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Review Article

Use of Blood, Urine or Saliva-based Biomarkers in the Diagnosis of Active Pulmonary Tuberculosis - Sample Type Matters

Ngiambudulu M Francisco[1,2,3]*

[1]*Curso de Análises Clínicas, Departamento de Ciências da Saúde, Instituto Superior Politécnico Kangonjo, Luanda, Angola*

[2]*Curso de Enfermagem, Departamento de Ciências da Saúde, Instituto Superior Politécnico Kangonjo, Luanda, Angola*

[3]*Curso de Ciências Farmacêuticas, Departamento de Ciências da Saúde, Instituto Superior Politécnico Kangonjo, Luanda, Angola*

***Corresponding Author:** Ngiambudulu M. Francisco, Departamento de Ciências da Saúde, Instituto Superior Politécnico Kangonjo, Luanda, Angola.

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Abstract

Novel molecular diagnostic tools that are rapid, accurate, cost-effective, and could be used at point-of-care are urgently required if we are to achieve the goals of the End TB Strategy and reduce TB incidence by 90% by 2035. Early and rapid diagnosis of TB can significantly improve therapeutic outcomes, patient survival rates and reduce recurrence. Studies have examined blood, urine, saliva and sputum to identify new TB biomarker candidates. However, no comparative evaluation of all four fluids as a diagnostic tool to identify active and latent TB has been reported.

In the present paper, I peer-reviewed publications from PubMed databases in all languages between 2000 - April 1, 2018.

Identifying a sample type, with appropriate microbial, immunologic or molecular biomarkers to accurately diagnose active TB or latent TB, particularly if the sample was easier to collect, such as saliva; offers a unique opportunity to reduce discomfort in patients via the provision of a noninvasive TB detection method.

With its ease of access, processing and the invasiveness of collection procedures for TB molecular diagnosis, putting effort and resources into the saliva biomarker investigation may yield a point-of-care molecular diagnostic test that could revolutionize the TB field.

Keywords: *Infection; Mycobacterium tuberculosis; Biomarkers; Molecular Diagnosis; Point-of-Care*

Introduction

The incidence of tuberculosis (TB) in 2019 was 10 million new cases of active TB [1]. Most new cases occurred in the South-East Asian (44%), African (25%) and Western Pacifc (18%) Regions with smaller proportions in the Eastern Mediterranean (8.2%), the Americas (2.9%) and European Region (2.5%) [1]. To reach the goals of the End TB Strategy to reduce the incidence of TB by 90% by 2035, requires new rapid and accurate diagnostic tools to enable the timely initiation of treatment and better treatment outcomes [2]. Currently, diagnosis of active TB relies on the evaluation of clinical symptoms, radiological evaluations, and detection of *Mycobacterium tuberculosis* (*M.tb*) in pulmonary samples of patient, usually sputum; however, microscopic detection of *M.tb* in sputum smears is the largely widely used method for diagnosing pulmonary active TB and for therapy response monitoring [3]. However, sputum smears are poorly sensitive, and it has been reported that a high percentage (20% - 66%) of active TB cases maybe sputum smear negative [4]. Nucleic acid amplification-based tests have been reported to be more sensitive for the diagnosing active TB [4], but do not distinguish between live

and dead *M.tb* and are thus not useful in the monitoring of therapy-mediated clearance of the pathogen [5]. Currently, sputum culture is recognized as the gold standard for active TB diagnosis and for monitoring therapy response, but it takes almost 3 to 6 weeks to obtain results [3]. Blood-based host signatures for diagnosing active TB or latent TB infection (LTBI) are attractive alternatives tests that rely on discriminating active TB from healthy individuals or other pulmonary diseases [5]. Existing and accessible blood-based tests, such as IFN-γ release assays (IGRAs), which measure IFN-γ^+ produced in response to stimulation with *M. tb*-specific antigens CFP10 and ESAT6, have been shown to be specific for *M.tb* infection [6,7]. However, studies have found that IGRAs tests (e.g. T-SPOT.TB or QuantiFERON), are unsuccessful to differentiate between active TB and LTBI [8,9], and are inadequate for monitoring treatment response [10].

