AN OVERVIEW OF TUBERCULOSIS TREATMENTS AND DIAGNOSTICS. WHAT ROLE COULD METABOLOMICS PLAY?

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Abstract: In 2001, the WHO declared TB a global emergency, as one third of the world’s population suffered from latent M. tuberculosis infection. Today, a decade later, millions of people still die worldwide as a result of this disease. The growing TB incidence may be ascribed to a variety of reasons, including, amongst others, the inadequacies associated with the currently available diagnostic methods and TB treatment regimes, especially when considering the growing MDR-TB and HIV epidemics. This review discusses and compares the various TB diagnostic and treatment approaches researched and developed to date, considering these with regards to their advantages and shortcomings. We additionally discuss the potential of a relatively new research approach, termed metabolomics, as a tool for new biomarker discovery, and consequently, better diagnostic approaches. Furthermore, this approach, when used alone or in combination with other “omics” techniques, allows for a better understanding of TB disease mechanisms, which may ultimately lead to improved treatment regimens, bringing us ever closer to eradicating this disease.

Key words: Tuberculosis, Treatment, Diagnostics, Metabolomics

INTRODUCTION

Annually, approximately 9 million new tuberculosis (TB) cases are recorded, and up to 2 million people die as a result of this disease. TB is most commonly localised in the lungs and is caused by the bacteria, Mycobacterium tuberculosis. Although this is a worldwide epidemic, Africa (30%) and Asia (55%) accounts for 85% of all global TB cases. When evaluating recorded adult deaths in low- and middle-income countries, TB is ranked third, after HIV/AIDS and ischemic heart disease [1]. These statistics are disturbing considering the fact that TB can be prevented and is, in most instances, a curable disease. From the 1950s to the 1980s, various first-line anti-TB drugs have been developed, and when using combinations of these, patients with drug-susceptible TB can successfully be cured within 6 months. Approximately 0.4 - 0.5 million annual TB cases are, however, multi-drug resistant (MDR) [2], requiring the use of second-line drugs, which are costly, have severe side effects and require almost 2 years of continual treatment, resulting in a dismal cure rate of only 50 – 70%. Since the 1980s, the rising HIV pandemic has become a significant obstacle in modern TB control. TB is considered to be the primary cause of death in most HIV infected patients, partly due to the increased TB infection rate in HIV patients, a weekend immune system, and the difficulties associated with diagnosing TB in HIV patients due to a lowered bacterial load in the lungs of these individuals [3,4].

In this overview, we describe the development and mechanism of action of current TB vaccination, diagnostic approaches, and treatment protocols, considering their advantages and drawbacks. We also discuss the potential of a number of newer treatment and diagnostic approaches recently developed. In order to fully understand the mechanisms by which these preventative, treatment and diagnostic approaches function, an understanding
of the pathophysiology of TB is required.

**PATHOPHYSIOLOGY OF TUBERCULOSIS**

TB is transmitted via aerosolized droplet nuclei, which are *M. tuberculosis* containing particles with a diameter of 1-5 μm. These droplets are produced and expectorated by patients with pulmonary TB during talking, singing, sneezing or coughing and can, due to their small particle size, stay airborne for many hours [5]. Additionally, these droplets can also be created in clinics and hospitals during sputum induction, aerosol treatments, aerosolization during bronchoscopy, or through tissue or sputum processing [6].

After inhalation by a previously uninfected individual, the infectious droplet nuclei lodge in the alveoli of the distal airways of the lung. Alveolar macrophages then engulf these nuclei, which sets of a cascade of events leading to either a successful suppression of the infection or the progression to active TB [7].

*M. tuberculosis* replicates slowly, but constantly, inside these macrophages and spreads to the hilar lymph nodes via the lymphatic system. In the majority of infected individuals, a cell-mediated immune reaction develops 2-8 weeks after infection. Consequently, necrotic, cheese-like granulomas, containing non-viable *M. tuberculosis*, are formed by activated T-lymphocytes and macrophages, which restrict the further replication and spread of the bacteria [8]. At the cellular level, *M. tuberculosis* infected macrophages interact with T-lymphocytes via a number of vital cytokines. Activated T-lymphocytes then release interferon-γ, another cytokine, which indirectly stimulates phagocytosis of *M. tuberculosis* inside these macrophages [9]. Interferon-γ also stimulates the release of tumor necrosis factor by these macrophages, which, in turn, aid in granuloma formation, and controls the extent of infection [10].

In a host with a strong cell-mediated immunity, a latent infection can be maintained in this form, and active disease may never occur. Even though small amounts of viable *M. tuberculosis* may be present in these granulomas, infected individuals (without active disease) cannot transmit the organism and are, hence, not infectious during this latent phase [6]. Active disease manifests when the host immune system cannot restrain *M. tuberculosis* replication. Although this primary developing disease most frequently occurs in the parenchyma of the mid and lower lung, it may spread throughout the body and can present in almost any organ system. Several factors may trigger the conversion of latent infection to active disease, with HIV co-infection being the single greatest risk factor for this. Other immunocompromising conditions increasing the risk for active disease development include; diabetes mellitus, chemotherapy, malnutrition, vitamin D or A deficiency, renal failure, and extensive corticosteroid therapy [5].

Due to the insidious onset of TB, symptoms might only occur in later stages of the disease. In the case of an advanced disease, clinical symptoms include cough, fever, night sweats, anorexia, weight loss, chest pain, hemoptysis, and dyspnea [11]. As inflammation and tissue necrosis develop, sputum, which is widely used as diagnostic material, is produced. Hemoptysis, or the coughing up of blood, is typically a result of preceding disease and does not automatically indicate active TB. Hemoptysis may manifest as a result of remaining tuberculous bronchiectasis (irreversible dilation of part of the bronchial tree), breakage of a dilated vessel in a cavity wall, bacterial or fungal infections in a residual cavity, and / or erosion of calcified lesions into the lumen of an airway. TB may cause severe respiratory failure, but dyspnea is atypical, except in the case of extensive disease [6,12]. These symptoms alone are, however, rather non-specific and cannot be used for accurately diagnosing TB, as these correlate with many other lung infections or malignant conditions [11].

This strong, cell-mediated immune response, induced by TB infection, forms the basis on which the currently used TB vaccination procedures function.

**TUBERCULOSIS VACCINATION**

The first TB vaccination, and successful immunization, was carried out in 1921. This vaccine, the Bacille Calmette-Guerin or BCG, an avirulent *M. bovis* strain, was attenuated for 13 years by serial passages into glycerol imbibed potato slices [13]. Due to the high demand, the original BCG strain was distributed globally, even before the establishment of an appropriate culture protocol. Therefore, numerous BCG strains, with a variety of antigenic and immunological differences, exist today in various parts of the world. Nevertheless, BCG is currently still
the most commonly used TB vaccine worldwide, and BCG immunization is mandatory in high-incidence TB areas [14]. Using 1264 published articles, Colditz et al. (1994) did a meta-analysis on the efficiency of the BCG vaccination. This study concluded that, when vaccinated with BCG, the risk to develop TB is reduced by only 50% [15]. Newer approaches in the development of TB vaccines are, like BCG, also based on the induction of a strong cell-mediated immune response, which can control bacterial replication and maintain infection in the latent phase. Some of these attempts include: attenuated \textit{M. tuberculosis} mutants; recombinant BCG strains; recombinant proteins; and DNA vaccines. However, due to the complexity of these studies, it is expected that the results of phase III trails will only be available by 2014-2015 [14].

