

**Elucidating the antimicrobial mechanisms of colistin
on *Mycobacterium tuberculosis* using
metabolomics**

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“God, grant me the serenity to accept the things I cannot change, the courage to change the things I can, and the wisdom to know the difference.”

-Reinhold Niebuhr

Table of Contents

Acknowledgements.....	6
Summary	7
Opsomming	8
List of tables and figures	10
Chapter 1: Preface.....	12
1.1 Background and motivation	12
1.2 Aim and objectives of the study	14
1.2.1 Aim	14
1.2.2 Objectives.....	14
1.3 Structure of article dissertation	14
1.4 Outcomes of the study	15
1.5 Author contributions	16
References	18
Chapter 2: Literature overview	20
2.1. Introduction	20
2.2. Pathophysiology of tuberculosis	20
2.3. Tuberculosis treatment	21
2.3.1 First-line medications.....	23
2.3.2 Second-line medications	25
2.3.3 New possible second-line anti-TB medications and regimen.....	26
2.3.4 Colistin.....	28
2.4. Metabolomics.....	31
2.4.1 An introduction to metabolomics in the context of drug- development research.....	31
2.4.2 Analytical methods most often used for metabolomics	34

2.4.3 Statistical approaches	35
2.4.4 The application of metabolomics towards drug investigations	37
2.5. Concluding remarks	44
References	45
Chapter 3: Elucidating the antimicrobial mechanisms of colistin sulfate on <i>Mycobacterium tuberculosis</i> using metabolomics	56
Abstract	56
Keywords	57
3.1. Introduction	57
3.2. Materials and methods.....	58
3.2.1. Cell culture	58
3.2.2. Whole metabolome extraction procedure and derivatization	60
3.2.3. GCXGC-TOFMS analyses	61
3.2.4. Data processing, clean-up and statistics	61
3.3 Results	62
3.4 Discussion	66
3.5 Concluding remarks	69
References	70
Chapter 4: Metabolomics of colistin methanesulfonate treated <i>Mycobacterium tuberculosis</i>	74
Abstract	74
Keywords	75
4.1 Introduction	75
4.2 Materials and methods.....	76
4.2.1 Cell culture	76
4.2.2 Whole metabolome extraction procedure and derivatization	78

4.2.3	GCXGC-TOFMS analyses	78
4.2.4	Data processing, clean-up and statistics	79
4.3	Results and Discussion.....	80
4.4	Concluding remarks	89
	References	90
	Chapter 5: Discussion and conclusion	96
5.1	Introduction	96
5.2	Summary of the main findings and future recommendations	96
	Chapter 6: Appendix (1-3).....	98
	Appendix 1	98
	Appendix 2.....	138
	Appendix 3.....	144

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Summary

In 2014, the WHO declared tuberculosis (TB) an epidemic, as an estimated 9 million people suffered from *Mtb* infection. Today, millions of mortalities are still reported worldwide as a result of this disease. This growing TB incidence may be ascribed to a variety of reasons, including, treatment failure, poor patient adherence, lack of new anti-TB drugs, and long treatment duration.

Despite the wide research on anti-TB drugs to date, the mechanisms of these drugs remain poorly understood. Colistin sulfate (CS) and colistin methanesulfonate (CMS) provide hope for a promising outcome as a new anti-TB drug, however its exact mechanism of action has not been explored. It is also unclear how colistin could provide the necessary treatment advances to the current six-month “directly observed treatment short-course” (DOTS) regimen. Thus, there is a need for new, sensitive and specific analytical techniques to elucidate the anti-bacterial effect of colistin on TB.

Considering this, we used GCxGC-TOFMS metabolomics and identified new metabolite markers for the purpose of confirming or elucidating both forms of colistin’s mechanisms of action against *Mycobacterium tuberculosis* (*Mtb*). The most significant observations were the unanimous flux in the metabolism of the colistin treated *Mtb* towards fatty acid synthesis and cell wall repair, confirming previous reports that colistin acts by disrupting the cell wall of mycobacteria. Accompanying this, is a subsequently elevated glucose uptake, since it serves as the primary energy substrate for the upregulated glyoxylate cycle, and additionally as a precursor for further fatty acid synthesis via the glycerolipid metabolic pathway.

In addition to the proposal of a number of new hypotheses, explaining various mechanisms of colistin, the mapping of the newly identified metabolite markers led to the confirmation of various previously suggested metabolic pathways and alterations thereof due to an assortment of perturbations. Therefore, this study significantly contributes to the characterisation of colistin, which may in the future lead to a new treatment protocol for TB, pertaining to the global TB epidemic.

Key words: Metabolomics; *Mycobacterium tuberculosis*; Colistin; Tuberculosis

Opsomming

Afrikaanse titel:

Toeligting van die antimikrobiële meganismes van colistien op *Mycobacterium tuberculosis* met behulp van metabolomika.

In 2014 het die WGO tuberkulose (TB) 'n epidemie verklaar, aangesien 'n beraamde 9 miljoen mense aan *Mtb*-infeksie gely het. Vandag word miljoene sterftes steeds wêreldwyd gerapporteer as gevolg van hierdie siekte. Hierdie groeiende TB-voorkoms kan toegeskryf word aan verskeie redes, insluitende behandelingsversaking, swak pasiëntafhanklikheid, gebrek aan nuwe anti-TB-middels en lang behandelingstydperk.

Ten spyte van die verskillende navorsing oor anti-TB medisyne tot op datum, bly die meganismes van hierdie middels swak verstaan. Colistiensulfaat (CS) en colistienmetansulfonaat gee aanleiding tot 'n belowende uitkoms as 'n nuwe anti-TB middel, maar die presiese meganisme van werking is nog nie ondersoek nie. Dit is ook onduidelik hoe colistien die nodige behandelingsvorderings kan bied aan die huidige 6 maande "regstreeks waargeneemde behandeling kort kursus" (DOTS) regime. Daar is dus 'n behoefte aan nuwe, sensitiewe en spesifieke analitiese tegnieke om die anti-bakteriese effek van colistien op TB te verhelder.

In die lig hiervan het ons GCxGC-TOFMS metabolomika gebruik en nuwe metaboliese merkers geïdentifiseer vir die bevestiging of beklemtoning van beide vorme van colistien se meganismes van werking teen *Mtb*. Die belangrikste waarnemings was die eenparige vloei in die metabolisme van die colistien behandelde *Mtb* na vetsuur sintese en selwand herstel, wat vorige verslae bevestig dat colistien die selwand van mycobacteria ontwrig. Gevolglik kom 'n verhoogde glukose opname voor, aangesien dit as die primêre energie substraat dien vir die opgereguleerde glyoksilaat siklus, en addisioneel as 'n voorloper vir verdere vetsuur sintese via die gliserolipied metaboliese weë.

Benewens die voorstel van 'n aantal nuwe hipoteses, wat verskeie meganismes van colistien uiteensit, het die kartering van die nuut geïdentifiseerde metaboliese merkers die verskeie voorheen voorgestelde metaboliese weë, en veranderinge daarvan as gevolg van 'n

verskeidenheid perturbasies, bevestig. Daarom dra hierdie studie aansienlik by tot die karakterisering van kolistien, wat in die toekoms tot 'n nuwe behandelingsprotokol vir TB kan lei, wat verband hou met die globale TB-epidemie.

Sleutelwoorde: Metabolomika; Mycobacterium tuberkulose; Colistien; tuberkulose

List of tables and figures

Table 1:1 the research team. 16

Table 3:1 The 21 metabolite markers that best explain the variance between the individually cultured *Mtb* samples in the absence (*Mtb*-Controls) and presence (*Mtb*-CS) of colistin sulfate. 65

Table 4:1 The 22 metabolite markers best explaining the variance between the individually cultured *Mtb* samples in the absence (*Mtb*-Controls) and presence (*Mtb*-CMS) of colistin methanesulfonate. 85

Figure 1-1 Global trends in the estimated number of incident TB cases and the number of TB deaths (in millions), 2000–2016. Shaded areas represent uncertainty intervals.....12

Figure 3-1 PCA differentiation using the GCxGC-TOFMS whole metabolome analysed data of the individually cultured *Mtb* in the absence (*Mtb*-Control) and presence (*Mtb*-CS) of colistin sulfate (32 mg/mL). The variances accounted for are indicated in parenthesis. 63

Figure 3-2 Venn diagram illustrating a multi-statistical approach for selecting the 21 metabolite markers best describing the variation detected between the individually cultured *Mtb* samples in the presence and absence of colistin sulfate. 64

Figure 3-3 Altered *Mtb* metabolome induced by treatment with colistin sulfate. The schematic representation indicates the 21 metabolite markers in bold and the confirmatory metabolites which were also elevated, but not necessarily significantly so, indicated in italics. Increase and decrease in the metabolite markers are indicated by ↑↓ respectively. 68

Figure 4-1 PCA differentiation of individually cultured *Mtb* in the absence (*Mtb*-control) and presence (*Mtb*-CMS) of colistin methanesulfonate (32 µg/mL) and analysed via GCxGC-TOFMS. The variances accounted for are indicated in parenthesis. 81

Figure 4-2 Venn diagram illustrating the multi-statistical selection criteria of the 22 metabolite markers best describing the variation between the individually cultured *Mtb* sample groups in the presence and absence of CMS..... 82

Figure 4-3 Metabolite markers best describing the variation in the metabolome of the CMS treated *Mtb* compared to that of *Mtb* cultured without CMS, are schematically represented in bold and those metabolites which were not necessarily significantly elevated using the statistical procedure selected, but still showed significance via considering their P-values, indicated in italics. Elevated and reduced concentrations of each metabolite marker indicated by either ↑ or ↓ respectively. 87

Figure 4-4 Pentose phosphate pathway indicating an elevated flux in the CMS treated *Mtb* towards glyceraldehyde-3-phosphate and fructose-6-phosphate, via the elevated erythrose and reduced arabinose concentrations. 88

Chapter 1: Preface

1.1 Background and motivation

According to the World Health Organization (WHO) (2015), one of the world's deadliest communicable diseases is tuberculosis (TB). TB is an airborne, infectious bacterial disease caused by *Mtb* and it usually affects the lungs (Floyd, 2014). In 2013, an estimated 9 million people developed TB, with an estimated 1.6 million mortalities that were reported. According to the WHO surveillance system report, 5% of all TB cases were multidrug-resistant TB (MDR-TB) in 2014 (WHO, 2016). The 2017 WHO TB report indicated the TB incidences and deaths among people with and without human immunodeficiency virus (HIV) as seen in Figure 1-1 (WHO, 2017). These statistics are rather disturbing considering the fact that TB can be prevented and is, in most instances, a curable disease.

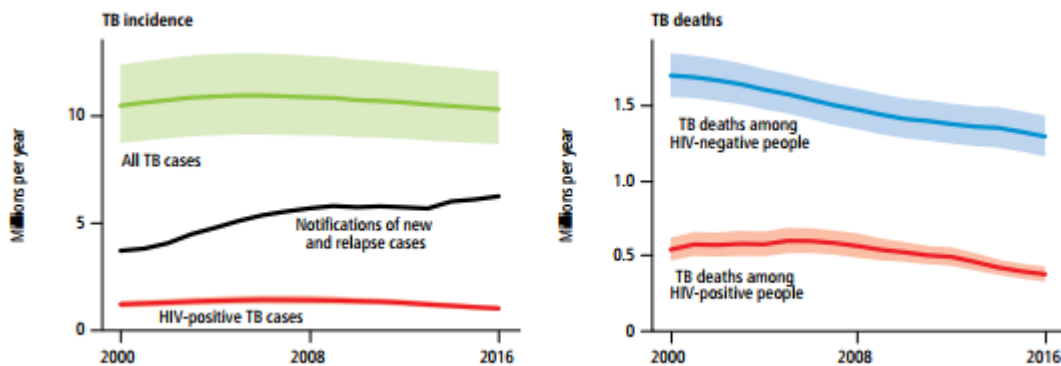


Figure 1-1 Global trends in the estimated number of incident TB cases and the number of TB deaths (in millions), 2000–2016. Shaded areas represent uncertainty intervals (WHO, 2017).

Patients with drug-susceptible TB can successfully be cured with a 6-month regimen (the DOTS programme), consisting of four first-line drugs, namely rifampicin, isoniazid, ethambutol and pyrazinamide (Kamfer, 2013). Although the success rate of the current drug-susceptible TB is 85%, it is far lower for MDR-TB (Raviglione, 2014). Therefore, a new, less toxic, faster-acting TB treatment approach is urgently needed to eradicate this disease.

Recently-introduced bedaquiline was the first new anti-TB drug in 45 years, targeted at treating MDR-TB. The lack of new anti-TB drugs over the years may be due to the poorly understood mechanisms of *Mtb*, but more likely the fact that interest in new TB drug development waned after the discovery of the frontline drugs, which were considered sufficient. (de Villiers & Loots, 2013). Colistin was one of the first antibiotics showing significant activity against gram-negative bacteria, hence making it a feasible candidate for investigation. Although colistin was discovered in the 1940s, it was only used for a short period due to its nephron- and neurotoxicity. Colistin methanesulfonate, however, can be inhaled which may serve as a means for getting around its toxicity to humans (Falagas et al., 2005). Considering the lack of second-line drugs for treating TB, and the need for shorter treatment protocols for drug susceptible TB, it is worth investigating colistin as a treatment option.

According to the literature, colistin interacts electrostatically with the gram-negative outer membrane of bacteria and competitively displaces divalent cations from the negatively charged phosphate groups of membrane lipids (Peterson et al., 1985). Insertion of colistin disrupts the outer membrane and lipopolysaccharides are released (Chen & Feingold, 1972). Additionally, electron microscopic results have demonstrated that membrane vesicles emerge from the surface of gram-negative bacteria in the presence of colistin (Lopes & Inniss, 1969). Colistin is likely to have the same effect on *Mtb*, allowing for a possible promising outcome (de Knecht, et al., 2017; Bax, et al., 2015; van Breda, et al., 2015; Cassir, et al., 2014; Rastogi, et al., 1986).

Considering this, a characterisation perspective is needed of colistin and its anti-TB mechanism. Hence, metabolomics, the relatively new research field, uses highly sensitive analytical techniques that identify and quantify all metabolites in a biological system (Dunn et al., 2005). For the past years, a variety of diseases have been characterized with the use of metabolomics, including TB (Schoeman et al., 2011). Considering the above-mentioned, metabolomics would serve an excellent characterisation perspective (de Villiers & Loots, 2013) on the drug mechanism of colistin. The investigation will contribute to existing scientific knowledge on colistin's drug mechanism, by clarifying the *Mtb*'s metabolomic profile when treated with colistin. According to Al-Khayyat A.A. & Aronson A.L. (1973) CS and CMS have different antibacterial activities, pharmacokinetics, and pharmacodynamics. Thus an understanding of the mechanisms of CS and CMS on *Mtb* is very important for interpreting results from metabolomic studies.

1.2 Aim and objectives of the study

1.2.1 Aim

The aim of this study is to use metabolomics to better characterize colistin sulfate and colistin methanesulfonate as possible anti-TB drugs.