In 2016, the WHO reviewed and recommended Four other diagnostic tests, all based on sputum sampling; the loop-mediated isothermal amplification test for active TB, also known as TB-LAMP, is a two line probe assays (LPAs) that is devised for the detection of resistance to the first line anti-TB drugs isoniazid and rifampicin, and also an LPA devised for the detection of resistance to second-line anti-TB drugs. Also, the lateral flow urine lipoarabinomannan assay (LF-LAM) can provide a timely diagnosis of TB and help to reduce TB mortality among people living with HIV. WHO has recommended use of this test since 2015, and a policy update was issued in 2019. While, a new diagnostic platform called GeneXpert Omni and a next-generation cartridge called Xpert Ultra are currently in development, their performance are being assessed by the WHO, and the assessment results are still anticipated [1]. All these tests still rely on patients being able to generate an adequate sputum sample, something we know is difficult for many patients, especially children and the elderly.

To meet the ambitious WHO goals of reducing the case of TB deaths by 95% and all new TB cases by 90% by 2035, two key issues need to be addressed. First, we need techniques that can rapidly and accurately detect TB. Second, this sample should be non-invasive to increase adherence of testing. In this review, I summarize recently published articles in the English literature on the performance outcomes of molecular signatures in the blood, urine and saliva as TB diagnostics. The potential use of these samples in clinical practice, diagnostic discovery, and the gaps in the literature, as well as areas warranting further investigation are identified. I will review the current state of blood, urine and salivary biomarker candidates as diagnostic tools for active tuberculosis.

Methods

This review was formulated after a review of peer-reviewed publications from PubMed databases in all languages between 2000-April 1, 2018. The following search terms were utilized 'biomarkers', tuberculosis', 'blood', 'urine', 'urinary', 'saliva', 'salivary' and '*Mycobacterium tuberculosis* DNA'.

Blood and urine versus salivary biomarker

Potential biomarkers for TB are increasingly being identified from multiple sample of patients, including blood, urine and saliva. Many factors can affect choice of sample, including characteristics of the infection, the volume of test sample required, concentration of the biomarker, stability of biomarker within the sample, but most importantly the patient's willingness to provide the sample. For instance, blood is a complex biofluid that is known to contain a wide variety of molecular constituents [11,12]. Blood-based host biomarkers for diagnosis of TB are increasingly being recognized. Blood has historically been the most frequently used sources of measurable TB biomarkers. The disadvantage of this is that blood requires collection of significant volumes, sterile sample collections and trained personnel and analysis can be problematic, physically intrusive and not cost-effective. Urine samples however, are non-invasive, but sample volumes are relatively large and easily obtainable [13]. Other advantages of urine sample are easing laboratory handling as well as processing, and the lower risk of nosocomial transmission to laboratory and healthcare workers [14]. Furthermore, this ease of collection increases patients of all ages the willingness to provide samples at each required time. For diagnosis, urine may contain several mycobacterial antigens, and the improved sensitivity of molecular reagents are key factors increasing the use of urine as a source of molecular diagnosis of TB [13,15].

However, the presence of *M.tb* DNA in urine remains controversial [13]. It is still unclear whether *M.tb* infects renal cells. Further studies that involve indicators of successful sample preparation are urgently required. TB patient blood and saliva samples contain intact cells, which serve as a reservoir for endogenous biomarker control. Regrettably, urine samples contain few intact live cells, making it difficult to find endogenous controls for this sample [13].

Saliva is an indispensable constituent of the digestive process and serves to instigate the breakdown of starches and lipids through endogenous enzymes. Previous studies have reported that salivary fluid contains a variety of microbial and molecular analytes [16-19]. These observations have enhanced the field of salivary diagnostics, and sparked investigations to identify of saliva-based biomarkers for infectious diseases [20,21]. Like in blood, salivary proteomic and transcriptomic markers have shown the most promising result in other disease model such as arthritis and cancer [22-25]. Recently, tremendous interest has been shown in the investigation of molecular signature in saliva of TB patients. Information obtained from oral immunologic biomarkers is important in utilizing saliva for diagnostic biomarkers. However, the mechanism by which these biomarkers come to exist in salivary fluids have not yet been understood.

Using saliva as a medium for host-signature TB diagnostic test development and therapy evaluation, will eases TB patient discomfort, providing a non-invasive method of TB detection. Comparatively, saliva samples carry numerous advantages over urine and blood samples: (i) Collection is unchallenging, while blood sampling requires trained medical personnel, and urine sampling requires patient's privacy, saliva procurement can be done by any individual, via self-collection; (ii) Sample procurement is relatively painless, without the tremendous discomfort most individuals endure from biopsies and repeated blood samples draws making it easier for patients to adhere in timely medical assessments and screenings; (iii) Saliva samples are easy to ship and store, because saliva does not clot, it does not require separation of component like blood; (iv) Saliva samples can be easily stored for long term, resulting in decreased overall costs for TB patients and health care providers [26]. It is still unclear whether, salivary fluid secretions contain factors that can stop the infectivity of TB disease or that may result in TB transmission.

