Tuberculosis 115 (2019) 126–139

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: www.elsevier.com/locate/tube

Review

The application of metabolomics toward pulmonary tuberculosis research

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ARTICLE INFO

Keywords:
Biomarkers
Chemometrics
Metabolites
Metabolomics
Sample matrix
Tuberculosis

ABSTRACT

In the quest to identify novel biomarkers for pulmonary tuberculosis (TB), high-throughput systems biology approaches such as metabolomics has become increasingly widespread. Such biomarkers have not only successfully been used for better disease characterization, but have also provided new insights toward the future development of improved diagnostic and therapeutic approaches. In this review, we give a summary of the metabolomics studies done to date, with a specific focus on those investigating various aspects of pulmonary TB, and the infectious agent responsible, *Mycobacterium tuberculosis*. These studies, done on a variety of sample matrices, including bacteriological culture, sputum, blood, urine, tissue, and breath, are discussed in terms of their intended research outcomes or future clinical applications. Additionally, a summary of the research model, sample cohort, analytical apparatus and statistical methods used for biomarker identification in each of these studies, is provided.

1. Introduction

Active pulmonary tuberculosis (TB) is highly contagious and is currently the leading cause of death globally from an infectious pathogen, resulting in approximately 1.4 million fatalities in 2017 alone. Furthermore, the World Health Organization (WHO) reported 10 million newly diagnosed TB cases in the same year, with the majority occurring in India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa [1]. Considering this, it is not surprising that TB research is still receiving much attention from academia, the medical community and governments, despite the discovery of the causative agent, *Mycobacterium tuberculosis* (*M.tb*), by Robert Koch as far back as 1882.

The development of improved diagnostic and treatment approaches for pulmonary TB relies heavily on gaining a better understanding of the general biology of *M.tb* and the underlying mechanisms of the disease. To this end, various metabolomics studies have been published describing new metabolic processes, virulence factors, gene function, and enzyme activities in *M.tb*, all of which contribute to a better understanding of the biology of this infectious organism [2–4]. Several research groups have also applied metabolomics specifically toward identifying new TB biomarkers for the primary purpose of developing improved TB diagnostic approaches [5,6] and treatment strategies [7–9].

2. Metabolomics

Metabolomics refers to the quantitative measurement of dynamic metabolic changes of a living system in response to a genetic change or physiological stimuli (including nutrients and drugs) [10]. These metabolic changes are measured via the identification, quantification, and characterization of the metabolome, the latter of which includes all small molecules (< 1500 Da) such as metabolic intermediates, hormones and other signaling molecules, as well as secondary metabolites found in a specific cell, organ, organism or biofluid. Metabolomics can, therefore, be used to characterize a specific metabolic phenotype related to an external or internal perturbation, such as, for example, a disease state [11]. The metabolite changes associated with the specific phenotype being investigated may be classified as characteristics of the perturbation, which, in the context of investigating a disease, could be used toward better disease characterization, diagnostics, treatment, and other clinical applications.

In practice, metabolomics experiments can be approached in an untargeted manner, where the variation between thousands of features, detected in different sample groups, are measured according to their relevant concentrations. These features can be defined as signal patterns recorded by the analytical apparatus (for example, a combination of the retention time and mass to charge [m/z] ratios, when collecting mass spectrometry [MS]-based metabolomics data), which can be an-

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https://doi.org/10.1016/j.tube.2019.03.003
Received 11 December 2018; Received in revised form 27 February 2019; Accepted 8 March 2019

notated by comparison to various commercial or in-house-generated metabolite mass spectra databases [12]. This approach has served exceptionally well and has enabled researchers to identify novel metabolite biomarkers for TB diagnostics and disease characterization [13–15], elucidate drug metabolism and drug action mechanisms [16,17], investigate mechanisms of drug side effects [18,19], elucidate drug resistance mechanisms [20,21], monitor treatment efficiency [22,23], and describe general cellular processes of *M.tb* [4,24]. Metabolomics studies can also be done using a semi-targeted approach, where hundreds of compounds are unambiguously identified and quantified by comparison to previously injected or isotopically labeled standards, while potentially detecting thousands of unknown or tentatively identified features simultaneously. Although the list of metabolites to be analyzed is defined prior to the analyses, the hypothesis might not necessarily be [12]. A few research groups have applied semi-targeted metabolomics to investigate the organic acid and lipid profiles of *M.tb* cultures and patient-collected biofluids, in an attempt to identify unique metabolite profiles for application to TB diagnostics [25,26] and the prediction of treatment outcome [7], as well as to elucidate drug resistance [27,28] and virulence mechanisms [29] of *M.tb*. When the aim of an experiment is to test a specific hypothesis, however, targeted metabolomics is typically applied, in which case the absolute concentrations of pre-defined compounds are measured [12,30]. Although targeted metabolomics approaches have been applied less frequently to TB investigations to date, this tactic has been tremendously valuable in cases where a specific biological process or function had to be clarified or proven. Examples of such cases include the verification of host drug activation [31], the discovery of enzymatic activities encoded by genes of unknown function [32], and the elucidation of the physiological functions of known enzymes [33].

The typical workflow of a metabolomics experiment aimed at new disease biomarker discovery, includes a series of successive steps, including the pre-analytical procedures, analytical analyses, data analyses and biological interpretation of the identified biomarkers [34,35]. In order to eventually identify reliable, biologically relevant biomarkers, all pre-analytical steps, including study design, sample collection, transport, storage, and sample preparation, should be carefully designed and executed. Poor sample quality, for example, may heavily bias the coefficient of variance of the final analytical results [34], and the lack of, or inappropriate use of sample normalization procedures could dramatically affect compound quantification [36]. Particulars of the methods to follow and pitfalls to avoid during the pre-analytical phase of a metabolomics investigation fall beyond the scope of this review, but have been discussed and evaluated in a number of publications [30,34–36].

Currently, the analytical measurement of the metabolome is mostly accomplished using nuclear magnetic resonance (NMR) and MS coupled to an array of separation systems [37,38]. NMR is appropriate for the detection of all hydrogen-containing compounds, since it determines the magnetic resonance of nuclei in a molecule, and it is considered an unbiased, robust, reproducible, non-destructive and selective analytical platform, which requires almost no sample pre-treatment. On the downside, however, NMR has a low sensitivity and is short of an analyte separation element [12,37]. MS is defined as the process of forming gaseous ions, with or without fragmentation, which are then characterized by their *m/z* ratios and respective relative abundances [38]. Direct MS infusion is a high-throughput method, requiring only 1 min for each sample analysis, and has been applied successfully in metabolomics studies, but it is not preferred for the analyses of complex biological samples such as blood and urine due to matrix interference. Prior chromatographic separation of analytes in such a complex sample matrix improves the identification and quantification of the metabolite components [38]. For metabolomics applications, the most commonly used hyphenated techniques include liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE) prior to MS analysis [37]. LC-MS is highly suited for the analyses of non-volatile, thermally unstable, high- or low molecular weight compounds with a high polarity range, and requires no prior derivatization of the metabolites. This technique is therefore commonly applied for the metabolomics analyses of biofluids such as blood and urine. GC-MS, on the other hand, is generally preferred for the separation and identification of volatile, thermally stable compounds, which necessitates the prior derivatization of metabolites with a subsequent analysis time penalty [38]. CE-MS is used for the analysis and identification of water-soluble and charged molecules, and provides high resolution power, with minimum sample volumes, making it a complementary analytical method to LC-MS and GC-MS. A disadvantage of CE-MS is that the electroosmotic flow results in migration time shifts throughout the analyses [37]. Other analytical apparatuses, such as Fourier-transform infrared spectroscopy, have also occasionally been implemented toward metabolomics research. Comprehensive discussions of the drawbacks and strengths of each of these methods have previously been reviewed in detail [12,37–39].

