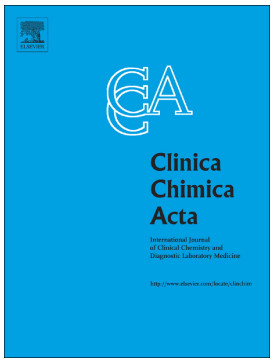

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Mass Spectrometry-Based Metabolomics for Tuberculosis Meningitis

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Abstract

Tuberculosis meningitis (TBM) is a prevalent form of extra-pulmonary tuberculosis that causes substantial morbidity and mortality. Diagnosis of TBM is difficult because of the limited sensitivity of existing laboratory techniques. A metabolomics approach can be used to investigate the sets of metabolites of both bacteria and host, and has been used to clarify the mechanisms underlying disease development, and identify metabolic changes, leadings to improved methods for diagnosis, treatment, and prognostication. Mass spectrometry (MS) is a major analysis platform used in metabolomics, and MS-based metabolomics provides wide metabolite coverage, because of its high sensitivity, and is useful for the investigation of *Mycobacterium tuberculosis* (Mtb) and related diseases. It has been used to investigate TBM diagnosis; however, the processes involved in the MS-based metabolomics approach are complex and flexible, and often consist of several steps, and small changes in the methods used can have a huge impact on the final results. Here, the process of MS-based metabolomics is summarized and its applications in Mtb and Mtb-related diseases discussed. Moreover, the current status of TBM metabolomics is described.

Key words: Metabolomics; Tuberculosis meningitis; Mass spectrometry; Diagnosis

1. Introduction

Tuberculosis meningitis (TBM) is an infectious disease of the nervous system caused by *Mycobacterium tuberculosis* (Mtb). TBM accounts for nearly 5% of all cases of extra-pulmonary tuberculosis [1], and it is associated with the highest rates of morbidity and mortality among all forms of tuberculosis [2]. Outcomes of patients often include substantial neurological sequelae or death [3, 4]. Early diagnosis and initiation of appropriate treatment at an optimal dose can greatly improve the clinical outcomes of TBM [5]; however, it is difficult to make a definitive diagnosis of TBM, because of its variable and nonspecific clinical presentation, and the low sensitivity of current diagnostic laboratory tests. Hence, there is an urgent need for diagnostic variables that can accurately identify TBM.

Metabolomics, an emerging science of the omics era, provides a new tool for the identification of novel diagnostic markers of TBM. This methodology is used to identify and quantify low-molecular-weight metabolites, and produces metabolite profiles which reflect the state of a given biological system [6]. Metabolites can change rapidly in response to even slight alterations in environmental stimuli [7], and key metabolites and related metabolic pathways that correlate with specific human diseases have potential for use as indicators or biomarkers. Recently, metabolomics has been applied in the identification of biomarkers for disease processes including Alzheimer's disease [8], Parkinson's disease [9], and multiple sclerosis [10].

Metabolomics is an advanced analytical tool that relies on the specific technique used for its implementation. A high throughput and sensitive analytical platform is needed to identify and quantify metabolites, and the primary approaches that have been applied are nuclear magnetic resonance (NMR) spectrometry and mass spectrometry (MS) [11-13] (Table 1). Although NMR is highly quantitative and reproducible, its low sensitivity has greatly limited its application in metabolomics. MS is commonly integrated with various chromatography systems, providing superior sensitivity and resolution compared with NMR [14]. MS can profile more metabolites in one analytical run; therefore, relative to NMR, MS is the dominant metabolomics platform.

Metabolomics has been widely used in tuberculosis research, and MS has become the technology of choice for this approach. To date, the metabolomics approach has been applied successfully to the investigation of TBM diagnosis; however, the number of studies remains limited. Although the majority of such investigations have used NMR, rather than MS, some have

begun to take an MS-based approach to obtain more specific metabolic markers. The high sensitivity and resolution of MS-based metabolomics will lead to its wide application in TBM research; for example, for identification of indicators or biomarkers of TBM. The objectives of this review were to introduce the uses and limitations of current MS-based metabolomic approaches for TBM research, describe recent advances in MS-based metabolomics for Mtb-related research, and discuss the prospects for the application of MS-based metabolomics for the development of TBM diagnostic approaches.