In spite of these advantageous aspects, the use of saliva as a molecular diagnostic fluid has yet to become a mainstream idea. Whereas, most analytes detected in the urine and blood are also found in salivary fluid, their level expressions may be distinctly different [26,27]. Blood-, urine- and salivary-constituents imply that these three biofluids are different and unique, but they may be linked on a molecular level. For this reason, there is therefore an important to know the most promising diagnostic sample type that helps to ensure early detection and prompt treatment.

Biomarker utility in clinical setting

A biomarker is a substance, structure, or biological process which objectively measures, accurately evaluates and reproducibly identifies a defined conditions in normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic intervention [28,29]. Biomarkers can exist in a multiple forms, including microbes, and -omics libraries such as transcriptome, proteome, metabolome, miRNAome complement of microRNAs, small non-coding microRNAs, epigenome or microbiome [26]. Biomarkers are an important and attractive tool in disease detection, diagnosis, prognosis, risk assessment, and therapy monitoring [30]. However, variations in their structure, concentration, function or mode of action can be associated with the onset, progression or even regression of a host-specific biological process or disease [26,31]. In regard to TB, although, there are still no specific biomarkers used in clinical practice, a number of potential biomarkers have been identified.

Understanding and comprehensively assessing the significance of an individual's host biosignature may be useful in determining the presence and exact location of a pathology [26]. Further, biomarkers may be useful measure of an individual response to treatment and thus useful to rapidly identify non responders, often a sign of drug-resistance or treatment non-compliance. Yoshizawa and colleague

2013, suggested that to develop a clinical biomarker, a number of milestones should be achieved: first, when performing preclinical testing, host-signatures should be developed using patient samples and validated at the *in vivo* or *in vitro* level in all cohort tests. Second, during the pilot or feasibility study, biomarker signatures should be tested in a small number of patient subpopulations to demonstrate they have the ability to differentiate TB patients from healthy subjects. Third, when validating biomarker, these host-signatures must be assayed accurately. Fourth, a proper statistical analysis must be performed to evaluate the discriminatory accuracy of the host-signature molecules in a large number of patient population. Fifth, following the reporting of a validated signature profile, enormous efforts should be made to critically investigate their respective immunological and biochemical functions during TB [26,32]. Sixth, most importantly, biomarkers must be able to discriminate diseased and healthy subject at an early stage. The time or stage of disease is a concern, since different biomarkers may be expressed at different disease stages; those that are regulated at early stage may not necessarily be detected at a late stage of disease. These challenges have meant that adequate biomarkers to discriminate clinical disease and predict response to therapy have not yet been confirmed.

Blood biomarker-based diagnostics of tuberculosis

Sample specimen collection and processing

In developing an ideal biomarker selection of sample for measurement is critically important. The difficulties in collecting an adequate sputum sample from all TB suspected individuals have been well documented. In addition, sample collection and processing poses a risk to health care workers. Blood collection requires a qualified flombist, needles and an extraction system and tubes that may contain anticoagulant, or RNA stabilizing reagent, depending on the type of biomarkers to be tested. Depending on the planned analysis, serum or plasma may need to be separated, requiring access to a centrifuge at a minimum. If cells are required, ficoll based separation will be required. Serum or plasma will usually need to be stored at -80 °C until use.

For RNA isolation from whole blood, blood will need to be collected with a stabilizing agent such as in a Tempus tube, or RNAlater tube. These tubes require specific RNA isolation kits, and well equipped laboratories for RNA extraction and analysis. The optimal sample for collection, whether that be whole blood, blood cells, serum or plasma remains to be confirmed. Ideally a point-of-care test such as the those now available for Malaria or HIV, that could be undertaken with just a pinprick of blood, with results within minutes would be ideal. Developing such a diagnostic test remains a major challenge for TB researchers.