**TUBERCULOSIS TREATMENT**

One of the greatest discoveries of the 20th century was that of antimicrobial drugs, which have ever since saved millions of lives and are still the major element in the treatment of infection. The first effective \textit{M. tuberculosis} antimicrobial, streptomycin, was tested as part of the first randomized clinical trial carried out from 1948 until 1988. The publication of the positive outcome of this trial forever changed the national TB treatment policy [16].

Today, many anti-TB drugs are available which can, based on their activity, be classified into 3 groups: those with bactericidal activity, those with sterilizing activity, and those preventing drug resistance. Bactericidal activity is the capacity of a drug to reduce the amount of actively dividing bacilli in the initial therapy stage. Although rifampicin and streptomycin have some bactericidal activity, the most potent bactericidal anti-TB drug is isoniazid [17]. Sterilizing activity, as in the case of rifampicin and pyrazinamide, is the ability of a drug to eliminate the putative subpopulation of dormant bacteria from which a clinical relapse can occur [18]. Most effective treatment regimes consist of two phases: the initial intensive phase, during which a combination of at least two bactericidal anti-TB drugs (isoniazid and rifampicin) are used to kill actively growing \textit{M. tuberculosis} populations, followed by a continuation phase for the elimination of intermittently dividing and dormant bacteria, using sterilizing drugs [5].

In an attempt to reduce the global burden of TB, the WHO formulated the Millennium Development Goals (MDGs). The target of the MDGs is to halve TB prevalence and death rates by 2015 compared to that in 1990, and furthermore to completely eliminate TB by 2050. To achieve this, the WHO developed the DOTS (directly observed treatment, short-course) strategy in the mid-1990s, which was recommended internationally and consequently expanded worldwide. DOTS is a 6 months therapy regimen consisting of an initial 2 month treatment phase with four first-line drugs (isoniazid, rifampicin, pyrazinamid and ethambutol), followed by a 4 months treatment phase with only isoniazid and rifampicin. The addition of direct observation therapy (DOT), where patients consume each dose of anti-TB drugs under supervision, to the treatment strategy is strongly recommended. This approach maximizes the probability of therapy completion, hence, limiting the emergence of drug-resistance [19]. More or less 90% of all drug-susceptible TB cases are cured when this regimen is fully adhered to. In the case of MDR-TB, with resistance to at least rifampicin and isoniazid, DOTS alone may not succeed. For these cases, the WHO recommends DOTS-plus, which includes adding second-line drugs to the conventional DOTS program. This regimen is, however, costly, takes up to 24 months, and has a much higher level of toxicity as a result of harmful second-line medication [1].

A brief overview of the mechanisms of action and adverse effects of the most commonly used first- and second-line TB drugs will now be discussed.

**First-line medications**

\textbf{Streptomycin:} In 1944, Selman Waksman discovered and isolated the first effective anti-TB drug, streptomycin, from \textit{Streptomyces griseus}, and for this, Waksman was awarded the Nobel Prize in 1952 for physiology and medicine [20].

Streptomycin is an aminoglycoside antibiotic which interferes with protein synthesis of the TB causing bacteria by inhibiting mRNA translation, resulting in the misreading of the genetic code, and consequently, cell membrane damage [21,22]. The exact binding site of streptomycin was found to be in the small 30S subunit of the ribosome, more specifically at ribosomal protein S12 (\textit{rpsL}) and 16S rRNA (\textit{rrs}) [23].

As early as 1946, only two years after its discovery, Klein and Kimmelman already reported the first streptomycin resistant mutants. These mutants could
be classified into two groups depending on whether or not they demonstrated a high or low level of resistance [24]. Ever since, numerous studies have investigated the underlying molecular mechanism of streptomycin resistance [25,26,27], but despite this, the molecular cause of this resistance is, however, still not yet completely understood.

Mutations within both the \textit{rrs} and \textit{rpsL} genes have been linked to a high-level streptomycin resistance [25,26]. Most of these resistance causing mutations leads to a hyper-accurate phenotype (increased accuracy of translation), compensating for the effect of the drug without having any effect on the interaction between the ribosome and the drug [28,29,30]. These mutations, nevertheless, have only been identified in just more than one half of all clinical streptomycin-resistant \textit{M. tuberculosis} isolates [31,32]. The underlying mechanisms of the other, low-level streptomycin resistant strains, is still, however, obscure.

Recently, Okamoto et al. (2007) identified a mutation within the gene, \textit{gidB}, conferring low-level resistance in 33\% of \textit{M. tuberculosis} isolates [30]. They furthermore confirmed that \textit{gidB} encodes for a conserved 7-methylguanosine (m7G) methyltransferase (GidB) specific for the 16S rRNA. Further studies on the precise function and role of GidB in the pathogenesis of \textit{M. tuberculosis} is, however, still unknown.

Apart from organisms developing resistance, other complications may also occur regarding the side effects of streptomycin treatment. Hypersensitivity is not unusual in patients treated with streptomycin, and may, depending on the severity, require treatment interruption. Transient dizziness and numbness around the mouth can occur after injections, but does regress and completely disappear when drug administration is stopped. With long-term treatment, chronic toxicity might lead to blackouts, ataxia, ringing in the ears, and even permanent deafness [1].

**Isoniazid:** Isonicotinic acid hydrazide, or isoniazid, is one of the most efficient anti-TB drugs used to inhibit mycobacterial growth. However, despite an immense number of studies dedicated to elucidating the mode of action of this drug, its exact mechanism is still largely unknown, mainly due to the fact that it my influence a variety of different mycobacterial cellular processes.

Early investigations indicated that, when exposed to isoniazid, \textit{M. tuberculosis} loses its acid-fast nature and viability, which led to the suspicion that the drug functions by altering the cell-wall lipids, specifically by inhibiting mycolic acid synthesis [33]. Several studies have ever since confirmed that isoniazid does indeed disturb cell-wall structure [34,35] and electronmicroscopy scanning of \textit{M. tuberculosis} exposed to isoniazid determined various altered morphological features [36]. Other modes of action of isoniazid include reactivity with bacterial proteins (tyrosine residues specifically) [37] and the formation of reactive oxygen radicals during the activation of the drug [38].