The constant improvement of the aforementioned analytical equipment, especially over the last decade, has led to the acquirement of more information and more complex datasets, and consequently, the application of advanced chemometric methods for processing this information is becoming a necessity [40]. In order to identify potential biomarkers from these high-throughput metabolite profiles, either univariate (parameter-by-parameter) or multivariate statistical techniques are typically used. Univariate methods, such as *t*-test and analysis of variance, reduce a large number of measured analytes to only those showing the strongest variations in response to the investigated perturbation. These methods, however, fail to differentiate sample groups when the alterations on a single molecule level are minor, and hence, various multivariate methods are additionally used, since these also consider dependency structures between individual analytes. The most commonly used multivariate methods for metabolomics applications include principle component analysis, partial least squares regression and cluster analysis [41]. Particulars of the different chemometric methods [40–42], as well as the most popular software tools for pre- and post-processing and statistical analyses of metabolomics data [12,43], have been reviewed.

In the current review, we specifically summarize the pulmonary TB-related metabolomics studies which have been done to date and provide the analytical and statistical methodologies used in each case. We also discuss the main research aims and general outcomes achieved. Particular focus on the specific metabolomics biomarkers identified in each study falls beyond the scope of this review, but these have been listed and discussed previously in terms of their potential application to TB diagnostics [5], therapeutics [7] and drug mechanism elucidation [9].

3. TB metabolomics studies done to date

Various sample matrices, including bacteriological cultures, sputum, blood (serum and plasma), urine, tissue, and breath, have been used in the quest to identify new metabolomics biomarkers. A summary of the advantages and disadvantages of each of these sample matrices in terms of biomarker identification and application to TB metabolomics investigations are listed in Table 1.

3.1. Bacteriological culture metabolomics studies

Although the metabolome of *M.tb* grown *in vitro* differs from that grown *in vivo*, many researchers still prefer to use bacteriological cultures in the initial stages of biomarker identification (Table 2), since the growth environment of the organism, and the induction of the specific perturbation being investigated, can be strictly controlled. Such biomarkers can, for example, be used to describe metabolome variations between various microbial species for later disease characterization or diagnostic purposes. In one such application, various TB causing and

Table 1
Advantages and disadvantages of various sample matrices typically used in TB metabolomics research [71,88,89].

Sample matrix	Advantages	Disadvantages
Bacteriological cultures	• Growth environment can be controlled • No background interference or metabolite quenching by sample matrix (e.g. no interfering host metabolites) • Easily accessible — can be cultured in a laboratory • *Mycobacterium* specific metabolites can easily be extracted (in high concentrations) • Relatively easy to replicate the study • Excellent repeatability with minimal inter-sample variation.	• Does not take *in vivo* adaptations of host or microbe into account • Slow growth rate of *Mycobacterium* — timely experiments • Culturing of highly infectious bacteria such as *M.tb* requires biosafety level 3 facilities
Sputum	• Collection is considered relatively non-invasive (in adults without HIV infection) • Standardized protocol for sample collection and storage • Directly from the source of infection • Extracted metabolome characterizes the altered metabolome of both the host and *Mycobacterium*	• Invasive sample collection procedures in children and HIV co-infected patients • Difficult to work with (due to high viscosity and lumpy consistency) • Requires isolation of bacterial cells or homogenization prior to metabolite extraction • The use of reducing agents for liquification for example, may alter metabolite structures • Can only be used for pulmonary TB investigations • Sample collection can be time-consuming • Lack of direct normalization techniques
Blood (serum or plasma)	• Standardized protocols for sample collection and storage • Extracted metabolome characterizes mostly the systematic changes due to an infection • Can be used to do metabolomics investigations of pulmonary and extra-pulmonary TB	• Invasive sample collection procedure • Difficult to determine origin of metabolic perturbation (although it is mostly ascribed to host response) • High lipoprotein content can overwhelm the metabolic profile • Contains a low concentration of *Mycobacterium* metabolites • Individual variation of excreted metabolites and the concentrations thereof due to various external factors, i.e. diet, disease, gender, etc.
Urine	• Non-invasive sample collection procedure • Large quantities can easily be obtained • Well-characterized protocols for sample collection and storage • Protein-free, hence minimal sample preparation prior to analysis • Extracted metabolome characterizes mostly host response to perturbation • Can be used to do metabolomics investigations of pulmonary and extra-pulmonary TB	• Contains few *Mycobacterium* specific metabolites • Inter-individual variation of excreted metabolites and the concentrations thereof due to various external factors, i.e. diet, disease, gender, etc. • Various factors interfere with the normalization of urine metabolite concentrations • Lack of direct normalization techniques
Lung tissue	• Reflects direct local changes due to infection	• Highly invasive collection procedures • Difficult to obtain • Only small quantities of sample can be collected • Difficult to process • Can only be used for pulmonary TB investigations
Breath	• Non-invasive sample collection procedure • Feasible in children, the elderly, and HIV co-infected patients • The ability to monitor volatile compounds	• Sampling procedures are not standardized • Samples contain very dilute concentration of characteristic metabolites • A sample cannot be aliquoted and therefore numerous samples have to be collected from each individual in order to repeat the analyses • Normalization of sample volumes and quantification of characteristic metabolites are problematic

non-TB mycobacteria could be differentiated on the basis of the variations in their GC-MS generated lipidome profiles. The identified biomarkers were used to build a classification model, which correctly identified two 'unknown' samples for each species, with probabilities ranging from 72 to 100%, indicating the potential diagnostic applications of metabolite biomarkers identified from bacteriological cultures [26].

Culture metabolomics has also been applied toward the elucidation of the metabolism and mode of action of various commonly used [31,44] and prospective [45,46] TB drugs. By using the latter approach, it was discovered that *para*-aminosalicylic acid, one of the first anti-infectives used to treat TB, actually acts as an alternative substrate for the folate metabolism in *M.tb*, as opposed to the inhibition of dihydropteroate synthase as previously proposed [47]. Using a similar metabolomics approach, colistin sulfate was determined to function as a TB drug via the disruption of the *M.tb* cell wall [16], a mode of action which has also been proposed for this drug in other organisms [48]. Recently, a high-throughput culture metabolomics approach was used toward the prediction of the mode of action of 196 potentially new antimicrobial compounds based on reference metabolite profiles generated from 62 compounds with known modes of action [49]. These and similar studies (Table 2), could be of high value in the development and testing new TB drugs.

Metabolomics studies of drug-resistant cell cultures have additionally allowed for the identification of characteristic biomarkers describing previously unknown mechanisms of drug resistance and the survival of these strains. An example of such an approach is an LC-MS-based metabolomics study which identified a number of lipidome changes in rifampicin-resistant *M.tb* strains, associated with an adapted iron uptake and virulence [28]. Their results additionally concurred with a preceding study where a culture-based GCxGC-TOFMS metabolomics approach led to the identification of biomarkers associated with the remodeling of the cell wall lipids in rifampicin-resistant *M.tb* strains [21]. A recent study elaborated further on the aforementioned findings by using an LC-MS metabolomics research approach to characterize the lipidome of macrophages infected with various rifampicin-resistant *M.tb* strains. The authors indicated that, in these strains, an over-expression of phthlocerol dimycocerosates (a group of cell wall lipids) results in the bypass of the interleukin 1 receptor of the type 1 signaling pathway, initiating the induction of interferon-β and a reprogramming of the macrophage metabolism [50].