Hematological microbiome for TB diagnostic

The microbiome field is now an emerging area of research that is starting to prove its importance. Disruption of a stable microbiome ecosystem in the circulatory system results in dysbiosis. This imbalance is associated with numerous diseases including inflammatory, cancerous and respiratory diseases [33-37]. Case-control studies showed that sputum microbial diversity differed by TB status [38,39]. Studies reported that the gut but most importantly lung microbiome are associated with TB infection and disease [40-44].

TB is a disease of a failed immune response. A number of genetic variations have been identified that increase an individual's susceptibility to *M.tb* infection [45,46]. Single-nucleotide polymorphisms (SNPs) in the host genome are also reported to impact microbiota composition [47]. These reports suggest that host genetics play a critical role in establishing and shaping the microbiota environment; additionally, pharmacologic and dietary factors have been reported to also alter microbial community structure [48,49]. Therefore, there is a great promise in evaluating and correlating the microbiome compositions with TB.

Blood transcriptomics for TB diagnosis

The study of transcriptomics involved the analysis of genome-wide gene expression, usually measured in a form of RNA transcript abundance when using gene chip microarrays or RNA sequencing analysis; most of the time, this expression can also be used in the expression of non-coding RNA and protein-coding genes and may contain more than 300,000 different transcripts [50,51]. Since the first report in 2007, over 20 studies have examined the transcriptional signature response to TB [52] (Table 1). Despite this large number of studies no diagnostic test for TB utilizing this technology has been developed yet, and several reasons may account for this. Unfortunately, several of these studies were especially designed with the intention of exploring the immunopathogenesis of TB [53-58] rather than identifying diagnostic biosignature that are capable of discriminating diseased and healthy subjects. While, other studies have aimed at evaluating therapy response in TB with an idea to find new surrogate markers of success to be used for both clinical management and in trials of new therapies [2,59,60]. Of those designed to derive biomarkers that would differentiate active TB from health or other pulmonary disease states, only a small proportion has a case definition of active TB based on microbiological test confirmation, validation of their signatures in independent cohorts or evaluation of the diagnostic accuracy of the biomarker [51].

Table 1: *Summary of blood-derived transcriptomics studies.*

CCTB: Culture-Confirmed TB; CNTB: Culture-Negative TB; HC, EP: Extrapulmonary; Healthy controls; LTBI: Latent TB Infection; OD: Other Diseases; TB: Active Tuberculosis; P: Pulmonary; N/A: Not Applicable; P: Positive; N: No; -: HIV Negative; +: HIV Positive, UD: Undefined.

Study	Country	Groups and (number)	Dataset GSE #	HIV (-/+)	Transcript biomarker identified/ validated
[2]	China	ATB (275) HC (290) OD (290)	N/A	- - -	2 genes (GBP5, KLF2)
[65]	India	TB (113) LTBI (56) HC (20)	74092	- - -	15 and 4 gene (GBP1, P2RY14, IFITM3, ID3)
[64]	USA	TB (109) LTBI Pneumonia	73408	-	47 (TB v Pneumonia), 119 (TB v LTBI or pneumonia) transcripts
[148]	Kenya, Malawi, South Africa	CCTB (95) CNTB (27) LTBI (68) OD (140) CCTB (51) CNTB (17) LTBI (0) OD (93)	39941	- - - - + + + +	51 transcript signature
[149]	China	TB (173) LTBI (148) HC (51)	54992	UD UD UD	C1q genes and proteins
[150]	South Africa	TB (21) HC (22)	50834	+ +	251 gene signature
[63]	Malawi, South Africa	TB (97) LTBI (83) OD (83) TB (97) LTBI (84) OD (92)	37250	- - - + + +	TB v LTBI 27 transcript signature. TB v OD 44 transcript signature

