specific to the TB CHILD populations and three had been specific to the TB NEW population. However, four miRNAs with a significant p-adj in individuals' samples had not been identified previously as significant in the pooled samples.

Further, the directions of the variation (increase or decrease) of the 16 miRNAs, which were found to be significant for differentiating H and PTB in the pooled analysis and also observed to be significant (*p-adj*< 0.05) in individual analysis (let-7e, miR-146a, miR-148a, miR-16, miR-192, miR-193a-5p, miR-25, miR-365, miR-451, miR-532-5p, miR-590-5p, miR-660, miR-885-5p, miR-223*, miR-30a) were compared. There was inconsistence of miRNAs observed between individual and pooled samples as indicated in Table 6. 11 miRNAs identified were similar in the expression directions in the pooled and individual samples between the two study populations. Moreover, three miRNAs varied in their expression level in African and European populations and one showed discordant in both populations. Five and three miRNAs found to be associated with only the African and European populations, respectively. However, a total of 15 miRNAs were identified as a significant signature for discriminating between H and PTB (let-7e, miR-146a, miR-148a, miR-16, miR-192, miR-193a-5p, miR-25, miR-365, miR-451, miR-532-5p, miR-590-5p, miR-660, miR-885-5p, miR-223*, miR-30e). This pattern of signature of miRNAs was identified from the combined analysis of pools and individuals samples.

Table 6; miRNA signature expression differences between PTB and H and between the two study populations. The table reports data from individual and pooled specimens.

	African			European			Combined				
miRNAs p-adj<0.05	**Individuals**	**Pools**		**Individuals**	**Pools**		**Individuals**	**Pools**		**Relevance in pooled specimens**	**To be considered for**
hsa-let-7e-4395517	⇩	⇩		⇩	⇩		⇩	⇩		African &Europeans and other race	Africans & Europeans
hsa-miR-146a-4373132	⇩	⇧	x	⇩	⇩		⇩	⇩		African only	Africans only
hsa-miR-148a-4373130	⇧	⇧		⇧	⇧		⇧	⇧		African &Europeans and other race	Africans & Europeans
hsa-miR-16-4373121	⇧	⇧		⇧	⇧		⇧	⇧		Europeans and other race only	Europeans only
hsa-miR-192-4373108	⇧	⇧		⇧	⇧		⇧	⇧		African &Europeans and other race	Africans & Europeans
hsa-miR-193a-5p-4395392	⇧	⇧		⇧	⇧		⇧	⇧		African &Europeans and other race	Africans & Europeans
hsa-miR-25-4373071	⇧	⇧		⇧	⇧		⇧	⇧		Europeans and other race only	Europeans only
hsa-miR-365-4373194	⇧	⇧		⇧	⇩	x	⇧	⇧		Europeans and other race only	Europeans only
hsa-miR-451-4373360	⇧	⇧		⇧	⇧		⇧	⇧		African &Europeans and other race	Africans & Europeans
hsa-miR-532-5p-4380928	⇧	⇩	x	⇧	⇧		⇧	⇧		African only	Africans only
hsa-miR-590-5p-4395176	⇧	⇧		⇧	⇧		⇧	⇧		African &Europeans and other race	Africans & Europeans
hsa-miR-660-4380925	⇧	⇧		⇧	⇧		⇧	⇧		African only	Africans only
hsa-miR-885-5p-4395407	⇧	⇧		⇧	⇧		⇧	⇧		African &Europeans and other race	Africans &Europeans
hsa-miR-223*-4395209	⇧	⇧		⇧	⇧		⇧	⇧		African only	Africans only
hsa-miR-30a-4373061	⇧	⇩	x	⇧	⇩	x	⇧	⇩	x	African &Europeans and other race	-
hsa-miR-30e-4395334	⇧	⇩	x	⇧	⇧		⇧	⇩	x	African &Europeans and other race	African only

Discrepancies between the two cohorts are marked by "x" and highlighted in grey

Table 7; Signature of miRNAs from individual samples showing different expression levels in healthy (H) and pulmonary active tuberculosis (PTB) subjects from the two study populations.

miRNA	TB NEW against TB CHILD (p-val)	H vs PTB (p-val)
has-miR-155-4395459	0,01375	0,40055
hsa-miR-126-4395339	0,02580	0,76655
hsa-miR-129-5p-4373171	0,02600	0,84640
hsa-miR-139-3p-4395424	0,00975	0,91465
hsa-miR-142-5p-4395359	0,00025	0,33310
hsa-miR-145-4395389	<0,00001	0,88630
hsa-miR-146b-5p-4373178	0,02525	0,10360
hsa-miR-148b-4373129	0,00170	0,84365
hsa-miR-150-4373127	0,01065	0,37600
hsa-miR-152-4395170	<0,00001	0,43940
hsa-miR-17-4395419	0,00630	0,50405
hsa-miR-184-4373113	0,00510	0,32595
hsa-miR-195-4373105	<0,00001	0,16715
hsa-miR-19a-4373099	0,04110	0,34870
hsa-miR-20b-4373263	0,00315	0,52460
hsa-miR-220c-4395322	0,00100	0,29480
hsa-miR-29c-4395171	0,02855	0,29935
hsa-miR-302c-4378072	<0,00001	0,55550
hsa-miR-324-3p-4395272	0,00805	0,75735
hsa-miR-331-3p-4373046	0,00455	0,43190
hsa-miR-374b-4381045	0,00745	0,36145
hsa-miR-423-5p-4395451	0,04475	0,15395
hsa-miR-485-3p-4378095	<0,00001	0,54070
hsa-miR-574-3p-4395460	0,04470	0,05040
hsa-miR-597-4380960	0,00025	0,98915
hsa-miR-628-5p-4395544	0,01450	0,32615
hsa-miR-744-4395435	0,00100	0,40245
hsa-miR-872-4395375	<0,00001	0,06135
hsa-miR-9-4373285	0,04985	0,21925
MammU6-4395470	0,00140	0,51640
hsa-miR-135a*-4395343	0,00020	0,22165
hsa-miR-509-3p-4395347	0,01160	0,19885
hsa-miR-645-4381000	0,00005	0,27560
hsa-miR-801-4395183	<0,00001	0,87435
hsa-miR-923-4395264	0,00080	0,06465
MammU6-4395470	0,00045	0,58190

hsa-let-7e-4395517	0,80890	0,00395 *
hsa-miR-10b-4395329	0,79615	0,0072 *
hsa-miR-127-5p-4395340	0,96665	0,00495 *
hsa-miR-130a-4373145	0,09690	0,02485
hsa-miR-146a-4373132	0,14045	0,0047 *
hsa-miR-148a-4373130	0,91780	<0,00001 *
hsa-miR-16-4373121	0,17145	0,00045 *
hsa-miR-185-4395382	0,47350	0,00685 *
hsa-miR-19b-4373098	0,42240	0,01720
hsa-miR-24-4373072	0,29355	0,04625
hsa-miR-25-4373071	0,18345	0,00065 *
hsa-miR-27a-4373287	0,19845	0,01235
hsa-miR-27b-4373068	0,12255	0,03670
hsa-miR-342-3p-4395371	0,77485	0,01620
hsa-miR-365-4373194	0,48025	0,00345 *
hsa-miR-374a-4373028	0,17845	0,03010
hsa-miR-376c-4395233	0,32505	0,04740
hsa-miR-451-4373360	0,50895	0,0082 *
hsa-miR-532-5p-4380928	0,09210	0,00155 *
hsa-miR-590-5p-4395176	0,46820	0,0039 *
hsa-miR-660-4380925	0,48025	0,00095 *
hsa-miR-885-5p-4395407	0,86025	0,003 *
hsa-miR-144*-4395259	0,63015	0,02595
hsa-miR-223*-4395209	0,79420	0,00025 *
hsa-miR-30a-4373061	0,14410	0,00065 *
hsa-miR-30a*-4373062	0,54630	0,03605
hsa-miR-30d-4373059	0,43060	0,00715
hsa-miR-30e-4395334	0,50865	0,0012 *
hsa-miR-106a-4395280	0,03535	0,01145
hsa-miR-125a-5p-4395309	0,00380	0,03295
hsa-miR-192-4373108	0,04850	0,001 *
hsa-miR-193a-5p-4395392	0,01675	0,00485 *
hsa-miR-212-4373087	<0,00001	0,02215
hsa-miR-483-5p-4395449	0,00120	0,03800
hsa-miR-518d-3p-4373248	0,03350	0,00155 *

Key: * miRNAs showing *a p-adj* <0.05. The 20 miRNA with significant *p-adj* are in bold and italic font.

3.9 Diagnostic performances of the serum miRNA signature identified.

Diagnostic performances of the miRNAs signature identified were assessed and determined by two models: RVM model, and AIC logistic regression analysis. The diagnostic performances of the two models were validated by the use of a LOOCV approach.

The 15 miRNAs signature identified to significantly differentiate H and PTB in the two study populations were used to classify the 56 individuals' subjects had diagnostic accuracy of 82% by RVM and 77% by logistic regression. Diagnostic accuracy of each population was determined in the miRNA signatures that were observed to be significantly specific to the African or European population. Thus, the diagnostic accuracy of the African –specific 12 miRNAs signature was 95% by RVM, and 100% by logistic regression, respectively. The diagnostic accuracy for European cohort specific 10 miRNAs signature was 83% by RVM and 81% by logistic regression. More parameters for diagnostic accuracy by two statistical models are miRNA signatures presented in Table 8 and Table 8. Moreover the areas under the curves (AUCs) for the regression logistic analyses are reported in Figure 10, Figure 11 and Figure 12.