More recently, it has been established that isoniazid is a pro-drug and requires oxidative activation by catalase-peroxidase KatG [39]. An isonicotinoyl radical, the activated form of isoniazid, then reacts with NAD, leading to the covalent adduct INH-NAD, which inhibits the \textit{M. tuberculosis} InhA enzyme [40]. InhA is an enoyl-acyl carrier protein, catalyzing the reduction of the trans double bond, conjugated to the carbonyl group of fatty acyl substrates, with NADH acting as the hydrogen donor for these reactions [41]. Inhibition of InhA will consequently block fatty acid elongation via the FAS II system, which is essential for mycolic acid synthesis, the main building blocks of the mycobacterial envelope [42].

Resistance to isoniazid entails various mutations (insertions, deletions and point mutations) in a number of genes. The major targets are the \textit{katG} and the coding and regulatory area of \textit{inhA}. Other mutations either have minor roles in isoniazid resistance or are compensatory due to the loss of catalase-peroxidase activity [43].

When recommended isoniazid doses are administrated, adverse effects are uncommon, unless the patients have a history of previous kidney or liver failure [19]. Hypersensitivity reactions may, however, occur in the first week of treatment. Peripheral neuropathy is the most common adverse effect (20\% of cases) of isoniazid treatment, especially in high-risk patients (pregnant women, alcoholic, malnourished, and diabetic patients), but can be prevented with a complementary low dose of vitamin B6 [1]. When isoniazid is used in isolation, hepatitis manifests in 0.6\% of cases, and when used in combination with rifampicin, the incidence increases to 2.7\%. This incidence increases with age and in
patients with previous liver disease. The fatality rate of isoniazid treated patients due to hepatitis is, however, less than 0.023% [19,44].

**Ethambutol:** Ethambutol is normally included in initial TB treatment regimes, especially when potential isoniazid resistance is expected [17]. Ethambutol is effective against intracellular and extracellular bacteria, however, the exact mode of action is still unknown. To date, several studies have been done in an attempt to explain this drugs mode of action, however, most of these analyses focused on its actions on altering the mycobacterial cell wall structure [45,46]. Takayama and Kilburn (1989) showed that ethambutol has an inhibitory effect on the transfer of arabinogalactan (arabinosyl transferases) into the *Mycobacterium* cell wall, which in turn leads to the accumulation of trehalose mono- and dimycolates [46]. Silve *et al.* (1993) additionally indicated that ethambutol inhibits the transfer of [D-14C]glucose into the D-arabinose fraction of arabinogalactan [47]. Consequently it was suggested that, due to inefficient arabinogalactan transfer into the cell wall, mycolic acids accumulate in these mycobacteria, leading to the previously observed bacterial declumping and morphological alterations following ethambutol treatment [48]. Furthermore, sequence analyses of ethambutol resistant, clinically isolated mycobacteria, showed that this resistance is primarily linked to a number of missense mutations in the ethambutol resistance determining region of the arabinosyl transferase encoding gene, *embB* [49]. Starks *et al.* (2009) determined that mutations in *embB* codon 306, in particular, are important indicators of ethambutol resistance, and may also be useful for confirming isoniazid resistance in 50-70% of clinical samples [50]. The exact role of the *embB* 306 mutation in the acquisition of isoniazid resistance is, however, still controversial.

A major adverse effect associated with ethambutol treatment, is retrobulbar optic neuritis, with symptoms including: the decline of visual sharpness (blurry vision), dyschromatopsia (colour blindness), and central scotoma (tunnel vision). These effects can be prevented in the case of low dosage treatment for a maximum period of 3 months [1].

**Rifampicin:** Rifampicin is a powerful anti-TB drug, reducing actively dividing and semi-dormant organisms. This drug primarily inhibits DNA-dependant RNA-polymerase, an enzyme essential for the transcription of RNA [51]. Resistance to rifampicin is mainly as a result of alterations in the β-subunit of RNA-polymerase due to mutations in the encoding *rpoB* gene. A variety of specific resistance-conferring mutations (accounting for more than 95% of rifampicin-resistant strains) have been described in the 81-bp region of *rpoB* known as the rifampicin-resistance determining region (RRDR) [52]. The majority of these are point mutations, and results in the replacement of aromatic with non-aromatic amino acids. These replacements lead to drug resistance by interrupting the forces that bind rifampicin to RNA polymerase. These mutations also impair the fitness of these bacteria, but the fitness can, however, be restored by secondary mutations [53].

Minor side effects associated with rifampicin treatment include: flu-like symptoms, abdominal pain, fatigue, ataxia, dyspnea, and anorexia and, in most cases, do not warrant the discontinuation of treatment. In combined-treatment procedures, however, exanthema (a rash-like reaction) can occur, which requires immediate discontinuation. Furthermore, cholestatic hepatitis occurs in 2.7% of patients treated with a combination of isoniazid and rifampicin and in up to 1.1% when rifampicin is combined with other anti-TB drugs [1,19,44].

**Pyrazinamide:** Pyrazinamide, together with isoniazid, rifampicin, and ethambutol, plays an important role in initial TB treatment regimes. As it is active in an acidic pH environment, hence, able to kill semi-dormant bacteria not killed by other TB drugs, the inclusion of pyrazinamide in these regimens shortens TB therapy form 9 to 6 months [19]. Even though pyrazinamide is widely used today, its specific mechanism of action, as is the case with most other TB medications, is still largely unknown. Pyrazinamide, a prodrug, is converted to its active form, pyrazinoic acid (POA) by the bacterial enzyme nicotinamidase/pyrazinamidase (PZase) [54], and it has been shown that various mutations in the PZase encoding gene, *pncA*, leads to pyrazinamide resistance in *M. tuberculosis* [55]. All bacteria are equipped with PZase, nevertheless, *M. tuberculosis* is uniquely susceptible to pyrazinamide. This susceptibility is due to a deficient pyrazinoic acid efflux mechanism in *M. tuberculosis*, in contrast to the natural pyrazinamide resistant *M. smegmatis*, which rapidly extrudes pyrazinoic acid out of the bacterial cell [56]. Various studies have implied that
Caceras by inhibiting Alr and Ddl [59]. Cycloserine blocks mycobacterial cell wall synthesis alanine:D-alanine ligase (Ddl) catalyzed reaction. D-dipeptide D-alanyl–D-alanine, is then formed in a D-branched during peptidoglycan biosynthesis [60]. The alanine racemase (Alr) in the initial step of alanine L-stereoisomer and is converted to D-alanine via D-peptidoglycan. Alanine is typically accessible as the components of the backbone of the bacterial cell wall, aminopimelate in, particular) are important D-Amino acids (D-alanine, D-glutamate, and D-inhibiting cell wall synthesis in mycobacteria [59]. Cyclic structural analogue of D-alanine, acts by drugs, D-cycloserine (D-4-amino-isoxazolidone), a D-Cycloserine: [1,44].