1.2.2 Objectives

The above-mentioned aim will be accomplished by completing the following objectives:

1. The development of the most optimal methodological approach for the metabolomics investigations of cultured samples.
2. The application of the relevant developed methodology in objective 1 to identify metabolite markers for the purpose of better characterizing colistin sulfate in treated *Mtb*.
3. The application of the relevant developed methodology in objective 1 to identify metabolite markers for the purpose of better characterizing colistin methanesulfonate in treated *Mtb*.

1.3 Structure of article dissertation

This article dissertation is a compilation of chapters written specifically to comply with the requirements of the North-West University, Potchefstroom Campus, South Africa, for the completion of the degree Magister Scientiae (Biochemistry) in article dissertation format. In order to ensure easy reading and a logical flow, all chapters contain their own introduction, materials and methods, results, discussion, conclusions and reference sections.

Chapter 1 gives a brief background of the conducted study, focusing on the aim and objectives. This chapter also discusses the structure of the article dissertation and the outcomes of the study, and clarifies the contributions and roles of each co-author and co-worker towards the completion of this study.

Chapter 2 provides an overview of the relevant literature required as a basis for understanding the results, discussion and conclusions in the chapters that follow. A part of this chapter has been published in the journal *Advances in Protein Chemistry and Structural Biology*.

Chapter 3 describes the use of a GC/GC-TOF/MS, metabolomics methodology for characterizing colistin sulfate (the anti-TB drug) treated *Mtb* specimen. The GC/GC-TOF/MS-generated data was consequently analysed using multivariate statistical data analysis (PCA and PLS-DA), in order to identify those metabolite markers contributing to colistin sulfate's mechanism of action. This chapter has been submitted to the journal *Tuberculosis*.

Chapter 4 describes the above-mentioned GC/GC-TOF/MS metabolomics approach, except for the characterization of (the anti-TB drug) colistin methanesulfonate- treated *Mtb* sample. The GC/GC-TOF/MS-generated data was consequently analysed using multivariate statistical data analysis (PCA and PLS-DA), in order to identify those metabolite markers contributing to the elucidation of colistin methanesulfonate's mechanism of action. This chapter has been submitted to the journal *Tuberculosis, and a brief communication*.

Chapter 5 is a comprehensive discussion and conclusion of the results obtained in Chapters 3 and 4. Additional recommendations and future research prospects, potentially emanating from this research, are discussed.

1.4 Outcomes of the study

The publications which originated from this study are attached in Appendix 1-3.

Manuscripts - Appendix 1-3

Koen, N., Du Preez, I., Loots du, T., 2016. Metabolomics and personalized medicine. *Adv. Protein Chem. Struct. Biol.* 102, 53–78.

Koen, N., van Breda, S., & Loots, D.T., 2018. Elucidating the antimicrobial mechanisms of colistin sulfate on *Mycobacterium tuberculosis* using metabolomics. *Tuberculosis*. 111, 14-19.

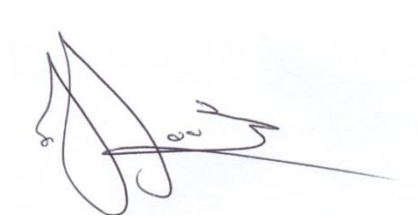
Koen, N; van Breda, S; Loots, D.T., 2018. Metabolomics of colistin methanesulfonate treated *Mycobacterium tuberculosis*. *Tuberculosis*. 111, 154-160.

1.5 Author contributions

The primary author/investigator of this dissertation in article format is Nadia Koen. The contributions of the co-authors, co-workers and collaborators towards this work, are summarized in Table 1-1.

The following is a statement from the primary investigator and supervisor, confirming their individual roles in the study and giving their permission that the data generated and conclusions made may form part of this article dissertation:

I declare that my role in this study, as indicated in Table 1-1, is a representation of my actual contribution, and I hereby give my consent that this work may be published as part of the M. Sc. article dissertation of Nadia Koen.



Prof. Du Toit Loots



Nadia Koen

Table 1:1 the research team.

Co-author	Co-worker	Contribution
Nadia Koen (B.Sc. Hons. Biochemistry)		Responsible, together with the study leader, for the conceptualizing, planning, execution, data analyses, and writing of the article dissertation, publications, and all other documentation associated with this study

Prof. Du Toit Loots
(Ph.D.
Biochemistry)

Study leader:
Conceptualized, coordinated and supervised all aspects of the study, including the study design, planning, execution, writing of the article dissertation, publications, and all other documentation associated with this study

Dr. Shane Vontelin van Breda (Ph.D. Biochemistry)	Provided and performed the culturing of cell samples, used in Chapter 3 and 4
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Dr. Ilse du Preez (Ph.D. Biochemistry)	Co-author on two review papers and responsible, together with the other co-authors, for developing and conceptualizing the review topic, working on data acquisition and drafting the article
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Mrs. Derylize Beukes Maasdorp (B.Sc. Biochemistry)	Assisted with sample analyses, as officially appointed laboratory manager
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Available at: http://apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf
[Accessed 13 02 2016].

Chapter 2: Literature overview

A part of this chapter has been published in *the Advances in Protein Chemistry and Structural Biology*

Koen, N., Du Preez, I., Loots du, T., 2016. Metabolomics and personalized medicine. *Adv. Protein Chem. Struct. Biol.* 102, 53–78.

2. 1. Introduction

Approximately 10.4 million new cases of tuberculosis (TB), caused by the bacteria *Mycobacterium tuberculosis* (*Mtb*), and 1.4 million deaths, are reported worldwide per annum (WHO, 2016). These alarming statistics on TB control globally is attributed to insufficient diagnostics, inadequate vaccination strategies, poor patient compliance to anti-TB treatment (due to accessibility to drugs, the drug side effects, and the long treatment duration), and the rapidly increasing drug-resistant strains of *Mtb* in many third world countries. In the current chapter, considering the title and aims of this investigation, a review of relevant literature will be given which includes current knowledge of the state of TB disease in general. Also discussed are TB treatment approaches and the drugs used for these purposes, considering their advantages and disadvantages. Additionally, we will specifically focus on colistin's antimicrobial mechanisms of action and the role of metabolomics in TB research.

2.2. Pathophysiology of tuberculosis

TB transmission occurs via the spread of aerosolized droplet nuclei, containing *Mtb* particles, which are expectorated during talking, sneezing or coughing by an individual with active pulmonary TB - and these droplets can remain suspended in the air for several hours (World Health Organization, 2015). Infection occurs when a non-infected individual inhales these droplet nuclei, which then traverse the respiratory passages and respiratory tract and bronchi, and finally

reach the alveoli of the lungs (Smith, 2003). *Mtb* is known to develop most frequently in the parenchyma of the lungs, yet it has been found to spread throughout the body, with the possibility of infecting any organ system (Miranda et al., 2012). After entering the alveoli, the host macrophages engulf these nuclei, which leads to a cascade of events that will result in either a suppression of the infection or the progression of the disease to active TB (Villarino et al., 1992). Once inside the macrophages, the *Mtb* slowly replicates and spreads via the lymphatic system to the hilar lymph nodes, inducing an immune response after 2-8 weeks of the infection. This immune response is characterised by activation of the T-lymphocytes and macrophages, which in turn may lead to the formation of necrotic granuloma containing non-viable *Mtb*, via host cytokines (Fremond et al., 2005), characterised by the release of interferon- γ from the activated T-lymphocytes (BoseDasgupta & Pieters, 2014). If the infected host has a high immunity, *Mtb* can be maintained in these granulomas indefinitely. However, an active disease state can occur at any time when the host's immune system becomes compromised (because of factors such as human immunodeficiency virus (HIV); diabetes mellitus; renal failure; extensive corticosteroid therapy; malnutrition and vitamin D or A deficiency) (Esmail et al., 2014), and it can hence no longer contain the *Mtb* in this non-replicative state (Smith, 2003).

The clinical symptoms of active TB, including: hemoptysis, coughing, night sweats, fever, chest pain, weight loss and dyspnea might only occur at a later stage of the disease progression because of the perfidious onset of TB. These symptoms are, however, not a confirmation of TB, but typically precede the disease and could correlate with many other diseases or infections in the lungs (Knechel, 2009).

2.3. Tuberculosis treatment

Conventional disease diagnostics generally entails a physician identifying a disease or abnormality on the basis of a physical examination of the symptomatic patient, with (or without) the additional use of standard diagnostic tests. These test include Xpert® MTB/RIF Ultra cartridge, critical concentrations for culture-based drug-susceptibility testing, etc. A positive diagnosis is normally followed by treatment using drugs produced on a large scale and administered at a standardized and universally-accepted dosage. These conventional drugs are developed to treat

general symptoms or the disease as determined by the mean results obtained over large population groups (Debas et al., 2006). However, it is crucial to understand that because of, for example, genetics and a variety of other factors such as individual diet, habits (e.g. smoking), gender etc., not all diseases affect all individuals in the same manner (Jirtle & Skinner, 2007), and neither do all individuals respond to treatment in the same way (Dworkina et al., 2014). This occurrence is clearly reflected by the increasing incidence of treatment failure and relapse, which is especially disturbing when considering their prevalence in life-threatening diseases such as TB and acquired immune deficiency syndrome (AIDS).

Currently, various anti-TB drugs are used for different aspects of drug activity, such as bacterial activity, sterilising activity, and prevention of drug resistance. These three categories of drug activity are included in the DOTS (directly observed treatment, short-course) regimen, which consists of four first-line drugs, namely: rifampicin; pyrazinamide; isoniazid; and ethambutol. The anti-bacterial activity is the ability of the drug to prevent or reduce the dividing bacilli in the initial stages of therapy. These drugs include ethambutol, rifampicin and streptomycin, ethambutol being the most potent of the three (Arbex et al., 2010). The second and third of the three categories are the sterilising activity and the prevention of drug resistance, which is the drug's ability to disrupt the putative subpopulation of TB, normally resulting in clinical relapse (Zhang et al., 2003). The DOTS strategy was formulated later, in the 90s, and is still recommended internationally today (WHO, 2016). The DOTS strategy consists of a six-month treatment regimen divided into two phases, the first being the initial intensive phase which uses all four first-line drugs to eliminate the actively growing *Mtb* populations. The second phase includes the sterilising activity to clear the intermittent dividing bacteria, using only isoniazid and rifampicin (Prideaux et al., 2015; WHO, 2016). However, because of poor patient adherence, drug resistance is emerging, limiting the success rate of the current first-line anti-TB drug treatment protocols (Telenti & Iseman, 2000). In the case of drug resistance in TB, also known as multi-drug resistant TB (MDR-TB) and extreme drug resistance (XDR-TB), the infecting mycobacteria is resistant to at least rifampicin and isoniazid. Subsequently, treatment using the more expensive second-line drugs with high toxicity is required, which takes up to 24 months to treat the patient with MDR-TB (WHO, 2016).

For the purpose of comprehensively describing all facets of the current knowledge of anti-TB drugs used today, apart from a discussion on the mechanisms and side effects of the current first-line anti-TB drugs, a brief and general discussion on current second line anti-TB drugs will also be given in this section. This will be followed by a brief description of two new anti-TB drugs currently

in development and being tested. This will then be followed by a comprehensive description of what is known about colistin sulfate (CS) and colistin methanesulfonate (CMS).

2.3.1 First-line medications

2.3.1.1 Streptomycin

The first successful antibiotic against TB (streptomycin) was discovered and isolated in 1943 by Selman Waksman, from *Streptomyces griseus*. Streptomycin's anti-TB activity includes the ability to inhibit protein synthesis, via inhibition of the *Mtb* mRNA translation, resulting in cell death (Bogen, 1948). This inhibition occurs specifically at ribosomal protein S12 (*rpsL*) and 16S rRNA (*rrs*), in the small 30S ribosomal subunit (Zhang et al., 2011). However, streptomycin-resistant mutants started forming as early as 1946, and were classified into two groups depending on their level (high or low) of resistance. Those with high levels of resistance to streptomycin are attributed to mutations on the *rrs* and *rpsL* genes (found in half the resistant *Mtb* isolates) (Jagielski et al., 2014). Low-level resistance, however, occurs in 33% of these resistant isolates of *Mtb*, characterised by a mutation in the *gidB* gene encoding 7-methylguanosine (m7G) and methyltransferase (GidB) for 16S rRNA (Okamoto et al., 2007). Apart from the rapidly forming resistance, streptomycin is associated with a number of side effects including: hypersensitivity, drowsiness, chronic toxicity, ataxia, blackouts, and hearing loss (WHO, 2016).

2.3.1.2 Isoniazid

Although its exact mechanism of action is largely still unknown, one of the most successful anti-TB drugs identified to date, for eliminating *Mtb*, is isoniazid. It is however suggested to act by compromising the acid-fast nature and viability of *Mtb*, by inhibition of mycolic acid synthesis and subsequently altering the *Mtb* cell-wall lipids (Nguyen, 2016). Confirming these hypotheses, several electron-microscopy scanning studies have shown morphological changes to *Mtb* (Takayama et al., 1973). Furthermore, a number of alternative drug mechanisms have been proposed, and includes it acting by the formation of free radicals during drug activation and

tyrosine reduction (protein activity) (Lü et al., 2010). Isoniazid is traditionally described as a pro-drug requiring oxidation via the peroxidase catalyzation process of KatG, to react with nicotinamide-adenine dinucleotide (NAD), which in turn inhibits the *Mtb InhA* enzyme (enoyl-acyl carrier protein) via the INH-NAD product (Bulatovi et al., 2002). Consequently, this inhibition of *InhA* results in the blockading of fatty acid elongation and subsequently mycolic acid synthesis (Takayama et al., 2005). Isoniazid-resistance has been characterised by various mutations, which mainly target the *katG* and *inhA* genes, and have been observed in 30% of all TB isolates (Miesel et al., 1998). Apart from the *Mtb* developing drug resistance to isoniazid, side effects of the drug in humans includes: hypersensitivity, peripheral neuropathy (prevented by vitamin B6) and hepatitis (Klostranec, 2012).

2.3.1.3 Ethambutol

During the early stages of TB treatment, ethambutol is used, especially when isoniazid resistance is detected, since ethambutol is shown to be very effective against intracellular and extracellular *Mtb*. Ethambutol functions by inhibiting the transfer of arabinogalactan into the *Mtb* cellular wall, resulting in a build-up of trehalose mono and dimycolates (Goude et al., 2009), and this as a result of the repression of (D-14C) glucose transmission into the D-arabinose portion of arabinogalactan (Umeno et al., 2005). Resistance of *Mtb* to ethambutol is shown to be due to common missense mutations in the arabinosyl transferase encoding gene (*embB*) of this organism (Umeno et al., 2005). Starks et al. (2009), further indicate specifically *embB* codon 306 are important indicators of ethambutol resistance, confirming that up to 50-70% of the clinical samples result in ethambutol resistance. However, the exact contribution made by *embB* codon to resistance ethambutol is disputed (Starks et al., 2009).