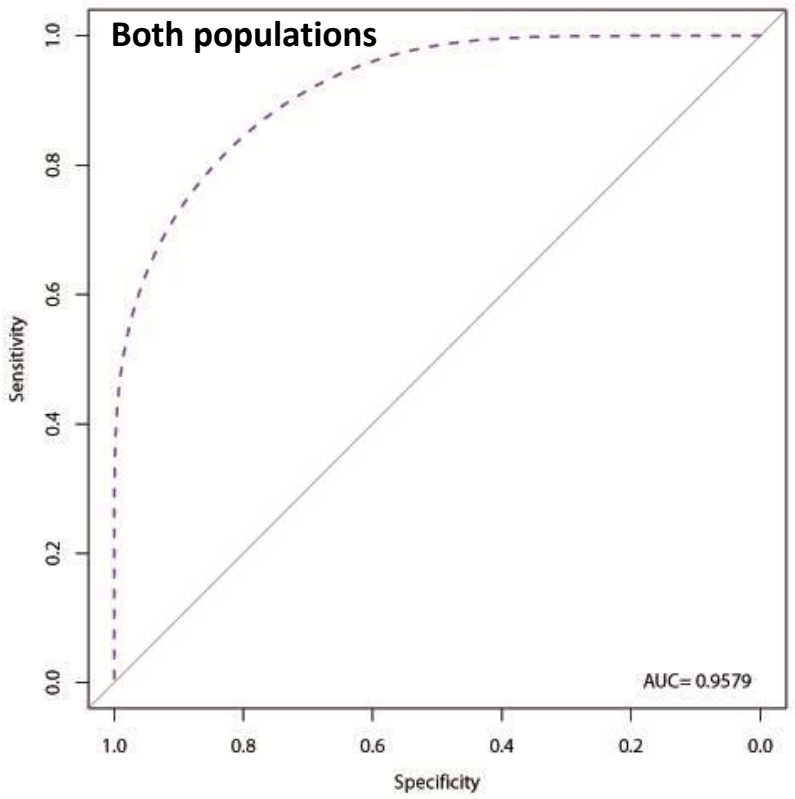


Figure 10;Area under the curve (AUC) of 0.9579 for 15 miRNA signature for both populations was calculated by Akaike information criterion (AIC) logistic regression.*The AIC model*

identified the best miRNA that were able to discriminate healthy individuals and active PTB from both African and European populations.

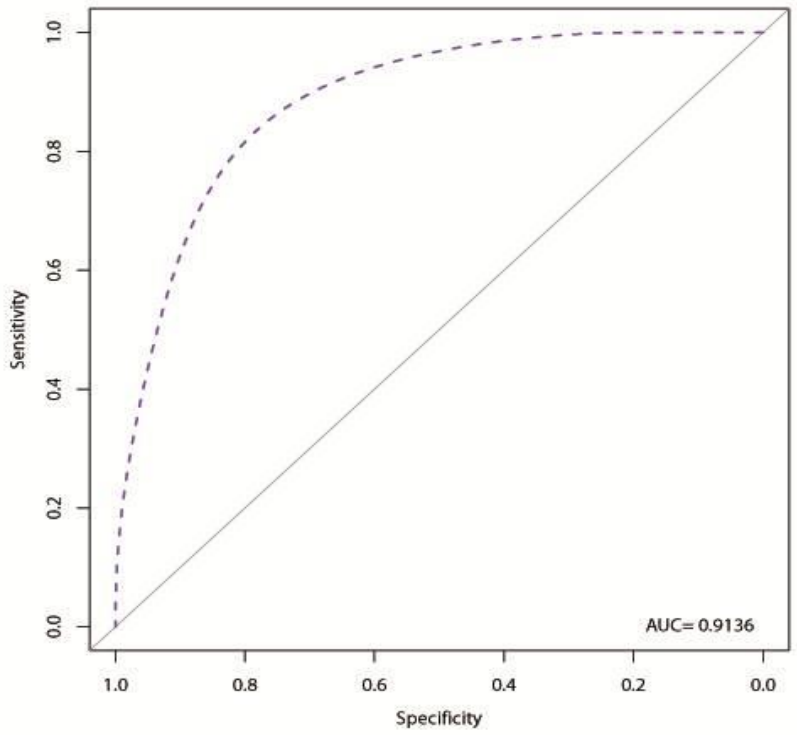


Figure 11;**Area under the curve (AUC) of 0.9136 for 10 miRNA signatures observed to be significant and specific for TB NEW cohort was calculated by Akaike information criterion (AIC) logistic regression**.

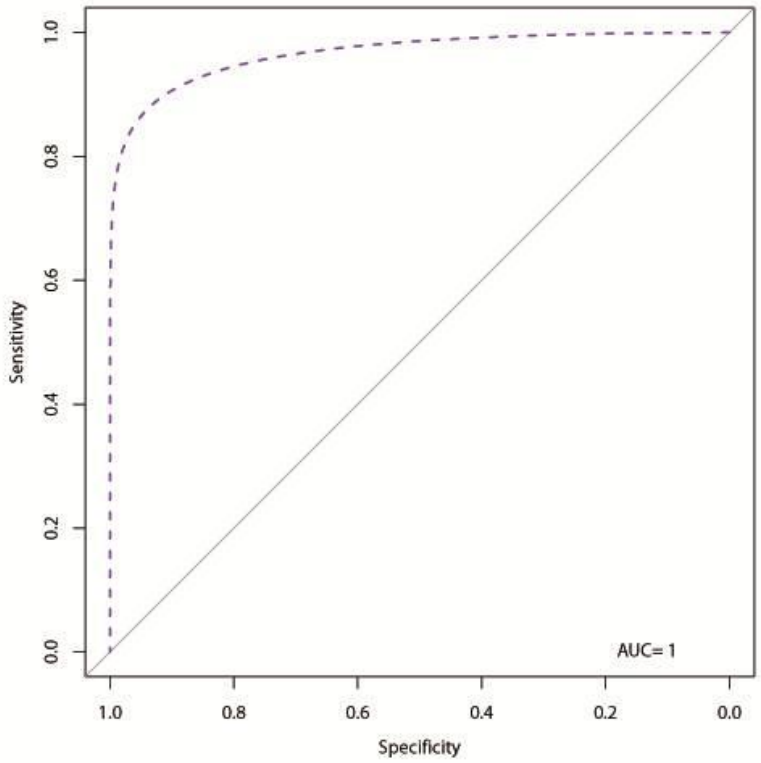


Figure 12; **Area under the curve (AUC) of 1 for 12 miRNA signatures observed to be significant in diagnostic performance and specific for African cohort was identified by Akaike information criterion (AIC) logistic regression**.

Table 8; Diagnostic performances of the serum miRNA signatures for discrimination between healthy and active PTB from the two study population obtained through Relevance Vector Machine (RVM) after validation with the leave-one-out cross validation (LOOCV).

Serum miRNA signature (No. of miRNAs)	Both population (15)	Italians and other race (10)	African(12)
n of individuals (H/PTB)	56 (28/28)	36 (18/18)	20 (10/10)
Sensitivity % (95% CI)	85.71 (68.51-94.30)	77.78 (54.78-91.00)	100.00 (72.25-100.00)
Specificity % (95% CI)	78.57 (60.46-89.79)	88.89 (67.20-96.90)	90.00 (59.58-98.21)
PPV % (95% CI)	80.00 (62.69-90.50)	87.50 (63.98-96.50)	90.91 (62.26-98.38)
NPV % (95% CI)	84.62 (66.47-93.85)	80.00 (58.40-91.93)	100.00 (70.08-100.00)
Diagnostic accuracy % (95% CI)	82.14 (70.16-90.00)	83.33 (68.11-92.13)	95.00 (76.39-99.11)
Likelihood ratio of a positive test % (95% CI)	4 (2.846-5.621)	7 (2.524-19.41)	10 (1.409-70.99)
Likelihood ratio of a negative test % (95% CI)	0.1818 (0.1087-0.3041)	0.25 (0.1508-0.4144)	0

Table 9; Diagnostic performances of the serum miRNA signatures for discrimination between healthy and active PTB from the two study population obtained through logistic regression model (AIC) after corrected with the leave-one-out cross validation (LOOCV).

Serum miRNA signature (No. of miRNAs)	Both population (15)	Italians and other race(10)	African(12)
n of individuals (H/PTB)	56 (28/28)	36 (18/18)	20 (10/10)
Sensitivity % (95% CI)	71.43 (52.94-84.75)	72.22 (49.13-87.50)	100.00 (72.25-100.00)
Specificity % (95% CI)	82.14 (64.41-92.12)	88.89 (67.20-96.90)	100.00 (72.25-100.00)
PPV % (95% CI)	80.00 (60.87-91.14)	86.67 (62.12-96.26)	100.00 (72.25-100.00)
NPV % (95% CI)	74.19 (56.75-86.30)	76.19 (54.91-89.37)	100.00 (72.25-100.00)
Diagnostic accuracy % (95% CI)	76.79 (64.23-85.90)	80.56 (64.97-90.25)	100.00 (83.89-100.00)
Likelihood ratio of a positive test % (95% CI)	4 (2.599-6.156)	6.5 (2.302-18.35)	undefined
Likelihood ratio of a negative test % (95% CI)	0.3478 (0.2672-0.4527)	0.3125 (0.2079-0.4696)	0

4. Discussion and Conclusion

4.1 Discussion

Our results show promising data on circulating miRNAs as diagnostic parameters for different disease states. We demonstrate that diagnostic performance for discrimination of active TB from healthy person in this proof-of-concept study is high. In this chapter, we will discuss the significantly expressed miRNAs detected, illustrate their relevance and compare to existing literature. The study is the first one to characterize miRNA signatures for TB disease classification from different geographic origins (Ajit 2012), and include a cohort of TB patients co-infected with HIV.

In this study, the focus was on the panels of miRNAs composed of multiple targets, rather than a single marker. To ascertain if serum miRNA signature can discriminate different categories of diseases, a strict stratification screening approach was used to minimize the number of possible confounding factors. The results from the pooled samples identified different serum miRNAs profiles. The observed distribution of miRNAs residuals could discriminate healthy (H) and pulmonary TB (PTB), latent TB infection (LTBI) and PTB, as well as PTB and PTB/HIV. The signatures identified were observed to a large extent in both populations of healthy and PTB subjects from African origin and European cohort.

Previously, the impact of geographic origin or genetic background on miRNA expression was not examined. The differences we observed could be due to number of reasons including:

(i) Differences in genetic background
(ii) Different environmental stimuli like concomitant infections
(iii) Co-morbidity /co-infection conditions: although concomitant disease was excluded as far as possible using a questionnaire, some of the conditions or diseases could have been missed.

Understanding the mechanism of transition from latent TB disease to active TB still remains challenging and incomplete. Multiple host factors are involved in this complex process (Barry, III et al. 2009). The described miRNA signatures in this study can discriminate active and latent TB, which may be related to the disease process and give insight into pathophysiology once the understanding is more complete.

Comparing our results to literature, there are however no similarities in the discriminatory miRNA signatures. This could be explained by differences in the technique and source of samples: in the mentioned study, not all microarray results could be confirmed by RT-PCR (Wang et al. 2011a). Since microarray analysis and real-time PCR are two methods with

different sensitivities and specificities, miRNAs expressed at low levels could not always be detected by both methods (Thai et al. 2007) hence comparisons of the results might not give the true picture of the biomarkers involved.