Despite its comparatively better mode of action for eliminating TB, pyrazinamide does have rather severe side effects. Pyrazinamide treatment may be associated with pruritus, exantheme, or rhabdomyolysis with kidney failure, and myoglobinuria. Pyrazinamide is, additionally considered to be the most hepatotoxic of all first-line anti-TB drugs and treatment should be replaced or, at least, temporarily discontinued when any of the above side effects are noticed. [1,44].

**Second-line medication**

**D-Cycloserine:** As is the case with many anti-TB drugs, D-cycloserine (D-4-amino-isoxazolidone), a cyclic structural analogue of D-alanine, acts by inhibiting cell wall synthesis in mycobacteria [59]. D-Amino acids (D-alanine, D-glutamate, and D-aminopimelatein, in particular) are important components of the backbone of the bacterial cell wall, peptidoglycan. Alanine is typically accessible as the L-stereoisomer and is converted to D-alanine via D-alanine racemase (Alr) in the initial step of alanine branching during petidoglycan biosynthesis [60]. The dipeptide D-alanyl–D-alanine, is then formed in a D-alanine:D-alanine ligase (Ddl) catalyzed reaction. D-cycloserine blocks mycobacterial cell wall synthesis by inhibiting Alr and Ddl [59].

Caceres et al. (1997) determined that the over expression of alr in M. smegmatis and M. bovis led to a D-cycloserine resistant phenotype [61]. This resistance was observed to a far higher degree than was seen during Ddl over expression, leading to the suspicion that the primary target of D-cycloserine might be Alr [62]. Furthermore, an increased sensitivity to D-cycloserine was observed when alrA [63] or ddl [64] was inactivated. Even though it is presumed that D-cycloserine resistance results due to mutations in alrA and ddl, the exact mechanism of resistance is still unclear [65].

Despite the fact that D-cycloserine is a successful antimycobacterial drug, it is rarely prescribed and only used in combined therapies due to its severe adverse effects. Neurological effects including: headaches, vertigo, memory deficiency and mental confusion only to name a few, in addition to psychiatric effects including: depressive and paranoid reactions, and psychotic states, are common in patients receiving D-cycloserine treatment [65]. These adverse effects are the result of D-cycloserine binding to neuronal N-methyl aspartate receptors [66], and the inhibition of the enzymes involved in the metabolism and synthesis of the neurotransmitter γ-aminobutyric acid [67].

**Ethionamide:** Being structurally similar to isoniazid, ethionamide is also considered a prodrug, requiring activation by the bacterial cell. When activated, ethionamide, like isoniazid, disrupts cell wall biosynthesis by inhibiting a mutual cellular target, the enoyl-acyl carrier protein, inhA [68,69]. Gene array studies, indicating similar patterns of gene expression induced by both isoniazid and ethionamide, verifies this shared site of action [70]. The fact that isoniazid resistance does not always result in ethionamide resistance, has led to the suspicion that different enzymes may activate these drugs. Little was known about this enzyme, until only recently, when two different research groups almost simultaneously identified etaA, a monoxygenase [71,72]. EtaA, a FAD-enclosing enzyme, oxidizes ethionamide to its analogous S-oxide, which, in turn, is oxidized to 2-ethyl-4-amidopyridine by an unstable oxidized sulfenic acid intermediate [69]. Resistance to ethionamide may occur due to various mutations in this activating enzyme, etaA, and the target, inhA [73].

Ethionamide is classified as a second-line drug due to its severe side effects. Extreme gastrointestinal associated complications, including intense salivation, nausea, loss of appetite, vomiting, and abdominal pain are common after ethionamide treatment. These symptoms can be reduced by administrating the drug with food or at bedtime. As is the case with isoniazid, ethionamide can cause hepatotoxicity, especially in patients with liver disease and a history of alcoholism. Neurological symptoms such as optic neuritis, anxiety, depression, and hallucinations have been reported in only 1-2% of all treated patients. Other adverse effects of ethionamide include: postural hypotension, alopecia, impotence, hypothyroidism, acne, and photosensitivity, only to name a few [65].

**Kanamycin and amikacin:** Since the discovery of streptomycin in 1944 [20], aminoglycosides have played a major role in TB therapy. Kanamyc in
explaining the lack of cross-resistance of an
treatment regimens [65,74]. These drugs, like streptomycin and other aminoglycosides, bind to the small 30S ribosomal subunit, in particular the 16S rRNA (rrs), consequently inhibiting translation and, hence, protein synthesis [75]. Information regarding the exact mechanism underlying kanamycin resistance in M. tuberculosis is scarce. What is known is that similar to streptomycin, mutations in the rrs gene, results in high-level kanamycin resistance, and some mutations may also cause cross-resistance to amikacin and other second-line drugs [74]. However, as much as 80% of clinical kanamycin resistant clinical isolates exhibit low-level resistance without rrs mutations or cross-resistance. Recently, Zaunbrecher et al. (2009), determined that in M. tuberculosis, an over expression of the survival protein, Eis, due to eis promotor mutations, is common in these low-level kanamycin resistant clinical isolates. Eis, an aminoglycoside acetyltransferase, inactivates both kanamycin and amacykin. Kanamycin is 3-times more efficient than amacykin as a substrate for Eis, explaining the lack of cross-resistance of an eis mutation to kanamycin [76].

Ototoxicity, or damage to the ear, is one of the most severe adverse effects of aminoglycosides. Hearing loss is a result of cranial nerve VIII damage, including cochlear and vestibular impairment. Instant discontinuation of aminoglycoside treatment is advised when ototoxicity occurs. As a result of their build-up in renal tubules, aminoglycosides may additionally cause toxic renal effects such as proteinuria, oliguria (low urine output), and decreased creatinine clearance. In rare occasions, aminoglycoside treatment might bring about hypersensitivity (extreme allergic reaction to the drug) or neuromuscular blockage, resulting in respiratory failure [65].

Fluoroquinolones: Nalidixic acid, the first quinolone medication, was acquired in the early 1960’s as an impurity during the production of quinine [77]. Many fluoroquinolone derivatives have ever since been tested for their antibacterial activity. Levofoxacin, sparflxacin, ofloxacin, and ciprofloxacin have shown to be extremely active against M. tuberculosis [78], and have therefore been used in TB treatment regimens since 1980’s [79].