[53]	United Kingdom, France	TB (35) Sarcoid (61) Pneumonia (14) Lung (16) Cancer HC (113)	42834	- - - - - -	144 transcripts TB v OD or HC
[151]	Venezuela	TB (9) LTBI (29) HC (25) Pneumonia (18)	41055	- - - -	116 gene signature TB v LTBI v HC. 9 and 10 gene subsets showed the similar predictive value.
[59]	South Africa	TB (27)	313486238	-	> 4000 genes differentially expressed during TB treatment
[56]	Germany	TB (8) LTBI (4) Sarcoid (18) HC (14)	34608	- - - -	691 genes differentially expressed TB v Sarcoid
[57]	Indonesia	TB (23) HC (23)	56153	-	460 genes TB v HC
[60]	South Africa	TB (37) LTBI (38)	40553	- -	664-transcript signature TB v LTBI, 320 transcript signature diminished at 2 weeks
[152]	USA	TB (5) LTBI (6) BCG vacc (5) HC (7)	N/A	- - - -	127 probe set
[153]	South Africa	TB (33) LTBI (34) HC (9)	25534	- - -	2048 transcripts TB V LTBI, 5 gene TB patients, of which five genes discriminate: (94% sensitivity and 97% specificity)
[55]	The Gambia	TB (46) LTBI (25) HC (37)	28623	- - -	UD
[154]	China	TB (46) LTBI (59) HC (26)	27984	- - -	3 gene signature (CXCL10, ATP10A and TLR6)
[62]	United Kingdom, South Africa	PTB (54) LTBI (69) HC (24) OD (96)	19491 19444 19443 19442 19439 22098	UD UD UD UD	393 transcript signature
[58]	Colombia	TB (1) LTBI (1) HC (1)	N/A	UD UD UD	UD
[155]	Germany	TB (37) LTBI (22) HC (15)	6112	- UD UD	3 gene signature lactoferrin, CD64, and RAB 33A
[52]	South Africa	TB (10) Cured TB (10) LTBI (10) Rec TB (10)	N/A	- - - -	9 gene signature for cure and risk of relapse

Recently, whole-blood transcriptomics research has significantly emerged ahead of metabolomics and proteomics for signature discovery in the diagnostic of TB, this, because of the recently reported result of well-established sample-processing pathways, that led in the development of accurate and rapid samplein-answer-out multiplex PCR platforms [61].

Many studies have identified differential gene signatures expression in active pulmonary TB patients compared with healthy controls and those with LTBI or other pulmonary diseases [2,51,53,56,59,60,62-66]. There has been relatively little work in the assessment of the specificity of TB-associated blood transcriptional biomarkers compared with other inflammatory or infectious pulmonary diseases. Recently, for an affordable diagnostic test, studies have sought to reduce the number of transcripts in a diagnostic biomarker, achieving as few as 2, 3 or 4 signature genes to differentiate active TB from healthy individuals with or without LTBI, or to discriminate active TB from other pulmonary infections or diseases, however, with variable accuracy [2,65,66]. Notwithstanding that the blood is rich in transcriptional signatures, published studies have found discrepancies in the expression of these biomarkers in TB patients (Table 1). Developing these signatures into a useable diagnostic tool requires further evaluation in larger cohorts across different ethnic and geographical locations and populations of different ages and co-morbidities.

Metabolomics in tuberculosis

Metabolomics are the metabolites produced by a cell, tissue, or organism, that are essential intermediate products of metabolic reactions catalyzed by various enzymes occurring within cells during health and disease state. So far, the primary goal of most published studies in TB metabolomics was to gain novel biological insights into the pathogenesis of TB rather than to explore their diagnostic application (Table 2). The diagnostic performance of potential candidate signatures has not yet been evaluated. Researchers interested in the diagnostic assessment of metabolomics cannot easily make use of the generated data, because data generated are not routinely deposited in public databases [51]. To the best of our knowledge, only one study has provided its raw metabolomic data as supplementary material into the public domain [67].

Table 2: *Blood-derived metabolomics studies.*
HC: Healthy Controls; LTBI: Latent TB Infection; OD: Other Diseases; TB: Active Tuberculosis; UD: Undefined; +: HIV Positive; -: HIV Negative; Y: Yes.

Study	Country	Groups and (Number)	HIV Status	Metabolomics biomarker identified
[156]	China	TB OD (110) HC	UD UD UD	Y
[67]	Hong Kong	TB (37) OD (30) HC (30)	UD UD UD	Y
[157]	China	TB (120) OD (146) HC (105)	UD UD UD	Y
[69]	Georgia	TB (17) HC (40)	UD UD	Y
[158]	China	TB (76) HC (56)	UD UD	Y
[159]	China	TB (136) Treated (6) HC (30)	- - -	Y
[70]	South Africa	TB (34) OD (61)	UD UD	Y
[68]	South Africa	TB (44) LTBI (46) HC (46)	- - -	Y