Although these miRNAs have been identified in this and other studies (Rajaram et al. 2011; Rasmussen et al. 2010; Sharbati et al. 2011; Wang et al. 2011b; Yi et al. 2012), further validation is required to answer the question as to whether these gene expression pattern are specific for TB or shared, at least in part, with diseases of similar pathophysiology but distinct etiology, and for analyzing their power to distinguish between LTBI and PTB when used as a diagnostic assay. Discrimination between PTB and other infections is crucial, since patients presenting for diagnosis will mostly be symptomatic due to a respiratory or other diseases, especially persons living with HIV/AIDS are at increased risk for infections. We analyzed miRNAs that can discriminate active TB co-infected with HIV against healthy individuals or active TB without HIV. Patterns of miRNAs were identified in pooled samples that could differentiate these conditions in HIV positive patients. Further validation in individual serum samples would have been necessary to assert the clinical value of our findings, but due to limitations in time and capacity these were not performed in this study. After comparing pooled samples, individual sample comparison showed that the levels of numerous miRNAs from both populations were significantly different. Our results support the hypothesis that the genetic background plays a role in influencing specific miRNA profiles.

There are similarities and differences observed between our results and previous reported studies, as summarized in table 10, however the one to one comparison was not possible due to the differences in analytical platforms and normalization process. For example, miRNAs belonging to the families let-7, miR-30, and miR-146 were found to be discriminatory of healthy and PTB in these two studies (Fu et al. 2011; Qi et al. 2012). The expression levels of miRNAs: miR-590-5p, miR-185, miR-660, let-7e, miR-25, miR-146a, and miR-885-5p found to be significantly different between healthy and PTB patients in the study reported by Qi and colleagues (Qi et al. 2012). However, the previous studies did not consider the genetic background of the subjects enrolled: the inclusion of two groups with different genetic background allowed us to better define serum miRNA signatures.

Table 10; Similarities and differences of miRNAs signature identified in this study and the miRNAs reported in the previous studies

miRNAs from this study	(Fu et al. 2011)	(Qi et al. 2012)
hsa-let-7e-4395517	let-7 family	let-7e, let-7 family
hsa-miR-146a-4373132	miR-146a	miR-146a
hsa-miR-148a-4373130	-	-
hsa-miR-16-4373121	-	-
hsa-miR-192-4373108	-	-
hsa-miR-193a-5p-4395392	-	miR-193 family
hsa-miR-25-4373071	-	miR-25
hsa-miR-365-4373194	miR-365 family	-
hsa-miR-451-4373360	-	-
hsa-miR-532-5p-4380928	-	miR-532 family
hsa-miR-590-5p-4395176	-	miR-590-5p
hsa-miR-660-4380925	-	miR-660
hsa-miR-885-5p-4395407	-	miR-885-5p
hsa-miR-223*-4395209	-	miR-223 family
hsa-miR-30e-4395334	miR-30 family	miR-30 family

Additionally, our study had more samples from different nationalities, where we used similar methodology: thus increase the likelihood of detecting many miRNAs that are expressed during active PTB which have not been reported elsewhere.

Despite the difference in methodologies, a signature of miRNA increase chances of detecting the diseases as compared to the use of a single miRNA. Using the 15 miRNA signature observed to be significant from pooled and individuals samples; diagnostic accuracy was between 77% and 82% by LOOCV approach and RVM respectively, whereas AUC was 0.90. In fact, diagnostic accuracy increased when classification of population based miRNAs signature was used; 81-83% for Italians and other races and 95-100% for African population. As mentioned earlier, serum miRNA signature showed less efficiency in classifying subjects belonging to the European population, which probably had a more diverse genetic background than the Ugandans and Tanzanians in the African population.

Moreover, miRNA expression can be altered in pathophysiological processes and could reflect and predict disease progression (Kim and Nam 2006). Additionally, the functions of most differentially expressed miRNAs are still largely unknown. The significant change in expression levels of these miRNAs detected in these two populations study, examples; the let-7 families suggests their involvement in regulating anti-TB immune response (Yi et al. 2012) or play an important role during pathogenesis of active TB and during infection.

4.2 Limitation and challenges

Inconsistent results from different studies, present a challenge when moving ahead in identifying miRNAs that will accurately identify PTB or risk of progression from LTBI to active PTB. It should be noted that differences in miRNA profiling methodologies, analytic approaches, sample-sizes, population types, methods of tissue archival, RNA extraction and others, are likely to affect findings in such studies.

Stringent selection criteria of the serum samples to be analysed, makes the generalization of the findings from this study limited. For examples, signature of miRNAs observed to be significantly expressed in PTB patient who are non-smokers might be different in similar case of PTB patient who smoke. However, for the proof of concept procedure, a homogenous population was critical. Further investigation of the significant signature of miRNAs detected in this study, should therefore consider comparing different pulmonary diseases to verify the diagnostic performance of these miRNAs. For future validation, methodologies should be kept homogenous with two studies, which will of course be challenging at a later stage when a technological platform suitable for point-of-care diagnostics will be introduced.

Patterns of biomarkers may be used for identifying groups of diseases, whereas others allow for differential diagnosis of distinct types of diseases, for example infectious or non-infectious diseases (Maertzdorf et al. 2012). Currently, biomarker-based differential diagnosis requires simultaneous measurement of more than one analyse which is not cost-effective for point of care diagnosis of active PTB. Focusing on differential biomarkers only might be the best solution at the moment but rather taking into account the shared ones, consequently an algorithm can be designed for screening patients with pulmonary symptoms (Maertzdorf et al. 2012).

Our findings have added to the knowledge on changes in miRNA expression profiles during PTB disease, and indicate potential for improving diagnosis, prognosis and surrogate marker for treatment. The understanding of miRNA pathophysiology in TB is still very limited. Several studies reported related phenotype in active TB patients which suggest that changes of these miRNA expression levels in active PTB may lead to the changes in immune cell profile and the alterations of the host immune response during TB disease (Wang et al. 2011a). For example, a previous study that used whole genome transcriptional profiling observed changes in expression levels of different cell types, such as macrophages and NK cells in samples from active and latent TB patients (Maertzdorf et al. 2011).Studies have also reported an increase in the proportion of CD14+,CD16+ inflammatory monocytes and a decrease in the proportion of CD4+ T cells, CD8+ T cells, and B cells in blood cells of patients with active TB (Berry et al. 2010). However, it is still not clear yet, whether this alteration of cellular composition and gene expression in active TB patients is regulated by miRNAs (Wang et al. 2011a).

Although these bio-signatures have been identified by several independent groups and possess the potential to discriminate latent *M. tuberculosis* infection and healthy individuals from active TB patients, the question still remains to be answered if these expressed gene signatures are specific for TB or shared, at least in part, with diseases of similar pathology but distinct aetiology (Maertzdorf et al. 2012). Currently, based on these finding and others from previously reported studies it is still difficult to synthesize the results to reach a conclusive opinion on the pattern of miRNAs to be used as biomarkers for PTB diagnosis. Despite advancement in molecular characterization of *M. Tuberculosis* (Smith 2003), yet very little is known about the molecular basis of bacteria-host interaction and molecular cellular mechanism during pathogenesis process and the of drug resistance. The ability of the *M. tuberculosis* to persist and multiply within alveolar macrophages after phagocytised forming the tubercle is the key to the pathogenic virulence (Houben, Nguyen, and Pieters 2006). During this era of biomarker development for TB diagnosis, understanding of host-pathogen interactions is crucial.

The immune system following alteration of miRNAs expression due to the diseases like sarcoidosis (SARC) which has significant similarities in immune activation with active PTB could have as well have a similar pathophysiological changes (Prince, Kheradmand, and Corry 2003). Despite the fact that, SARC is a non-communicable disease of unknown etiology; clinical symptoms and histological presentation of a patients with pulmonary SARC are very similar to PTB including granulomatous structures in the lung (Marchiori et al. 2011; Prince et al. 2003). For future studies, it will be interesting to compare miRNA signatures between SARC and TB patients. The complexity of interaction between genes and miRNAs, involved in disease phenotypes needs to be clarified in more depth to gain deeper understanding the pathologic mechanisms of causal or development of the disease (Schadt 2009). ‘‘Maertzdorf and colleague described a first attempt to gain a detailed insight into similarities and differences between the TB and SARC and reported miRNA expressions with a highly similar pattern in both diseases. Such pattern in patho-physiologically similar diseases as described suggests a potential future use in differential diagnosis of different lung diseases ’’ (Maertzdorf et al. 2012). However, more research has to be done to define the unique biomarkers for a specific disease.

4.3 Conclusions and recommendation

For the past decades, tuberculosis continues to be a major risk to the global population. It is a mysterious communicable infectious disease regardless the major advancement on research on control programmes. ‘‘Of the various research efforts in diverse directions for the control of tuberculosis, recently identified involvement of miRNA in mycobacterial infection has also nourished the hopes for better understanding of pathogenesis, and for developing a new class of sensitive and accurate diagnostic and prognostic biomarkers and possible new therapeutics for tuberculosis’’ (Singh et al. 2013).

‘‘Although a number of miRNAs have been identified as biomarkers to differentiate active TB and latent TB from healthy individuals´´(Fu et al. 2011; Wang et al. 2011a), ‘‘it is not clear whether such biomarkers are specific for TB or shared by other diseases´´(Singh et al. 2013). ‘‘Therefore, along with developing reliable miRNA - based biomarkers, future work is needed also to discover biomarkers for the prediction of relapse, resistance and treatment response on account to provide better treatment as well as to facilitate the testing of new drugs’’ (Singh et al. 2013). Currently, this area is in its infancy stage and need more attention and more works towards understanding the complex regulation of miRNA in tuberculosis, and for developing signatures to be used for the effective control and management of tuberculosis. The accurate and rapid diagnosis as well as ability to monitor the treatment response is very crucial for effective control and management of tuberculosis.

Our findings show both common and different disease-related miRNA expressed levels in active TB and active TB/HIV, LTBI and H individuals. Pulmonary TB pathogenesis is a complex process and could involve interplay between genes, miRNAs, and immunological system. miRNAs that are significantly expressed in active TB are patterns of both miRNAs involved in non-specific inflammatory processes and specific disease manifestations. But again, miRNAs that are expressed following the lung damages; lung cancer, active TB and other inflammations due to bacteria or virus infection suggest a potential future use in differential diagnostic tool of different lung diseases. The validation process will consider the striking unique patterns and similarity in expression profile of miRNAs in active TB against LTBI, H individuals and PTB with co-morbidity like PTB/HIV for better understanding the underlying mechanisms of pathology. This consideration could greatly benefit the definition of true biomarkers for active TB and other co-infection and or related pathogens with similar pathogenesis as results development of new array based diagnostic tools, which discriminate not only active TB disease status from healthy individuals but also between different diseases of similar pathology.