2. Methods for TBM diagnosis

The gold standard for TBM diagnosis relies on the identification of Mtb in the cerebrospinal fluid (CSF). As it is rapid and affordable, smear microscopy remains the most common diagnostic approach [15, 16]; however, it is notoriously insensitive, despite numerous improvements [17, 18]. Bacterial culture is more sensitive than smear microscopy [19]; however, identification of Mtb from CSF by culture generally takes weeks [20], which is too slow for clinical decision-making purposes. Nucleic acid amplification tests (NAATs) are molecular diagnostic methods with potential to overcome the inadequacies of smear microscopy and bacterial culture. These methods aim to detect specific Mtb DNA sequences in clinical specimens and culture [21, 22], and the use of Xpert MTB/RIF, a commercial cartridge-based nucleic acid amplification test, has been endorsed by the World Health Organization. This test targets the Mtb *rpoB* gene, and can rapidly and simultaneously detect Mtb and rifampicin resistance in clinical specimens. Xpert MTB/RIF has high specificity for TBM; however, its sensitivity is highly variable [23-25] and it is considered a sub-optimally accurate approach to TBM diagnosis.

Given the difficulty in detecting Mtb in CSF, there has been interest in whether specific immune responses can aid TBM diagnosis. Interferon-γ release assays (IGRAs), based on the ability of T lymphocytes to release interferon-γ when stimulated with Mtb-specific antigens, was an important diagnostic approach for TBM over the past decade, and was the subject of a systematic review [26] which demonstrated its limited effectiveness for TBM diagnosis [27, 28]. In addition, it is not suitable for patients with HIV-TBM co-infection.

Detection of specific molecules, such as lipoarabinomannan (LAM), in CSF has the potential to be exploited as an alternative approach for TBM diagnosis. Some investigations have suggested

that the LAM assay has high sensitivity [29]; however, others indicate that it is less sensitive in clinical than in laboratory settings [30], and more research is needed to evaluate its diagnostic value.

The limited sensitivity of TBM diagnostic approaches has led to screen for diagnostic markers, and a number of studies have attempted to detect different biomacromolecules within the CSF to address this issue [31-34]. Many of these approaches appeared promising in preliminary studies; however, some have failed to be translated into routine clinical care.

3. MS-based metabolomics: methodology and processes

Although metabolomics is regarded as an unbiased approach for identification and quantification of all low molecular weight metabolites, current analytical techniques are unable to achieve this goal. The great complexity of metabolites, including their concentrations, sources, and physico-chemical properties, represent significant challenges for metabolomics studies [35]. As mentioned above, NMR and MS are the main analytical platforms used for metabolomics, and the higher sensitive of MS makes it an attractive alternative to NMR. MS-based metabolomics studies commonly include four steps: sample preparation, extraction of metabolites, analysis of metabolites, and data analysis(Fig. 1).

3.1. Sample preparation

Sample preparation is a critical step in ensuring the quality of data generated by metabolomics assays. The primary purpose of sample preparation is to eliminate enzymatic effects and remove extraneous components (such as microbes and cells), since the enzymatic activity and metabolism of cells or microbes in samples can continue after they are collected. Samples should be prepared as soon as possible after collection to ensure that the state of metabolites is as similar as possible to that at the moment of collection [36]. Low temperature is also required to reduce the enzymatic activity and metabolism of cells and microbes.