2.3.1.4 Rifampicin

As previously mentioned, rifampicin is a sterilization anti-TB drug, which reduces the dividing activity of the semi-dormant and putative subpopulation *Mtb*, by inhibiting the DNA-dependent RNA-polymerase from transcribing RNA (Nakamura & Yura, 1976). Resistance to rifampicin is

due to the onset of mutations in the encoding *rpoB* gene, which results in variation in the β -subunit of RNA-polymerase by replacing the aromatic with non-aromatic amino acids (Cai et al., 2017). Minor drug-related side effects experienced by the TB patient include: abdominal pain, flu-like symptoms, dyspnea, fatigue, anorexia, and ataxia. However, more severe side effects might arise with combinational treatment with isoniazid, which include cholestatic hepatitis and exanthema (Lawrence Flick Memorial Tuberculosis Clinic, 1998).

2.3.1.5 Pyrazinamide

Due to pyrazinamide's acidic pH, it is effective in neutralizing semi-dormant bacteria surviving the aforementioned TB drugs (Drew, 2017). The addition of this drug in a multi-faceted drug-administration protocol resulted in the successful reduction of TB therapy duration, from nine to six months (Mitnick et al., 2009). *Mtb* is uniquely vulnerable to pyrazinamide, due to an absence of a pyrazinoic acid efflux mechanism (Ramirez-Busby & Valafar, 2015). Studies suggest that pyrazinoic acid kills *Mtb*, not because it has a specific bacterial target, but because of its effectiveness against *Mtb*'s weak acid features (Baer et al., 2015). Further studies by Zhang et al. (2003) suggest that pyrazinoic acid de-energizes the bacterial membrane, resulting in membrane collapse (Zhang et al., 2003; Dillon, et al., 2017; Rosen, et al., 2017; Gopal, et al., 2017). *Mtb* resistance to pyrazinamide is characterised by mutations in the PZase coding gene (*pncA*) (Gopal, et al., 2017; Gopal, et al., 2016). Pyrazinamide is extremely hepatotoxic and characterised by rather severe side effects in the treated TB patient and these include: pruritus, exanthema, kidney failure and myoglobinuria. It is suggested that treatment with pyrazinamide should be briefly discontinued when any of the above-mentioned side effects occur (Kamfer, 2013; Yee, et al., 2017).

2.3.2 Second-line medications

The standardised regimen for treating MDR-TB includes using various second-line drugs (D-cycloserine, ethionamide, kanamycin and amikacin, and fluoroquinolones). However, these drugs are characterised by extremely high toxicities, long treatment durations, and high costs (Mitchison

& Davies, 2008). Furthermore, the STREAM study (Standardised Treatment Regimen of Anti-TB Drugs for Patients with MDR-TB), supported by the United States Agency for International Development (USAID), led to the first anti-TB second-line drug trial in Ethiopia, South Africa, Vietnam and Mongolia. STREAM uses a shortened drug regimen consisting of two phases, the first being the standardized care (as previously described), with a slight modification (gatifloxacin has been replaced by moxifloxacin), and a second phase which entails a nine-month bedaquiline treatment (Riya Moodley, 2016).

Although the recommendations made by the WHO, regarding second-line anti-TB drug treatment protocols, have proven to be very effective, recent studies have shown unwanted combined interactions - between these anti-TB drugs with one another, and with many other medications that these patients may be consuming for other ailments (Arbex et al., 2010). Apart from these unwanted interactions, there is also evidence that efficacy and uptake may also be altered/influenced (Cascorbi, 2012). The second-line anti-TB drug side effects, and extent to which they are experienced, is however dependent on a number of other factors also, including: the age of the patient, nutritional status, dosage, time of administration, and preceding diseases and dysfunctions (Ramappa & Aithal, 2013).

2.3.3 New possible second-line anti-TB medications and regimen

The abovementioned disadvantages associated with the current first-line and second-line anti-TB drugs described above, in addition to known interactions between the current anti-TB drugs and antiretroviral drugs (taken by HIV positive patients), are some of the many reasons why there is still urgent need for the discovery of new anti-TB drugs (Lange et al., 2014). The only new anti-TB drugs approved over the last 50 years include delamanid and bedaquiline (Zumla et al., 2013).

2.3.3.1 Delamanid

The new drug delamanid is a novel drug of the dihydro-nitroimidazole currently recommended for adults for a maximum of six months. Delamanid is thought to primarily inhibit synthesis of methoxy-mycolic and keto-mycolic acid, which are components of the mycobacterial cell wall and recently,

the WHO approved the use of delamanid for children above 6 years of age, pharmacokinetic data having been made available (WHO, 2016). Two studies reported evidence of higher success rates at the end of treatment. However, cardio-toxicity research on the drug is still low (Harausz et al., 2016). In fact, the combinational therapy of delamanid and bedaquiline may induce high toxicity (Pym et al., 2016). Two studies reported paediatric TB cases cured with a delamanid-containing regimen (D'Ambrosio et al., 2014).

2.3.3.2 Bedaquiline

Bedaquiline is a new active substance against TB which blocks the enzyme ATP synthase inside *Mtb*. By doing so, the bacteria is unable to produce energy, which in turn gives the patient the ability to improve in health (Field, 2015). According to Pym et al. (2016), bedaquiline has increased the cure rates of MDR-TB. Additionally, bedaquiline's regimen led to positive outcomes during clinical patient cohort with MDR-TB (Pym et al., 2016).

2.3.3.3. NIX-TB

A new anti-TB drug combination, currently undergoing clinical drug trials, is Nix-TB, which consists of a completely new multi-drug combination of pretomanid, bedaquiline and linezolid. It is the first of its kind to potentially result in a shortened treatment duration (Gualano et al., 2016). It has the advantage of being administered orally and requires fewer pills, in addition to possibly curing MDR-TB in six to nine months (Sloana & Lewis, 2016).

2.3.4 Colistin

Polymyxins were originally discovered during the 1940s, and isolated from *Bacillus polymyxa*, a spore-forming soil bacteria (Falagas et al., 2005). One of these polymyxins, called colistin (polymyxin E), is a cationic cyclic decapeptide, synthesised and isolated from *B. polymyxa colistinus* (Satlin & Jenkins, 2017). Colistin has previously been used for generally treating gram-negative infections; however, due to its nephro- and neurotoxic side effects, it was replaced with less toxic antibiotics in the 1970s (Grill & Maganti, 2011). Because recent studies have indicated increased reports of MDR strains of various bacteria, including *Pseudomonas aeruginosa* (Beceiro et al., 2013), *Acinetobacter baumannii* (Lin & Lan, 2014) and *Mtb* (Smith et al., 2013), there has been a renewed interest in colistin, thanks to its gram-negative killing capacity.

Colistin can be sub-classified into two groups, 1. colistin sulfate (CS) and 2. colistin methanesulfonate (CMS). The first can be used topically (Jain, et al., 2014) and orally, and the latter used parentally, but both are less toxic in an inhaled form (Antoniou & Cojocaru, 2012). Furthermore, it has been reported that CMS is less toxic than CS (Al-Shaer et al., 2014).

2.3.4.1 Chemistry of the different polymyxin entities

Colistin contains a cyclic heptapeptide ring of amino acids (D- and L-) with a tripeptide side chain (positively charged) (Velkov et al., 2013). This side chain enables colistin to bind covalently to the fatty acid groups on the bacteria (Gao et al., 2016). Colistin can occur as a number of different polymyxin structures, differing by their fatty acid and amino acid contents, the most common of which are colistin A (polymyxin E1) and colistin B (polymyxin E2), and the less common polymyxin E3 and E4, polymyxin E7 and polymyxin E8 (Gallardo-Godoy et al., 2016). The major structure resulting in the antimicrobial properties of colistin A, is its lariat structure (Kline et al., 2001). Due to the hydrophobic fatty acid basic properties and moiety of colistin's five γ -amino groups ($P_{ka} \approx 10$), colistin displays amphipathic behaviour (Li et al., 2005), hence colistin is able to be equally soluble in water and the lipid membranes of various cells. A non-active pro-drug of colistin can be synthesized via a reaction of the γ -amino groups of colistin with formaldehyde, followed by reaction with sodium bisulfate (Bergen et al., 2006). According to Li et al., (2005), CMS can be converted

to colistin, and various other sulfomethylated colistin-related compounds. CMS was found least stable in an isotonic phosphate buffer and human serum at 37°C, whereas CMS rapidly forms colistin and then slowly degrades. CMS is most stable in water, where it takes much longer to be converted to colistin. Interestingly, no colistin is formed when CMS is dissolved in Mueller–Hinton broth at 37°C, and the reduction in live *P. aeruginosa* observed suggests that this sulfomethyl derivative of colistin, has antimicrobial activity in its native form also (Li et al., 2005).

2.3.4.2 Antimicrobial mechanism of action

Previous studies have shown that colistin interacts electrostatically with the outer membrane of gram-negative bacteria, via the interaction between the cationic fatty acid side chains of colistin with the lipopolysaccharide (LPS) of the bacterial membrane (Catchpole et al., 1997), subsequently displacing divalent cations (magnesium and calcium) from the bacterial cell membrane lipids (phosphate groups), disrupting the cell membrane and increasing its permeability (Clifton et al., 2015). According to Landman et al. (2008), gram-negative bacteria are likely to be more susceptible to hydrophobic antimicrobials because of this disruption in the membrane permeability, an important observation when considering using colistin in combination with other antimicrobial agents (hydrophobic antibiotics). It has also been suggested that the bacteria's metabolic activity has no influence on the degradation of colistin, and hence bacterial resistance to the drug is slow (Bialvaei & Kafil, 2015).

2.3.4.3 Resistance to colistin

Although bacterial resistance to colistin is lower, compared to that of other antimicrobials, a number of studies have shown that resistance can occur, and they have suggested the mechanisms associated with this. Resistance by *P. aeruginosa* was reported, in cystic fibrosis patients using high concentrations of inhaled colistin (Field, 2015). The mechanisms for developing resistance to these polymyxins are variations of lipid A with less cationic binding sites in the bacterial LPS, reducing the amount of available surface charges and consequently reducing their binding capacity for colistin (Field, 2015). These observations were confirmed from colistin

studies done on bacteria in culture media with reduced amounts of Mg^{2+} , which in turn results in PhoP activating the *pmrCAB* locus, the latter of which is responsible for reduction in the cationic binding sites in *Salmonella enterica serovar Typhimurium* (Ortwine et al., 2015). According to Landman et al. (2008), when culturing *P. aeruginosa* with colistin, acidic phospholipids are converted to neutral phospholipids in its outer membrane, subsequently leading to a neutralizing effect on the biological properties of the endotoxins as well as cytoplasmic leakage, which causes the bacteria to become more susceptible to hydrophobic antimicrobials.

2.3.4.4 Combined antibiotics activity

The main reason for initially approaching the treatment of various diseases with a combination-treatment approach, using CMS and other antibiotics, was to prevent the reoccurrence of the bacteria post treatment, as well as to prevent possible resistance from developing against the primary antibiotic (Chi et al., 2012). Additionally, a combination-treatment approach, considering the mechanism by which colistin acts, would be expected to allow for successful treatment outcomes using lower dosages of the antibiotics (and therefore less toxicity) and shorter therapy duration.

CMS, administered in combination with ciprofloxacin, has been previously used for treating cystic fibrosis patients with an aggressive MDR *P. aeruginosa* infection, at the Danish cystic fibrosis centre (Høiby, 2011). The successful prevention of chronic *P. aeruginosa* occurred in 85% of the patients and, for over 15 years of use, there has been minimal resistance to colistin in these cases (Cassir et al., 2014). Furthermore, a combination-therapy approach of CMS with rifampicin in patients with MDR *A. baumannii* infection, resulted in the successful treatment of 64% of the treated patients with a very low incidence of any side effects in vitro (Lee et al., 2013). Landman et al. (2008), reviewed a number of studies using colistin in combination with rifampicin, and reported a success rate of a hundred percent when treating *A. baumannii* and *P. aeruginosa*, similarly as to when using a combination treatment using polymyxin B, imipenem and minocycline. Furthermore, various studies done on isolates of *K. pneumoniae*, *S. maltophilia* and *S. marcescens*, indicated a high antibacterial rate when using rifampicin in combination with these polymyxins. Zeidler et al. (2013), described the successful treatment of various *Candida* species, using a combination treatment strategy using colistin and echinocandin. It was proposed that

echinocandin weakens the cell wall, facilitating the colistin's action on the cell membranes. Prior to this, Garonzik et al. (2011), suggested the combination therapy using CMS and CS for treating patients (with moderate to good renal functions) for organisms with MIC ≥ 1.0 $\mu\text{g/ml}$, since inadequate colistin plasma levels are obtained in these patients when CMS monotherapy was applied (Garonzik et al., 2011). All these studies confirm that combinational treatment strategies of anti-TB drugs with CMS or CS could have promising outcomes.

2.3.4.5 Colistin's activity against mycobacteria

Colistin's antibacterial activity has been previously investigated against *M. aurum* and, via electron microscopy, was suggested to function via disruption of the cytoplasmic membrane of the infectious bacteria (patchy and diffused polysaccharide outer layer) (David & Rastogi, 1985). More recently, colistin antibacterial activity was determined for *Mtb* at an MIC of 5 $\mu\text{g/ml}$. However, a combinational-treatment approach using sub-lethal concentrations of colistin, was suggested by the authors (Keren et al., 2011). Prior to these *Mtb* investigations, a number of studies were done determining the effects of colistin on other mycobacteria strains, describing the efficacy (positive for *M. fortuitum* but no inhibition for *M. chelonae*), using the disc diffusion and broth dilution tests (Flores et al., 2005). Harris & Keane (2010) recently indicated that polymyxins have inhibited the release of tumour necrosis factor via LAM, neutralizing the cytokine response associated with cachexia (Harris & Keane, 2010), which is an important consideration in the context of treating TB, a disease associated with cachexia (Tazi & Errihani, 2010).

2.4. Metabolomics

2.4.1 An introduction to metabolomics in the context of drug- development research

Conventional disease diagnostics generally entails a physician identifying a disease or abnormality on the basis of a physical examination of the symptomatic patient, with (or without)

the additional use of standard diagnostic tests. A positive diagnosis is normally followed by treatment using drugs produced on a large scale and administered at a standardized and universally-accepted dosage. These conventional drugs are developed to treat general symptoms or the disease as determined by the mean results obtained over large population groups (Debas et al., 2006). However, it is crucial to understand that due to, for example, genetics and a variety of other factors such as individual diet, habits (e.g. smoking), gender etc., not all diseases affect all individuals in the same manner (Jirtle & Skinner, 2007), and neither do all individuals respond to treatment in the same way (Dworkina et al., 2014). This occurrence is clearly reflected by the increasing incidence of treatment failure and relapse, which is especially disturbing when considering their prevalence in life-threatening diseases such as TB and AIDS. Although this variation between individuals might not be obvious in the initial clinical presentation of the disease, it is most likely still detectable on a molecular scale. Several researcher groups have subsequently shifted their focus to the development of medicine, which uses the molecular information of an individual, as dictated by his or her genome, transcriptome, proteome, and metabolome (Redekop & Mladsi, 2013) to develop patient-specific diagnostics and drugs. This information can also be used to determine/predict treatment response, prior to and during the treatment regimen, in an attempt to lower the incidence of treatment failure or relapse (Salari, 2009), and also to optimize drug dosages, in an attempt to prevent or lessen the severity of the drug-related side effects (Lecea & Rossbach, 2012).