In a process of attempting to validate and link miRNAs to PTB pathogenesis or any certain biological processes, first; unique and common miRNAs based on their expression levels can be clustered. Clustered miRNAs with correlating expression patterns can reveal functional relations to biological processes to the underlying disease pathology. Secondly, standardizations of the miRNAs identification process; thus, type of sample to be used, collection, storage, amplification method and analysis will be crucial in the process. Normalization and selection of stable endogenous control is equally important. Lastly, although these miRNAs have been identified and reported by several independent groups to possess the potential signature to discriminate active TB, to answer the question as whether these expression miRNA signatures are specific for TB or shared, comparative analysis of serum miRNAs in patients with active

pulmonary, Latent TB, PTB/HIV and other most common lung diseases and TB that are on treatment has to be considered. Such design methodology could provide a more promising approach to identify a unique miRNAs to be used as biomarker diagnoses pulmonary disease, including active TB.

More research needs to be done to address some of the following challenges:

i. Standardizing the methodology in type of the samples to be used to isolate total RNAs, the use of high throughput and sensitive technique for analysis of miRNAs, stable endogenous controls to be used, improvements in data are analysis and presentation. The reported results have to repeated and reproducible.
ii. ''As intracellular bacteria, *M. Tuberculosis* depends on the tolerance of host immune system for its survival and replication, which makes it susceptible to the host gene-regulatory mechanisms. Silencing via host miRNA might be a mechanism human macrophage employs to defend against intracellular pathogens such as *M. tuberculosis''*(Guo et al. 2010). Previous reported targets observed to be regulated by miRNAs need further re-assessed; for examples immune cells (dendritic cell, macrophages, interferon-γ, few to mention) (Banchereau and Steinman 1998; Wallet, Sen, and Tisch 2005),genes or cell related to *M. tuberculosis* growth, virulence or drug resistance. A pattern of genes and miRNAs with correlating expression levels, which could indicate a possible functional relationship and reveal particular biological processes involved in pulmonary diseases can be created. The selection has to focus on the miRNAs with significance, stable and reproducible in the biological processes.
iii. Determine the main source of miRNAs involved; if originating from the host or pathogens and determine which ones play the main role. But again, should be able to differentiate miRNAs that are specific to TB disease and those changes in expression levels following inflammation of the lung: for example; inflammation due to pneumonia, asthma, or chronic pulmonary obstructive diseases. There are also pathogens or conditions that have similar pathways of pathogenesis with TB like sarcodosis causing chronic inflammatory disease. Described commonalities as well as unique signatures in miRNAs expression profiles of one or more distinct inflammatory pulmonary diseases not only have considerable implications for the design of TB biomarkers as a diagnostic tool but they also provide insights into biological processes underlying chronic inflammatory disease entities of different etiology.
iv. The results have to be validated in the large population of different geographic origins in blinded methodology, choosing the correct cut off point is also a challenge in results presentation. What changes in expression levels of these biomarkers are significant and accurate correlated with the present diseases or etiology?. To ensure similarities of the results, the cut off point for low or high expression needs to be determined.

Ideally, these expected results require simultaneous measurement of different miRNAs and target genes or immune cells, to result in meaningful conclusion which will be able to tell accurately biomarkers which are specific to diseases, conditions and should in all population regardless of their genetic differences. We conclude that miRNAs biomarker profiles not only contain disease-specific signatures but also provide insight into biological processes shared by different diseases or transition of LTBI to active TB. This promising approaches of using miRNAs as biomarkers for diagnosis might also be useful to predict treatment outcome of TB patients and early detection of resistance TB strains which can guide proper management of the patients.

Literature Cited

1. Aderaye, G., J. Bruchfeld, G. Aseffa, Y. Nigussie, K. Melaku, Y. Woldeamanuel, D. Asrat, A. Worku, H. Gaegziabher, M. Lebaad, and L. Lindquist. 2007. Pneumocystis jiroveci pneumonia and other pulmonary infections in TB smear-negative HIV-positive patients with atypical chest X-ray in Ethiopia. Scand.J.Infect.Dis. 39(11-12):1045-1053.
2. Agranoff, D., D. Fernandez-Reyes, M.C. Papadopoulos, S.A. Rojas, M. Herbster, A. Loosemore, E. Tarelli, J. Sheldon, A. Schwenk, R. Pollok, C.F. Rayner, and S. Krishna. 2006. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. Lancet 368(9540):1012-1021.

3. Ajit, S.K. 2012. Circulating microRNAs as biomarkers, therapeutic targets, and signaling molecules. Sensors.(Basel) 12(3):3359-3369.
4. Amanatidou, V., G. Syridou, M. Mavrikou, and M.N. Tsolia. 2012. Latent tuberculosis infection in children: diagnostic approaches . Eur.J.Clin.Microbiol.Infect.Dis. 31(7):1285-1294.

5. Balasubramanian, R., S. Sivasubramanian, V.K. Vijayan, R. Ramachandran, M.S. Jawahar, C.N. Paramasivan, N. Selvakumar, and P.R. Romasundararn. 1990. Five year results of a 3-month and two 5-month regimens for the treatment of sputum-positive pulmonary tuberculosis in south India. Tubercle 71(4):253-258.

6. Banchereau, J. and R.M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392(6673):245-252.

7. Barry, C.E., III, H.I. Boshoff, V. Dartois, T. Dick, S. Ehrt, J. Flynn, D. Schnappinger, R.J. Wilkinson, and D. Young. 2009. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat.Rev.Microbiol. 7(12):845-855.

8. Bas, A., G. Forsberg, S. Hammarstrom, and M.L. Hammarstrom. 2004. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes . Scand.J.Immunol. 59(6):566-573.

9. Benes, V. and M. Castoldi. 2010. Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. Methods 50(4):244-249.

10. Benjamini Y, Y.D. 2001. The control of the false discovery rate in multiple testing under dependency . Ann Stat 29:1165-1188.

11. Bernardo, B.C., F.J. Charchar, R.C. Lin, and J.R. McMullen. 2011. A MicroRNA Guide for Clinicians and Basic Scientists: Background and Experimental Techniques. Heart Lung Circ.

12. Berry, M.P., C.M. Graham, F.W. McNab, Z. Xu, S.A. Bloch, T. Oni, K.A. Wilkinson, R. Banchereau, J. Skinner, R.J. Wilkinson, C. Quinn, D. Blankenship, R. Dhawan, J.J. Cush, A. Mejias, O. Ramilo, O.M. Kon, V. Pascual, J. Banchereau, D. Chaussabel, and A. O'Garra. 2010. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 466(7309):973-977.

13. Bloomston, M., W.L. Frankel, F. Petrocca, S. Volinia, H. Alder, J.P. Hagan, C.G. Liu, D. Bhatt, C. Taccioli, and C.M. Croce. 2007. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 297(17):1901-1908.

14. Boehme, C.C., P. Nabeta, D. Hillemann, M.P. Nicol, S. Shenai, F. Krapp, J. Allen, R. Tahirli, R. Blakemore, R. Rustomjee, A. Milovic, M. Jones, S.M. O'Brien, D.H. Persing, S. Ruesch-Gerdes, E. Gotuzzo, C. Rodrigues, D. Alland, and M.D. Perkins. 2010. Rapid molecular detection of tuberculosis and rifampin resistance. N.Engl.J.Med. 363(11):1005-1015.

15. Bolstad, B.M., R.A. Irizarry, M. Astrand, and T.P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias . Bioinformatics. 19(2):185-193.

16. Braun AC, W.U.H.S. 2012. Classification in High-Dimensional Feature Spaces—Assessment Using SVM, IVM and RVM With Focus on Simulated EnMAP Data . IEEE Journal of Selected Topics in Applied Earth Observations and Remote Sensing 5(2):436-443.

17. Brodersen, P. and O. Voinnet. 2009. Revisiting the principles of microRNA target recognition and mode of action. Nat.Rev.Mol.Cell Biol. 10(2):141-148.

18. Calin, G.A. and C.M. Croce. 2006. MicroRNA signatures in human cancers. Nat.Rev.Cancer 6(11):857-866.

19. Calin, G.A., M. Ferracin, A. Cimmino, L.G. Di, M. Shimizu, S.E. Wojcik, M.V. Iorio, R. Visone, N.I. Sever, M. Fabbri, R. Iuliano, T. Palumbo, F. Pichiorri, C. Roldo, R. Garzon, C. Sevignani, L. Rassenti, H. Alder, S. Volinia, C.G. Liu, T.J. Kipps, M. Negrini, and C.M. Croce. 2005. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N.Engl.J.Med. 353(17):1793-1801.

20. Castoldi, M., S. Schmidt, V. Benes, M. Noerholm, A.E. Kulozik, M.W. Hentze, and M.U. Muckenthaler. 2006. A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). RNA. 12(5):913-920.

21. Chen, C., D.A. Ridzon, A.J. Broomer, Z. Zhou, D.H. Lee, J.T. Nguyen, M. Barbisin, N.L. Xu, V.R. Mahuvakar, M.R. Andersen, K.Q. Lao, K.J. Livak, and K.J. Guegler. 2005. Real-

time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33(20):e179.

22. Chen, X., Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, Y. Zhang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, J. Wang, K. Zen, J. Zhang, and C.Y. Zhang. 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 18(10):997-1006.

23. Comstock, G.W., V.T. Livesay, and S.F. Woolpert. 1974. The prognosis of a positive tuberculin reaction in childhood and adolescence. Am.J.Epidemiol. 99(2):131-138.

24. Corbett, E.L. 2003. HIV and tuberculosis: surveillance revisited . Int J.Tuberc.Lung Dis. 7(8):709.

25. Corbett, E.L., S. Charalambous, K. Fielding, T. Clayton, R.J. Hayes, K.M. De Cock, and G.J. Churchyard. 2003. Stable incidence rates of tuberculosis (TB) among human immunodeficiency virus (HIV)-negative South African gold miners during a decade of epidemic HIV-associated TB . J.Infect.Dis. 188(8):1156-1163.