Fluoroquinolones circulate throughout the body and have the extraordinary property of functioning intracellularly, reaching mycobacteria inside the macrophages and, hence, leading to a potent treatment outcome [80]. Fluoroquinolones function by inhibiting DNA gyrase (Gyr, a type II topoisomerase), an enzyme essential for reducing the tension when double-strand DNA is unwound during DNA replication, recombination and expression. This inhibition prevents supercoiling of the DNA, leading to uncontrolled mRNA synthesis, exonuclease production, protein synthesis and chromosome degradation, due to free DNA ends [80,81]. When used as a monotherapy, mycobacteria quickly develop resistance to fluoroquinolones due to mutations in the DNA gyrase enzyme (gyr). Other resistance conferring mechanisms include: 1) the presence of an efflux system, actively releasing the drug form the bacterial cell or, 2) an altered cell membrane structure, leading to fluoroquinolone impermeability and thus reducing diffusion of the drug into the cell [80]. Cross-resistance between fluoroquinolones and other TB-drugs however, does not occur, and even though cross-resistance between various fluoroquinolones has been described [80,81], moxifloxacin and levofloxacin have been used to successfully treat patients resistant to ofloxacin. Furthermore, a study done in India showed that oxifloxacin, in combination with various first-line drugs, may be effective as a three month, ultra-short course TB treatment regime [82].

Gastrointestinal effects are the most common side effects associated with fluoroquinolone treatment. These effects including; vomiting, anorexia, diarrhea, and abdominal pain, arise in 3 - 17% of treated patients. A small amount (0.9 - 11%) of patients receiving fluoroquinolone treatment may also develop insomnia, tremors, and headaches. Skin rash, erythema, and pruritus may occur in 0.4 - 2.2% of fluoroquinolone treated patients [65].

Table 1 gives a summary of the modes of action, cellular targets, and resistance conferring genes of the drugs used for TB treatment. Although the WHO recommended treatment regimes are highly effective, various studies have demonstrated unwanted interactions between the different anti-TB drugs and between anti-TB drugs and other medications used by TB patients [19].

Three of the four first-line anti-TB drugs in the DOTS program are possibly hepatotoxic and depending on
In addition to the severe side effects resulting from these drug interactions, serum drug concentrations are also altered, thereby reducing their efficiency [44]. Severe effects due to anti-TB drugs do, however, emerge because of several factors, and the extent of these effects are influenced by, amongst others, the age of the patient, nutritional status, dosage, time of administration, and pre-existing diseases or dysfunctions (such as HIV co-infection, liver or kidney impairment, and alcoholism) [1,19,44]. Changes in first-line treatment procedures are mainly as a result of severe adverse effects, leading to the use of more toxic, less active, and more expensive second-line TB drugs, usually accompanied by increased hospitalizations and home visits [83]. These side effects additionally contribute to patients disrupting or ceasing treatment, leading to higher levels of treatment failure and, hence, acquired resistance [44].

The shortcomings of current anti-TB drugs and TB treatment regimes are not entirely responsible for the case detection rate of 61% in mid-2010, which fell far short of the global target of 70% [3]. Despite the need for faster acting, less toxic TB treatment procedures, we also urgently require innovative, sensitive and rapid diagnostic approaches, which can manage the concurrent HIV epidemic and rising incidence of MDR-TB cases.

**TUBERCULOSIS DIAGNOSTICS**

The emergence of MDR-TB, and the rising HIV pandemic, has challenged the conventional TB diagnostic methods, and the development of new, accurate, sensitive and quick TB diagnostic approaches are now crucial. In the past few years, innovative, high-tech diagnostic procedures such as molecular techniques and rapid cultures have entered the market. These tests are, however, still only commercially used in high-income countries, where the TB incidence is low. Due to their complexity and high costs, these tests are not yet suited for high-burden, low-income settings [84].

The underlying mechanism of the currently available and more recently developed TB diagnostic procedures, considering each of their advantages and disadvantages will subsequently be discussed.

**Tuberculin skin test:** The tuberculin skin test (TST) is based on the delayed-type hypersensitivity reaction produced in *M. tuberculosis* infected individuals, when antigenic compounds (purified protein derivative [PPD], acquired from heat-killed *M. tuberculosis*) are injected intra-cutaneously into the forearm of an individual [6,84]. T-cells formed as a result of present or prior infection, then travel to the infected skin area where they release lymphokines. The induction of local vasodilatation, fibrin deposition,
edema and the accumulation of other inflammatory cells, as a result of these lymphokines, cause a thickening of the skin at the injection site, which can be measured [6]. The reported sensitivity and specificity of this technique, however, varies due to number of reasons. For instance, in a study conducted by Al et al. (2000), patients with a TST with a size smaller than the 5 mm threshold, were found to be less likely to have active TB, however, cases above the threshold were not necessarily indicative of active disease [85]. Additionally, patients with varying disease states gave almost identical results, considering the size and shape of the TST. Furthermore, false-positives regularly occur in patients who were previously vaccinated with BCG or are infected with other, non-tuberculous mycobacteria. False-negative reactions, on the other hand, may also occur due to a variety of technical factors, for example, in patients with compromised immune systems due to immunosuppressive drugs (i.e. steroids), AIDS, cancer, age (newborns and adults over 65 years), and supplementary bacterial, fungal or viral infections [86,84].

**Cytokine detection assay:** A newer approach used for the detection of latent TB infection, also making use of the cell-mediated immune response, is the cytokine detection assay. In this case, circulating lymphocytes (extracted from a patients blood), are exposed to mycobacterial antigens for 6 to 24 hours. *M. tuberculosis* infected lymphocytes recognize these antigens and subsequently produce cytokines, mostly interferon-gamma (INF-γ), which can be measured [84]. The first INF-γ release assays (IGRAs) used PPD as the antigen of choice. More recently developed tests, however, rely on antigens which are more specific to *M. tuberculosis*, such as early secreted antigen target (ESAT)-6 and culture filtrate protein (CFP)-10 [87]. Currently, there are two commercially available IGRAs termed QUANTIFERON®-TB Gold and T-SPOT-TB. Several studies indicated that these assays have a high specificity (>95%), but a lower, variable sensitivity (75-97%), and also, results between the two IGRAs vary, limiting their efficacy in routine clinical settings [87,88]. Considering that both TST and the cytokine detection assay detect markers for possible infection only, follow-up diagnostics are required to determine if the patient with an infection does in fact have active TB.

**Radiographic methods:** For the diagnosis of active pulmonary TB, chest X-rays (CXRs) can be classified as either 1) typical of TB, with i) the occurrence of nodular, alveolar, or interstitial infiltrates predominantly affecting the zones above the clavicles or upper zones of the lung, or ii) the presence of cavitations affecting the upper zones or the apical segment of the lower lobes of the lungs; 2) compatible with TB in the case of atelectasis, enlarged hilar nodes, pleural exudate, mass lesion, miliary or pneumonic lesion, or; 3) atypical (all other patterns, including normal CXR) [89]. As summarized by the WHO [84], X-rays alone are still widely used as an important tool in TB diagnostics, despite the fact that various studies have proven that TB shows no unique radiographic patterns [6]. Also, numerous other lung diseases have a similar radiographic appearance, which can easily mimic TB [90], leading to over diagnosis when used alone. When using culture as the reference test, Van Cleeff et al. (2005) reported the sensitivity and specificity of CXRs as 80% and 67% respectively [91], whereas Arslan et al. (2010) reported these values to be 73% and 94% respectively [92], which is unsatisfactory. CXR can thus be useful for the identification of abnormalities in the lungs, but to ascertain the tubercular aetiology further tests, such as bacteriology, are an absolute necessity [84].