Culture metabolomics has also become an increasingly popular tool for the elucidation of various general biological processes of *M.tb*. Comparative lipidomics, for example, has contributed to comprehensive lipid databases, allowing for chemotaxonomic analyses of mycobacteria, which in turn have resulted in the identification of inter-strain

Table 2
Bacteriological culture metabolomics studies investigating various aspects of TB.

Research model	Metabolome fraction	Analytical apparatus	Statistical methods	Reference
Disease characterization and diagnostics				
M.tb	Volatile organic compounds	ATD-GC-MS	Fuzzy logic; hierarchical cluster analysis; PCA; K-nearest neighbor; PLS path modeling; SIMCA	[85]
M.tb; *M. bovis*; *M. bovis* (Bacillus Calmette–Guérin); *M. avium*; *M. fortuitum*; *M. chelonae*; *M. abscessus*	Culture headspace	SPME-GC-MS	Visual comparison of chromatograms	[86]
M.tb	Culture headspace	GCxGC-TOFMS	Random forest; volcano plot; AUC of ROC	[90]
Various mycobacterial strains	Total metabolome	THM-GC-MS	PLS-DA; AUC of ROC	[66]
M.tb; *M. kansassi*; *M. avium*; *M. bovis*; *Pseudomonas aeruginosa*	Lipids, total metabolome	GC-MS	PCA; PLS-DA	[91]
M.tb; *M. kansassi*; *M. avium*; *M. bovis*	Lipids	GC-MS	PCA; PLS-DA	[26]
Various mycobacterial strains	Mycolic acids	Multiple reaction monitoring-MS	Two-tailed Mann-Whitney; PCA	[92]
M.tb; *M. avium*; *M. bovis* (Bacillus Calmette–Guérin); *M. chelonae*; *M. fortuitum*; *M. kansassi*	Culture supernatant	UHPLC-ESI-QTOFMS	PCA; PLS-DA; one-way ANOVA; *t*-test	[93]
Elucidation of drug metabolism and drug action mechanisms				
M. smegmatis grown in D-cycloserine	Total metabolome	^{1}H NMR	PCA	[94]
M. smegmatis grown in various drugs	Total metabolome	^{1}H NMR	OPLS-DA; PCA	[95]
M. smegmatis grown in D-cycloserine	Total metabolome	^{1}H NMR	Targeted approach	[46]
M.tb grown in isoniazid	Total metabolome	LC-MS	Targeted approach	[31]
M.tb grown in isoniazid, streptomycin, and rifampicin (respectively)	Total metabolome	LC-MS	Hierarchical cluster analysis	[96]
M.tb grown in para-aminosalicylic acid	Total metabolome	LC-MS	Targeted approach	[47]
M. smegmatis grown in 62 drugs with previously known function and 212 drugs with previously unknown mechanisms of action	Total metabolome	LC-QTOFMS	Multiple linear regression scheme; entropy-based measure of similarity; iterative hypergeometric test	[49]
M.tb wild-type and econazole-resistant strains grown in econazole	Total metabolome	UPLC-MS; GC-MS	Welch's two sample *t*-test; two way ANOVA	[97]
M.tb grown in colistin sulfate	Total metabolome	GCxGC-TOFMS	PCA; PLS-DA; effect size; *t*-test	[16]
M.tb grown in colistin methanesulfonate	Total metabolome	GCxGC-TOFMS	PCA; PLS-DA; effect size; *t*-test	[98]
M.tb grown in D-cycloserine	Polar metabolites	LC-MS	Targeted approach	[45]
M.tb grown in pyrazinamde 5-Cl-pyrazinamide, *n*-propyl pyrazinoate and pyrazinoic acid (respectively)	Lipids	Reverse-phase HPLC	Targeted approach	[99]
M. vaccae grown in ethambutol	Lipids	GC-MS	Targeted approach	[44]
Elucidation of drug resistance mechanisms				
Rifampicin-resistant *M.tb*	Total metabolome	UPLC-MS	PCA; ANOVA	[100]
Rifampicin-resistant *M.tb*	Total metabolome	GCxGC-TOFMS	PCA; effect size	[21]
Rifampicin-resistant *M.tb*	Lipids	GC-MS	PCA; PLS-DA	[27]
Rifampicin-resistant *M.tb*	Lipids	UHPLC-ESI-QTOFMS	*t*-test; fold change	[28]
Rifampicin-resistant *M.tb*-infected macrophages	Lipids	UPLC-QTOFMS	Two-tailed *t*-test; one-way ANOVA; two-way ANOVA	[50]
Ethambutol-resistant *M.tb*	Specific compounds	HPLC-MS	Targeted approach	[101]
Isoniazid-resistant *M.tb*	Total metabolome	GCxGC-TOFMS	PCA; PLS-DA	[20]
Multi-drug-resistant *M.tb* before and after treatment	Total metabolome	LC-MS/MS	PCA; PLS-DA	[102]
M. smegmatis (natural drug resistance mechanisms)	Total metabolome	HRMAS NMR	PCA; OPLS-DA; Mann-Whitney *U* test; volcano plots, fold change	[103]
General biological processes of *M.tb*				
M.tb grown on 13C-labeled carbon substrates	Total metabolome	LC-TOFMS	Targeted approach	[53]
M.tb grown on 13C-labeled carbon substrates	Total metabolome	LC-MS	Targeted approach	[55]
M.tb grown on cholesterol	Total metabolome	LC-MS; GC-MS	Welch's test	[54]
M.tb, M. smegmatis, M. bovis (Bacillus Calmette–Guérin) cultured with NaCl	Lipids	NMR; LC-MS; MALDI-TOFMS; HPLC-QTOFMS	Targeted approach	[62]
M.tb cultured in oxygen limited conditions	Lipids	HPLC-QTOFMS	Lipid database search	[61]
M.tb, M. smegmatis, M. bovis (Bacillus Calmette–Guérin)	Lipids	HPLC-ESI-QTOFMS	Lipid database development; paired *t*-test	[51]
M.tb	Lipids	HPLC-ESI-QTOFMS	Lipid database development	[52]
M.tb wild-type and mutants	Lipids	HPLC-QTOFMS	Lipid database search	[60]
Hyper and hypo virulent Beijing *M.tb*	Total metabolome	GCxGC-TOFMS	PCA; PLS-DA; Mann-Whitney *U* test; effect size	[4]
Virulent *M.tb*; *M. bovis* (Bacillus Calmette–Guérin)	Lipids	HPLC-QTOFMS	Corrected *p*-value; fold change	[56]
M. smegmatis knock-out	Total metabolome	GC-MS	PCA; PLS-DA; Mann-Whitney *U* test; effect size	[57]
M. smegmatis knock-out	Total metabolome	GCxGC-TOFMS	PCA; PLS-DA; *t*-test; effect size	[104]
M.tb knock-out	Total metabolome	LC-MS	Targeted approach	[33]
M.tb knock-out	Total metabolome	UHPLC-QTOFMS	Fold change; *t*-test	[24]
M. bovis (Bacillus Calmette–Guérin), *M.tb* and *M.tb icl* knock-down	Total metabolome	LC-MS	Targeted approach	[63]
M.tb knock-out	Total metabolome Lipids	LC-MS HPLC-MS	Contrast-based analysis of significance	[59]
Wild-type *M.tb* and *mce1* operon mutant	Lipids	LC-MS/MS	Fold-change	[58]
M.tb knock-out	Lipids	LC-MS	Targeted approach; ANOVA	[105]
Purified protein of *M.tb*	Total metabolome	LC-MS; ^{1}H NMR	Targeted approach	[32]
Culture-infected macrophage	Total metabolome	DIMS	Fold change; *t*-test	[106]