Once recent study showed that at least 20 serum metabolites were required to differentiate between active pulmonary TB patients, healthy control subjects and LTBI individuals with an accuracy of 97% [68]. Changes found in the metabolome of patients with active TB consist of differences in the abundance of specific host-derived metabolites, but most importantly the presence of compounds derived from *M.tb* itself (e.g. cell wall lipids) or when including confirmed active TB patients on anti-TB therapy [51,69,70]. Additionally, because the metabolic profile is structured by a number of environmental factors such as dietary intake, comorbidities, medication and stress response [71], careful matching of case and control subjects will be required during biomarker discovery to minimize metabolite noise. Haas., *et al*. (2016) stated that the number of metabolites that has been tested so far, has varied significantly between published studies, ranging from 34 to > 21,000 studies, depending on the analytical test or technique used. Thus, the approach on metabolomics measurement in the discovery of TB biomarker still faces many unresolved issues, such as data standardization, quality control, reproducibility, but also validation [72]. These problems highlight a critical need for further additional well-designed studies aimed at specifically discovering diagnostic biomarkers.

Proteomics measurement during tuberculosis

Proteomics is a large-scale analysis of proteins in the cell organism and a rapidly growing field of molecular biology [73]. Several published studies have examined the possible diagnostic potential of proteomic fingerprinting to discover diverse disease conditions between active pulmonary TB versus healthy state, latent TB infection (LTBI) or other pulmonary diseases [51,74-78]. Proteomics is the study that involve combined set of proteins expressed by different cells or organism during inflammatory response or infection at any given time [51]. At least, up to 1 million different proteins are thought to encompass the human proteome. The discovery of proteomics-based host-signatures establish an essential part of inflammatory or infectious disease research due to their influence on disease prevention, diagnosis and treatment [79]. Although, different tests such as ELISA are used to analyze protein, proteomic studies predominantly use mass spectrometry [80].

Proteomics-based approaches can be used to discover proteins as biomarkers that can serve as mean to compare protein levels in a blood fluid (serum or plasma) of diseased or healthy subjects, or they can also be used to detect the presence of pathogen antigens [79]. The first study to show that the serum proteins could distinguish active pulmonary TB from both healthy controls and other pulmonary diseases identified a combination of four biomarkers made of C-reactive protein, serum amyloid A, neopterin and transthyretin using conventional immunoassays such as ELISA [74]. Similarly, we have recently shown that C reactive protein can be used as a surrogate marker for monitoring anti-TB therapy [2]. Blood serum and plasma are enthusiastically being studied as an important and rich source of protein biomarkers, and over 15 studies have been reported involving active TB versus LTBI or healthy controls and other diseases (Table 3). Each of this study has identified different number and type of proteins using blood from individuals of different ethnicity, suggesting that sample type matters.

Table 3: *Blood-derived proteomics studies.*

EP: Extrapulmonary; HC: Healthy Controls; LTBI: Latent TB Infection; N/A: Not Applicable; ND: Not Defined; OD: Other Diseases; P: Pulmonary; TB: Active tuberculosis; SP: Smear Positive; SN: Smear Negative; +: HIV Positive; -: HIV Negative; UD: Undefined.

Study	Country	Groups and (Number)	HIV Status	Number of protein biomarker identified/validated
[78]	USA	TB (37)	-	8 proteins
		LTBI (34)	-	
		OD (19)	-	
		HC (20)	-	
		TB (10)	+	
		LTBI (23)	+	
		OD (26)	+	
		HC (16)	+	

[77]	China	TB (122) Treated (91) Cured (59) HC (122)	- - - -	2 proteins
[160]	China	SP-TB (49) SN-TB (66) HC (80)	- - -	58 proteins
[161]	China	TB (40) OD (80) HC (40)	- UD UD	3 proteins
[76]	China	LTBI (71) HC (75)	- -	14 proteins
[162]	China	TB (76) HC (56)	-	4 proteins
[163]	South Korea	TB (26) HC (31	UD UD	1 protein
[164]	Uganda	TB (39) Responder (19) Non-responder (20)	- - -	5 proteins
[165]	China	TB (180) OD (120) HC (90)	- - -	1 protein
[166]	Uganda	TB (39) Treated (39)	- -	11 proteins
[167]	China	TB (129) LTBI (36) OD (69) HC (30)	UD UD UD UD	1 protein
[75]	Peru	TB (151) OD (-LTBI) (44) OD (+LTBI) (53) OD all (110)	UD UD UD UD	ND
[168]	China	TB (80) OD (36) HC (32)	- - -	3 proteins
[169]	China	TB (37) EP-TB (81) OD (35) HC (40)	- - - -	ND
[170]	Japan, Vietnam	TB (39) HC (63)	-	4 proteins
[171]	China	SP-TB (51) SN-TB (36) OD (13) HC (55)	- - - -	ND
[74]	Angola, Uganda, The Gambia, United Kingdom	TB (197) OD (168) HC (25)	+/- +/- +/-	4 proteins