**Microscopic examination:** Despite recent advantages in TB diagnostics, smear microscopy, as first demonstrated by Robert Koch in 1882, is still the most commonly used screening method for the detection of mycobacteria in clinical sputum specimens [93]. This quick (less than 2 hours), simple and low cost technique is based on the acid-fastness of mycobacteria, and thus the ability of these organisms to retain dye after treatment with an acid-alcohol solution [6]. The characteristic mycolic acids present in the cell walls of all mycobacteria are responsible for this colour reaction, hence, limiting the ability of the method for species identification and drug susceptibility testing. Also, these fatty acids do persist when the bacteria dies, and therefore, this technique cannot discriminate a current, active disease state, from previous *Mycobacterium* infection [94]. Furthermore, numerous quantitative studies have shown that high amounts of bacilli (5000 – 10 000 bacteria mL⁻¹) are required for the detection of bacteria using smear microscopy tests [95], leading to a sensitivity of no more than 35 – 70%. This method has on occasion, however, been reported to detect only 20 – 30% of all TB cases [96], in less advanced disease states, TB-HIV co-infection, and in children [84]. Nevertheless, although a negative smear does not rule out mycobacterium infection, a positive result almost verifies a diagnosis, resulting
in a technique specificity of more than 95%. This high specificity is limited only by cross-contamination, due to environmental mycobacterium, non-tuberculous mycobacterium, and technical inadequacies [94].

**Bacteriological culture:** The WHO considers bacteriological culture as the gold standard for TB diagnosis, as it detects over 80% of TB cases accurately, with a reported specificity of close to 100% [97,84]. However, it has been reported that negative sputum cultures may occur in 15-20% of adult pulmonary TB cases. Additionally, despite immense anti-contamination procedures, false-positive culture results have been reported to occur in 1-4% of all cases, due to a transfer of bacilli from TB-positive to TB-negative samples during laboratory handling [98].

Either solid or liquid media can be used to grow cultures. Solid media including; egg based media (e.g. Löwenstein–Jensen), or agar based media (e.g. Middlebrook 7H10), are most commonly used due to their low costs. This approach, however, has a diagnostic time of 4-6 weeks [99]. Growth in liquid media is considerably faster (2-4 weeks) and far more sensitive, but does have the disadvantage of higher contamination rates (8-10%) when compared to solid media (3-5%) [94]. The development of automated culture systems, such as BACTEC 460 and mycobacterial growth indicator tube (MGIT) systems was a major improvement in mycobacterium culture diagnosis [6]. Hanna et al. (1999) reported a multicenter evaluation of the BACTEC MGIT 960 and BACTEC 460 systems in comparison to solid culture media for mycobacteria isolation. They found that traditional solid media (Löwenstein–Jensen slopes and Middlebrook 7H11 plates) was capable of a *M. tuberculosis* recovery rate of 79%, in a mean time of 24.1 days, in comparison with BACTEC 460 TB and BACTEC MGIT 960, with recovery rates of 90% and 77%, and mean times of 15.2 and 14 days, respectively. A combination of solid media and BACTEC 460 gave the best *M. tuberculosis* recovery rate (97%) [100]. As an added advantage, the above-mentioned methods can differentiate between various clinically important *Mycobacterium* species, including non-tuberculous *Mycobacterium* [101]. These automated systems are, however, expensive and requires expensive infrastructure and maintenance, and skilled and experienced staff [84].

Bacteriological culture is also the conventionally used method for drug susceptibility testing (DST) in TB. When using solid media, the growth of *M. tuberculosis* in the presence of anti-TB medication can be detected by one of three methods including: proportions, resistance ratios, or absolute concentrations [43]. In the proportions method, which is considered the reference standard, the growth of organisms on a drug-free media is compared to the growth on media containing an anti-TB drug. The resistance ratio method, on the other hand, determines the minimum inhibitory concentration of a drug-susceptible, reference strain, as compared to the patient’s strain. Lastly, the absolute concentrations method utilizes media containing various dilutions of the anti-TB drug in order to determine the lowest concentration of the medication necessary to inhibit growth. These results are, however, only available within 2-3 months after sample collection [84].

The gold standard for culture DST, for both first- and second line medication, is the semi-automated radiometric BACTEC 460 TB system [102,103], where bacilli are cultivated in liquid media containing various concentrations of anti-TB drugs. This method has been used effectively for over 25 years, and has reduced the time required for DST to a mere 4-13 days [102]. Nevertheless, the BACTEC 460 TB system makes use of radioactivity, leading to many concerns regarding safety during use and disposal after use. To overcome this problem, the BD BACTEC MGIT 960 SIRE (testing for Streptomycin, Isoniazid, Rifampin, and Ethambutol) assay has been developed and has shown to be an excellent alternative to the radiometric assay [103]. This automated, non-radiometric method uses fluorometric technology to accurately detect the consumption of O₂ in the presence of anti-TB medication, leading to a diagnostic result within 4-12 days [102]. It does, however, suffer the disadvantage of higher contamination rates than the BACTEC 460 TB assay [102,103]. This contamination may be due to either the richness of the medium (which is unlikely to change due to the methods grow detection principle) or the use of screw caps instead of rubber septa, which could be resolved in time [103]. These methods are considered extremely expensive and require highly trained personnel, a limitation to developing countries [84]. A newer liquid-media-based DST method, the microscopic observation drug susceptibility (MODS) assay, is however, more affordable and does not require radioactive isotopes or fluorescent indicators.
With MODS, inverted-light microscopy is used to detect early growth of *M. tuberculosis* as strings and tangles of bacterial cells in Middlebrook 7H9 broth medium in the absence or presence of anti-TB drugs, with a diagnostic result in less than two weeks [104]. Six to eight mL of sputum per specimen is, however, required for this analysis, which is already difficult to obtain from adults, not to mention children and immuno-suppressed (TB/HIV co-infected) patients. Further disadvantages associated with this method are; the fact that it is an indirect method for the detection of *M. tuberculosis*, requires daily microscopic observations, and does not allow for mycobacterial species identification [43].

Colourimetric methods used for DST in TB are based on the reduction of an oxidation–reduction indicator, which is added to liquid culture medium after the exposure of *M. tuberculosis* to various anti-TB medications. A colour change, proportional to the number of viable bacteria, is indicative of drug-resistance [105]. These tests are mainly performed on clinical isolates and hence, do not exclude the critical culturing waiting period. Some of these methods have, nevertheless, been tested directly on sputum samples with a sensitivity and specificity ranging between 88% and 100%. These studies did, however, only focus on the detection of rifampicin- and isoniazid-resistant TB, using highly infectious samples (positive with more than 10 acid-fast bacilli per microscopic field) [106]. A big concern of colourimetric tests is the biohazard of aerosol generation due to the manipulation of microtitre plates. Also, phase III and IV diagnostic trails are still required before the clinical implementation of these methods can be considered [105].