TB has undoubtedly been one of the most topical issues over the past decade, and several research fields have joined hands in using metabolomics for the potential to transform clinical practice and treatment efficacy. Traditionally, genomics was considered the most important approach for determining variation and the development of antibiotics (Jain, 2009). However, several intermediate processes occur between the genotype and disease phenotype in the “omics” cascade, which may influence disease outcome or treatment response, and includes transcription, translation, and metabolism. Furthermore, various other factors, such as environmental influences and age, may also play a role in the disease phenotype, a phenomenon which genotyping is not able to characterize or explain. The elucidation of antimicrobial activities requires a holistic view of all molecular variation that may differentiate individuals, and researchers are therefore shifting their focus - from using exclusively genetics methodologies, for instance, to a systematic/integrative “omics” approach. “Omics” is a general term used to describe the study of all genes (genomics), transcription of these genes (transcriptomics), translation into their

respective proteins (proteomics), and all the resulting metabolite changes (metabolomics), and is aimed at acquiring large-scale data sets from a single and/or multiple samples (Wheelock et al., 2013). These “omics” research fields, alone or in combination, have shown to be valuable for the identification of new disease biomarkers for the purpose of elucidating disease mechanisms and the development of treatment regimes.

As per definition, metabolomics is the nonbiased identification and characterization of “all” the small molecular compounds (metabolites) in a biological system, using highly-sensitive analytical techniques, in combination with bio-informatics (Dunn et al., 2005). The metabolome, which is a collective term for all the metabolites in a specific biological system/sample, is the ultimate downstream result of genes, transcription, and translation, and will therefore reflect changes to the genome, transcriptome, and proteome, in addition to that caused by a disease state or other environmental factors. The identification of the main differences between the metabolomes of two sample cohorts (drug vs no-drug controls, for example) is a starting point for the discovery of new drug metabolite biomarkers in order to elucidate mechanism of action. Additionally, a comparison of various cohorts with individuals showing variation to disease or response to treatment can also be done in order to identify markers associated with this type of variation.

The extraction and analysis of metabolites from a sample or sample group can be done in an untargeted or semi-targeted manner. Untargeted metabolomics aims to extract and detect all metabolites (known and unknown, from all metabolite classes), i.e. the total metabolome, as per the definition of metabolomics. Semi-targeted metabolomics approaches, however, are focused on the analysis of specific fractions of the metabolome or a subclass of metabolites, such as only the lipids or organic acids for instance. Sample preparation methods for untargeted metabolome analyses are simple, and the generated metabolite profiles can provide researchers with a good general picture of the effect of the investigated perturbation on the overall metabolome. However, these methods tend to have a lower sensitivity and detection limit, when compared to that of the semi-targeted approach which provides simpler metabolite profiles, representing specific metabolic pathways (Wishart, 2010). The choice of the sample preparation method will also depend on the analytical apparatus selected, and whether an untargeted or semi-targeted approach is required. When using nuclear magnetic resonance (NMR) spectrometry, for instance, chemicals such as ethanol and hexane should be avoided, as these solvents are also deuterated and will therefore result in multiple resonances and subsequently interference (Dunn et al., 2005). Currently, there is no single analytical apparatus available with the capacity to identify all the

metabolites extracted from a sample and, therefore, when doing untargeted metabolomics, a combination of a number of different analytical approaches is recommended. However, this may not always be a viable option in a particular laboratory, as it is dependent on instrument availability. In instances with limited analytical capacity, a lot can still be done in the context of untargeted metabolomics. For instance, derivatization of a sample prior to gas chromatography–mass spectrometry (GC–MS) analysis, in addition to appropriate column selection, can serve well in the detection of a large portion of the metabolome during a single analytical run. Each analytical technique comes with its own set of molecular preferences, advantages and limitations, as will be discussed below.

2.4.2 Analytical methods most often used for metabolomics

The most commonly used analytical approaches for metabolome data acquisition include the use of various chromatographic techniques, most commonly gas chromatography (GC), or liquid chromatography (LC), coupled to various different options of mass spectrometry (MS) detectors, and Nuclear magnetic resonance (NMR). Without prior separation or derivatization, LC–MS is considered to be the apparatus with the potential to detect the largest variety of metabolites present in a specific sample. However, the derivatization of sample extracts makes GC–MS an even - if not better - contender, considering the availability of spectral data for GC–MS compound identification. Furthermore, although LC–MS is ideal for the analysis of polar and ionic compounds, it has a lower chromatographic resolution and higher running costs in comparison. Additionally, a great advancement in GC–MS technology was the development of the GCxGC system, which separates metabolites in two dimensions, on the basis of not only volatility but also polarity, thereby reducing the amount of co-eluting peaks and enhancing the resolution of the eluting metabolites (Marriott & Shellie, 2002). GC–MS analysis also requires smaller sample volumes when compared to that required for LC–MS and NMR, but because these samples undergo metabolite separation and derivatization, they are non-recoverable after GC analysis. Another downside to GC is the rather long analysis times required for compound separation, and that the identification of “unknown” metabolites (those compounds detected with mass fragment patterns not in the commercial libraries) is rather complex.

NMR spectroscopy is based on the principle of detecting metabolites according to the signals produced by their proton content, allowing for straightforward metabolite identification (Bonhommea et al., 2014). This relatively fast method (2–3 min per sample), is mostly used for the detection of polar metabolites and is non-destructive to the sample. NMR instrumentation is, however, rather expensive, requires large sample volumes and has a lower sensitivity when compared to other techniques (Halket et al., 2005). Subsequent to sample analysis, one of the most important steps for generating data, which can be used for metabolomics, is the extraction of reliable data matrixes from the complex chromatographic and mass spectrometric outputs, for subsequent statistical analyses and biomarker selection. This course of action includes peak detection, peak de-convolution, peak alignment, compound quantification, and identification, among various other steps. Most of the analytical methods described above come with their own software packages, specifically designed for this purpose (such as ChromaTOF for the Leco GCxGC-TOFMS), whereas other universal software packages, such as MET-IDEA, are also freely available for use for processing data generated from a variety of different commercially available analytical techniques (Broeckling et al., 2006). However, because each of these packages comes with their own advantages and limitations, most researchers prefer to use a combination of software packages, in addition to manual inspection, in order to obtain the optimum data matrix for statistical data analysis and biomarker identification.

2.4.3 Statistical approaches

The increasing complexity of the data matrixes obtained from the analytical equipment used in metabolomics studies has led to the use of various multivariate chemometric data analysis methods for biomarker identification/ extraction from these data sets. In order to get an overview of the data, certain unsupervised methods can be used to highlight trends in the data and grouping or differentiation of various sample sets, and to additionally identify potential outlier samples and batch effects. When employing these unsupervised methods, samples are not assigned to specific groups (for example, disease and control) prior to the statistical analysis, allowing the analyst to determine whether or not the samples are naturally differentiated or grouped based on their analyzed metabolite profiles. For this purpose, principle component analysis (PCA) is the method most commonly used. PCA reduces the dimension of the input data matrix by calculating a

weighted sum (score) of the compound (metabolite) concentrations detected in each sample and expresses these in terms of principal components (PCs), with PC1 describing the most variation in the data, PC2 the next highest variation, and so on. These PCs subsequently serve as coordinates on a scatter plot and provide an overview of the samples and how they relate to each other on the basis of their analyzed metabolomes. Other chemometric methods - such as self-organizing maps, hidden Markov models, and canonical correlation - can also be implemented in this initial, exploratory stage for the same purpose (Madsen et al., 2010; Trygg et al., 2007). If those samples, belonging to a specific group, do in fact assemble and group together, supervised methods where individual samples are allocated to their respective sample groups before the analyses, can be applied for the purpose of identifying potential biomarkers best describing the variation detected. Partial least squares discriminant analysis (PLS-DA) is one such method, which uses group membership information to build a discrimination model. The variable influence on the projection (VIP) parameter, which is a weighted sum of the squares of the PLS-DA weights, gives an indication of the importance of the metabolite to the prediction model, and can therefore be used to identify those metabolites which are most characteristic of a specific sample group, or those metabolites which vary the most between the specified groups. The metabolites with the highest VIP scores are then ranked and can be used to identify potential biomarkers. Similar supervised classification models also used for biomarker identification include, but are not limited to, soft independent modelling of class analogy and support vector machines. The technical details of these chemometric methods fall beyond the scope of this review, but the authors suggest the review by Trygg et al. (2007) for a more detailed description of these.

Various software packages and Web-servers, such as MetaboAnalyst (Xia et al., 2009), have been developed specifically for researchers with limited statistical knowledge, to perform these essential chemometric analysis on metabolomics data. Although these tools are helpful, most metabolomics research groups still prefer to use qualified biostatisticians, with knowledge of the underlying mathematical programming, for mining the relevant biomarkers from these complex data sets. In these instances, more traditional statistical packages such as Statistica and “R” are used for the analysis of the data in the context of the specific biological question. Identified biomarkers can subsequently be used to explain individual variation in disease and treatment response, by interpreting this as the context of known metabolic pathways, and/or prior genomic, proteomic and transcriptomic data. Furthermore, individual biomarkers or combinations thereof (biosignatures) can be used for diagnostic purposes; the latter can be achieved by building a

prediction model, such as a classification tree. In the light of this, metabolomics is considered an important tool for the development of new anti-TB drugs.

2.4.4 The application of metabolomics towards drug investigations

Before treatment strategies can be tailored to a unique response to therapy, it is important to understand the general xenobiotic metabolism and underlying mechanisms of the proposed drug. For this purpose, pharmacometabonomics can be applied in a number of ways: (1) the comparison of the changes in xenobiotic metabolite concentrations of the treated cell line versus the control cell line (Čuperlović-Culf et al., 2010), (2) comparison of infectious cell cultures by comparison to those incubated in the presence of the drug and those in the absence of the drug, or the presence of the drug carrier (Covalciuc et al., 1999), (3) and the synthesis and screening of the modeled drug for ADMET (absorption, distribution, metabolism, elimination and toxicity) (Yang & Marotta, 2012). These methodologies have been implemented to investigate the metabolism of various nutrients, drugs, and other xenobiotics, using a variety of analytical equipment and bioinformatics strategies (Lan & Jia, 2010). In addition to drug-derived metabolites originating directly from xenobiotic metabolism, these drug-exposure signatures will also include drug-induced alterations to normal metabolism, representing the cell line's altered metabolic state in response to the treatment. In one such instance, Wang et al., (2011), characterized metabolites to differentiate pathways that operate in a living cell, which was then used to evaluate differences between diseased and healthy organisms, and provided information on the underlying cause of disease. The pharmacometabonomics can be implemented to verify or complement drug mechanisms proposed by other omics approaches (Wang et al., 2011). Lorenz et al., (2011), applied this approach to investigate metabolites in adherent mammalian cells using the clonal β -cell line INS-1 as a model sample. The utility of this methodology demonstrated a precise metabolite measurement associated with step changes in glucose concentration that evoked insulin secretion in the clonal β -cell line INS-1 (Lorenz et al., 2011). A study by Dewar et al. (2010), investigated the metabolic differences between chronic myelogenous leukemic cell lines, MyL, and MyL-R. They demonstrated a clear differentiation in the metabolite profiles of drug-resistant and sensitive cells, with the biggest difference being an elevation of creatine metabolites in the imatinib-resistant MyL-R cells (Dewar et al., 2010). Previous studies have linked the xenobiotic metabolism of drugs

to the production of reactive oxygen species (ROS), and therefore this group proposed that this is responsible for the drug action (Tiziani et al., 2009). In order to prove this hypothesis, and potentially other previously unknown drug mechanisms, the group applied NMR metabolic profiling to three AML cell lines treated with BEZ and MPA. From the generated biosignatures, they were able to identify changes to TCA cycle intermediates (more specifically alterations to the conversion of α -ketoglutarate to succinate), which are consistent with ROS action.

In order to minimize the influence of individual variation on the resultant metabolite profiles, however, many of these studies were done on samples collected from homogeneous patient populations or animal models kept under identical conditions, thereby ensuring that the metabolites emanating as biomarkers are, in fact, related to the xenobiotic metabolism exclusively, with little or no individual variation due to either genetic or environmental factors. Various external factors - such as age, stress, diet, gut microbes (microbiome), medication, lifestyle, and disease, in addition to genetic factors including gender, epigenetics, and polymorphisms in genes encoding for xenobiotic-metabolizing components such as enzymes, transporters, receptors, and ion channels - can also influence xenobiotic metabolism and account for individual variation (Johnson et al., 2012). Clayton et al. (2009) demonstrated the application of pharmacometabonomics toward a better understanding of these variables by analyzing pre- and post-dose urinary metabolites of patients on acetaminophen (paracetamol), using NMR spectrometry. When analyzing the pre-dose profiles of these individuals, considering the levels of the excreted drug-derived metabolites, they found high levels of pre-dose p-cresol sulfate, which correlated with low post-dose ratios of acetaminophen sulfate to acetaminophen glucuronide. The ratio of these derivatives, which indicate the extent to which acetaminophen is metabolized through two major phase 2 conjugating processes (O-sulfonation and glucuronidation), is known to be a site and indicator of individual variation in response to paracetamol. The group finally concluded that in patients with high levels of gut microbiome-mediated p-cresol generation, competitive p-cresol O-sulfonation reduces the capacity to sulfonate acetaminophen, which in turn results in an increased likelihood of drug-induced hepatotoxicity. This study subsequently proves the capacity of metabolomics to identify individual variation in xenobiotic metabolism, related to a variation in individual patient environment.

Pharmacometabonomics can also be implemented as an informative tool, assisting pharmacogenomics in the investigation of genome-related variation in drug metabolism (Johnson et al., 2012). With this goal, Ji et al. (2011) investigated urine metabolites of individuals with major

depressive disorder undergoing therapy with selective serotonin reuptake inhibitors (SSRIs), citalopram and escitalopram. On average, 40% of patients generally do not respond to this treatment and previous pharmacogenomic studies failed to identify potential polymorphisms which could be used for the prediction of a SSRI treatment outcome. From the obtained metabolite profiles, elevated glycine levels were identified to be associated with a decreased treatment response, and after subsequent pharmacogenomics studies, polymorphisms in the glycine dehydrogenase gene were identified. These results show that metabolomics can additionally play a significant role in supporting or initiating pharmacogenomics studies, with the intention of identifying genetic factors related to individual variation in drug metabolism.

Considering these and several other studies on the topic (de Carvalho, Darby, Rhee, & Nathan, 2011; Halouska et al., 2007; Lu, Deng, Li, Wang, & Li, 2014; Wang et al., 2013), it is evident that the identification of drug-exposure metabolites can play a significant role in the elucidation of drug mechanisms and the influence of individual variation on these, which in turn can contribute to the development of more effective drugs, or the positive adjustment of drug dosages and treatment schedules, on the basis of the individual differences detected and explained.