Research model: ***M.tb***: *Mycobacterium tuberculosis;* **NaCl**: sodium chloride; **icl**: isocitrate lyase; *Analytical apparatus*: **ATD-GC-MS**: automated thermal desorption gas-chromatography mass spectrometry; **DIMS**: direct injection mass spectrometry; **GC-MS**: gas-chromatography mass spectrometry; **GCxGC-TOFMS**: two dimensional gas-chromatography time-of-flight mass spectrometry; **^{1}H NMR**: proton nuclear magnetic resonance; **HPLC**: high-performance liquid-chromatography; **HPLC-MS**: high-performance liquid-chromatography mass spectrometry; **HRMAS NMR**: high-resolution magic angle spinning nuclear magnetic resonance; **LC-MS**, liquid-chromatography mass spectrometry; **SPME-GC-MS**: solid-phase microextraction followed by gas chromatography-mass spectrometry; **TMS-GC-MS**: thermally-assisted hydrolysis and methylation gas chromatography-mass spectrometry **UHPLC-ESI-QTOFMS**: ultra-high-performance liquid-chromatography electrospray ionization time-of-flight quadrupole mass spectrometry; **UHPLC-QTOFMS**: ultra-high-performance liquid-chromatography time-of-flight quadrupole mass spectrometry; **UPLC-MS**: ultra-performance liquid-chromatography mass spectrometry. *Statistical methods*: **ANOVA**: analysis of variance; **DA**: discriminate analysis; **OPLS**: orthogonal partial least squares; **PCA**: principal component analysis; **PLS**: partial least squares; **SIMCA**: soft independent modeling of class analog.

Table 3
Sputum metabolomics studies investigating various aspects of **TB**.

Research model	Sample cohort (no. of samples)	Metabolome fraction	Analytical apparatus	Statistical methods	Reference
Disease characterization and diagnostics					
Human	TB+ (136); TB- (471)	Headspace	Electronic nose	PCA; PLS-DA; LDA	[69]
Human	TB- control (6); TB- spiked with *M.tb* (6)	Total metabolome	GCxGC-TOFMS	PCA; PLS-DA	[64]
Human	TB+ (61); TB- (34)	Total metabolome	GCxGC-TOFMS	PCA; PLS-DA; *t*-test, fold change	[14]
Human	TB+ (various *Mycobacterium* strains) (202); cultures of various *Mycobacterium* strains (338)	Mycolic acids	HPLC-FL	Computer-driven pattern recognition models	[107]
Human	TB+ (15); TB- (17)	Mycolic acids	ESI-MS/MS	*t*-Test; multiple reaction monitoring pairs	[108]
Human	TB+ (7); TB- (11)	Mycolic acids	THM-GC-MS	Targeted analysis	[65]
Human	TB+ (9); TB- (23)	Lipids	SPE-THM-GC-MS	Targeted analysis; decision tree	[67]
Human	TB+ (25); TB- (87)	Lipids	SPE-THM-GC-MS	Targeted analysis	[68]
Human	TB- spiked with *M.tb* (4)	Lipids	SPE-THM-GC-MS	Targeted analysis	[109]

Sample cohort: **TB-**: TB symptoms, but negative for disease (clinical control); **TB+**: active tuberculosis disease; ***M.tb***: *Mycobacterium tuberculosis*. *Analytical apparatus*: **ESI-MS/MS**: electrospray ionization tandem mass spectrometry; **GCxGC-TOFMS**: two dimensional gas-chromatography time-of-flight mass spectrometry; **HPLC-FL**: High-performance liquid chromatography with fluorescence detection; **SPE**: solid phase extraction; **THM-GC-MS**: thermally-assisted hydrolysis and methylation gas chromatography mass spectrometry. *Statistical methods*: **LDA**: linear square discriminant analysis; **PCA**: principal component analysis; **PLS-DA**: partial least squares discriminant analyses.

lipidome variations [51,52]. This approach has also been applied toward characterizing the metabolic adaptations of *M.tb* during growth on a variety of different substrates including glucose [53], cholesterol [54], and multiple carbon sources [55]. Virulence factors [4,56], gene function [57,58], enzyme activities [24,59], and the adaptive metabolism [60–63] of *M.tb* have also been investigated using culture metabolomics methods.

Apart from their various direct applications, the identified bacteriological biomarkers can also be indicative of what to expect when analyzing more complex samples, where mycobacterial metabolites are present in lower quantities and possibly masked by the matrix-associated background noise, such as when using sputum. When comparing the untargeted GCxGC-TOFMS metabolomics data of *M.tb*-spiked sputum (collected from TB-negative individuals) to that of non-spiked sputum controls, 19 metabolites which varied significantly between the sample groups were identified. A targeted analysis using these 19 compounds indicated their capacity to clearly differentiate between patient-collected TB-positive sputum samples and TB-negative controls [64]. This study proves the value of using *M.tb* cultures for biomarker identification when aiming to directly apply these toward clinical diagnostics using patient-collected sputum. Details of all the TB-related metabolomics studies done to date using bacteriological cultures are given in Table 2.

It is important to note that, although bacteriological culture metabolomics has contributed to our fundamental knowledge of *M.tb* and other *Mycobacterium* species, the clinical application of such biomarkers is, however, limited to the identification of metabolites originating directly from the infectious bacteria. Metabolome variations related to the *in vivo* growth of mycobacteria, or changes in the host metabolome in response to the infection, can only be determined via the investigation of patient-collected biofluids or tissue samples.

3.2. Sputum metabolomics studies

The most commonly used sample matrix in clinical pulmonary TB research to date is sputum. This mucus-like biofluid originates directly from the area of infection, i.e. the airways of the lungs. A metabolic profile of a sputum sample obtained from an infected patient consequently comprises metabolites originating from both the bacteria and the human host [14], unless the bacteria are removed beforehand, allowing for the separate analyses of the bacterial and host metabolomes. However, even when the mycobacteria are removed, the host metabolome would still contain the mycobacterial excretome. Despite the many advantages and the popularity of using sputum in other research disciplines, it has only been used in a limited number of metabolomics studies to date, where the aims are restricted to disease characterization and diagnostics (see Table 3).

In an untargeted GCxGC-TOFMS metabolomics approach, novel metabolite biomarkers in TB-positive patient sputum were identified, which shed light on the adaptations of *M.tb* due to *in vivo* growth and the host's response to infection, thereby generating a number of new hypotheses related to the disease mechanisms. Briefly, the identified metabolome variations indicated the presence of a citramalate cycle in *M.tb* and the interaction of this cycle with an up-regulated glyoxylate cycle upon infection, which supported previous findings suggesting an increased use of fatty acids and glutamate as alternative energy sources *in vivo*. These results also led to the proposed alternative mechanism of hydrogen peroxide production, the inhibition of the electron transport chain due to oxidative stress, and elevated concentrations of neurotransmitters in the host, which were associated with several TB symptoms [14].