**High performance liquid chromatography:** For species identification, high performance liquid chromatography (HPLC) assays can be completed in a few hours, and have sensitivities and specificities of almost 100%. This technique can identify and distinguish over 50 *Mycobacterium* species, based on their unique sets of mycolic acids and β-hydroxy-α-fatty acids [107]. HPLC cannot, however, distinguish between *M. tuberculosis* and *M. bovis* and requires at least 10^6 pure culture organisms for a diagnostic result, hence not excluding the delay of bacteriological culture [6].

**Nucleic acid amplification:** A newly developed TB diagnostic approach, nucleic acid amplification tests (NATs), enzymatically amplifies regions of bacterial DNA specific to the *M. tuberculosis* complex. The most widely used NATs are polymerase chain reaction (PCR), transcription mediated amplification (TMA) and strand displacement amplification (SDA) [84]. Several commercial NATs tests are available, which can be used directly on sputum or other clinical samples [109]. In a study conducted by Catanzaro *et al.* (2000), the clinical performance of a NAT, approved by the Food and Drug Administration in 1995, the enhanced *Mycobacterium tuberculosis* Direct (E-MTD) test, was investigated. The sensitivity of the E-MTD test for low, intermediate, and high clinical TB suspicion was 83%, 75%, and 87% respectively, with a corresponding specificity of 97%, 100% and 100%. The positive prediction values of the E-MTD test were 59% (low TB suspicion), 100% (intermediate TB suspicion), and 100% (high TB suspicion) vs. 36%, 30%, and 94% respectively for smear microscopy, hence, confirming NATs to be potentially helpful for the diagnosis of early stage TB [109]. Furthermore, Pounder *et al.* (2010), developed a genomic deletion assay based on multiplex PCR with melting temperature analysis, to differentiate between six clinically important *Mycobacterium* species, based on the regions of difference (RDs) in the their complete genome sequences. Using a set of 3 primers for each RD, they correctly identified 96% of *Mycobacterium* isolates in pure culture [110]. However, because sequence variation may occur at primer binding sites during evolution, a reselection of the target sequence may be required in future.

Genotyping methods have also been developed for DST of all first-line and most second-line TB drugs by detecting specific resistance linked mutations in target genes of *M. tuberculosis*. PCR-restriction fragment length polymorphism (PCR-RFLP) analysis is a rapid, low-cost method for the detection of polymorphisms at mutated codons of mostly INH and EMB resistant *M. tuberculosis* strains [111,112]. However, due to the presence of PCR inhibitors in sputum samples, researchers still prefer to use pure cultures as the genomic DNA source for PCR amplification, hence, this diagnostic approach still suffers the culturing waiting period [111]. Direct DNA sequencing is the most realistic method to use if all resistant strains have mutations in a specific region of a single target gene (as is the case with the RRDR in the *rpoB* gene of rifampicin-resistant strains). DNA sequencing is, however, impractical when analysing large amounts of specimens, particularly in developing countries [43]. Espasa *et al.*
al. (2005) evaluated the potential of real-time PCR for rifampicin and isoniazid susceptibility testing in clinical samples. The sensitivity of the test ranged from 30.4 to 35.3% in smear-negative samples and 95.1 to 99.2 % in smear-positive samples, with a 100% specificity. The detection limit of real-time PCR for detecting target mutations in clinical samples was found to be 1.5 X 10^3 CFU/mL, compared to 10 CFU/mL in culture [113]. Furthermore, genetic alterations in the target gene sequences were absent in 30% of the isoniazid-resistant isolates, hence, resulting in the low sensitivity of this diagnostic approach.

Commercially available NATs, nevertheless, suffer the further disadvantages of high costs and the need for high tech infrastructure and well-trained personnel. The high incidence of false-positive results due to laboratory cross-contamination, also limits its performance under field conditions [84,109].

Serology: The use of serology (detection of antibodies, antigens and immune complexes) in TB diagnostics has, up to now, failed largely in providing the necessary sensitivity and specificity for effective clinical use [84]. Perkins et al. (2003), for example, evaluated the performance of a commercial immuno-chromatographic test kit (ICT Tuberculosis), employing five recombinant M. tuberculosis antigens. This kit correctly identified only 64.2% of the smear-positive and of the 46.3% smear-negative, culture confirmed-TB patients [114]. Using a serological approach for TB diagnostics is challenging, as the various stages of the disease e.g. exposure, latent infection, active disease and severe disease, each have their own antibody patterns. Therefore, when developing such a test, one must consider the use of antigens expressed in all stages of the disease by employing, for instance, cocktails of multiple antigens [108]. Results may also vary due to the lack of reproducibility in antigen purifying methods and, additionally, since environmental mycobacteria can cross-react with antibodies in samples, false positive results frequently occur. Therefore, the use of serological TB diagnostic methods is not yet recommended by the WHO [84].

Phage assay: Currently, the only commercially available phage assay for the detection of TB, FASTPlaqueTB, makes use of mycobacteriophages (mycobacteria infecting viruses) to signify the incidence of viable M. tuberculosis in clinical samples. When using bacteriological culture as the reference test, Muzaffar et al. (2002) determined the sensitivity and specificity of this assay to be 87.4% and 88.2% for smear-positive sputum samples as opposed to 67.1% and 98.4% respectively in smear-negative, culture confirmed cases [115]. Another study, conducted in South Africa, confirmed the poor sensitivity (48.7%) of this approach in smear-negative, culture confirmed specimens [116]. Although these assay test results can be achieved in 48 hours and is specific for M. tuberculosis, it missed other infectious Mycobacterium spp., and is not able to detect drug resistance [84].

When weighing up the advantages and disadvantages of the above-mentioned TB-diagnostic approaches, it is obvious that no test currently available meets all the specifications of sensitivity, specificity, speed, safety, robustness, training simplicity, and cost. Hence, the world is in need for a new, innovative TB diagnostic method, which will overcome current limitations, and can easily be implemented in low-income, high-burden counties.

WHERE TO FROM HERE?

The metabolome is the ultimate downstream result of genome transcription, and may be described as a compilation of all the metabolites (small molecular compounds), present in a specific cell or organism, participating in metabolomic reactions during normal cell function, growth, and maintenance. Metabolomics is ‘the non-biased identification and quantification of all these metabolites in a biological system’, using highly sensitive analytical procedures [117]. During active disease and perhaps even latent infection, the infectious organisms, such as TB causing bacilli, disturb the host’s biochemical networks and provoke alterations in the quantity and types of metabolites present [118]. Using specialized techniques, this response to pathological stimuli can be measured over time. This approach is the foundation for the discovery of new metabolomic biomarkers for diagnosing disease, better describing the mechanisms of drug resistance, elucidating drug mechanisms and monitoring treatment approaches.