2.4.4.1 Metabolomics and understanding response to treatment

Despite the elucidation of drug mechanisms, pharmacometabolomics can also be used to investigate and predict an individual's response to treatment. Statins, for example, are commonly prescribed for patients with increased levels of LDL-cholesterol and risk for cardiovascular disease, despite the substantial individual variation in response to this therapy. Trupp et al. (2012) investigated this occurrence using GC–MS metabolomics analyses of patient plasma samples prior to, and 6 weeks after, simvastatin treatment onset. A number of metabolites were identified implicating genetic, gut microbiome and various environmental factors, contributing to the variation in simvastatin response. Additionally, responders and non-responders to the drug could be differentiated, based on their baseline metabolite profiles; and the most significant compounds responsible for this differentiation were identified. These metabolite variations could be correlated to different treatment responses and subsequently described the mechanisms related to the individual variation to this therapy. These markers additionally have the potential to be implemented pre-clinically, to identify those patients who would/would not benefit from simvastatin

treatment, prior to commencing treatment. Using the same approach, Wei et al. (2013) built a prediction model, based on four identified serum metabolite biomarkers, in order to predict the outcome of breast cancer neoadjuvant chemotherapy. The model was able to predict complete response (disappearance of all tumor deposits) versus stable disease (tumor reduction less than 50%) with 100% specificity and 80% sensitivity (AUC of 0.95). Despite their prognostic value, the biomarkers identified in these studies also show promise in the development of new, more efficient drugs, and also to sub-classify patients during clinical trials.

The same approach has been used to explain and predict variation to treatment response in patients diagnosed with various infectious diseases. Das et al. (2015) investigated the influence of anti-TB drug treatment on the urine metabolic profiles of TB patients at various treatment intervals. A clear treatment-dependent trend could be seen on the PCA, as the metabolite profiles of each consecutive treatment interval shifted closer to that of healthy controls, with profiles of clinically cured patients very closely resembling that of the control group. With these profiles, one might be able to build a prediction model for treatment outcome, provided that profiles of patients with failed treatment outcomes are also incorporated. These profiles may additionally give clues as to why certain patients fail to respond to TB treatment within the recommended 6-month therapy regime, and by using a similar approach, metabolite biomarkers predicting treatment failure or relapse might also be identified. When investigating infectious diseases in this manner, it is important to bear in mind that individual variation to drug treatment outcomes can be a result of the host's variable response to the drug, or the pathogen's resistance to the drug, and therefore both factors should be considered when identifying biomarkers reflecting treatment response. A more recent study using metabolomics for elucidating the mechanisms pertaining to treatment failure was conducted by Luies et al., (2017), using patient-collected urine. The treatment failure group was characterized by an imbalance in the gut microbiome, abnormalities in the long-chain fatty acid β -oxidation pathway, a mitochondrial trifunctional protein defect, and a compromised insulin secretion (Luies et al., 2017).

Furthermore, in a second investigation, a model was built in order to predict treatment outcome to first-line anti-TB drugs using urine collected at time of diagnosis. The predictive ability of the model was assessed based on a ROC curve, and achieved an AUC of 0.94 (95% CI 0.84–1) and cross-validated well in a leave-one-out context, with an AUC of 0.89 (95% CI 0.7–1). (Luies et al., 2017). Using a GC–MS metabolomics approach, Du Preez and Loots (2012) investigated rifampicin resistance in pulmonary TB by comparing the fatty acid metabolomes of two *Mtb* strains, with

resistance conferring mutations on different locations on the *rpoB* gene, to a fully susceptible wild-type parent strain. All three groups showed a clear differentiation when doing PCA, and a number of metabolites indicating a decreased synthesis of various 10-methyl branched-chain fatty acids and cell wall lipids, and an increased use of the shorter-chain fatty acids as carbon sources, were identified as markers in the drug-resistant strains. In addition, the *rpoB* S531L mutant, previously reported to occur in well over 70% of all clinical rifampicin-resistant *Mtb* strains, showed a better capacity for using these alternative energy sources, compared to the less frequently detected *rpoB* S522L mutant. This study therefore shows that pharmacometabonomics has the power to not only detect metabolome changes related to pathogen-induced drug resistance, but it can also differentiate between the various genotypes leading to the observed phenotype. The clinical use of these identified markers can significantly contribute to the development of improved treatment approaches, thereby bettering treatment outcomes in patients with drug-resistant TB. When developing new therapeutic approaches, it is also important to realize that not all individuals will respond to an infection in a similar fashion, and although infected with identical pathogen strains, treatment outcomes may vary. Through metabolomics, markers can be identified in order to explain and predict this phenomenon. Loots (2016) investigated rifampicin resistance in pulmonary TB by using a two-dimensional GC-coupled time-of-flight MS metabolomics approach, in order to identify the most significant metabolite markers associated with resistant *Mtb* strains. Metabolites associated with an *rpoB* mutation were identified, and subsequently explained how these organisms have managed to survive despite the mutation in their *rpoB* gene (Loots, 2016). Just prior to this, Loots (2014) used an identical metabolomics research approach to identify potential new metabolite markers associated with a *katG* mutation which results in isoniazid resistance in *Mtb*. It was determined that the isoniazid-resistant strains experienced an increased susceptibility to oxidative stress and adapted to this by upregulation of metabolic pathways associated in both the uptake and use of alkanes and fatty acids, and the synthesis of compounds directly involved in reducing oxidative stress, including an ascorbic acid degradation pathway (Loots, 2014).

2.4.4.2 Metabolomics and understanding drug toxicology

Several metabolomics studies have identified universal, nonspecific biomarkers, related to general toxicity of various different drugs, which are unrelated to the specific drug type, drug mechanism, or site of action. These metabolites, including reduced concentrations of TCA-cycle intermediates and hippurate, are a reflection of secondary side effects related to the ingestion of these drugs and include general changes to energy demand or energy metabolism, and changes to gut microbiota (Keun, 2006). Additionally, many metabolomics studies have also identified general markers related to hepatotoxicity (Holmes et al., 1992; Robertson et al., 2000; Schnackenberg, Dragan, Reily, Robertson, & Beger, 2007) and nephrotoxicity (Garrod et al., 2005; Lenz, Nicholson, Wilson, & Timbrell, 2000). These markers are especially important in the preclinical drug development phase, considering that liver and kidney damage are the two major reasons for drug withdrawal from the market. Although knowledge of these general toxicity markers is of importance, metabolomics also has the capacity to identify markers related to specific drug mechanisms and side effects to vital organs and, by using blood and urine for biomarker detection, eliminates the need for intrusive procedures for monitoring these outcomes (such as tissue biopsy, for instance). Sumner, Burgess, Snyder, Popp, and Fennell (2010) detected urinary markers related to abnormalities in inositol, carbohydrate, glycerolipid, and glyoxylate metabolism, correlating to hepatic microvesicular lipid accumulation (MVLA), a histopathological side effect related to the treatment of TB with isoniazid. They propose that, if validated, these metabolite changes can be used to develop a non-invasive method for the early detection of MVLA. A study entitled *Consortium for Metabonomic Toxicology (COMET)* was one of the most extensive pharmacometabolomics investigations conducted to date. The aim of COMET was to build models for the prediction of organ toxicity (mainly liver and kidney), from NMR spectra obtained from rodent urine and serum, from multiple toxicity studies (Lindon et al., 2003). A validation of these markers indicated that these methods could predict liver and kidney toxicity with specificities of 100% and 77%, and sensitivities of 41% and 67%, respectively (Ebbels et al., 2007). These models are now being implemented by the six pharmaceutical companies involved in the consortium, in pre-clinical studies.

Another important general drug-related adverse effect to be considered is the change in the patient's intestinal microbiota composition, in response to antibiotic treatment. Pharmacometabonomics has also contributed significantly to this intensely studied research area,

indicating that 87% of all metabolites in the intestinal metabolome are influenced by antibiotic treatment. Furthermore, a disturbance in a number of metabolic pathways, including bile acid, eicosanoid, and steroid hormone synthesis, were identified subsequent to antibiotic treatment (Antunes et al., 2011). In addition to better describing the molecular mechanisms resulting in general adverse effects such as hepatotoxicity, nephrotoxicity, and intestinal microbiota response to antibiotic treatment, metabolomics has also been used to investigate the mechanisms of more specific drug-related side effects associated with specific treatment regimens. To this end, Loots, Wiid, Page, Mienie, and Helden (2005) evaluated the effects of the combined anti-TB drug, Rifater, on the metabolic profiles of Sprague-Dawley rats. The identified biomarkers indicated elevated oxidative stress levels in the animal models receiving treatment, and the metabolite profiles closely resembled that of human patients suffering from a multiple acyl-CoA dehydrogenase deficiency (MADD). These findings indicated that Rifater treatment could be linked to an inhibition of the electron transport chain flavoproteins, and the group subsequently indicated that this MADD metabolite profile, and hence the associated drug-related side effects, could be corrected by the co-administration of melatonin. From these and other studies, it is evident that metabolomics can assist in a better description and understanding of the general and specific side effects related to various drugs, which may contribute to the development of safer treatment approaches, or the expansion of therapeutic strategies for the prevention or early management of these occurrences. Once again, it is important to consider all factors which may influence these metabolite changes, including the previously mentioned environmental and genetic factors, when identifying those biomarkers related to toxic insult (Beger et al., 2010). Although adverse side effects are not uncommon for many prescription drugs, especially those targeting life-threatening disease states such as cancer for instance, it can also occur in drugs which are widely recognized as safe and are normally well tolerated by most patients. As discussed earlier, the largest contributor to the variation in individual patient xenobiotic metabolism, and hence their toxicology phenotypes, are polymorphisms in genes encoding for xenobiotic-metabolizing enzymes. However, various other factors have been identified that contribute to this, including the patient's health status (hepatic dysfunction, inflammation, infection, and cancer), drug-drug interactions, and exposure to contaminants such as heavy metals, all of which can influence the activities of these drug-metabolizing enzymes (Chen et al., 2007). Although pharmacogenomics is the primary approach used for the investigation of such variation, metabolomics studies have also shown to be useful in the elucidation of the mechanisms leading to these phenotypes. For example, a number of

genetics studies have linked a polymorphism in the CYP2D6 with an excessive hypotensive response to the antihypertensive drug; debrisoquine. When investigating this phenomenon using LC–MS metabolomics, metabolite profiles from urine could differentiate the treatment response phenotypes (poor metabolizers vs. extensive metabolizers). As expected, debrisoquine was significantly higher in the profiles of poor responders, whereas the products of drug metabolism: 4-hydroxy-debrisoquine, and two-open ring products of debrisoquine (2-(guanidinoethyl) benzoic acid (I) and 2-(guanidinomethyl) phenyl acetic acid (II)), were detected in higher levels in the profiles of the good responders. The two CYP2D6 genotypes could thus be identified using biomarkers determined by metabolomics, proving that pharmacometabonomics has the capacity to identify individual variation in drug-related side effects, originating from these polymorphisms (Zhen et al., 2006). In a similar fashion, metabolomics can also be implemented to investigate other factors leading to this variation, thereby paving the way for the development of more effective medicines, with a lowered incidence of drug-induced side effects.

2.5. Concluding remarks

Considering the above literature review, due to treatment failure, poor patient adherence, lack of new anti-TB drugs, and long treatment duration, it is clear that the TB epidemic is still one of the largest complications, emphasising the urgency for new, faster, less toxic TB treatment regimens. A possible anti-TB drug candidate is the antibiotic colistin methanesulfonate (CMS), an inactive prodrug of colistin sulfate (CS), also known as polymyxin E (Ortwine et al., 2015). Colistin has previously been shown to have high anti-bacterial activities against *P. aeruginosa*, *A. baumannii*, and *Klebsiella pneumoniae*, and additionally shown to be resistant to these organisms developing drug tolerance (Catchpole et al., 1997). Furthermore, metabolomics has been recently shown to be a useful research tool for investigating new drug mechanisms. Due to its capacity for characterising the metabolome (one of the research subdivisions in the omics cascade), the observed phenotype associated with given perturbation, which in the case of this study would be colistin, can be observed. Considering this, the aim of the study was to use a metabolomics approach, in order to identify the metabolite markers associated with CS and CMS, *Mtb*, and for the purpose of elucidating its antibacterial mechanisms of action.

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Chapter 3: Elucidating the antimicrobial mechanisms of colistin sulfate on *Mycobacterium tuberculosis* using metabolomics

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Abstract

Considering the disadvantageous of first line anti-tuberculosis (TB) drugs, including poor patient adherence, drug side effects, the long treatment duration and rapidly increasing microbe resistance, alternative treatment strategies are needed. Colistin sulfate (CS), a polymyxin antibiotic, considered a last-resort antibiotics for treating multidrug-resistant *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter*, has antimicrobial activity towards mycobacteria, and could serve as a possible anti-TB drug.

Using GCxGC-TOFMS metabolomics, we compared the metabolic profiles of *Mycobacterium tuberculosis* (*Mtb*) cultured in the presence and absence of CS, to elucidate the mechanisms by which this drug may exert its antimicrobial effects.

The principal component analysis of the metabolite data indicated significant variation in the underlying metabolite profiles of the groups. Those metabolites best explaining this differentiation, were acetic acid, and cell wall associated methylated and unmethylated fatty acids, and their alcohol and alkane derivatives. The elevated glucose levels, and various glyoxylate and glycerolipid metabolic intermediates, indicates an elevated flux in these metabolic pathways.

Since all the metabolites identified in the colistin treated *Mtb* indicates an increase in fatty acid synthesis and cell wall repair, it can be concluded that CS acts by disrupting the cell wall in *Mtb*, confirming a similar drug action to other organisms.

Keywords

Colistin sulfate, *Mycobacterium tuberculosis*, Tuberculosis, Metabolomics, Treatment, Antibiotics

3.1. Introduction

Tuberculosis (TB), is an infectious bacterial disease caused by the organism *Mycobacterium tuberculosis* (*Mtb*) and usually affects the lungs (Floyd, 2014). The World Health Organisation (WHO) reports TB to be one of the world's deadliest communicable diseases, resulting in the death of up to 2 million people per annum. Furthermore, TB is considered the leading cause of death among people living with HIV (World Health Organization, 2015). TB is currently treated using the 6 month "directly observed treatment short-course" (DOTS) regimen, consisting of the four first-line drugs: rifampicin, isoniazid, ethambutol and pyrazinamide (Palmer, Chan, Dieckmann, & Honek, 2012). In patients with drug-susceptible TB, this regimen reportedly has a 1 – 4 % failure rate, and 7 % of the patients with a successful treatment outcome, reportedly relapse within 24 months (Dye, et al., 2005). The WHO has additionally reported 5% of all TB cases have multidrug-resistant TB (MDR-TB) (World Health Organization, 2015), which requires treatment using second-line anti-TB drugs (Zhenkun, 2010). These second line drugs are not only more expensive, but also have severe side effects, and an even longer treatment duration (approximately 2 years) (Baths, Roy, & Sing, 2011). These complexities, in addition to the fact that current anti-TB drugs have cross-reactions and interactions with HIV-antiretroviral therapy, emphasise the need for researching and developing new anti-TB drugs or alternative therapeutic approaches.