A fully automated TB diagnostic method, detecting two characteristic *M.tb* mycolic acids in sputum, was developed using a thermally-assisted hydrolysis and methylation (THM) GC-MS metabolomics approach [65]. The method was later improved upon by including 20 mycolic acids into a classification model, making it more specific for *M.tb* [66], and the addition of a solid phase extraction (SPE) prior to THM-GC-MS allowed for a further simplification the method [67]. Further refinement of this technique, via the inclusion of only five of the aforementioned mycolic acids, resulted in a diagnostic approach which could correctly classify 80% of the TB-positive patient sputum samples and 98% of TB-negative sputum samples (collected from patients suspected of having TB), from a total sample cohort of 112 [68]. This progressive series of targeted metabolomics studies show the capacity of metabolomics toward the identification of biomarkers which can be used for the development of fast TB diagnostic methods for detecting *M.tb* directly from patient-collected sputum.

The metabolomics analyses of sputum are, however, multifaceted (requiring preprocessing and homogenizing steps) and could be rather time-consuming. In one study, where these sputum sample preparation steps were bypassed, the headspace volatile organic compounds (VOCs) of TB-positive and TB-negative patient sputum samples were analyzed using two electronic nose apparatuses (A and B). After data extraction and pattern recognition analyses, they were able to diagnose TB with a sensitivity of 68%, specificity of 69% and accuracy of 69% using electronic nose apparatus A, and 75%, 67%, and 69%, respectively, when using electronic nose apparatus B [69]. The limitation of this electronic nose technology is, however, that the differentiation (diagnosis) of

samples relies on the "smell" prints detected by an array of sensors, and therefore the identities of these VOCs cannot be determined for future application to breath analyses, for example.

Although non-invasive protocols can be employed for sputum collection in non-HIV adults, these are usually inefficient when trying to collect sputum from children, the elderly, and HIV co-infected patients, where more invasive methods are traditionally used [70]. On the upside, since sputum is presently the preferred sample matrix for TB diagnostics, "left-over" sample material can easily be obtained from diagnostic clinics for research purposes, provided that the necessary informed consent and ethical approval have been granted beforehand. When using such samples, researchers should ensure that the sample collection and storages procedures used (including the collection tubes, the addition of anticoagulants or antiseptics, etc.) comply with that prescribed for metabolomics studies.

3.3. Blood metabolomics studies

Although blood (plasma and serum) samples are regarded as comparatively more homogeneous, less viscose and generally easier to process than sputum, it also requires a certain degree of processing to facilitate the removal of high-molecular-weight proteins and lipoprotein particles, which will otherwise interfere with accurate metabolomics analyses [71]. Blood samples typically contain little or no *M.tb*, and for that reason, very low concentrations of metabolites originating directly from the infectious bacteria are generally detected in this sample matrix. Hence, the biomarkers identified from blood cannot be used toward the elucidation of *in vivo* growth mechanisms of the bacteria, nor the mode of action of TB drugs. On the other hand, the biomarkers obtained when doing metabolomics using blood is an excellent reflection of the disease phenotype since it reveals systemic alterations in the host due to the infection and the treatment thereof. TB research groups have therefore successfully used blood samples for metabolomics investigations where the aim of experiment was to describe the host pathology and host mechanisms of defense against *M.tb*, to monitor TB disease progression, for TB diagnostic and therapeutic monitoring purposes, and for the investigation of mechanisms related to drug side effects, as summarized in Table 4.

With the aim of disease characterization, untargeted metabolomics analyses were able to differentiate between serum samples collected from uninfected healthy individuals, *M.tb*-infected healthy individuals (latent infection) and patients with active TB. Changes to various metabolic pathways, collectively indicating an anti-inflammatory response, could be associated with TB. Also, a number of other metabolome variations, such as those indicating fibrotic lesions, were detected for active TB, but not for the latent infection state. Additionally, 20 metabolites were identified as differentiating active TB from latently infected or healthy individuals, showing the potential diagnostic capacity of these serum metabolic signatures [72]. Likewise, when characterizing the serum and plasma metabolomes of HIV-negative, TB-exposed individuals, metabolite biosignatures were identified which could predict the progression to active TB (3–24 months post-exposure) with a sensitivity of 69% and specificity of 75%, as early as 12 months prior to a diagnosis of active TB disease [73]. The clinical implementation of such a prognostic model could play a significant role in the prevention and transmission of the disease.

In metabolomics studies, urine is largely considered to be the preferred sample matrix for drug metabolism phenotype (DMP) studies. The use of blood, for example, can lead to unreliable results, because many of the important altered human and drug metabolites are largely undetectable using standard methods, either due to covalent protein-binding of these compounds or due to their short half-lives. In some cases, significant metabolites can be inferred by downstream metabolites if these are not protein-bound, but since these contribute to such a small portion of the total DMP, it would not be accurate to identify or quantify these for such purposes [74]. Blood has however been successfully used as a sample matrix for the investigation of toxicity mechanisms of TB drugs. In one such study, serum metabolome changes in rats induced by pyrazinamide, one of the four first-line TB drugs recommended by the WHO, and its two metabolites: pyrazinoic acid (POA) and 5-hydroxy pyrazinoic acid (5-OH-POA), were recently identified [75]. Drug administration resulted in a change in the concentrations of 19 metabolites associated with inflammation and oxidative stress. Interestingly, a larger degree of metabolome variations was identified when the pyrazinamide metabolites were administered compared to the parent drug, with 5-OH-POA contributing to an overall higher level of toxicity than POA. The biological interpretation of the resultant metabolite markers led to a better understanding of the mechanisms related to pyrazinamide-induced hepatotoxicity, which may in time result in the development of improved, less toxic treatment strategies for TB. In a related study, global metabolomics analyses also led to the identification of variations in the serum metabolome induced by the conventional four-drug combination treatment regimen recommended by the WHO, in TB patients infected with either *M.tb* or *M. africanum*. Results showed similar metabolic profiles before treatment in both patient groups, but a more pronounced decline in pro-inflammatory metabolites in the *M.tb*-infected patients after treatment, in comparison to those infected with *M. africanum* [23]. These observations might not only be indicative of intrinsic host factors related to TB susceptibility or the efficiency of TB treatment on the two lineages, but also shows the capacity of metabolite profiling in the development of personalized therapeutic approaches for TB.

Blood metabolomics has also been implemented toward the investigation of TB drug resistance. Discrepancies between the LC-MS-generated plasma metabolite profiles of 23 patients with multi-drug-resistant TB (MDR-TB) and those of 45 patients with drug-susceptible TB indicated an upregulation of *M.tb* cell wall N-glycan-specific metabolites in the MDR-TB group [76]. This proves the capacity of metabolomics using patient-collected blood for identifying biomarkers which can be used to characterize or diagnose drug resistance in TB patients.

3.4. Urine metabolomics studies

Urine is mostly free of proteins, making the processing thereof comparatively easier than sputum and blood. However, certain metabolites, such as urea and various sugars, may interfere with organic acid and amino acid profiles, especially when using GC-based analytical methods; thus a certain degree of sample preparation and metabolite extraction is required when using urine for metabolomics [77]. Furthermore, in contrast to other sample matrices, metabolite concentrations in urine are largely influenced by individual variation in diet and fluid intake. Therefore, normalization approaches, the most common of which is the use of urinary creatinine concentration, are necessary for determining the amount of urine and reagents required for each individual sample during metabolite extraction and analysis, as well as for the later quantification of the identified metabolites [78]. Similar to blood, urine also contains very low concentrations of metabolites originating directly from the infectious bacteria, but greatly reflects those metabolites altered in the host due to the infection, making this sample matrix ideal for disease characterization and diagnostics (Table 5). For the purpose of finding novel biomarkers for TB, the metabolite profiles of urine collected from patients with active TB were compared to that of healthy controls. Five metabolites characterizing these samples groups were identified, and the combination of these showed diagnostic promise, with receiver-operator characteristic (ROC) area-under-the-curve (AUC) values greater than 80% when applied to a blinded validation cohort consisting of patients with active TB and others with non-TB pulmonary diseases [15]. This study proves the capacity of metabolomics for identifying biomarkers from urine for their use in the development of new TB diagnostic protocols from a sample matrix collected in a non-invasive manner.