26. Corbett, E.L., B. Marston, G.J. Churchyard, and K.M. De Cock. 2006. Tuberculosis in sub-Saharan Africa: opportunities, challenges, and change in the era of antiretroviral treatment. Lancet 367(9514):926-937.

27. Cuevas, L.E., R. Browning, P. Bossuyt, M. Casenghi, M.F. Cotton, A.T. Cruz, L.E. Dodd, F. Drobniewski, M. Gale, S.M. Graham, M. Grzemska, N. Heinrich, A.C. Hesseling, R. Huebner, P. Jean-Philippe, S.K. Kabra, B. Kampmann, D. Lewinsohn, M. Li, C. Lienhardt, A.M. Mandalakas, B.J. Marais, H.J. Menzies, G. Montepiedra, C. Mwansambo, R. Oberhelman, P. Palumbo, E. Russek-Cohen, D.E. Shapiro, B. Smith, G. Soto-Castellares, J.R. Starke, S. Swaminathan, C. Wingfield, and C. Worrell. 2012. Evaluation of tuberculosis diagnostics in children: 2. Methodological issues for conducting and reporting research evaluations of tuberculosis diagnostics for intrathoracic tuberculosis in children. Consensus from an expert panel. J.Infect.Dis. 205 Suppl 2:S209-S215.

28. Deo, A., J. Carlsson, and A. Lindlof. 2011. How to choose a normalization strategy for miRNA quantitative real-time (qPCR) arrays . J.Bioinform.Comput.Biol. 9(6):795-812.

29. Dheda, K., J.F. Huggett, J.S. Chang, L.U. Kim, S.A. Bustin, M.A. Johnson, G.A. Rook, and A. Zumla. 2005. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal.Biochem. 344(1):141-143.

30. Doherty, T.M., R.S. Wallis, and A. Zumla. 2009. Biomarkers of disease activity, cure, and relapse in tuberculosis. Clin.Chest Med. 30(4):783-96, x.

31. Drobac, P.C., S.S. Shin, P. Huamani, S. Atwood, J. Furin, M.F. Franke, C. Lastimoso, and C.H. del. 2012. Risk factors for in-hospital mortality among children with tuberculosis: the 25-year experience in Peru . Pediatrics 130(2):e373-e379.

32. Dye, C., S. Scheele, P. Dolin, V. Pathania, and M.C. Raviglione. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA 282(7):677-686.

33. Dye, C. and B.G. Williams. 2010. The population dynamics and control of tuberculosis. Science 328(5980):856-861.

34. Fine, P.E., J. Bruce, J.M. Ponnighaus, P. Nkhosa, A. Harawa, and E. Vynnycky. 1999. Tuberculin sensitivity: conversions and reversions in a rural African population. Int.J.Tuberc.Lung Dis. 3(11):962-975.

35. Foulds, J. and R. O'Brien. 1998. New tools for the diagnosis of tuberculosis: the perspective of developing countries. Int.J.Tuberc.Lung Dis. 2(10):778-783.

36. Friend, J.H., S. Mason, A.D. Harries, F.M. Salaniponi, and F. Neuhann. 2005. Management and outcome of TB suspects admitted to the medical wards of a central hospital in Malawi. Trop.Doct. 35(2):93-95.

37. Fu, Y., Z. Yi, X. Wu, J. Li, and F. Xu. 2011. Circulating MicroRNAs in Patients with Active Pulmonary Tuberculosis. J.Clin.Microbiol. 49(12):4246-4251.

38. Gandhi, N.R., A. Moll, A.W. Sturm, R. Pawinski, T. Govender, U. Lalloo, K. Zeller, J. Andrews, and G. Friedland. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa . Lancet 368(9547):1575-1580.

39. Gil-Setas, A., L. Torroba, J.L. Fernandez, V. Martinez-Artola, and J. Olite. 2004. Evaluation of the MB/BacT system compared with Middlebrook 7H11 and Lowenstein-Jensen media for detection and recovery of mycobacteria from clinical specimens . Clin.Microbiol.Infect. 10(3):224-228.

40. Good P.I. 2005. Permutation, parametric, and bootstrap tests of hypotheses . Springer:336.

41. Guo, W., J.T. Li, X. Pan, L. Wei, and J.Y. Wu. 2010. Candidate Mycobacterium tuberculosis genes targeted by human microRNAs. Protein Cell 1(5):419-421.

42. Hammond, S.M. 2006. MicroRNAs as oncogenes. Curr.Opin.Genet.Dev. 16(1):4-9.

43. Hargreaves, N.J., O. Kadzakumanja, C.J. Whitty, F.M. Salaniponi, A.D. Harries, and S.B. Squire. 2001. 'Smear-negative' pulmonary tuberculosis in a DOTS programme: poor outcomes in an area of high HIV seroprevalence. Int.J.Tuberc.Lung Dis. 5(9):847-854.

44. Hatherill, M., M. Hanslo, T. Hawkridge, F. Little, L. Workman, H. Mahomed, M. Tameris, S. Moyo, H. Geldenhuys, W. Hanekom, L. Geiter, and G. Hussey. 2010. Structured approaches for the screening and diagnosis of childhood tuberculosis in a high prevalence region of South Africa . Bull.World Health Organ 88(4):312-320.

45. Hawken, M., L. Nganga, H. Meme, J. Chakaya, and J. Porter. 1999. Is cough alone adequate to screen HIV-positive persons for tuberculosis preventive therapy in developing countries? Int.J.Tuberc.Lung Dis. 3(6):540-541.

46. Hawken, M.P., D.W. Muhindi, J.M. Chakaya, S.M. Bhatt, L.W. Ng'ang'a, and J.D. Porter. 2001. Under-diagnosis of smear-positive pulmonary tuberculosis in Nairobi, Kenya . Int J.Tuberc.Lung Dis. 5(4):360-363.

47. Hesseling, A.C., S. Kim, S. Madhi, S. Nachman, H.S. Schaaf, A. Violari, T.C. Victor, G. McSherry, C. Mitchell, and M.F. Cotton. 2012. High prevalence of drug resistance amongst HIV-exposed and -infected children in a tuberculosis prevention trial . Int J.Tuberc.Lung Dis. 16(2):192-195.

48. Hong Kong Chest Service/British Medical Research Council. 1991. Controlled trial of 2, 4, and 6 months of pyrazinamide in 6-month, three-times-weekly regimens for smear-positive pulmonary tuberculosis, including an assessment of a combined preparation of isoniazid, rifampin, and pyrazinamide. Results at 30 months. Am.Rev.Respir.Dis. 143(4):700-706.

49. Horsburgh, C.R., Jr. 2004. Priorities for the treatment of latent tuberculosis infection in the United States. N.Engl.J.Med. 350(20):2060-2067.

50. Houben, E.N., L. Nguyen, and J. Pieters. 2006. Interaction of pathogenic mycobacteria with the host immune system. Curr.Opin.Microbiol. 9(1):76-85.

51. Iorio, M.V., M. Ferracin, C.G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Menard, J.P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Nenci, G.A. Calin, P. Querzoli, M. Negrini, and C.M. Croce. 2005. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 65(16):7065-7070.

52. Jacobsen, M., D. Repsilber, A. Gutschmidt, A. Neher, K. Feldmann, H.J. Mollenkopf, A. Ziegler, and S.H. Kaufmann. 2007. Candidate biomarkers for discrimination

between infection and disease caused by Mycobacterium tuberculosis. J.Mol.Med.(Berl) 85(6):613-621.

53. Keeler, E., M.D. Perkins, P. Small, C. Hanson, S. Reed, J. Cunningham, J.E. Aledort, L. Hillborne, M.E. Rafael, F. Girosi, and C. Dye. 2006. Reducing the global burden of tuberculosis: the contribution of improved diagnostics. Nature 444 Suppl 1:49-57.

54. Kim, V.N. 2005. MicroRNA biogenesis: coordinated cropping and dicing. Nat.Rev.Mol.Cell Biol. 6(5):376-385.

55. Kim, V.N. and J.W. Nam. 2006. Genomics of microRNA. Trends Genet. 22(3):165-173.

56. Kirschner, M.B., S.C. Kao, J.J. Edelman, N.J. Armstrong, M.P. Vallely, Z.N. van, and G. Reid. 2011. Haemolysis during sample preparation alters microRNA content of plasma . PLoS.One. 6(9):e24145.

57. Kopreski, M.S., F.A. Benko, and C.D. Gocke. 2001. Circulating RNA as a tumor marker: detection of 5T4 mRNA in breast and lung cancer patient serum. Ann.N.Y.Acad.Sci. 945:172-178.

58. Krzywinski, M., J. Schein, I. Birol, J. Connors, R. Gascoyne, D. Horsman, S.J. Jones, and M.A. Marra. 2009. Circos: an information aesthetic for comparative genomics . Genome Res. 19(9):1639-1645.

59. Lawn, S.D. and G. Churchyard. 2009. Epidemiology of HIV-associated tuberculosis. Curr.Opin.HIV.AIDS 4(4):325-333.

60. Lawn, S.D. and A.I. Zumla. 2011. Tuberculosis. Lancet 378(9785):57-72.

61. Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, and V.N. Kim. 2003. The nuclear RNase III Drosha initiates microRNA processing . Nature 425(6956):415-419.

62. Lesho, E., F.J. Forestiero, M.H. Hirata, R.D. Hirata, L. Cecon, F.F. Melo, S.H. Paik, Y. Murata, E.W. Ferguson, Z. Wang, and G.T. Ooi. 2011. Transcriptional responses of host peripheral blood cells to tuberculosis infection. Tuberculosis.(Edinb.) 91(5):390-399.

63. Liam, C.K. and B.G. Tang. 1997. Delay in the diagnosis and treatment of pulmonary tuberculosis in patients attending a university teaching hospital. Int.J.Tuberc.Lung Dis. 1(4):326-332.

64. Liu, C.G., G.A. Calin, B. Meloon, N. Gamliel, C. Sevignani, M. Ferracin, C.D. Dumitru, M. Shimizu, S. Zupo, M. Dono, H. Alder, F. Bullrich, M. Negrini, and C.M. Croce.

2004. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. Proc.Natl.Acad.Sci.U.S.A 101(26):9740-9744.

65. Liu, J. 2008. Control of protein synthesis and mRNA degradation by microRNAs. Curr.Opin.Cell Biol. 20(2):214-221.

66. Liu, Y., X. Wang, J. Jiang, Z. Cao, B. Yang, and X. Cheng. 2011. Modulation of T cell cytokine production by miR-144* with elevated expression in patients with pulmonary tuberculosis. Mol.Immunol. 48(9-10):1084-1090.