Colistin sulfate (CS), a polymyxin antibiotic discovered in the 1940s, is a cyclic peptide with a hydrophobic tail, and was one of the first antibiotics with significant activity against gram-negative bacteria (Ortwine, Kaye, Li, & Pogue, 2015), in particular *Pseudomonas aeruginosa* (Sabuda, et al., 2008), *Acinetobacter baumannii* (Quresh, et al., 2015) and *Klebsiella pneumonia* (Poudyal, Howden, & Bell, 2008). Colistin sulfate was proposed to function by binding electrostatically to the lipopolysaccharides and phospholipids on the outer cell membrane of these gram negative bacteria, and subsequently displace the membrane cations (magnesium and calcium) from the

phosphate groups of these membrane lipids, creating pores, and subsequently causing cell death (Falagas & Vardakas, 2014). Using *M. aurum*, David and Rastogi (1985), additionally indicated that colistin sulfate has an effect on the cytoplasmic membrane of mycobacteria, and indicated a resultant cell leakage in experiments using *M. avium* (Rastogi N. , Potar, Henrotte, Franck, & David, 1988), *M. aurum*, *M. xenopi* and *M. smegmatis* (Rastogi, Potar, & David, 1986), as a consequence of cell wall disruption. Considering this evidence, colistin sulfate would also be expected to have similar effects on *Mtb*. Korycka-Machala et al., (2001) subsequently showed an increased cell wall permeability in *Mtb* following colistin sulfate treatment, and Van Breda (2015) and Bax et al., (2015), that colistin sulfate also allows for elevated first line TB drug uptake in *Mtb*, as a result of this. Since most of this evidence pertaining to the anti-bacterial mechanisms of colistin sulfate in mycobacteria has been done from a histological and genomics research perspective, research using other “omics” disciplines are also required to understand this drug better, and its possible application to treating TB. Metabolomics is one of the latest additions to the “omics” technologies, and defined as an unbiased identification and quantification of all metabolites present in a sample (disease or treatment related), using advanced analytical techniques, and statistical analysis and bioinformatics, to identify the most important biomarkers for describing a perturbation (Berg, Tymoczko, & Stryer, 2007). We used a two dimensional gas chromatography coupled time-of-flight mass spectrometry (GCxGC-TOF/MS) metabolomics approach, to identify those metabolite markers best differentiating *Mtb* cultured in the presence and absence of colistin sulfate, for the purpose of confirming or elucidating its mechanism of action against *Mtb*.

3.2. Materials and methods

3.2.1. Cell culture

The cell cultures were prepared in the presence and absence of colistin sulfate, as described by van Breda et al., (2015), with slight modifications. Briefly, *Mtb* H37Ra ATCC 25177 (obtained from Ampath Pathology Laboratory Support Services, Centurion, Gauteng, South Africa) was swabbed onto Middlebrook 7H10 agar (Becton Dickinson, Woodmead, Gauteng, South Africa),

supplemented with 0.5% v/v glycerol (Saarchem, Krugersdorp, Gauteng, South Africa), and enriched with 10% v/v oleic acid, albumin, dextrose, catalase (OADC) (Becton Dickinson). Our reasons for selecting a H37Ra strain in this experiment, was due to the fact that the original description of the effects of polymyxins by Rastogi et al., (1986), used H37Ra, and a recent publication by Bax et al., (2015), described similar results using H37Rv as to what van Breda et al., (2015), described for H37Ra.

The stock culture was prepared after three weeks of incubation at 37 °C, by suspending the cells in 1 x phosphate buffered saline (PBS) (Sigma Aldrich, Kempton Park, Gauteng, South Africa) containing 0.05% v/v Tween 80 (Saarchem) to a McFarland standard of 3. Aliquots of 1 mL were stored at -80 °C in cryovials, containing 20% v/v glycerol (Saarchem). By using the TB Ag MPT64 Device (KAT Medical, Roodepoort, Gauteng, South Africa), the presence of *Mtb* was confirmed, and the purity was determined by swabbing 100 µL of culture media onto tryptic soy agar (Merck, Darmstadt, Germany) and incubating at 37 °C for 48 h. Before experimental investigations, a cryovial of the stored aliquots was allowed to thaw to room temperature, vortexed and swabbed onto Middlebrook 7H10 agar. Plates were sealed in Ziploc bags and incubated at 37 °C until mid-log growth was reached (approximately 10 – 14 days).

The mid-log growth culture was suspended to a McFarland standard of 1 (using Sauton media (van Breda et al., 2015)); approximately 1×10^7 colony-forming units (CFU)/mL. The cell suspension (195µL) was then added to each well in a 96 well microtiter plate (Eppendorf). The antimicrobials were added to final concentrations of 0 µg/mL and 32 µg/mL colistin sulfate respectively, and the plate was sealed using sterile ziploc bags, and incubated at 37 °C for 24 hours. The mixture in each well was subsequently transferred to Eppendorf tubes up to a volume of 1 mL. The 10 samples containing 32 µg/mL colistin sulfate and 7 samples containing no colistin sulfate, were centrifuged at 10000 x g for 1 min and showed no difference in the amounts of viable CFU/mL. The supernatant was removed and pellet rinsed and resuspended in 1 x PBS (without Tween 80) and then stored at -80°C.

In the current investigation it is important to note, that the reason Sauton media was used, is because other media, such as Middlebrook 7H9 for instance, contains the following components which antagonize the effects of polymyxins 1. BSA (forms complexes with polymyxins) (Liu, Tyo, Martinez, Petranovic, & Nielsen, 2012), 2. Mg^{2+} and Ca^{2+} (Chen & Feingold, 1972) (D'Amato, Capineri, & Marchi, 1975), and hence it was important to use media where physiological

concentrations of these divalent cations can be controlled, i.e., cation-adjusted to 10-12.5mg Mg^{2+} /L and 20-25mg Ca^{2+} /L (Falagas & Kasiakou, 2005) (Landman, Georgescu, Martin, & Quale, 2008), 3. Na^+ (Hancock & Sahl, 2006) (Ramón-García, et al., 2013), and 4. Catalase, since the latter is an antioxidant which would inhibit polymyxin induced Fenton reaction mechanisms (Sampson, et al., 2012). Furthermore, it was important to substitute glycerol with 0.2% w/v glucose, since lower than normal MICs have been previously observed for *Mtb* when glycerol was used as the only carbon source (Pethe, et al., 2010), and with 0.05% v/v Tween 80, since *Mtb* requires the fatty acids present within Tween 80 for growth (Schaefer & Lewis, 1965) (Smith, Zahnley, Pfeifer, & Goff, 1993).

3.2.2. Whole metabolome extraction procedure and derivatization

Prior to GCxGC-TOFMS analysis, 0.5 mg of each of the individually cultured *Mtb* sample pellets described above were weighed into an Eppendorf tube, followed by the addition of 50 μ L 3-phenylbutyric acid (0.0175 μ g/mL) (Sigma-Aldrich (St. Louis, MO, USA)) as internal standard. Chloroform, methanol (Burdick and Jackson brands (Honeywell International Inc., Muskegon, MI, USA)) and water were added in a ratio 1:3:1, vortexed for 1 min and then placed in a vibration mill (Retsch, Haan, Germany) with a 3 mm carbide tungsten bead (Retsch) for 5 min at 30 Hz/s. Each sample was then centrifuged for 10 min at 10 000xg and the supernatants transferred to a GC sample vial, and subsequently dried under a nitrogen stream. Each extract was derivatized using 20 μ L methoxyamine hydrochloride-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Sigma-Aldrich (Darmstadt, Germany)) (containing 15 mg/mL pyridine) at 50 °C for 90 min, followed by silylation using 40 μ L MSTFA with 1 % trimethylchlorosilane (TMCS) at 50 °C for 60 min. These extracts were then transferred to a 0.1 mL insert in a clean GC sample vial and capped, prior to GCxGC-TOFMS analysis (Meissner-Roloff, Koekemoer, Warren, & Loots, 2012).

3.2.3. GCxGC-TOFMS analyses

2.3 GCxGC-TOFMS analyses

The samples (1 μ L) were analysed in random sequence, using a Pegasus 4D GCxGC-TOFMS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA), TOFMS (LECO Africa) and Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany), in a splitless ratio. The necessary quality control (QC) samples were also analyzed at regular intervals in order to correct for any batch effects and also monitor the performance of the analysis over time. A Rxi-5Sil MS primary capillary column (30 m, 0.25 μ m film thickness and 250 μ m internal diameter), and a Rxi-17 secondary capillary column (1.2 m, 0.25 μ m film thickness and 250 μ m internal diameter) were used for GC compound separation. Helium was used as a carrier gas at a flow of 1 mL/min with the injector temperature held constant at 270 °C for the entire run. The primary column temperature was set at 70 °C for 2 min, and then increased at a rate of 4 °C/min to a final temperature of 300 °C, at which it was maintained for a further 2 min. The temperature of the secondary oven was programmed at 85 °C for 2 min, then increased at a rate of 4 °C/min to final temperature of 305 °C, at which it was maintained for a further 4.5 min. The acquisition voltage of the detector was 1700 V and the filament bias -70 eV. A mass range of 50–800 m/z was used for the mass spectra, at an acquisition rate of 200 spectra/s.

3.2.4. Data processing, clean-up and statistics

Mass spectral deconvolution (at a signal to noise ratio of 20), peak alignment and peak identification, were done on the collected mass spectra using ChromaTOF software (version 4.32). Identical mass spectra of the compounds in each of the samples were aligned, if they displayed similar retention times. Compounds were identified by comparison of their mass fragment patterns and retention times, to that of libraries compiled from previously injected standards.

Following the data processing steps described above, a standardized metabolomics data clean-up procedure was conducted (Smuts, Der Westhuizen, Francois, Louw, & al., 2013). Normalization of each of the detected compounds was done using the total useful MS signal (TUS)

(Chen, et al., 2013) and by calculating the relative concentration of each compound, using the internal standard as a reference. A 50% filter was applied in order to remove those compounds showing more than 50% zero values within both groups (Lutz, Sweedler, & Wevers, 2013) and the QC samples used to correct for any batch effects, using quantile equating (Wang, Kuo, & Tseng, 2012). Additionally, a 50% QC coefficient of variation (CV) filter was applied (Godzien, Alonso-Herranz, Barbas, & Armitage, 2014), and all zero-values were replaced by a value determined as half of the smallest concentration (i.e. the detection limit) detected in the entire data set, as these may be due to low abundance rather than being absent (Piotr, Xu, & Goodacre, 2014).

The data were subsequently analysed using a variety of multi- and univariate statistical methods, using a web based software package supported by the Metabolomics Society: MetaboAnalyst (based on the statistical package “R”; version 2.10.0), and included principal components analysis (PCA) (Buydens, et al., 2009), partial least squares–discriminant analysis (PLS–DA) (Cho, et al., 2008), a t-test and effect size calculations (Smith, Is it the sample size of the sample as a fraction of the population that matters?, 2004).

3.3 Results

Figure 3-1 shows clear PCA differentiation between the individually cultured *Mtb* samples in the presence and absence of colistin sulfate. This natural differentiation of the samples of each of the sample groups can be ascribed to the variation in the total metabolite profiles of each, as determined by GCxGC-TOFMS. The total variance explained by the first two principal components (PCs) (R²X cum) was 48.4% of which PC1 contributed to 37.5% and PC2 10.9%, respectively.

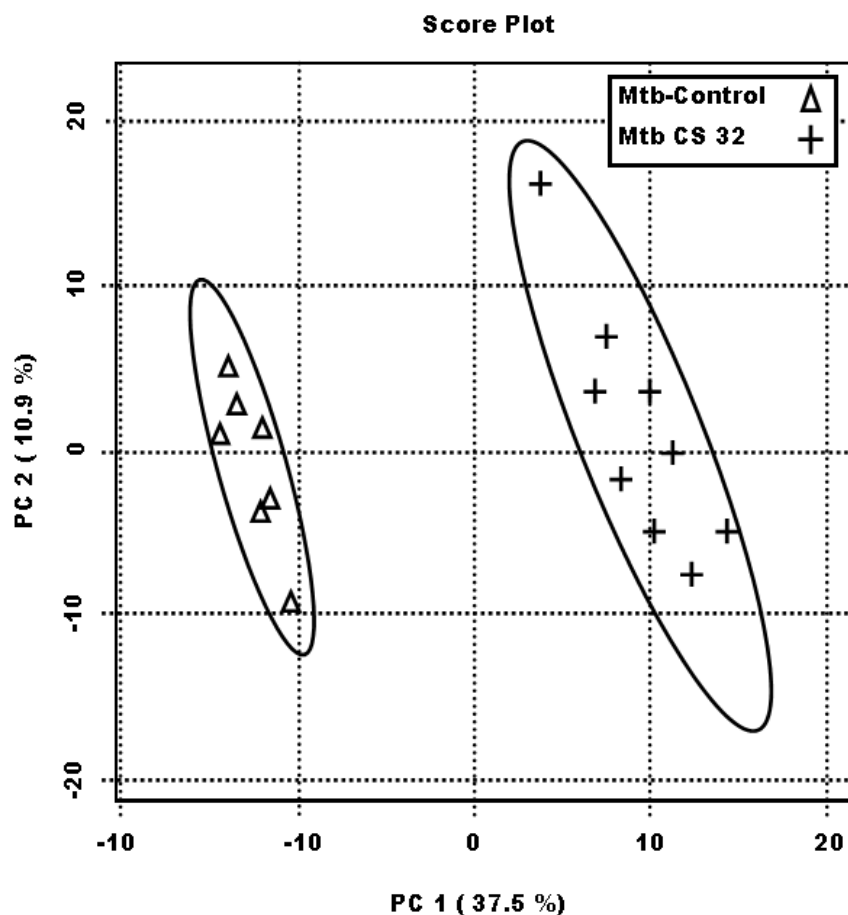


Figure 3-1 PCA differentiation using the GCxGC-TOFMS whole metabolome analysed data of the individually cultured *Mtb* in the absence (*Mtb*-Control) and presence (*Mtb*-CS) of colistin sulfate (32 µg/mL). The variances accounted for are indicated in parenthesis.

Subsequently, those metabolites that contributed most to this differentiation were selected on the basis of complying with all of the following criteria: a PCA modelling power > 0.5 (Buydens et al., 2009), a PLS-DA VIP value > 1 (Cho et al., 2008), a t-test P-value < 0.05 and an effect size > 0.5 (Smith, 2004).