Table 4
Blood metabolomics studies investigating various aspects of TB.

Research model	Sample cohort (no. of samples)	Metabolome fraction	Analytical apparatus	Statistical methods	Reference
Disease characterization and diagnostics					
Mice: serum	TB+ (10); healthy control (10)	Total metabolome	^{1}H NMR	PCA; OPLS-DA; *t*-test	[83]
Guinea pigs: serum	TB+ (40); healthy control (10)	Total metabolome	HRMAS NMR	PCA; AUC of ROC	[82]
Human: serum	TB+ (44); latent TB (46); healthy control (46)	Total metabolome	UPLC-MS/MS; GC-MS	*t*-test; Wilcoxon rank sum test; ANOVA; random forest classifier	[72]
Human: serum	TB+ (38); healthy control (39)	Total metabolome	^{1}H NMR	PCA; OPLS-DA	[110]
Human: serum	TB + before treatment (16); TB + after treatment (6); healthy control (10) Validation: TB+ (120); healthy control (120)	Total metabolome	GC-TOFMS	OPLS; *t*-test	[111]
Human: serum	TB+ (120); healthy control (105); lung cancer (51); pneumonia (45); chronic obstructive pulmonary disease (28); bronchiectasis (22)	Total metabolome	UPLC-MS	OPLS-DA; AUC of ROC	[112]
Human: serum	TB+ (20); healthy control (18)	Total metabolome	LC-MS	PCA; PLS-DA; fold change; *t*-test; Pearson correlation	[113]
Human: serum	TB+ (28); Sarcoidosis (35); HC (54)	Total metabolome	^{1}H NMR	OPLS-DA; *t*-test	[114]
Human: serum	TB+ (10); TB- (10)	Lipids	GC-MS	Direct observation of unique compounds	[115]
Human: plasma; serum	Two years after TB exposure: TB+ (97); TB- (338)	Total metabolome	UPLC-MS/MS; GC-MS	Random forest machine learning; generalized boosted models; neural networks; elastic-net logistic regression; AUC	[73]
Human: plasma	TB+ (17); healthy control (17)	Total metabolome	LC-MS	*t*-test; Fisher exact test; hierarchical cluster analysis; PCA; Pearson correlation	[116]
Human: plasma	TB+ (17); healthy control (16)	Total metabolome	LC-MS	Wilcoxon rank sum test; Fisher exact test; linear model for microarray data; Benjamini-Hochberg false discovery rate correction; logistic regression analysis	[117]
Investigation of drug side effects					
Rats: serum	Healthy control receiving pyrazinamide (60)	Total metabolome	^{1}H NMR	PCA; OSC-PLS-DA; fold change; *t*-test	[18]
Rats: serum	Healthy control receiving pyrazinamide (24)	Total metabolome	^{1}H NMR	PCA; PLS-DA; Mann-Whitney *U* test	[75]
Elucidation of drug resistance mechanisms					
Human: plasma	Multi-drug-resistant TB+ (23); drug-susceptible TB+ (45)	Total metabolome	LC-MS	Simultaneous logistic regression models	[76]
Prediction of treatment outcome and monitoring of treatment efficiency					
Human: serum	*M.tb*+ (14) and *M. africanum*+ (12) patients receiving DOTS	Total metabolome	UHPLC-ESI-MS/MS	ANOVA; *t*-test; Mann-Whitney *U* test; Wilcoxon matched-pairs signed rank test; Fisher's exact test	[23]
Human: plasma	TB + receiving DOTS (66)	Total metabolome	LC-MS	Individual regression models	[118]

Sample cohort: **DOTS**: directly observed treatment, short-course; ***M.tb*+**: *Mycobacterium tuberculosis*-infected; **TB-**: TB symptoms, but negative for disease (clinical control); **TB+**: active tuberculosis disease. *Analytical apparatus*: **GC-MS**: gas-chromatography mass spectrometry; **GC-TOFMS**: gas-chromatography time-of-flight mass spectrometry; **^{1}H NMR**: proton nuclear magnetic resonance; **HRMAS NMR**: high-resolution magic angle spinning nuclear magnetic resonance; **LC-MS**, liquid-chromatography mass spectrometry; **UHPLC-ESI-MS/MS**: ultra-high-performance liquid-chromatography electrospray ionization tandem *mass spectrometry*; **UPLC-MS/MS**: ultra-performance liquid chromatography tandem mass spectrometry; **UPLC-MS**: ultra-performance liquid-chromatography mass spectrometry. *Statistical methods*: **ANOVA**: analysis of variance; **AUC of ROC**: area under the curve of the receiver operating characteristic curve; **DA**: discriminate analysis; **OPLS**: orthogonal partial least squares; **OSC-PLS**: orthogonal signal correction-partial least square; **PCA**: principal component analysis; **PLS**: partial least squares.

Table 5
Urine metabolomics studies investigating various aspects of TB.

Research model	Sample cohort (no. of samples)	Metabolome fraction	Analytical apparatus	Statistical methods	Reference
Disease characterization and diagnostics					
Human	TB+ (117); healthy control (37); chronic obstructive pulmonary disease (7); other pulmonary diseases (5)	Volatile organic compounds	GC-MS	PCA; PLS-DA; OPLS-DA	[119]
Human	TB+ (197); TB- (201)	Volatile organic compounds	Colorimetric sensor array	PCA; SVM	[120]
Human	TB+ (21); TB- (21)	Total metabolome	GC-MS	PCA; PLS-DA; fold change; *t*-test; AUC of ROC	[13]
Human	TB+ (102); TB- (102) Validation: TB+ (50); TB- (50)	Total metabolome	HPLC-TOFMS	Mixed model and factor analysis methodology (RRmix)	[15]
Human	TB+ (46); healthy control (30)	Organic acids	GCxGC-TOFMS	PCA; PLS-DA; effect size; *t*-test	[25]
Elucidation of drug metabolism and drug action mechanisms					
Rats	Healthy control receiving pyrazinamide (5)	Specific predetermined compounds	HPLC	Targeted approach; fold change	[121]
Mice	*pxr*-null (5–7), wild-type (5–7), *PXR*-humanized (5–7) receiving rifampicin	Total metabolome	UPLC-TOFMS	PCA; PLS-DA; OPLS-DA	[122]
Human and mice	TB+ (8); TB + mice receiving isoniazid (8)	Total metabolome	LC-MS	Targeted approach	[31]
Human	Healthy control receiving isoniazid (6); healthy control receiving rifampicin (12)	Total metabolome	UPLC-TOFMS	PCA; OPLS-DA	[17]
Human	healthy control receiving rifampicin (12)	Total metabolome, Steroids	UPLC-QTOFMS; GC-MS	PCA; PLD-DA; OPLS-DA; *t*-test; Benjamini-Hochberg false discovery rate correction; Pearson correlation, UPGMA	[123]
Human	TB + receiving DOTS (20); healthy control receiving ethambutol (2)	Total metabolome	GC-TOFMS	PCA; Pearson correlation; pattern hunter	[79]
Investigation of drug side effects					
Rats	Healthy control receiving DOTS (6)	Organic acids	GC-MS	ANOVA; AUC of ROC; Tukey post-hoc comparisons	[124]
Rats	Healthy control receiving isoniazid (6)	Total metabolome	^{1}H NMR	PCA; PLS projection to latent structures	[125]
Rats	Healthy control receiving isoniazid (10)	Total metabolome	^{1}H NMR	PCA; PLS-DA; OPLS-DA	[126]
Mice	Wild-type (7); Cyp2e1-null (7) receiving isoniazid	Total metabolome	LC-MS	PCA; *t*-test	[127]
Human	TB + before treatment (25); TB + receiving DOTS with no DILI (49); TB + receiving DOTS with DILI (11)	Total metabolome	UPLC-MS	PCA; OPLS-DA; ANOVA; correlation analysis	[19]
Prediction of treatment outcome and monitoring of treatment efficiency					
Human	TB + receiving DOTS (87)	Total metabolome	LC-MS	*t*-test; ANOVA; logistic regression; PCA	[22]
Human	TB+ with successful (26) or unsuccessful (15) treatment outcome	Organic acids	GCxGC-TOFMS	PCA; PLS-DA; Mann-Whitney *U* test; fold change	[81]
Human	TB+ with successful (21) or unsuccessful (10) treatment outcome	Organic acids	GCxGC-TOFMS	Effect size; Mann-Whitney *U* test; fold change (with a logistic regression model)	[80]
Human	TB+ with successful treatment outcome (15); failed treatment (8); recurrence (12); healthy control (14)	Targeted approach	LC-MS	Relative abundance of target compound	[128]