Since TB was discovered in the 1800’s, characterization, diagnosis and treatment relied solely on the identification and traits of the causative organism in clinical samples. Ever since then, technical advances in the detection limits of various
analytical techniques have made it possible to diagnose TB from samples containing very low concentrations of bacilli. These analytical techniques do, however, go hand-in-hand with elevated levels of complexity and costs [84]. Metabolomics not only considers the metabolome of the infected organism, but also measures changes in the host metabolism due to infection. Consequently, new biomarkers may be used, not only diagnostically, but also to better characterize disease mechanisms in the host and to monitor treatment outcomes. In the past few years, metabolomics applications for the characterization of a variety of diseases has been described, and these include, amongst others: coronary heart disease [119]; type 2 diabetes [120]; epithelial ovarian cancer [121]; Huntington disease [122]; hypertension [123]; liver cancer [124]; liver failure due to hepatitis B infection [125]; meningitis and ventriculitis [126] Parkinson’s disease [127, 128]; pre-eclampsia [129,130], and schizophrenia [131]. Recently, two groups have investigated the potential use of metabolomics for TB diagnostics.

Pavlou et al. [132] completed a pilot study to test the potential of an electronic nose, which uses 14 conducting polymer sensor arrays, to identify *M. tuberculosis* in culture and patient collected sputum samples. Using this approach, they were able to discriminate between *M. tuberculosis* and sterile cultures, with a 100% prediction value, and also, between closely related cultures (*M. avium, P. aeruginosa, M. tuberculosis*, and *M. tuberculosis* + *M. scrofulaceum*) with a prediction value of 96%. They furthermore built a discriminant model using 36 patient collected sputum samples (*M. tuberculosis* (6), *M. avium* (8), *P. aeruginosa* (8), mixed infection (6) and control (6)), which correctly identified 90% of the unknown samples used to validate this method [132]. The same research group then further investigated this approach, applying it to 330 culture proven, HIV tested sputum samples. They detected TB with a sensitivity of 89% and a specificity of 91% at a detection limit of 1 x 10^4 bacteria mL^-1, which is comparable to smear microscopy. As the nature of sensor volatiles is not yet fully understood, the characteristics of these volatiles can, however, not be identified or quantified, which may be a limitation to using this sensor array technology [133].

Consequently, Phillips et al. [134] tested the hypothesis that volatile organic compounds (VOCs) in breath might lead to the identification of new TB biomarkers using a GCMS metabolomics approach. This hypothesis was made based on the fact that mycobacteria produce unique VOC patterns *in vitro*, and the observation that increased oxidative stress in TB patients may lead to distinct VOC patterns. In this study, they compared the VOC patterns in the headspace of *M. tuberculosis* cultures to that of sterile growth media. Headspace VOCs were captured on sorbent traps, which were analyzed by automated thermal desorption, gas chromatography and mass spectroscopy (ATD/GC/MS). A set of 130 VOCs, mainly benzene derivatives, naphthalene, and alkanes, were constantly detected exclusively in the *M. tuberculosis* cultured samples [134]. Following the same approach, breath VOCs from 42 patients, suspected of pulmonary TB, were analyzed. Accordingly, this method could distinguish between hospitalized patients (with suspected TB) and healthy controls with a 100% specificity and 100% sensitivity, and between TB culture positive and negative patients with a specificity of 78,9% and sensitivity of 95,7%. Using the characteristic VOCs, this approach was used to diagnose 226 patient collected sputum samples with a reported sensitivity of 84% and specificity of 64,7%, and a positive prediction value of 76%, when compared to smear microscopy results, 68% for sputum culture and 66% for chest X-rays [135]. This study shows the potential of metabolomics towards less invasive TB-diagnostic procedures and may, furthermore, assist in the diagnosis of children and HIV patients, who usually experience difficulty in supplying adequate sputum for current diagnostic procedures. Additionally, these procedures are relatively quick, taking merely a few hours to attain a diagnostic result, in contrast to the current gold standard, bacterial culture, which can take anything from 2-6 weeks [99].

The fact that the final outcome of alterations in the genome results in an altered metabolite profile, additionally makes metabolomics an excellent functional genomics tool [136]. In 2000, Fiehn et al. used a metabolomics approach to identify distinct metabolic profiles for four different *Arabidopsis* genotypes, including two homozygous ecotypes and a mutant of each [137]. Since then, several other studies have documented alterations in the metabolite profiles of a number of bacterial species, due to genetic perturbations [136]. Considering this, metabolomics may additionally be used to identify biomarkers specific to drug-resistant TB, contributing to a
better understanding of the underlying mechanisms of drug action and drug resistance, and additionally, potentially resulting in better MDR-TB diagnosis and treatments.

Furthermore, concentrations of newly identified metabolomics biomarkers may be used to monitor treatment outcomes, or may potentially be used for the early detection of relapses. This would enhance treatment strategies and may consequently prevent / lower the incidence of drug-resistance due to non-adherence. Metabolomics may additionally be used to test the performance of new anti-TB drugs using these biomarkers. An example of this is reported by Loots et al. (2005), using a metabolomics approach to investigate the effect of combined anti-TB drug therapy (using Rifater, a combination of rifampicin, isoniazid, and pyrazinamide) and melatonin (an antioxidant) on the organic acid and free radical profiles of rats. They indicated that Rifater treatment results in increased hydroxyradicals and abnormal organic acids, characteristic of a multiple acyl-CoA dehydrogenase defect (MADD). Furthermore, co-administering melatonin dramatically reverses these side effects [138]. This metabolomics approach was the first of its kind to indicate an altered metabolism due to potential electron-transport chain inhibition by anti-TB mediation, and the prevention thereof using the antioxidant, melatonin. In a similar study, Huo et al. (2009) successfully differentiated serum metabolic profiles of type 2 diabetes mellitus patients treated with metformin hydrochloride from profiles of untreated patients. They, furthermore, identified a number of compounds, which were characteristic of metformin hydrochloride therapy, as potential, treatment-induced, biomarkers [139]. These studies prove the capacity of metabolomics as a tool to elucidate drug mechanisms and better treatment.

**CONCLUSION**

Considering the above information, it is clear that the still growing TB epidemic is fuelled by the inadequate performance of currently used TB treatment and diagnostic procedures [140]. Thus, the world is in urgent need of new, less toxic, faster acting TB treatment procedures alongside more sensitive, rapid TB diagnostic methods, which are able to cope with the growing incidence of MDR-TB and the co-existing HIV epidemic. The relatively new research field of metabolomics may lead to new biomarker identification and a holistic view of intra-host changes during TB infection, active disease, and treatment. This added information may potentially open the door to a new era in TB research, diagnostics, and drug development. The identification of novel TB-biomarkers might be challenging, but should earnestly be pursued in an attempt to eradicate this worldwide pandemic.

**REFERENCES**