67. Lo, Y.M., N.B. Tsui, R.W. Chiu, T.K. Lau, T.N. Leung, M.M. Heung, A. Gerovassili, Y. Jin, K.H. Nicolaides, C.R. Cantor, and C. Ding. 2007. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. Nat.Med. 13(2):218-223.

68. Lu, J., G. Getz, E.A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B.L. Ebert, R.H. Mak, A.A. Ferrando, J.R. Downing, T. Jacks, H.R. Horvitz, and T.R. Golub. 2005. MicroRNA expression profiles classify human cancers. Nature 435(7043):834-838.

69. Madebo, T. and B. Lindtjorn. 1999. Delay in Treatment of Pulmonary Tuberculosis: An Analysis of Symptom Duration Among Ethiopian Patients. MedGenMed.:E6.

70. Maertzdorf, J., D. Repsilber, S.K. Parida, K. Stanley, T. Roberts, G. Black, G. Walzl, and S.H. Kaufmann. 2011. Human gene expression profiles of susceptibility and resistance in tuberculosis. Genes Immun. 12(1):15-22.

71. Maertzdorf, J., J. Weiner, III, H.J. Mollenkopf, T. Bauer, A. Prasse, J. Muller-Quernheim, and S.H. Kaufmann. 2012. Common patterns and disease-related signatures in tuberculosis and sarcoidosis. Proc.Natl.Acad.Sci.U.S.A 109(20):7853-7858.

72. Marais, B.J., A.C. Hesseling, R.P. Gie, H.S. Schaaf, D.A. Enarson, and N. Beyers. 2006. The bacteriologic yield in children with intrathoracic tuberculosis. Clin.Infect.Dis. 42(8):e69-e71.

73. Marais, B.J., C.C. Obihara, R.M. Warren, H.S. Schaaf, R.P. Gie, and P.R. Donald. 2005. The burden of childhood tuberculosis: a public health perspective. Int J.Tuberc.Lung Dis. 9(12):1305-1313.

74. Marais, B.J., M.C. Raviglione, P.R. Donald, A.D. Harries, A.L. Kritski, S.M. Graham, W.M. El-Sadr, M. Harrington, G. Churchyard, P. Mwaba, I. Sanne, S.H. Kaufmann, C.J. Whitty, R. Atun, and A. Zumla. 2010. Scale-up of services and research priorities for diagnosis, management, and control of tuberculosis: a call to action. Lancet 375(9732):2179-2191.

75. Marchiori, E., G. Zanetti, M.M. Barreto, F.T. de Andrade, and R.S. Rodrigues. 2011. Atypical distribution of small nodules on high resolution CT studies: patterns and differentials. Respir.Med. 105(9):1263-1267.

76. Martinez, A., S. Balandrano, A. Parissi, A. Zuniga, M. Sanchez, J. Ridderhof, H.B. Lipman, and B. Madison. 2005. Evaluation of new external quality assessment guidelines involving random blinded rechecking of acid-fast bacilli smears in a pilot project setting in Mexico Int J.Tuberc.Lung Dis. 9(3):301-305.

77. McNerney, R. and P. Daley. 2011. Towards a point-of-care test for active tuberculosis: obstacles and opportunities. Nat.Rev.Microbiol. 9(3):204-213.

78. McNerney, R., M. Maeurer, I. Abubakar, B. Marais, T.D. McHugh, N. Ford, K. Weyer, S. Lawn, M.P. Grobusch, Z. Memish, S.B. Squire, G. Pantaleo, J. Chakaya, M. Casenghi, G.B. Migliori, P. Mwaba, L. Zijenah, M. Hoelscher, H. Cox, S. Swaminathan, P.S. Kim, M. Schito, A. Harari, M. Bates, S. Schwank, J. O'Grady, M. Pletschette, L. Ditui, R. Atun, and A. Zumla. 2012. Tuberculosis diagnostics and biomarkers: needs, challenges, recent advances, and opportunities. J.Infect.Dis. 205 Suppl 2:S147-S158.

79. McShane, H. 2005. Co-infection with HIV and TB: double trouble. Int.J.STD AIDS 16(2):95-100.

80. Mendelson, M. 2007. Diagnosing tuberculosis in HIV-infected patients: challenges and future prospects. Br.Med.Bull. 81-82:149-165.

81. Menzies, D. 1999. Interpretation of repeated tuberculin tests. Boosting, conversion, and reversion. Am.J.Respir.Crit Care Med. 159(1):15-21.

82. Menzies, D., M. Pai, and G. Comstock. 2007. Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. Ann.Intern.Med. 146(5):340-354.

83. Mestdagh, P., T. Feys, N. Bernard, S. Guenther, C. Chen, F. Speleman, and J. Vandesompele. 2008. High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. Nucleic Acids Res. 36(21):e143.

84. Meyer, S.U., M.W. Pfaffl, and S.E. Ulbrich. 2010. Normalization strategies for microRNA profiling experiments: a 'normal' way to a hidden layer of complexity? Biotechnol.Lett. 32(12):1777-1788.

85. Miotto, P., G. Mwangoka, I.C. Valente, L. Norbis, G. Sotgiu, R. Bosu, A. Ambrosi, L.R. Codecasa, D. Goletti, A. Matteelli, E.N. Ntinginya, F. Aloi, N. Heinrich, K. Reither, and D.M. Cirillo. 2013. miRNA signatures in sera of patients with active pulmonary tuberculosis. PLoS.One. 8(11):e80149.

86. Mistry, R., J.M. Cliff, C.L. Clayton, N. Beyers, Y.S. Mohamed, P.A. Wilson, H.M. Dockrell, D.M. Wallace, P.D. van Helden, K. Duncan, and P.T. Lukey. 2007. Gene-expression patterns in whole blood identify subjects at risk for recurrent tuberculosis. J.Infect.Dis. 195(3):357-365.

87. Mitchell, P.S., R.K. Parkin, E.M. Kroh, B.R. Fritz, S.K. Wyman, E.L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K.C. O'Briant, A. Allen, D.W. Lin, N. Urban, C.W. Drescher, B.S. Knudsen, D.L. Stirewalt, R. Gentleman, R.L. Vessella, P.S. Nelson, D.B. Martin, and M. Tewari. 2008. Circulating microRNAs as stable blood-based markers for cancer detection. Proc.Natl.Acad.Sci.U.S.A 105(30):10513-10518.

88. Nana-Sinkam, S.P., M.G. Hunter, G.J. Nuovo, T.D. Schmittgen, R. Gelinas, D. Galas, and C.B. Marsh. 2009. Integrating the MicroRNome into the study of lung disease. Am.J.Respir.Crit Care Med. 179(1):4-10.

89. Nelson, P.T., D.A. Baldwin, L.M. Scearce, J.C. Oberholtzer, J.W. Tobias, and Z. Mourelatos. 2004. Microarray-based, high-throughput gene expression profiling of microRNAs. Nat.Methods 1(2):155-161.

90. Newton, S.M., A.J. Brent, S. Anderson, E. Whittaker, and B. Kampmann. 2008. Paediatric tuberculosis. Lancet Infect.Dis. 8(8):498-510.

91. O'Connell, R.M., D.S. Rao, A.A. Chaudhuri, and D. Baltimore. 2010. Physiological and pathological roles for microRNAs in the immune system. Nat.Rev.Immunol. 10(2):111-122.

92. O'Grady, J., M. Maeurer, P. Mwaba, N. Kapata, M. Bates, M. Hoelscher, and A. Zumla. 2011. New and improved diagnostics for detection of drug-resistant pulmonary tuberculosis. Curr.Opin.Pulm.Med. 17(3):134-141.

93. Oglesby, I.K., N.G. McElvaney, and C.M. Greene. 2010. MicroRNAs in inflammatory lung disease--master regulators or target practice? Respir.Res. 11:148.

94. Olivier, I. and d.T. Loots. 2012. A metabolomics approach to characterise and identify various Mycobacterium species. J.Microbiol.Methods 88(3):419-426.

95. Park, N.J., H. Zhou, D. Elashoff, B.S. Henson, D.A. Kastratovic, E. Abemayor, and D.T. Wong. 2009. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. Clin.Cancer Res. 15(17):5473-5477.

96. Parry, C.M. 1993. Sputum smear negative pulmonary tuberculosis. Trop.Doct. 23(4):145-146.

97. Perkins, M.D. 2000. New diagnostic tools for tuberculosis. Int.J.Tuberc.Lung Dis. 4(12 Suppl 2):S182-S188.

98. Perkins, M.D. and J. Cunningham. 2007. Facing the crisis: improving the diagnosis of tuberculosis in the HIV era. J.Infect.Dis. 196 Suppl 1:S15-S27.

99. Pradervand, S., J. Weber, J. Thomas, M. Bueno, P. Wirapati, K. Lefort, G.P. Dotto, and K. Harshman. 2009. Impact of normalization on miRNA microarray expression profiling . RNA. 15(3):493-501.

100. Prince, J.E., F. Kheradmand, and D.B. Corry. 2003. 16. Immunologic lung disease. J.Allergy Clin.Immunol. 111(2 Suppl):S613-S623.

101. Qi, Y., L. Cui, Y. Ge, Z. Shi, K. Zhao, X. Guo, D. Yang, H. Yu, L. Cui, Y. Shan, M. Zhou, H. Wang, and Z. Lu. 2012. Altered serum microRNAs as biomarkers for the early diagnosis of pulmonary tuberculosis infection. BMC.Infect.Dis. 12:384.

102. Rajaram, M.V., B. Ni, J.D. Morris, M.N. Brooks, T.K. Carlson, B. Bakthavachalu, D.R. Schoenberg, J.B. Torrelles, and L.S. Schlesinger. 2011. Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. Proc.Natl.Acad.Sci.U.S.A 108(42):17408-17413.

103. Rasmussen, K.D., S. Simmini, C. Abreu-Goodger, N. Bartonicek, G.M. Di, D. Bilbao-Cortes, R. Horos, L.M. Von, A.J. Enright, and D. O'Carroll. 2010. The miR-144/451 locus is required for erythroid homeostasis. J.Exp.Med. 207(7):1351-1358.

104. Risso, D., M.S. Massa, M. Chiogna, and C. Romualdi. 2009. A modified LOESS normalization applied to microRNA arrays: a comparative evaluation . Bioinformatics. 25(20):2685-2691.