Sample cohort: **TB+**: active tuberculosis disease; **TB-**: TB symptoms, but negative for disease (clinical control); **DILI**: drug-induced liver injury; **DOTS**: directly observed treatment short-course. *Analytical apparatus*: **GC-MS**: gas-chromatography mass spectrometry; **GC-TOFMS**: gas-chromatography time-of-flight mass spectrometry; **GCxGC-TOFMS**: two dimensional gas-chromatography time-of-flight mass spectrometry; **^{1}H NMR**: proton nuclear magnetic resonance; **HPLC-MS**: high-performance liquid-chromatography mass spectrometry; **HPLC-TOFMS**: high-performance liquid-chromatography time-of-flight mass spectrometry; **LC-MS**, liquid-chromatography mass spectrometry; **UPLC-MS**: ultra-performance liquid-chromatography mass spectrometry; **UPLC-QTOFMS**: ultra-performance liquid-chromatography quadrupole time-of-flight mass spectrometry; **UPLC-TOFMS**: ultra-performance liquid-chromatography mass spectrometry. *Statistical methods*: **ANOVA**: analysis of variance; **AUC of ROC**: area under the curve of the receiver operating characteristic curve; **DA**: discriminate analysis; **OPLS**: orthogonal partial least squares; **PCA**: principal component analysis; **PLS**: partial least squares; **SVM**: support vector machines; **UPGMA**: unweighted pair group method, using the arithmetic mean.

Table 6
Tissue metabolomics studies investigating various aspects of TB.

Research model	Sample cohort (no. of samples)	Metabolome fraction	Analytical apparatus	Statistical methods	Reference
Disease characterization and diagnostics					
Mice: Lung, liver, spleen	TB+ (10); healthy control (10)	Total metabolome	^{1}H NMR	PCA; OPLS-DA; *t*-test	[83]
Guinea pigs: Lung	TB+ (40); healthy control (10)	Total metabolome	HRMAS NMR	PCA; AUC of ROC	[82]
Investigation of drug side effects					
Mice: Liver	TB + receiving isoniazid (136)	Total metabolome	^{1}H NMR	OPLS-DA; *t*-test	[129]
Rats: Liver	TB + receiving pyrazinamide (30)	Total metabolome	^{1}H NMR	PCA; OSC-PLS-DA, fold change; *t*-test	[18]
Determination of drug distribution					
Human: Lung	Multi-drug-resistant TB + receiving rifampicin, isoniazid, pyrazinamide and moxifloxacin (15)	Drug metabolites	LC-MS/MS; MALDI-MSI	Two-tailed *z*-test; two-tailed unpaired *t*-test	[84]
General biological processes of *M.tb*					
Mice: Lung	Healthy control infected with various *M.tb* mutants (10 per group)	Lipids	FT-ICR MS	Targeted approach	[29]

Sample cohort: **TB+**: active tuberculosis disease. *Analytical apparatus*: **FT-ICR MS**: Fourier transform ion cyclotron resonance mass spectrometry; **GC-MS**: gas-chromatography mass spectrometry; **^{1}H NMR**: proton nuclear magnetic resonance; **HRMAS NMR**: high-resolution magic angle spinning nuclear magnetic resonance; **LC-MS/MS**: high pressure liquid chromatography coupled to tandem mass spectrometry; **MALDI-MSI**; matrix-assisted laser desorption/Ionization mass spectrometry imaging. *Statistical methods*: **AUC of ROC**: area under the curve of the receiver operating characteristic curve; **DA**: discriminate analysis; **OPLS**: orthogonal partial least squares; **OSC-PLS**: orthogonal signal correction-partial least square; **PCA**: principal component analysis.

Since urine directly reflects those metabolites excreted by the liver and kidneys, it additionally provides direct information regarding changes to an individual as a result of a toxic insult, and can be useful for determining the mechanisms of drug metabolism and drug action [79], drug-related side effects [19] and the prediction of treatment outcome [80] (see Table 5). In an untargeted GC-MS-based metabolomics study, the DMP of TB patients receiving the WHO-recommended combined-drug TB therapy regimen, consisting of treatment with isoniazid, rifampicin, ethambutol, and pyrazinamide, was investigated. Three of the four drugs, and most of their known metabolites were detected in the urinary metabolomes of this patient cohort. The calculated metabolic ratios of the parent drugs and their metabolites significantly contributed to an improved understanding of the DMP induced by TB treatment. Subsequent correlation analyses, in addition to the manual prediction of the drug catabolism, led to the identification of a novel ethambutol metabolite, thereby expanding on the known xenobiotic metabolism of this drug [79]. In a similar approach, comparing the urinary metabolomes of TB patients before and after receiving this four-drug TB treatment regimen, a number of host-associated metabolic pathways which were altered by drug administration were identified. The degree of variation, furthermore, differed between patients with drug-induced liver injury (DILI) and non-DILI patients, indicating that superoxide generation can aggravate the hepatotoxic effect induced by the oral administration of combined TB drugs. These distinct urinary metabolome profiles were additionally used to predict the degree of hepatotoxicity, a finding which could in future be implemented clinically toward the administration of personalized treatment [19]. Inter-individual variation in the host response to TB treatment is not only limited to discrepancies in the degree and presentation of toxicity but can also lead to treatment failure in some patients. To this end, in an attempt to better characterize treatment failure, the urinary organic acid profiles of active TB patients collected before treatment onset, who either had a successful (n = 26) or unsuccessful (n = 15) treatment outcome after therapy was completed, were compared. Clear differentiation of the compared groups was evident, and the identified metabolite markers were indicative of a potential imbalanced gut microbiome, abnormalities in the long-chain fatty acid β-oxidation pathway, a mitochondrial trifunctional protein defect, increased interferon-γ, and compromised insulin secretion in the treatment failure group [81]. In a follow-up study, the urinary metabolomes of these patients were further evaluated using a combination of univariate statistical approaches, together with a logistic regression model. This approach led to the identification of two specific metabolites related to a gut microbiota imbalance, which achieved a ROC AUC of 95% for predicting treatment failure to first-line TB drugs at the time of diagnosis, before treatment onset [80].