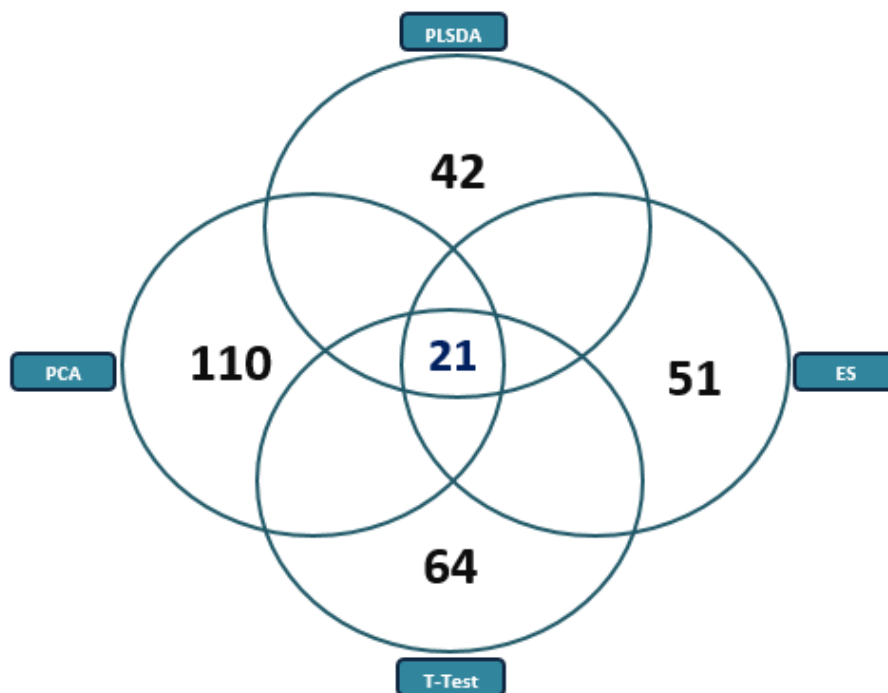


Figure 3-2 Venn diagram illustrating a multi-statistical approach for selecting the 21 metabolite markers best describing the variation detected between the individually cultured *Mtb* samples in the presence and absence of colistin sulfate.

Figure 3-2 is a summary of the number of metabolite markers selected by each of the univariate and multivariate statistical approaches described above, as well as the selection of the 21 metabolites listed in Table 3:1, considered most important for explaining the variation detected.

Table 3:1 The 21 metabolite markers that best explain the variance between the individually cultured *Mtb* samples in the absence (*Mtb*-Controls) and presence (*Mtb*-CS) of colistin sulfate.

	<i>Mtb</i> controls:		Mtb treated with colistin sulfate						
Metabolite name	Average concentration		Average concentration		PCA	PLS-DA	Effect sizes	<i>t</i> -test	<i>Fold change</i>
(Chempid ID)	(mg/g cells)		(mg/g cells)		(Power)	(VIP)	(<i>d</i> -value)	(<i>P</i> -value)	(log2)
	(standard error of the mean)		(standard error of the mean)						
Octanoic acid (NSC 5024 [DBID])	0.205	(0.192)	0.019	(0.010)	0.787	1.423	0.966	>0.001	-3.43
Hexadecanoic acid (NSC 5030 [DBID])	0.860	(0.176)	1.259	(0.198)	0.948	1.224	2.022	0.001	0.55
Octadecenoic acid (NSC931 [DBID])	>0.001	(>0.001)	0.021	(0.005)	0.861	1.165	4.580	0.003	4.39
Eicosenoic acid (4445895)	>0.001	(>0.001)	0.006	(0.007)	0.842	1.181	0.930	0.002	2.59
Hexacosanoic acid (NSC 4205 [DBID])	>0.001	(>0.001)	0.059	(0.038)	0.804	1.244	1.541	0.001	5.88
Methyldecanoic acid (LMFA01020090)	0.013	(0.004)	0.017	(0.004)	0.822	1.238	0.994	0.001	0.39
Methyldodecanoic acid (4445769)	0.045	(0.011)	0.115	(0.025)	0.855	1.123	2.794	0.004	1.35
Methyltetradecanoic acid (NSC 189699 [DBID])	0.317	(0.021)	0.789	(0.261)	0.893	1.073	1.810	0.007	1.32
Sebacic acid (NSC 19492 [DBID])	0.001	(0.001)	0.004	(0.003)	0.898	1.151	1.278	0.003	2.00
Tetradecanol (NSC 4194 [DBID])	0.016	(0.003)	0.059	(0.019)	0.982	1.516	2.251	>0.001	1.88
2-Ethyl-2-Methyl-Tridecanol (921600091)	0.006	(0.001)	0.044	(0.033)	0.809	1.245	1.159	0.001	2.87
5-Nonanol (NSC4552 [DBID])	0.001	(0.001)	0.023	(0.004)	0.965	1.054	6.168	0.001	4.52
Hexadecane (NSC 172781 [DBID])	0.002	(0.003)	0.031	(0.029)	0.978	1.555	1.024	>0.001	3.95
Octadecane (NSC 172781 [DBID])	0.020	(0.011)	0.115	(0.028)	0.878	1.197	3.446	0.002	2.52
Octacosane (NSC 5549 [DBID])	>0.001	(>0.001)	0.016	(0.016)	0.993	1.611	0.979	>0.001	4.00
Methyltetradecane (NSC 172781 [DBID])	0.005	(0.004)	0.044	(0.012)	0.664	1.090	3.186	0.006	3.14
Glucose (NSC4552 [DBID])	0.195	(0.187)	0.366	(0.170)	0.819	1.470	0.913	>0.001	0.91
Oxalate (c0017 [DBID])	>0.001	(>0.001)	0.006	(0.006)	0.863	1.266	0.954	0.001	2.59
Glycerol (NSC 9230 [DBID])	0.063	(0.012)	0.082	(0.011)	0.891	1.173	1.469	0.002	0.38
Monopalmitin (110006)	0.165	(0.058)	0.216	(0.056)	0.987	1.614	0.874	>0.001	0.39
Propyl myristate (AI3-31609 [DBID])	0.014	(0.003)	0.023	(0.006)	0.991	1.626	1.328	>0.001	0.72

3.4 Discussion

As previously mentioned, treatment with colistin sulfate results in a structural disruption of the cell wall in *Mtb* (Bax, et al., 2015). The metabolite markers detected in the colistin sulfate treated *Mtb* in the current metabolomics investigation confirms this, and additionally indicates that *Mtb* attempts to rectify this by upregulation of its fatty acid synthesis pathways for subsequent cell wall repair. Accompanying this is an upregulation of glycolysis which will be described in detail below and summarized in Figure 3-3.

In Table 3:1, 15 out of the 21 metabolite markers identified by the statistics described above, are directly linked to elevated fatty acid biosynthesis and subsequently also cell wall synthesis. These included hexadecanoic acid, octadecenoic acid, eicosanoic acid and hexacosanoic acid, all of which are known to form methyl-branched chain fatty acids and ultimately the mycolic acids (Shimakata, Iwaki, & Kusaka, 1984) (Du Preez & Loots, 2012), an important component of arabinogalactan (AG) in the cell wall core of *Mtb* (Kaur, Guerin, Škovierová, Brennan, & Jackson, 2009). Additionally, although not detected as part of the 21 metabolite markers, decanoic acid (0.100 vs. 0.219 µg/ml; $P < 0.05$), dodecanoic acid (0.421 vs. 0.592 µg/ml; $P < 0.05$) and octadecanoic acid (0.491 vs. 0.888 µg/ml; $P > 0.05$) were also found to be elevated, further supporting this. Another important observation was the elevated levels of the methylated branched fatty acids (methyldecanoic acid, methyl dodecanoic acid and methyltetradecanoic acid), in the colistin sulfate treated group comparatively, which in turn not only serve as substrates for mycolic acid synthesis, but also function as hydrophobic modulators of the host's cellular immune function, and various virulence factors in the microbe (Lee, VanderVen, Fahey, & Russell, 2013). These methylated fatty acids are proposed to be formed by 3 possible routes: 1. fatty acid methylation via S-adenosylmethionine (SAM) functioning as the methyl donor (Du Preez & Loots, 2012), 2. methylmalonyl-CoA derived polyketide synthase complexes, originating from propionyl-CoA and malonyl-CoA (Duncan & Garton, 2007) and 3. acetyl-CoA metabolism to butyric acid, which in turn reacts with propionyl-CoA (Massey, Sokatch, & Conrad, 1976).

Further substantiating these results, are the presence of various alcohols (tetradecanol, nonanol and 2-ethyl-2-methyltridecanol) and alkanes (hexadecane, octadecane, octacosane and methyltetradecane) corresponding to the aforementioned fatty acids (Park, 2004). Additionally, although not detected using the marker selection process described above, decanol (0 vs. 0.018

µg/ml; $P < 0.05$), decane (0.006 vs. 0.012 µg/ml; $P > 0.05$), dodecane (0 vs. 0.007 µg/ml; $P > 0.05$), eicosane (0.083 vs. 0.172 µg/ml; $P < 0.05$), tetracosanol (0.001 vs. 0.006 µg/ml; $P > 0.05$), tetracosane (0 vs. 0.01 µg/ml; $P < 0.05$), methylhexacosane (0.132 vs. 0.611 µg/ml; $P > 0.05$), tetramethylhexadecanol (0.001 vs. 0.023 µg/ml; $P < 0.05$) and methylhexadecane (0 vs. 0.01 µg/ml; $P > 0.05$)), were also seen to occur in elevated amounts, further confirming these mechanisms.

Another important observation supporting the unanimous metabolic flux observed in this study towards fatty acid biosynthesis and cell wall repair, is the significantly elevated concentrations of glucose, glycerol and monopalmitic acid. According to de Carvalho (2010), *Mtb*'s central carbon metabolism is able to co-catabolise multiple carbon sources for energy (de Carvalho, et al., 2010). Considering the colistin sulfate treated *Mtb*'s need to preferably utilize fatty acids towards cell wall repair, one would expect that this organism would subsequently resort to glucose, which was freely available in the growth media, as the primary energy substrate, in conjunction with an upregulated glyoxylate cycle (Badejo, et al., 2013), substantiated in this investigation by the elevated glucose and oxalic acid detected (Coad, Friedman, & Geoffrion, 2012). Furthermore, as shown in Figure 3-3, various intermediates of glycolysis, can additionally serve as substrates for fatty acid biosynthesis, including acetyl-CoA, as previously mentioned, and glyceraldehyde-3-phosphate (G-3-P) via glycerol (Berg, Tymoczko, & Stryer, 2007), the latter of which is supported by elevations in monopalmitic acid and the glycerol present in the growth media.

3.5 Concluding remarks

This study, is the first of its kind to use a metabolomics research approach in order to identify biomarkers explaining the antibacterial mechanisms of colistin sulfate against *Mtb*, and additionally shows the capacity of metabolomics for identifying metabolite markers which can be used to better understand or confirm drug action. The fatty acid metabolite markers identified in the colistin sulfate treated *Mtb*, shows a metabolic flux towards fatty acid synthesis and cell wall repair. Furthermore, glucose uptake is increased, serving as the preferential energy source (as opposed to fatty acids which are now preferentially being used for cell wall repair) to fuel an upregulated glyoxylate cycle, and additionally as a precursor for further cell wall fatty acid synthesis via the glycerolipid metabolic pathway. Considering this, it can be concluded that colistin sulfate acts by disrupting the cell wall in *Mtb*, confirming a similar drug action as that seen in other organisms.

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Chapter 4: Metabolomics of colistin methanesulfonate treated *Mycobacterium tuberculosis*.

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Abstract

Over the last 5 years, there has been a renewed interest in finding new compounds with anti-TB action. Colistin methanesulfonate or polymyxin E, is a possible anti-TB drug candidate, which may in future be used either alone or in combination to the current 6 month “directly observed treatment short-course” (DOTS) regimen. However its mechanism of action has to date not yet been fully explored, and only described from a histological and genomics perspective. Considering this, we used a GCxGC-TOFMS metabolomics approach and identified those metabolite markers characterising *Mycobacterium tuberculosis* (*Mtb*) cultured in the presence of colistin methanesulfonate, in order to better understand or confirm its mechanism of action. The metabolite markers identified indicated a flux in metabolism of the colistin methanesulfonate treated *Mtb* towards fatty acid synthesis and cell wall repair, confirming previous reports that colistin acts by disrupting the cell wall of mycobacteria. Accompanying this, is a subsequently elevated glucose uptake, since the latter now serves as the primary energy substrate for the upregulated glyoxylate cycle, and additionally as a precursor for further fatty acid synthesis via the glycerolipid metabolic pathway. Furthermore, the elevated concentrations of those metabolites associated with pentose phosphate, valine, threonine, and pentanediol metabolism, also confirms a shift towards glucose utilization for energy production, in the colistin methanesulfonate treated *Mtb*.

Keywords

Colistin methanesulfonate, *Mycobacterium tuberculosis*, Tuberculosis, Metabolomics, Treatment, Antibiotics

4.1 Introduction

In 2015, an approximated 10.5 million new cases of tuberculosis (TB) was reported globally, which subsequently contributed to 1.4 million deaths [1]. Tuberculosis is caused by the infectious organism *Mycobacterium tuberculosis* (*Mtb*), a mycobacteria bacillus which mainly targets the lungs [2]. Currently, the WHO approved treatment approach entails a 6 months combination treatment approach which is called the “directly observed treatment short-course” (DOTS) regimen [3]. According to the annual WHO report, a significant improvement to current treatment strategies is going to be a challenge, however the identification of new anti-TB drug candidates and or alternative treatment regimens, might be a plausible option for speeding up treatment duration and subsequently lowering the TB prevalence globally [4,5]. Although there are currently a number of new potential anti-TB drugs undergoing phase II and III preclinical trials, delamanid and bedaquiline are the only two new anti-TB drugs to have been approved over the last 50 years. These drugs, however, are currently only used for treating adults with MDR-TB, and considered as last option medications, when no other alternatives prove to be effective [6]. Considering this, there is still urgent need for new TB drugs and alternative TB treatment approaches.

Another possible anti-TB drug candidate is the antibiotic colistin methanesulfonate (CMS), an inactive prodrug of colistin sulfate (CS), also known as polymyxin E [7]. CMS has previously been shown to have high anti-bacterial activities against *P. aeruginosa*, *A. baumannii*, and *Klebsiella pneumoniae*, and additionally shown to be resistant to these organisms developing drug tolerance [8]. CMS is produced via a reaction from commercially synthesised CS with formaldehyde and sodium bisulphite, resulting in the subsequent addition of a sulfomethylated group to the primary amine groups of CS [9]. The original reason for modifying CS in this manner is that the resulting CMS is considered less toxic when administered parenterally [10]. When administered, a hydrolysis

reaction occurs, where CMS in an aqueous solution forms both CS and various partially sulfomethylated derivatives of CS [11]. Apart from the varying toxicity characteristics of CS and CMS, these two forms of the drug show different pharmacokinetic characteristics [12–14]. A study conducted by Plachouras et al., 2009, indicated that colistin concentrations increase slowly after the administration of CMS in critically ill patients, reaching a steady state after 2 days, suggesting benefits of treatment commencement with a loading dose [15]. Various colistin derivatives have also been proposed to promote first line anti-TB drug uptake, by creating pores in the outer membrane of *Mtb*, after binding electrostaticly to the outer cell membrane lipopolysaccharides and phospholipids [16]. Very little data however exists describing the antimicrobial action of CMS against *Mtb*, which has been described to date, was attained solely from a histological or genomics approach. Metabolomics, the latest addition to the “omics” family, is defined as an unbiased identification and quantification of all metabolites present in a sample, using highly specialised analytical procedures and a statistical analysis / bioinformatics, by which the most important metabolites characterising a perturbation (or drug) can be identified [17]. In this investigation, we extracted the intracellular metabolome of *Mtb* cultured in the presence and absence of 32 µg/ml CMS, and analysed these extracts using a 2 dimensional gas chromatography time of flight mass spectrometry (GCxGC-TOFMS) metabolomics approach, for the purpose of identifying those metabolite markers best characterising the changes to the *Mtb* metabolome induced by CMS.