105. Roldo, C., E. Missiaglia, J.P. Hagan, M. Falconi, P. Capelli, S. Bersani, G.A. Calin, S. Volinia, C.G. Liu, A. Scarpa, and C.M. Croce. 2006. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J.Clin.Oncol. 24(29):4677-4684.

106. Salani, R., B. Davidson, M. Fiegl, C. Marth, E. Muller-Holzner, G. Gastl, H.Y. Huang, J.C. Hsiao, H.S. Lin, T.L. Wang, B.L. Lin, and I. Shih. 2007. Measurement of cyclin E genomic copy number and strand length in cell-free DNA distinguish malignant versus benign effusions. Clin.Cancer Res. 13(19):5805-5809.

107. Salibian-Barrera M, Y.V. 2008. High breakdown point robust regression with censored data . Ann Stat 36:118-146.

108. Santin, M., L. Munoz, and D. Rigau. 2012. Interferon-c Release Assays for the Diagnosis of Tuberculosis and Tuberculosis Infection in HIV-Infected Adults: A Systematic Review and Meta-Analysis. PLoS ONE 7(3):e32482.

109. Scarparo, C., P. Piccoli, A. Rigon, G. Ruggiero, P. Ricordi, and C. Piersimoni. 2002. Evaluation of the BACTEC MGIT 960 in comparison with BACTEC 460 TB for detection and recovery of mycobacteria from clinical specimens . Diagn.Microbiol.Infect.Dis. 44(2):157-161.

110. Schadt, E.E. 2009. Molecular networks as sensors and drivers of common human diseases. Nature 461(7261):218-223.

111. Selwyn, P.A., D. Hartel, V.A. Lewis, E.E. Schoenbaum, S.H. Vermund, R.S. Klein, A.T. Walker, and G.H. Friedland. 1989. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. N.Engl.J.Med. 320(9):545-550.

112. Sharbati, J., A. Lewin, B. Kutz-Lohroff, E. Kamal, R. Einspanier, and S. Sharbati. 2011. Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection. PLoS.One. 6(5):e20258.

113. Sharma, S.K., S. Mohanan, and A. Sharma. 2012. Relevance of latent TB infection in areas of high TB prevalence. Chest 142(3):761-773.

114. Singh, Y., V. Kaul, A. Mehra, S. Chatterjee, S. Tousif, V.P. Dwivedi, M. Suar, K.L. Van, W.R. Bishai, and G. Das. 2013. Mycobacterium tuberculosis controls microRNA-99b (miR-99b) expression in infected murine dendritic cells to modulate host immunity. J.Biol.Chem. 288(7):5056-5061.

115. Sioud, M. and O. Rosok. 2004. Profiling microRNA expression using sensitive cDNA probes and filter arrays. Biotechniques 37(4):574-580.

116. Smith, I. 2003. Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence. Clin.Microbiol.Rev. 16(3):463-496.

117. Squire, S.B., A.K. Belaye, A. Kashoti, F.M. Salaniponi, C.J. Mundy, S. Theobald, and J. Kemp. 2005. 'Lost' smear-positive pulmonary tuberculosis cases: where are they and why did we lose them? Int.J.Tuberc.Lung Dis. 9(1):25-31.

118. Steingart, K.R., H. Sohn, I. Schiller, L.A. Kloda, C.C. Boehme, M. Pai, and N. Dendukuri. 2013. Xpert(R) MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane.Database.Syst.Rev. 1:CD009593.

119. Steinhoff, C. and M. Vingron. 2006. Normalization and quantification of differential expression in gene expression microarrays . Brief.Bioinform. 7(2):166-177.

120. Suzuki, H.I., K. Yamagata, K. Sugimoto, T. Iwamoto, S. Kato, and K. Miyazono. 2009. Modulation of microRNA processing by p53. Nature 460(7254):529-533.

121. Swarup, V. and M.R. Rajeswari. 2007. Circulating (cell-free) nucleic acids--a promising, non-invasive tool for early detection of several human diseases. FEBS Lett. 581(5):795-799.

122. Takamizawa, J., H. Konishi, K. Yanagisawa, S. Tomida, H. Osada, H. Endoh, T. Harano, Y. Yatabe, M. Nagino, Y. Nimura, T. Mitsudomi, and T. Takahashi. 2004. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 64(11):3753-3756.

123. Thai, T.H., D.P. Calado, S. Casola, K.M. Ansel, C. Xiao, Y. Xue, A. Murphy, D. Frendewey, D. Valenzuela, J.L. Kutok, M. Schmidt-Supprian, N. Rajewsky, G. Yancopoulos, A. Rao, and K. Rajewsky. 2007. Regulation of the germinal center response by microRNA-155. Science 316(5824):604-608.

124. Thomson, J.M., J. Parker, C.M. Perou, and S.M. Hammond. 2004. A custom microarray platform for analysis of microRNA gene expression. Nat.Methods 1(1):47-53.

125. Tomankova, T., M. Petrek, and E. Kriegova. 2010. Involvement of microRNAs in physiological and pathological processes in the lung. Respir.Res. 11:159.

126. Tricarico, C., P. Pinzani, S. Bianchi, M. Paglierani, V. Distante, M. Pazzagli, S.A. Bustin, and C. Orlando. 2002. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal.Biochem. 309(2):293-300.

127. Wallet, M.A., P. Sen, and R. Tisch. 2005. Immunoregulation of dendritic cells. Clin.Med.Res. 3(3):166-175.

128. Wallis, R.S., P. Kim, S. Cole, D. Hanna, B.B. Andrade, M. Maeurer, M. Schito, and A. Zumla. 2013. Tuberculosis biomarkers discovery: developments, needs, and challenges . Lancet Infect.Dis. 13(4):362-372.

129. Wallis, R.S., M. Pai, D. Menzies, T.M. Doherty, G. Walzl, M.D. Perkins, and A. Zumla. 2010. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. Lancet 375(9729):1920-1937.

130. Walzl, G., K. Ronacher, W. Hanekom, T.J. Scriba, and A. Zumla. 2011. Immunological biomarkers of tuberculosis. Nat.Rev.Immunol. 11(5):343-354.

131. Wang, C., S. Yang, G. Sun, X. Tang, S. Lu, O. Neyrolles, and Q. Gao. 2011a. Comparative miRNA expression profiles in individuals with latent and active tuberculosis. PLoS.One. 6(10):e25832.

132. Wang, R., N. Li, Y. Zhang, Y. Ran, and J. Pu. 2011b. Circulating MicroRNAs are promising novel biomarkers of acute myocardial infarction. Intern.Med. 50(17):1789-1795.

133. Wang, T., X. Qian, Z. Wang, L. Wang, L. Yu, Y. Ding, and B. Liu. 2009. Detection of cell-free BIRC5 mRNA in effusions and its potential diagnostic value for differentiating malignant and benign effusions. Int.J.Cancer 125(8):1921-1925.

134. Weinmann, L., J. Hock, T. Ivacevic, T. Ohrt, J. Mutze, P. Schwille, E. Kremmer, V. Benes, H. Urlaub, and G. Meister. 2009. Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. Cell 136(3):496-507.

135. Weyer, K., S. Carai, and P. Nunn. 2011. Viewpoint TB diagnostics: what does the world really need? J.Infect.Dis. 204 Suppl 4:S1196-S1202.

136. WHO. WHO. Global tuberculosis control-surveillance, planning,financing: WHO report 2008, document WHO/HTM/TB/2008.393.Geneva: World Health Organization, 2008.

137. WHO. WHO. Global tuberculosis control. Geneva: World Health Organization, 2010. 2010a. http://whqlibdoc.who.int/publications/2010/ 9789241564069_eng.pdf (accessed Dec 15, 2010).

138. WHO. 2010b. WHO. The global plan to stop TB 2011–2015: transforming the fight towards elimination of tuberculosis. Geneva: World Health Organization, 2010. http://whqlibdoc.who.int/publications/2010/9789241500340_eng.pdf.

139. WHO. 2010c. World health statistics 2010. Geneva: World Health Organization, 2010. http://www.who.int/whosis/whostat/EN_WHS10_Full.pdf.

140. WHO. 2011. WHO. Global tuberculosis control. Geneva: World Health Organization, 2011. http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf.

141. WHO. WHO. Global tuberculosis control. Geneva: World Health Organization, 2013. 2013. http://whqlibdoc.who.int/publications/2010/ 9789241564069_eng.pdf (accessed Dec 15, 2010).

142. WHO. WHO. Global tuberculosis control. Geneva: World Health Organization, 2014. 2014. http://whqlibdoc.who.int/publications/2010/ 9789241564069_eng.pdf (accessed April 22, 2015).

143. Wiesen, J.L. and T.B. Tomasi. 2009. Dicer is regulated by cellular stresses and interferons. Mol.Immunol. 46(6):1222-1228.

144. Williams, A.E., S.A. Moschos, M.M. Perry, P.J. Barnes, and M.A. Lindsay. 2007a. Maternally imprinted microRNAs are differentially expressed during mouse and human lung development. Dev.Dyn. 236(2):572-580.

145. Williams, A.E., M.M. Perry, S.A. Moschos, and M.A. Lindsay. 2007b. microRNA expression in the aging mouse lung. BMC.Genomics 8:172.

146. Williams, B.G. and C. Dye. 2003. Antiretroviral drugs for tuberculosis control in the era of HIV/AIDS. Science 301(5639):1535-1537.

147. Wu, J., C. Lu, N. Diao, S. Zhang, S. Wang, F. Wang, Y. Gao, J. Chen, L. Shao, J. Lu, X. Zhang, X. Weng, H. Wang, W. Zhang, and Y. Huang. 2012a. Analysis of microRNA expression profiling identifies miR-155 and miR-155* as potential diagnostic markers for active tuberculosis: a preliminary study. Hum.Immunol 73(1):31-37.

148. Wu, J., C. Lu, N. Diao, S. Zhang, S. Wang, F. Wang, Y. Gao, J. Chen, L. Shao, J. Lu, X. Zhang, X. Weng, H. Wang, W. Zhang, and Y. Huang. 2012b. Analysis of microRNA expression profiling identifies miR-155 and miR-155* as potential diagnostic markers for active tuberculosis: a preliminary study. Hum.Immunol. 73(1):31-37.

149. Xie, Y., N.W. Todd, Z. Liu, M. Zhan, H. Fang, H. Peng, M. Alattar, J. Deepak, S.A. Stass, and F. Jiang. 2010. Altered miRNA expression in sputum for diagnosis of non-small cell lung cancer. Lung Cancer 67(2):170-176.