3.5. Tissue metabolomics studies

Metabolomics analysis of patient-collected lung tissue directly reflects local changes in the host due to an *M.tb* infection or TB drug ingestion and is therefore considered to be an excellent medium for pulmonary TB biomarker identification. The collection of such samples from humans does, however, involve a highly invasive collection procedure, and for that reason, most metabolomics studies aimed at identifying TB-related metabolic changes in the lungs to date, have been done using TB-infected animal models (Table 6). In one such study, high-resolution magic angle spinning (HRMAS) NMR-generated lung metabolome profiles of *M.tb*-infected guinea pigs could clearly be distinguished from those of naïve controls. The metabolite markers characterizing *M.tb*-infected lung tissue indicated hypoxia in the necrotic lung lesions and a host response to the oxygen radical production. The abundances of these metabolites were also seen to change in accordance with the progression of infection [82]. In a similar study, characteristic metabolite markers were identified from lung, spleen and liver tissue of *M.tb*-infected mice. In this case, metabolite precursors of membrane phospholipids, phosphocholine and phosphoethanolamine, in addition to metabolite markers from glycolysis, amino acid metabolism, nucleotide metabolism and the anti-oxidative stress response, were altered as a result of infection [83]. Although the aforementioned tissue-based metabolomics studies better explained the host's response to infection and the associated disease mechanisms, these distinct metabolome profiles cannot be used diagnostically due to the aforementioned invasive manner of sample collection.

Tissue collected from *M.tb*-infected animal models has also been used in metabolomics studies aimed at investigating various aspects of TB drugs (Table 6). In one such study, the effects of sub-acute hepatotoxicity induced by pyrazinamide, on the liver metabolomes of rats, was explored. The identified metabolite markers reflected elevated oxidative stress and changes in the purine, energy and NAD metabolic pathways in a dose-dependent and gender-specific manner, thereby shedding new light on previously unknown mechanisms of pyrazinamide toxicity [18].

A recent study, which might be considered a scientific breakthrough, used a metabolomics approach to determine the quantitative and spatial distribution of TB drugs within the major lesion types in the

Table 7
Breath metabolomics studies investigating various aspects of TB.

Research model	Sample cohort (no. of samples)	Metabolome fraction	Analytical apparatus	Statistical methods	Reference
Disease characterization and diagnostics					
Human	TB + (23); TB- (19); healthy control (59)	Volatile organic compounds	ATD-GC-MS	Fuzzy logic; hierarchical cluster analysis; PCA; K-nearest neighbor; SIMCA; PLS path modeling	[85]
Human	TB + (10); TB- (10)	Volatile organic compounds	SPME-GC-MS	Targeted approach; unpaired *t*-test	[87]
Human	TB + (30); TB- (196)	Volatile organic compounds	ATD-GC-MS	AUC of ROC; Monte Carlo simulations; WDA models	[130]
Human	TB + (130); healthy control (121)	Volatile organic compounds	ATD-GC-MS	Monte Carlo simulations	[131]
Human	TB + (50); TB- (50)	Volatile organic compounds	GC-MS	SVM	[132]
Human	TB + (51); healthy control (20)	Volatile organic compounds	Electronic nose	Mann-Whitney *U* test; Kruskal-Wallis; PCA; K-nearest neighbor	[133]
Human	TB + (15); healthy control (15) Validation: TB + (34); TB- (114); healthy control (46)	Volatile organic compounds	Electronic nose	Artificial neural network; ROC	[134]
Primate (macaques)	*M.tb*- (9); *M.tb* + (9)	Volatile organic compounds	GCxGC-TOFMS	Random forest; Volcano plot; AUC of ROC; Mann-Whitney *U* test (with Benjamini-Hochberg false discovery rate correction)	[90]

Sample cohort: **TB-**: TB symptoms, but negative for disease (clinical control); **TB +**: active tuberculosis disease. *Analytical apparatus*: **ATD-GC-MS**: automated thermal desorption gas-chromatography mass spectrometry; **GC-MS**: gas-chromatography mass spectrometry; **GCxGC-TOFMS**: two-dimensional gas-chromatography time-of-flight mass spectrometry; ***M.tb* +**: *Mycobacterium tuberculosis*-infected; ***M.tb*-**: *Mycobacterium tuberculosis*-uninfected; **SPME-GC-MS**: solid-phase microextraction followed by gas chromatography-mass spectrometry. *Statistical methods:* **AUC of ROC**: area under the curve of the receiver operating characteristic curve; **PCA**: principal component analysis; **PLS**: partial least squares; **SIMCA**: soft independent modeling of class analogy; **SVM**: support vector machines; **WDA**: weighted digital analysis.

lungs of patients with TB. The importance of this approach lies in its possible applications toward the discovery and testing of improved antibiotic regimens for TB, in particular to that of sterilizing drugs [84].

3.6. Breath metabolomics studies

In theory, a more feasible diagnostic option in comparison to tissue (and perhaps even blood and urine), would be to use breath, which is considered a lung-derived biological specimen that can be collected in a non-invasive manner, even from children and HIV co-infected patients. Considering this, various research groups have successfully characterized the breath metabolite profiles of TB patients (Table 7). In a pilot study, the most abundant VOCs, which were uniquely detected in the headspace of *M.tb* bacteriological cultures in comparison to sterile culture media, were identified using sorbent traps and GC-MS analyses. In the same study, breath VOCs were also identified from 59 healthy controls and 42 hospitalized patients with suspicion of TB, of which 23 were diagnosed as *M.tb*-positive and 19 as *M.tb*-negative. Markers of oxidative stress were elevated in all hospitalized patients, and pattern recognition analyses could distinguish between the breath profiles of the “sick” individuals and healthy controls with a 100% specificity and 100% sensitivity. Similarly, *M.tb*-positive and *M.tb*-negative patients could be differentiated with a sensitivity of 95.7% and specificity of 78.9%. Interestingly, two characteristic TB breath VOC markers, including 1-methyl-naphthalene and 1,4-dimethyl-cyclohexane, were also identified as markers for *M.tb in vitro* [85]. Using a similar metabolomics approach, applying SPE-GC-MS analyses, four distinct volatile markers (*p*-anisate, methyl nicotinate, methyl phenylacetate, and *o*-phenylanisole) were identified for *M.tb* and *M. bovis* grown *in vitro* [86]. Since it is well-known that nicotinic acid is abundantly produced by *M.tb*, this compound (which is detected as methyl nicotinate after *in situ* derivatization) was selected for *in vivo* validation, and as a result, statistically significant variations of this compound in the breath of TB-positive patients compared to healthy controls, were detected [87]. These studies highlight the potential similarities between the VOCs produced by *M.tb in vitro*, and those present in the breath of TB patients, an observation which might add value to the future development of a fast, non-invasive, point-of-care diagnostic method for TB.

4. Concluding remarks

The application of metabolomics analyses to a number of different biological sample matrices, using a spectrum of analytical equipment and statistical methodologies, has led to an exponential rise in the number of newly identified TB biomarkers over the last decade. These biomarkers, in turn, have been used toward an improved understanding of *M.tb* biology and the TB disease state, the elucidation of drug action, drug resistance, and drug toxicity mechanisms, monitoring of disease progression and prediction of treatment outcome. However, although metabolomics can be regarded as a powerful research tool, the actual clinical implementation of these are still limited, and the validation and commercialization of the identified biomarkers need to be prioritized over the course of the next decade.

Declarations of interest

None.

Author contribution statement

ID conceived and designed the study. ID and LL researched and wrote distinct sections of the review. DL did scientific and language editing of the manuscript. All authors read and approved the final version of the manuscript.

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