Urinary biomarker-based diagnostics of tuberculosis

Sample collection

Given the requirements for obtaining blood samples for biomarkers, urine samples are being increasingly investigated for their biomarker potential in the diagnosis of infectious diseases including TB. Urine samples typically have large volumes of dilute analyte concentrations [81]. Systematic approaches for detecting antigens of *M.tb* in samples of human urine have been evaluated [82-84], and some of these mycobacterial antigens have been identified in the active TB patients urine [83,85-87]. Thus, increasing the potential to develop a urine based diagnostic for TB disease.

Molecular microbial tuberculosis diagnostics

Urinary microbial metabolites, such as Lipoarabinomannan (LAM), can also be of use [88]. This glycolipid comprises up to 15% of the total mycobacterial weight and is recognized as a virulence factor that has been released from metabolically active or degrading *M.tb.* However, LAM size is variable, ranging from 6 - 34 kDa [89-91]. A study involving 141 patients with confirmed active TB, and also number of 172 patients with no proven active TB all showed that urine LAM test was positive in only 17/141 and 1/172 patients, respectively [92]. Thus, the utility of LAM in urine as a diagnostic biomarker remains to be proven.

A meta-analysis and systematic review on the diagnosis of active TB using the commercial Clearview™ TB ELISA kit (Inverness Medical Innovations), reported an estimation of sensitivity and specificity in microbiologically confirmed TB cases of 13 - 93 and 87 - 99%, respectively [93]. When this test was used in five studies on HIV-positive subgroups, sensitivity of 3 - 53% was reported, with a systematically higher sensitivity observed in advanced immunosuppression.

Metabolics

Urine contains the most important sizable fraction of metabolites of microbial origin and human metabolome, and it requires minimal processing effort for analysis methods, including LC-MS [94-97]. Studies have exploited the use of metabolic flux in oncology, diabetes and cardiovascular disease [98-100], and it is therefore evident that metabolomic approaches provide a direct measure of human systems biochemical profile [101-103].

Nevertheless, little is known regarding urinary metabolites status during tuberculosis. A recent study has revealed a relatively small molecule metabolic biomarker that differentiated patients with active TB prior to the commencement of treatment from those successfully responding to therapy at very early stage (one month) after the commencement of treatment [104].

Proteomics

Urine is a less complex sample than plasma, containing approximately 2000 proteins [105,106]. Proteome from the urine can change significantly over time, as urine sample collected in the morning has been reported to contain more proteins than those collected in the afternoon and evening [107]. In addition, human urinary proteome can differ between healthy individuals, particularly between men and women, and specific protein levels of an individual may vary at different time points due to the effect of exercise, lifestyle and diet.

The composition of protein in the urine are however, relatively more stable compared to other biofluids, including serum and plasma, which are prone to proteolytic degradation during and after sampling [108]. Urine secretion is one of the steps of urine formation, and it is a consequence of blood filtration, but urine contains also proteins secreted from tubules and kidney specific cells [109-114]. The filtration of serum proteins is based on their size and charge at the glomeruli [115]. Because of disparities in sensitivity and availability of several

proteomic techniques, therefore, substantial efforts have been made to find the most appropriate method to analyze the expression of specific proteins biomarker in urine [116]. One study showed that urine proteomics study is an important platform to identify urinary excreted peptides and proteins from different stages of disease or therapy to reveal their quantity, biological functions and interactions [117]. Thus, indicating that proteomic approaches could suggest the mechanism of the disease and novel therapeutic targets [118-121]. Recently, urinary proteomics have become an important and efficient approach for biomarker discovery in TB disease [122]. For example, Paris., *et al*. found that urine lipoarabinomannan glycan protein levels in patients with HIV-negative and confirmed active pulmonary tuberculosis correlated with severity of disease [123], while Pollock and co-workers recently discovered a unique *M.tb* protein in the urine of active TB patients [124] that may have biomarker potential.

Salivary diagnostics

Sample collection

Saliva consists of almost 98% of water, but it contains also other substances, such as mucus, electrolytes, various enzymes and antibacterial compounds [125]. Saliva fluid is abundantly produced in individuals of all age groups, and at average, human produces it at a range of 0.3 to 7 mL per minute, and always has about up to 1 ml in the oral cavity [125]. Most importantly, saliva sample collection is simple and noninvasive [126].