The types of samples used for TBM metabolomics include biofluids and Mtb cultures. For culture samples, some investigations have suggested that cell structure should be preserved during the sample preparation process, since damage to the cell membrane can lead to metabolite leakage[36]. Currently, the majority of TBM metabolomics studies have used clinical biofluid

samples, including serum, urine, and CSF. These sample are usually collected from patients during routine diagnostic workup, and there are several factors that influence the quality of clinical samples, including the collection tubes used, the addition of anticoagulants or antiseptic, the occurrence of hemolysis, and other operations performed during collection [37-39]. Moreover, perturbations during biofluid sample transportation and storage can also influence their subsequent analytical fidelity [40]. It is easy to introduce variations during sample preparation that can affect subsequent data analysis. Thus, the use of standard operation procedures (SOPs) is important, as these can ensure data quality, particularly for clinical samples, and those collected in large numbers.

3.2. Metabolite Extraction

The strategy for metabolite extraction depends on the analytical purposes and metabolites concerned [41]. For isolation of specific metabolites, retention of those of interest should be maximized while other substances are removed. For metabolic profile analysis, metabolite extraction tends to aim to reduce the impact on metabolites, and obtain good metabolite coverage and repeatability.

Liquid phase extraction is the most common method of metabolite extraction and is usually achieved by a single or two-phase extraction. The methanol-water system is the classical extraction system for single phase extraction [42], while chloroform-methanol-water is most frequently used for two-phase systems [43]. Polar metabolites and non-polar metabolites can be extracted simultaneously and separated into different phases. Although the two fractions must be analyzed independently, two-phase extraction provides greater coverage of with higher concentration metabolites; however, some metabolites may split between both phases, which may reduce their concentrations and prevent their detection. In addition, the use of either a single- or two-phase extraction involves multiple steps, including solvent additions, mixing, and centrifugation, and each step has the potential to introduce variation into the extraction protocol. Moreover, the large volumes of extraction solvents, such as methanol and chloroform, involved in the liquid extraction process can also represent a problem for their toxicity and disposal [44].

3.3. Metabolite analysis

High quality metabolite data relies on high-throughput and high-sensitivity analysis platforms. For MS-based metabolomics these mainly include gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) (Table 2).

GC-MS is equipped with a modern capillary GC column which is typically tens of meters long. Such long columns provide high peak capacity, good resolution, and reproducible separation. An electron impact ionization source provides GC-MS systems with a stable metabolite fragmentation pattern. There is also a standard database of metabolites at the standardized ionization energy of 70 eV [45]. However, the drawbacks of GC-MS are also significant. GC-MS systems are only compatible with stable volatile components, while the majority of metabolites cannot be directly analyzed by GC-MS because of their temperature-sensitivity or non-volatility. Although non-volatile metabolites can be analyzed by derivatization (e.g., alkylation and silylation) [46], some may produce several derivatives, which can interfere with subsequent analyses.

Currently, the most popular analytical platform for metabolomics is LC-MS. In contrast to GC-MS, LC-MS is primarily used to analyze non-volatile metabolites. The flexible option of columns with different separation mechanisms makes LC-MS a versatile tool for metabolite analysis. Reversed-phase LC (RP-LC) columns are the most common type of LC column, and are primarily used to separate metabolites with moderate- and low-polarity. As an effective complement to RP-LC, hydrophilic interaction LC (HILIC) can easily achieve the separation of highly-polar metabolites [47, 48]. In addition, the resolution and peak capacity of LC columns is significantly improved with reduced packing size. Simultaneously, the analysis time for a single run has been greatly reduced. The major disadvantage of LC-MS based metabolomics is the lack of a standard database of metabolites. As the ionization modes differ greatly among different instruments and no standard retention index has been developed to date [49], LC-MS data from the same substances are inconsistent, leading to difficulties in identification of metabolites.