150. Yanaihara, N., N. Caplen, E. Bowman, M. Seike, K. Kumamoto, M. Yi, R.M. Stephens, A. Okamoto, J. Yokota, T. Tanaka, G.A. Calin, C.G. Liu, C.M. Croce, and C.C. Harris. 2006. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9(3):189-198.

151. Yi, Z., Y. Fu, R. Ji, R. Li, and Z. Guan. 2012. Altered microRNA signatures in sputum of patients with active pulmonary tuberculosis. PLoS.One. 7(8):e43184.

152. Zar, H.J., D. Hanslo, P. Apolles, G. Swingler, and G. Hussey. 2005. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. Lancet 365(9454):130-134.

153. Zou, K.H., W.J. Hall, and D.E. Shapiro. 1997. Smooth non-parametric receiver operating characteristic (ROC) curves for continuous diagnostic tests. Stat Med. 16(19):2143-2156.

154. Zumla, A., I. Abubakar, M. Raviglione, M. Hoelscher, L. Ditiu, T.D. McHugh, S.B. Squire, H. Cox, N. Ford, R. McNerney, B. Marais, M. Grobusch, S.D. Lawn, G.B. Migliori, P. Mwaba, J. O'Grady, M. Pletschette, A. Ramsay, J. Chakaya, M. Schito, S. Swaminathan, Z. Memish, M. Maeurer, and R. Atun. 2012. Drug-resistant tuberculosis--current dilemmas, unanswered questions, challenges, and priority needs. J.Infect.Dis. 205 Suppl 2:S228-S240.

155. Zumla, A. and W.W. Yew. 2011. New tools for achieving tuberculosis control: progress and reflexion. Int J.Tuberc.Lung Dis. 15(3):285-286.

Appendix 1;Questionnaires for TB patients and healthy control

Questionnaire – TB Patients

Filled by _____ **e-mail** _____

Patient's name and Surname _____ **Sex (M/F)** _____

ID_____ **Birth date** _____ **Country of birth** _____

Pregnancy status: □ Y (week: _____) □ N □ Not applicable

Smoker? □ Y □ N

Enrolment criteria:

□ Active pulmonary TB (culture positive for MTB)

□ Active extra-pulmonary TB (culture positive for MTB)

Specify: _____

□ Latent TB infection (test TIGRA positive, recent contact of AFB-positive active TB cases)

□ Latent TB infection (test TIGRA positive, non recent contact of AFB-positive active TB cases)

□ Patients with MTB-HIV co-infection (test TIGRA positive, culture negative for MTB)

□ HIV patients with active TB (culture positive for MTB)

Test TIGRA: □ Positive □ Negative □ To be done

If to be done: ( ) withdrawing available ( ) Not available

PPD: □ Positive (induration mm: _____) □ Negative □ Not available

HIV: □ Positive □ Negative

If Positive provide cell count (whenever available): _____

AIDS: □ Positive □ Negative

Case history

- Diabetes □ N □ Y
- Renal failure □ N □ Y
- Silicosis □ N □ Y
- Sarcoidosis □ N □ Y
- Cancer □ N □ Y Specify: _____
- **Transplantation**

  □ N □ Y Specify: _____
- **Intravenous drugs use**

  □ N □ Y Specify: _____ N of months: _____
- **Antirheumatic drugs use (including steroids and biological drugs)**

  □ N □ Y Specify: _____ N of months: _____
- **Antiretroviral therapy**

  □ N □ Y Specify: _____ N of months: _____
- **Anti-TB therapy**

  □ N □ Y Specify: _____ N of months: _____
- **Immunosuppressive drugs**

  □ N □ Y Specify: _____ N of months: _____
- **Other:**

_____

Questionnaire – Controls

Filled by _____ **e-mail** _____

Patient's name and Surname _____ **Sex (M/F)** _____

ID_____ **Birth date** _____ **Country of birth** _____

Pregnancy status: □ Y (week: _____) □ N □ Not applicable

Smoker? □ Y □ N

Enrolment criteria:

□ Control (culture negative for MTB, test TIGRA negative, PPD negative. HIV negative)

□ HIV-positive with negative history of exposure to TB (culture negative for MTB, test TIGRA negative, PPD positive)

□ pulmonary infection, non tubercular (culture negative for MTB, test TIGRA negative, PPD negative, HIV negative)

□ pulmonary infection, viral (culture negative for MTB, test TIGRA negative, HIV negative)

Test TIGRA: □ Positive □ Negative □ To be done
If to be done: ( ) withdrawing available ( ) Not available

PPD: □ Positive (induration mm: _____) □ Negative □ Not available

HIV: □ Positive □ Negative
If Positive provide cell count (whenever available): _____

AIDS: □ Positive □ Negative

Case history

- Diabetes □ N □ Y
- Renal failure □ N □ Y
- Silicosis □ N □ Y
- Sarcoidosis □ N □ Y
- Cancer □ N □ Y Specify: _____

- **Transplantation**
  □ N □ Y Specify: _____
- **Intravenous drugs use**
  □ N □ Y Specify: _____ N of months: _____
- **Antirheumatic drugs use (including steroids and biological drugs)**
  □ N □ Y Specify: _____ N of months: _____
- **Antiretroviral therapy**
  □ N □ Y Specify: _____ N of months: _____
- **Anti-TB therapy**
  □ N □ Y Specify: _____ N of months: _____
- **Immunosuppressive drugs**
  □ N □ Y Specify: _____ N of months: _____
- **Other:**

_____

Curriculum Vitae

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EDUCATION

2010 to date	PhD student at Ludwig Maximilian University
2004-2006	Masters of Veterinary Medicine- Molecular Epidemiology.
1998-2003	Bachelor of Veterinary Medicine –Sokoine University of Agriculture

WORK EXPERIENCE

Dec 2014 to date	Biomedical Thematic group leader
July 2012 to date	IHI- Bagamoyo Branch scientific advisor
Oct 2006 – to date	Research ScientistIfakara Healthy Institute
2009 to 2011	**CO-PI**Epidemiology and Management of Tuberculosis
2009 to date	**CO-PI-** A phase III, RTS,Scandidate vaccine against malaria disease caused by *P. falciparum* infection, across diverse malaria transmission settings in Africa.
2007 -2008	**Project leader-** Malaria Specimen Bank project
2004 - Aug 2004	Research Associate at Molecular biology laboratory in Mother-offspring Malaria study in Muheza DDH and SBRI, Seattle
June 2003- Jan2004	Research assistant at TARP II and IHEPRUCA Project at SUA.

PUBLICATIONS

Mwangoka, GW, et al, 2008; **Congenital *Plasmodium falciparum* infection in neonates in Muheza District, Tanzania**

Grace w. Mwangoka, et al, 2009;,**The Ifakara Healthy Institute's Bagamoyo Research and Training Centre: a well-established clinical Trials site in Tanzania**.

Selidji T. Agnandji, KwakuPokuAsante,JohnLyimo, Johan Vekemans, Solange S. Soulanoudjingar, Ruth Owusu, MwanajaaShomari,Amanda Leach, Jose Fernandes, David Dosoo, Maria Chikawe, SaadouIssifou, Kingsley Osei-Kwakye, Marc Lievens, Maria Paricek, Stephen Apanga, **Grace Mwangoka**, Blaise Okissi, et al. 2010; Evaluation of the Safety and Immunogenicityof the RTS,S/AS01E Malaria Candidate Vaccine WhenIntegrated in the Expanded Program of Immunization

Asante KP, Abdulla S, Agnandji S, Lyimo J, Vekemans J, Soulanoudjingar S, Owusu R, Shomari M, Leach A, Jongert E, Salim N, Fernandes JF, Dosoo D, Chikawe M, Issifou S, Osei-Kwakye K, Lievens M, Paricek M, Möller T, Apanga S, **Mwangoka G**, Dubois MC, et. al**(2011)**. **Safety and efficacy of the RTS,S/AS01(E) candidate malaria vaccine given with expanded-programme-on-immunisation vaccines: 19 month follow-up of a randomised, open-label, phase 2 trial**

Albertini A, Djalle D, Faye B, Gamboa D, Luchavez J, Mationg ML**, Mwangoka G**, Oyibo W, Bennett J, Incardona S, Lee E **(2011)**. Preliminary enquiry into the availability, price and quality of malaria rapid diagnostic tests in the private healthy sector of six malaria-endemic countries. Trop Med IntHealthy

Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, Methogo BG, Doucka Y, Flamen A, Mordmüller B, Issifou S, Kremsner PG, Sacarlal J, Aide P, Lanaspa M, Aponte JJ, Nhamuave A, Quelhas D, Bassat Q, Mandjate S, Macete E, Alonso P, Abdulla S, Salim N, Juma O, Shomari M, Shubis K, Machera F, Hamad AS, Minja R, Mtoro A, Sykes A, Ahmed S, Urassa AM, Ali AM, **Mwangoka G**, Tanner M, et al. (2011). RTS,S Clinical Trials Partnership. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N Engl J Med. 2011

Grace Mwangoka, et al, . 2013; 12:Experience and challenges from clinical trials with malaria vaccines in Africa

Salim Abdulla, Nahya Salim, Francisca Machera, Richard Kamata, Omar Juma, MwanajaaShomari, SulendeKubhoja, Ali Mohammed, **Grace Mwangoka**, et al, 2013; Randomized, controlled trial of the long term safety, immunogenicity and efficacy of RTS,S/AS02$_D$ malaria vaccine in infants living in a malaria-endemic region

Paolo Miotto *, **Grace Mwangoka*** et al, 2013; miRNA Signatures in Sera of Patients with Active Pulmonary Tuberculosis

List Publication from this study

Paolo Miotto *, **Grace Mwangoka*** et al, 2013; miRNA Signatures in Sera of Patients with Active Pulmonary Tuberculosis

Affidavit

MWANGOKA,GRACE

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Street

255, BAGAMOYO

Zip code, town

TANZANIA

Country

I hereby declare, that the submitted thesis entitled

SERUM microRNAs AS BIOMARKER FOR ACTIVE

Thesis Title

AND LATENT TB INFECTION IN IMMUNOCOMPETENT

Thesis Title (cont.)

AND IMMUNODEFICIENT HOSTS

Thesis Title (cont.)

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I further declare that the electronic version of the submitted thesis is congruent with the printed version both in content and format.

Place, Date

Signature PhD Student