Molecular diagnostic tests that use host biosignature of infectious or inflammatory markers could be an interesting valuable diagnostic, not only in adults patients, but most importantly in individuals such as children who have difficulties in providing good quality sputum samples [127] and those with extrapulmonary TB disease; particularly if based on more easily obtainable biological samples such as saliva, and developed into a diagnostic rapid point-of-care test [128,129]. In the TB context, a novel salivary biosignature set comprising of fibrinogen, ECM-1, HCC1, IL-1β and IL-23 showed 88.9% (95% CI, 76.7 - 99.9%) sensitivity and 89.7% (95% CI, 60.4 - 96.6%) specificity, regardless of HIV infection status; and could potentially be of use in the diagnosis of TB and monitoring of response to therapy [130].

Oral microbiome in tuberculosis

It is still unclear evidence whether a microbial profile derived from saliva could be used as a predictive marker of inflammation or disease [131], especially in TB. It is predicted that oral microbial diagnostic has potential value beyond assessing diseases of the oral cavity [26]; therefore, the microbial salivary profile may serve as an indicative of infection, including TB.

Transcriptomics

It is widely believed that the secretions of human saliva may not only harbor RNA molecules, but many other substances, therefore, these may serve as a highly promising source of differentiating gene signatures. A study using the saliva of healthy individuals and other disease models, including those with Sjogren's syndrome and cancer, identified more than 3,000 species of mRNA and over 300 miRNAs [132-135]. Salivary transcriptomic analysis may yield valuable information regarding individual pathological conditions. Eguchi and colleagues [136] used PCR to detect transcriptomics in the oral cavity of TB patients, showing the rates of detection by PCR from denture plaque, mixed saliva and caries lesions from sample of TB patients were 100%, 98.0%, 92.0%, and 89.0%, respectively. While the detection rates by culture method were 0%, 17.3%, 2.0%, and 0%, respectively [137]. Further analysis is required to validate these results and determine the potential of transcriptome derived from salivary fluid as a noninvasive and viable source of disease-specific biosignatures.

Proteomics

Large collections of diverse proteins are found in salivary fluid, but each with different biological functions. Human saliva has been actively investigated as a source of protein biomarkers [138]. A study reported that salivary protein biomarkers are capable of discriminating healthy from diseased individuals [139].

Previous studies have also shown discriminatory protein profiles in other disease models, such as AIDS, oral cancer, mammary gland carcinoma, diabetes, and periodontal disease [140-145]. In TB research, a study by Jacobs., *et al*. identified 9 markers in saliva, consisting of IL-1β, IL-9, IL-10, IL-15, MCP-1, MIP-1β, granzyme A, serum amyloid A and ferritin that changed as a result of TB treatment, suggesting they may be useful prognostic indicators [146]. Another recent study by Namuganga and colleagues also indicated the biomarker potential of saliva proteins [147]. The use of human salivary proteins as biomarkers of TB requires further investigation.

Conclusion

There is a tremendous unmet need for a non-invasive molecular diagnostic tools to aid the eradication of TB disease. Culture positive sputum continues to be the gold standard for TB diagnosis, but difficulties with obtaining samples and identifying TB are clear. Blood and urine are rich in potential biomarkers, but salivary biomarkers are of growing interest. With their ease of access, viability and a non-invasivity, saliva remains of interest to determine its suitability as a biomarker for the creation of a non-invasive molecular point-of-care test for the diagnosis of TB. In summarizing the most recent studies to identify a biomarker of TB, it is clear that sample type (involving the homogeneity of the population), co-morbidities, robustness of the assays (with the inclusion of new technology such as artificial intelligence) and reproducibility all need to be examined in larger cohorts. While there has been a rapid increase in our understanding of the analytes that may serve as a biomarker of TB, however, much work remains to be undertaken. This field presents exciting opportunities for the evaluation of new biomarkers in different sample types including urine and saliva. With its ease of access, putting effort and resources into the saliva biomarker investigation may yield a point-of-care molecular diagnostic test that could revolutionize the TB field.

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Author Contributions Statement

N.M.F wrote the first draft and final version of the manuscript.

Conflict of Interest Statement

The author has no conflict of interest with any organization or with the subject matter or materials discussed in the manuscript.

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