To improve metabolite coverage, analysis platforms with higher resolution and larger peak capacity are needed. Multidimensional chromatography is one potential solution. The basis of multidimensional chromatography is the integration of several chromatographic systems with different separation mechanisms. It can achieve better separation and resolution than one-dimensional chromatography. Thus, it is suitable for the analysis of complex biological matrices [50, 51]. For example, GC×GC-time-of-flight (TOF) MS integrates the advantages of

high resolution and peak capacity offered by two-dimensional GC systems with the high detection accuracy of high-resolution TOF MS. At present, GC×GC-TOF-MS is considered the best tool for analysis of volatile metabolites [52]. Multidimensional LC-MS is also envisaged to have a broader potential for application in metabolomics research than multidimensional GC-MS; however, the mobile phase of the LC system is related to the separation mechanism, and the large volume of the liquid mobile phase is difficult to remove during the analysis. These problems mean that multidimensional LC-MS is rarely implemented.

3.4. Data analysis

Data analysis often includes several steps, including raw data processing, statistical analysis, and metabolite identification. The raw datasets of MS-based metabolomics cannot be analyzed or identified directly, because they contain noise, random variations, and invalid data. Common data processing pipelines include data filtering, feature detection, feature alignment, and normalization [36]. After data processing, raw datasets are transformed into data matrices, containing large numbers of m/z ion fragment values, along with corresponding retention times and intensity information. These data are correlated with specific metabolites. Subsequently, statistical analysis is employed to capture the bulk of the variation between samples and generate a representative, biologically meaningful dataset. Traditional statistical measures (e.g., t-test, ANOVA, artificial neural networks, principal component analysis, and partial least squares discriminant analysis) are usually employed [53, 54].

The identification of metabolites relies on databases [55]. Currently, there are several online databases used for metabolite identification (Table 3). Although these databases have provided a large amount of MS metabolite data, there are still many metabolites that cannot be retrieved; thus, the establishment of a dedicated database of specific metabolites is recommended.

4. Application of MS-based metabolomics

4.1. Specific metabolism of Mtb

To date, most metabolomic research into TBM has focused on the pathogen causing the condition (Mtb), rather than the disease itself. Unlike the majority of microbes, Mtb has a specific life cycle [56-58], and MS-based metabolomics provides a new tool for investigation of the

specific biochemical signatures of each stage in the life of Mtb.

Mtb exhibits a high degree of metabolic plasticity, termed metabolic adaptation, to enable its adaption to changes in the host environments. Metabolic adaptation is considered an important factor contributing to difficulties in the diagnosis and treatment of tuberculosis, and specific metabolic features of Mtb may underlie the processes of metabolic adaption.

In the context of microbial metabolic activity, carbon substrates and their catabolites are important sources of carbon and energy, respectively. ^{13}C-based metabolomic profiling indicates that Mtb has a distinctive carbon metabolic pathway, and can catabolize multiple carbon sources simultaneously *in vitro* to achieve enhanced monophasic growth, whereas most bacteria consume individual carbon substrates in a preferred sequence and grow with diauxic kinetics [59]. In addition, Mtb catabolizes each carbon source via different pathways and with distinct metabolic fates. Within its host, Mtb mainly relies on fatty acid metabolism to both establish and maintain chronic infections; however, co-catabolism of multiple carbon substrates is a major determinant of Mtb pathogenicity [60]. In addition, the tricarboxylic acid cycle of Mtb also exhibits corresponding changes during adaptation to, and recovery from, hypoxia-induced dormancy [61].

4.2. Functions of genes and enzymes

To date, genomics research has not completely revealed all of the functions of Mtb genes, with many remaining undefined [62, 63]. In addition, a significant number of orphan enzyme activities have not been ascribed to any gene. Similarly, the roles of many enzymes in the metabolic networks of Mtb are also unknown. The application of a type of MS-based metabolomics, known as activity based metabolomics profiling (ABMP), has successfully allowed investigation of the function of these genes and enzymes [64]. ABMP allows unbiased discovery of enzymatic activities encoded by genes of known function, and applies chromatography mass spectrometry to analyze the impact of recombinant enzymes on homologous cellular extracts as physiological libraries of potential substrates and products [65]. For example, a putative oxoglutarate decarboxylase component of the TCA cycle (Rv1248c) and a nucleotide phosphatase (Rv1692) were reassigned as 2-hydroxy-3-oxoadipate synthase and a D,L-glycerol 3-phosphate phosphatase, respectively, using ABMP [66, 67]. In addition, the enzyme encoded by Rv1205 has been found to play an important role in protecting Mtb against nitric oxide [68].

The isocitrate lyases (ICLs) of Mtb are a class of enzymes known to be involved in fatty acid metabolism; however, knowledge of their biochemical roles in metabolic networks was lacking. An investigation using ABMP demonstrated that methylisocitrate lyase (MCL), an ICL of Mtb, connected the tricarboxylic acid (TCA) and methyl citrate cycles. Lack of MCL resulted in defects of membrane potential and intrabacterial pH [69]. Mtb is believed to primarily rely on fatty acid catabolism to survive in its host, while co-metabolism of carbohydrate is also indispensable for its survival. Using ABMP, enzymes involved in the glycolytic pathway, such as glucokinase and pyruvate kinase, have been identified to have important roles in the infection and survival phases of the Mtb life cycle [60, 70]. In addition, ABMP has also been used to investigate the roles of genes in the regulation of lipid metabolism in Mtb [71-73].

4.3. Drug-resistance of Mtb

Currently, because of the misuse of anti-TB drugs, drug-resistant Mtb has become prevalent. As rifampicin and isoniazid are the first-line medications for the prevention and treatment of tuberculosis, the main types of drug-resistant Mtb are those with rifampicin- and isoniazid-resistance. Genetic evidence indicates that mutation of the *rpoB* gene is the main cause of rifampicin-resistant Mtb; however, the metabolic alterations caused by such mutations are unclear. Metabolic profiling analysis demonstrated that *rpoB* mutation mainly induces changes in lipid metabolism [74, 75]. The *rpoB* mutant exhibits significant upregulation of multiple enzymes involved in the biosynthesis of lipids, leading to a decrease in the synthesis of cell wall lipids, and an increase in the consumption of short chain fatty acids as an energy source. Moreover, these changes were more obvious among the more common resistant mutant strains. A recent study has provided more details about the metabolic changes in rifampicin-resistant Mtb [76]. The key reason for the metabolic changes may be mRNA instability. The function of aconitase is transferred from energy production and growth to mRNA binding and stability, followed by an increased dependency on fatty acids as an alternative energy source. In addition, a number of other metabolic changes have been confirmed as additional survival responses that maintain/remodel the cell wall.

Isoniazid-resistance was induced in Mtb by *katG* mutations, which also led to a series of metabolic changes [77]. Mutations of *katG* in Mtb increase the susceptibility of the bacteria to

oxidative stress. Isoniazid-resistant strains exhibit increased uptake and use of alkanes and fatty acids as sources of carbon and energy. Moreover, *katG* mutant strains also synthesize a series of compounds directly involved in the reduction of oxidative stress.

4.4. Toxicity of anti-tuberculosis drugs

Beside their anti-tuberculosis effects, anti-tuberculosis drugs are well known for their toxicity [78]. The toxicity of primary anti-tuberculosis drug, including isoniazid, pyrazinamide, and ethambutol, is thought to be associated with their *in vivo* metabolites, and has greatly limited the treatment of TBM. Currently, drug toxicity metabolomics studies have mainly investigated the following aspects: i) searching for novel metabolites *in vivo*; ii) identification of metabolites with primary roles in induction of toxicity; and iii) determining the role of enzymes in drug-induced toxicity.

Isoniazid, a first-line medication for treatment of tuberculosis, is notorious for its toxicity. The metabolomics approach has been applied to investigate the metabolic changes associated with isoniazid-induced central nervous system (CNS) toxicity and hepatotoxicity [79, 80]. Although the metabolites involved in these processes were thought to be well-established, metabolomics research was able to identify seven novels metabolites and hydrazones in human urine [81], considered potential hepatotoxic metabolites.

Metabolomics has also been used to evaluate the role of CYP2E1, which is thought to be involved in isoniazid-induced hepatotoxicity. The effects of CYP2E1 are independent of the toxicity caused by isoniazid, but related to the isoniazid-induced cholestasis through enhancement of bile acid accumulation and mitochondria β-oxidation [82]. Commonly, one drug may have several potentially toxic metabolites. The metabolomics approach can be used to determine the toxicity indices of these metabolites. For example, metabolomics has been used to study the hepatoxicity of pyrazinamide, demonstrating that pyrazinoic acid (PA) and 5-hydroxy pyrazinoic acid (5-OHPA), metabolites of pyrazinamide in liver, may cause different degrees of hepatotoxicity, and that 5-OHPA is the main factor that induces hepatoxicity [83].

4.5. Drug modes of action

The increasing prevalence of drug-resistant Mtb globally has necessitated both a better

understanding of the physiological activities of pre-existing clinically effective drugs [84, 85] and the development of antibiotics with novel mechanisms of action [86]. However, metabolomics studies of major drugs targeting viable Mtb have shown that, irrespective of the primary target or mode of action, these drugs elicited similar metabolic responses [84] and the drug metabolism phenotypes (DMPs) of patients in the same geographical areas were similar [87]. These results emphasize the importance of the development of antibiotics with novel mechanisms of action. Lipid biosynthesis pathways and transporters are considered potential new targets for the development of anti-tuberculosis agents [88, 89]. Inhibition of peptidoglycan (PG) biosynthesis and cross-linking, which are proven antibiotic targets in multiple bacterial pathogens, are also novel targets for anti-tuberculosis drug development. An MS-based metabolomics study of D-cycloserine (DCS), a second-line antibiotic that inhibits Mtb peptidoglycan synthesis, identified its target enzymes. The results indicated that DCS can inhibit both alanine racemase (Alr) and d-alanine-d-alanine ligase (Ddl), but mainly Ddl [90]. The results were also validated by NMR-based metabolomics [91]. MS-based metabolomics research has also indicated that an upstream enzyme in the early stage of PG biosynthesis, glutamate racemase, may be a useful target for anti-tuberculosis drugs [92]. In addition, metabolomics has been used to evaluate drugs and regimens for TB treatment [93] and predict treatment outcomes [94].

4.6. Discovery of tuberculosis biomarkers

The value of metabolomics in the diagnosis of tuberculosis (TB) has drawn the attention of researchers. A large number of metabolomics studies have been performed to screen for novel biomarkers, and the majority were performed using MS platforms. These investigations have been well summarized and reviewed [95-97]. TB-associated changes in metabolite profiles can be examined in different specimens, including blood, urine, sputum, culture, and breath, greatly reducing the limitations imposed by individual types of specimens.

5. MS-based metabolomics of TBM

Studies of the metabolomics of TBM are at a very early stage, compared with those of tuberculosis more generally. Since TBM is associated with high rates of morbidity and mortality in young children, the first applications of metabolomics have been in pediatric cases with TBM [98,

99]. Metabolic profiling has indicated that TBM induces changes in energy metabolism. The catabolism of glucose is perturbed and the concentration of lactate elevated in CSF. A hypothesis termed the "astrocyte-microglia lactate shuttle" (AMLS) was used to explain this metabolic change. Subsequent targeted metabolomics research has analyzed the source of lactate [100], demonstrating that only the L-enantiomer of lactic acid was found in CSF from pediatric patients with TBM, indicating that lactic acid was solely a product of the host response to infection. A recent study analyzed the amino acid profile in the CSF of pediatric cases with TBM [101]. Five amino acids, namely alanine, asparagine, glycine, lysine, and proline, were significantly elevated. These elevations were thought to be linked to ammonia, because of the increased nitrite levels in TBM. These results all originated from metabolomics analyses of CSF; however, CSF is not a suitable specimen for the diagnosis of pediatric cases with TBM, since its collection involves an invasive procedure. In contrast, urine is an ideal non-invasive specimen. Based on urine metabolic profiling, four potential markers have been identified in pediatric cases with TBM [102]. These metabolites, including methylcitric, 2-ketoglutaric, quinolinic, and 4-hydroxyhippuric acids, exhibit excellent diagnostic ability and also provide prognostic insight for children with TBM.

It is of more interest to distinguish TBM from other forms of meningitis (viral, bacterial, and cryptococcal) than to study the metabolic differences between healthy people and patients with TBM. A recent study attempted to differentiate between bacterial meningitis (BM) and TBM using metabolic profiling [103]. TBM and BM could be differentiated by 3-hydroxyisovalerate, isobutyrate, and formate in CSF samples, or using alanine in serum, or valine and acetone in urine specimens. These results indicate that metabolites can be used to differentiate between TBM and BM; however, the metabolic markers identified in different specimens may not be consistent. In fact, there are already tests that can accurately diagnose bacterial meningitis. The difficulty of TBM diagnosis primarily lies in distinguishing between TBM and viral meningitis (VM), since CSF from TBM and VM patients has similar characteristics, including clear appearance, negative cultures for typical bacterial pathogens, normal white blood cell count, and pleocytosis with mononuclear predominance [104, 105]. Attempts to distinguish between TBM and VM by metabolic profiling [106] identified significant metabolic differences between TBM and VM, with 25 metabolites identified as potential metabolic markers. These potential metabolic markers included nine acid acids, namely glycine, tyrosine, L-glutamine, L-serine, L-threonine, L-valine,

alanine, aspartate and asparagine. Several other important metabolites were also identified, such as glucose, lactate, lipoprtein, glycerine, malonic acid and malonate. This research indicated that the metabolic changes included differences in the amino acid, carbohydrate, lipid, and nucleoside metabolism. Currently, metabolic profiling analysis mainly relies on NMR-based metabolomics, particularly for CSF metabolic profiling. To date, only one study of CSF metabolic profiling using an MS platform has been reported [107], possibly because MS is a complex analytical technique, CSF is difficult to obtain, and MS may require multiple analyses of the same CSF sample. MS will likely be more broadly applied for metabolomics research of TBM if other, more easily acquired specimens, can be used, rather than CSF.

6. Challenges and outlook

Although metabolomics has been applied to TBM research, the primary aim of most investigations has been to gain biological insights into TBM pathogenesis, rather than to determine diagnostic value. In addition, the independent studies that have been performed lack cross-validation and robust overlap, due to differences in the experimental processes used. The large amount of unidentifiable metabolite data generated by such studies often results in potential metabolic markers being missed and makes comparisons between investigations, or the reproduction of findings, difficult. Moreover, the small sample sizes used, in most studies to date has led to problems with validation of results.

Although the field is in its infancy, there is great potential for the development of MS-based metabolomics for TBM in the future. Its high sensitivity increases the potential for identification of specific metabolic markers. As trace metabolites can be detected by MS, MS-based metabolomics provides wide coverage of metabolites. Well-designed studies aimed toward the discovery of diagnostic markers are required. Moreover, large studies are necessary to account for heterogeneous genetic backgrounds and environmental factors. In addition, identification of markers in non-invasive specimens will be more suitable for clinical application than those from CSF.

7. Conclusions

Current diagnostic approaches are limited by their speed, sensitivity, and specificity of

detection. There remains a need for novel biomarkers to improve the diagnosis of TBM. Although the initial application of MS-based metabolomics research focused on TBM-related basic research, such as metabolic network and enzyme activity, MS-based metabolomics is a promising approach for identification of metabolic markers of TBM. Its sensitivity may allow superior biomarker screening, compared with NMR-based metabolomics; however, the field is still in the early stages of establishment. The limited results to date demonstrate the great potential of TBM for diagnostic application, and MS-based metabolomics is expected to provide additional diagnostic information in the future.

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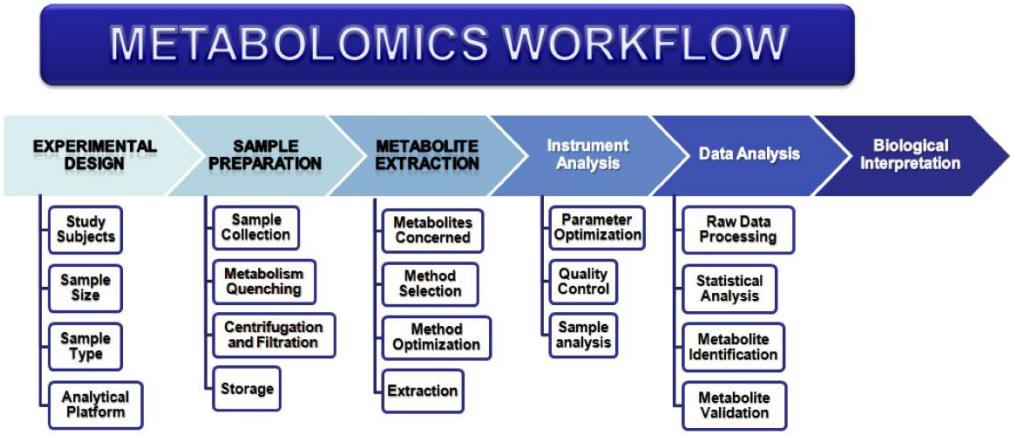

Fig. 1 General workflow for metabolomics.

Table 1 Summary of advantage and limitations of NMR and MS techniques

	MS	NMR
Sensitivity	High	Low
Reproducibility	Moderate	Very high
Type of sample	Liquid	Liquid and tissue
Sample preparation	Complex	Simple
Sample recovery	Destructive	Non-destructive
Metabolite identification	Easy	Difficult
Metabolite database	Only uniform for GC-MS	Uniform
Number of know identifiable metabolites	Thousands of metabolites in a single measurement	Hundreds of metabolites in a single measurement
Common techniques	GC-MS, LC-MS	1H-NMR, two-dimension NMR

Table 2 Comparison of GC-MS and LC-MS

	GC-MS	LC-MS
Metabolite properties	Volatile metabolites, Derivative non-volatile metabolites	Non-volatile metabolites
Column length	Several meters	Several centimeters
Resolution	High	Low
Reproducibility	Low	High
Mobile phase	He,N_2	Water, methanol, acetonitrile
Ionization	Electron ionization	Electrospray ionization
Mass analyzers	Q, QQQ, Ion trap, TOF	TOF, Q-TOF, IT-TOF, Orbitrap
Database	Standard database	Non-standard database
Metabolite identification	Easy	Difficult

Q, Quadrupole.

QQQ, Triple quadrupole.

TOF, time of flight.

IT: ion trap.

Table 3 Online databases for MS-based metabolomics.

Database	Type	Spectrum
Pubchem	Common chemicals	N/A
Chemispider	Common chemicals	N/A
HMDB	Metabolomics	GC-MS, LC-MS, MS/MS
METLIN	Metabolomics	LC-MS, LC-MS/MS
Massbank	MS database	GC-MS, LC-MS, LC-MS/MS
LipidMAP	Lipids	N/A
KEGG	Metabolic pathway	N/A

Highlights

- Metabolomics approach is an effective method for TBM research.
- Metabolites contributing to discrimination may be potential diagnostic markers.
- MS-based metabolomics is useful for the investigation of *Mycobacterium tuberculosis* and related diseases.
- In the future, MS-based metabolomics could be used to provide additional diagnostic information.