4.2 Materials and methods

4.2.1 Cell culture

As described by van Breda et al., (2015), the cell cultures were prepared in the presence and absence of CMS, with slight modifications. Briefly, *Mtb* H37Ra ATCC 25177 (obtained from Ampath Pathology Laboratory Support Services, Centurion, Gauteng, South Africa) was swabbed onto Middlebrook 7H10 agar (Becton Dickinson, Woodmead, Gauteng, South Africa), supplemented with 0.5% v/v glycerol (Saarchem, Krugersdorp, Gauteng, South Africa), and enriched with 10% v/v oleic acid, albumin, dextrose, catalase (OADC) (Becton Dickinson). The reasons for selecting a H37Ra strain in this experiment, was due to the fact that the original description of the effects of polymyxins

by Rastogi et al., (1986), used H37Ra, and a recent publication by Bax et al., (2015), described similar results using H37Rv as to what van Breda et al. (2015) described for H37Ra [16,18,19] .

The stock culture was prepared after three weeks of incubation at 37 °C, by suspending the cells in 1 x phosphate buffered saline (PBS) (Sigma Aldrich, Kempton Park, Gauteng, South Africa) containing 0.05% v/v Tween 80 (Saarchem) to a McFarland standard of 3. Aliquots of 1 mL were stored at -80 °C in cryovials, containing 20% v/v glycerol (Saarchem). By using the TB Ag MPT64 Device (KAT Medical, Roodepoort, Gauteng, South Africa), the presence of *Mtb* was confirmed, and the purity was determined by swabbing 100 µL of culture media onto tryptic soy agar (Merck, Darmstadt, Germany) and incubating at 37 °C for 48 h. Before experimental investigations, a cryovial of the stored aliquots was allowed to thaw to room temperature, vortexed and swabbed onto Middlebrook 7H10 agar. Plates were sealed in Ziploc bags and incubated at 37 °C until mid-log growth was reached (approximately 10 – 14 days).

The mid-log growth culture was suspended to a McFarland standard of 1 (using Sauton media [16]); approximately 1×10^7 colony-forming units (CFU)/mL. The cell suspension (195µL) was then added to each well in a 96 well microtiter plate (Eppendorf). The antimicrobials were added to final concentrations of 0 µg/mL and 32 µg/mL CMS respectively, and the plate was sealed using sterile ziploc bags, and incubated at 37 °C for 24 hours. The mixture in each well was subsequently transferred to Eppendorf tubes up to a volume of 1 mL. The 10 samples containing 32 µg/mL CMS and 7 samples containing no CMS, were centrifuged at 10000 x g for 1 min and showed no difference in the amounts of viable CFU/mL. The supernatant was removed and pellet rinsed and resuspended in 1 x PBS (without Tween 80) and then stored at -80°C.

In the current investigation it is important to note, that the reason Sauton media was used, is because other media, such as Middlebrook 7H9 for instance, contains the following components which antagonize the effects of polymyxins 1. BSA (forms complexes with polymyxins) [20], 2. Mg²⁺ and Ca²⁺ [21,22], and hence it was important to use media where physiological concentrations of these divalent cations can be controlled, i.e., cation-adjusted to 10-12.5mg Mg²⁺/L and 20-25mg Ca²⁺/L [23,24], 3. Na⁺ [25,26], and 4. Catalase, since the latter is an antioxidant which would inhibit polymyxin induced Fenton reaction mechanisms [27]. Furthermore, it was important to substitute glycerol with 0.2% w/v glucose, since lower than normal MICs have been previously observed for *Mtb* when glycerol was used as the only carbon source [28], and with 0.05% v/v Tween 80, since *Mtb* requires the fatty acids present within Tween 80 for growth [29,30].

4.2.2 Whole metabolome extraction procedure and derivatization

Prior to GCxGC-TOFMS analysis, 0.5 mg of each of the individually cultured *Mtb* sample pellets described above were weighed into an Eppendorf tube, followed by the addition of 50 μ L 3-phenylbutyric acid (0.0175 μ g/mL) (Sigma-Aldrich (St. Louis, MO, USA)) as internal standard. Chloroform, methanol (Burdick and Jackson brands (Honeywell International Inc., Muskegon, MI, USA)) and water were added in a ratio 1:3:1, vortexed for 1 min and then placed in a vibration mill (Retsch, Haan, Germany) with a 3 mm carbide tungsten bead (Retsch) for 5 min at 30 Hz/s. Each sample was then centrifuged for 10 min at 10 000xg and the supernatants transferred to a GC sample vial, and subsequently dried under a nitrogen stream. Each extract was derivatized using 20 μ L methoxyamine hydrochloride-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Sigma-Aldrich (Darmstadt, Germany)) (containing 15 mg/mL pyridine) at 50 °C for 90 min, followed by silylation using 40 μ L MSTFA with 1 % trimethylchlorosilane (TMCS) at 50 °C for 60 min. These extracts were then transferred to a 0.1 mL insert in a clean GC sample vial and capped, prior to GCxGC-TOFMS analysis [31].

4.2.3 GCXGC-TOFMS analyses

The samples (1 μ L) were analysed in random sequence, using a Pegasus 4D GCxGC-TOFMS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA), TOFMS (LECO Africa) and Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany), in a splitless ratio. The necessary quality control (QC) samples were also analyzed at regular intervals in order to correct for any batch effects and also monitor the performance of the analysis over time. A Rxi-5Sil MS primary capillary column (30 m, 0.25 μ m film thickness and 250 μ m internal diameter), and a Rxi-17 secondary capillary column (1.2 m, 0.25 μ m film thickness and 250 μ m internal diameter) were used for GC compound separation. Helium was used as a carrier gas at a flow of 1 mL/min with the injector temperature held constant at 270 °C for the entire run. The primary column temperature was set at 70 °C for 2 min, and then increased at a rate of 4 °C/min to a final temperature of 300 °C, at which it

was maintained for a further 2 min. The temperature of the secondary oven was programmed at 85 °C for 2 min, then increased at a rate of 4 °C/min to final temperature of 305 °C, at which it was maintained for a further 4.5 min. The acquisition voltage of the detector was 1700 V and the filament bias -70 eV. A mass range of 50–800 m/z was used for the mass spectra, at an acquisition rate of 200 spectra/s.

4.2.4 Data processing, clean-up and statistics

Mass spectral deconvolution (at a signal to noise ratio of 20), peak alignment and peak identification, were done on the collected mass spectra using ChromaTOF software (version 4.32). Identical mass spectra of the compounds in each of the samples were aligned, if they displayed similar retention times. Compounds were identified by comparison of their mass fragment patterns and retention times, to that of libraries compiled from previously injected standards.

Following the data processing steps described above, a standardized metabolomics data clean-up procedure was conducted [32]. Normalization of each of the detected compounds was done using the total useful MS signal (TUS) [33] and by calculating the relative concentration of each compound, using the internal standard as a reference. A 50% filter was applied in order to remove those compounds showing more than 50% zero values within both groups [34] and the QC samples used to correct for any batch effects, using quantile equating [35]. Additionally, a 50% QC coefficient of variation (CV) filter was applied [36], and all zero-values were replaced by a value determined as half of the smallest concentration (i.e. the detection limit) detected in the entire data set, as these may be due to low abundance rather than being absent [37].

The data were subsequently analysed using a variety of multi- and univariate statistical methods, using a web based software package supported by the Metabolomics Society: MetaboAnalyst (based on the statistical package “R”; version 2.10.0), and included principal components analysis (PCA) [38], partial least squares–discriminant analysis (PLS–DA) [39], a t-test and effect size calculations [40].

4.3 Results and Discussion

Figure 4-1 shows clear PCA differentiation of the individually cultured *Mtb* samples in the presence and absence of CMS, using the collected GCxGC-TOFMS metabolomics data. The total amount of variance explained by the first two principal components (PCs) (R²X cum) was 55.9%, of which PC1 accounted for 43.4%, and PC2 accounted for 12.5%. Subsequently, by compliance with all of the following criteria: a PCA modelling power > 0.5 [38], a PLS-DA VIP value > 1 [39], a t-test P-value < 0.05 and an effect size > 0.5 [40], the metabolites that contributed most to this differentiation were selected (Figure 4-2) and listed in Table 4:1. These metabolite markers were mapped on a metabolic chart as indicated in Figure 3 and discussed below. As indicated, the metabolomics investigation of the cultured *Mtb* in the presence and absence of CMS, led to the identification of various significantly altered metabolite markers. Glucose uptake was increased in the CMS treated *Mtb*, as the preferential energy source (as opposed to fatty acids which are now preferentially being used for cell wall repair) to fuel an upregulated glyoxylate cycle, and substrate further cell wall fatty acid synthesis via the glycerolipid metabolic pathway. However, the CMS treated *Mtb*, also showed comparatively elevated metabolites associated with pentose phosphate, valine, threonine, and pentanediol metabolism. These results confirm that CMS disrupts the *Mtb* cell membrane, and that these bacteria attempt to compensate for this via upregulation of various metabolic pathways related to cell wall repair.

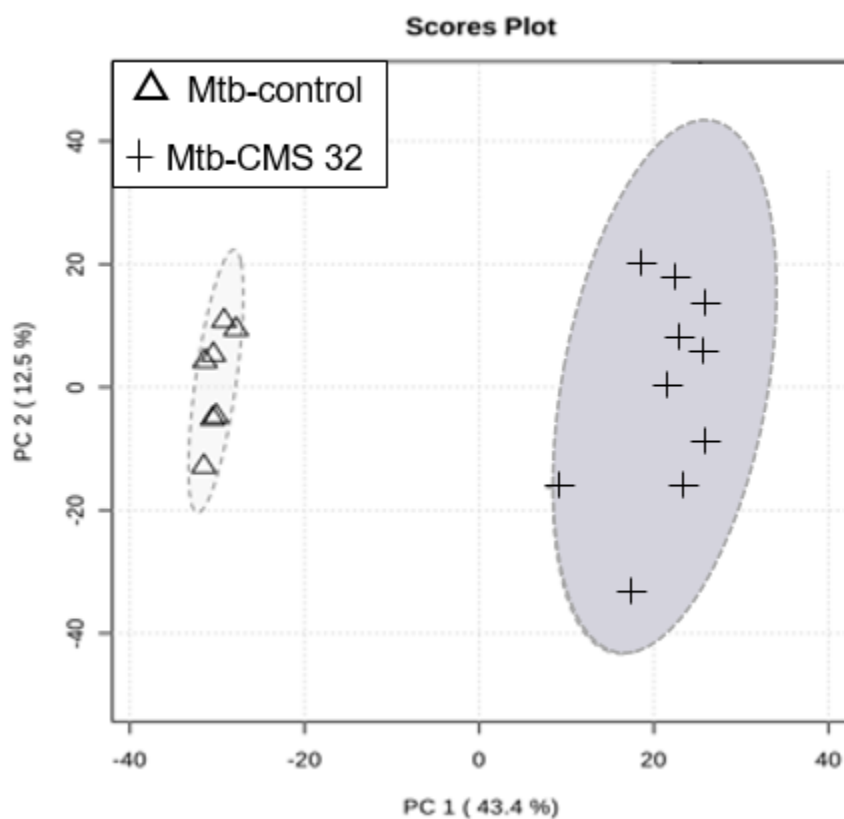


Figure 4-1 PCA differentiation of individually cultured *Mtb* in the absence (*Mtb*-control) and presence (*Mtb*-CMS) of colistin methanesulfonate (32 µg/mL) and analysed via GCxGC-TOFMS. The variances accounted for are indicated in parenthesis.

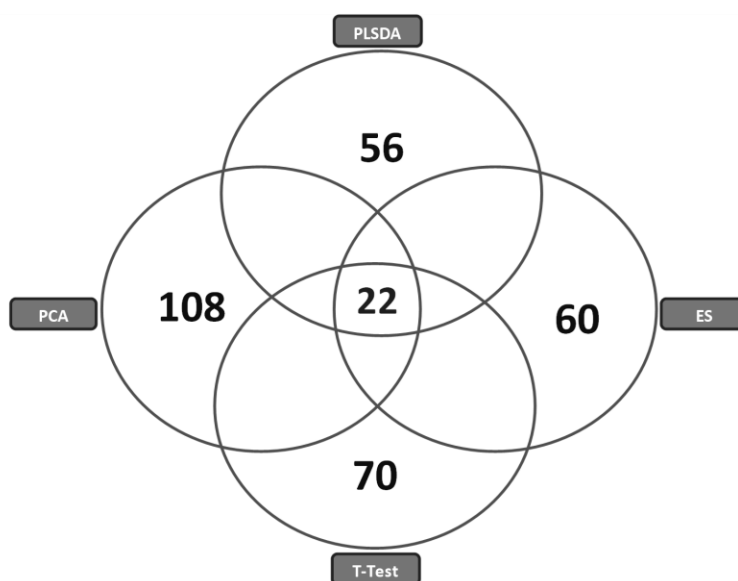


Figure 4-2 Venn diagram illustrating the multi-statistical selection criteria of the 22 metabolite markers best describing the variation between the individually cultured *Mtb* sample groups in the presence and absence of CMS.

Colistin has been previously reported to have an antimicrobial activity, which function by binding electrostatically to the lipopolysaccharides and phospholipids on the outer cell membrane of these gram negative bacteria, subsequently displacing the membrane cations (magnesium and calcium) from the phosphate groups of their membrane lipids, subsequently creating pores, which results in cell death [41]. This was supported by our previous metabolomics work on the topic, which showed that elevated fatty acid synthesis and cell wall repair mechanisms are activated in the CS treated *Mtb* [42]. As previously described by Bax, et al. (2015) and van Breda, et al. (2015), the CMS used in the current investigation, also forms colistin once administered, and hence, would also be expected to result in a structural disruption of the *Mtb* cell wall via the same mechanism as to when CS is administered. This is supported by the elevated levels of the cell wall associated with methylated and unmethylated fatty acids (methyladipic acid, methyl dodecanoic acid, methyltridecanoic, octadecenoic acid) and their fatty acid associated alcohols and alkanes [43] (tetramethylhexanodecanol, octacosane, octadecane, tetradecanol, and hentriacontane (Table 4:1). Additionally, although not selected using the markers selection statistics approach defined in the methods section, methyl octadecenoic acid (0.49 vs. 0.42 µg/ml; $P > 0.05$), hexadecanoic acid (0.859 vs. 0.857 µg/ml; $P > 0.05$), octadecanoic acid (22.45 vs. 8.07 µg/ml; $P > 0.05$), tetracosanoic acid (24.54 vs. 14.15 µg/ml; $P > 0.05$), decanol (0.02 vs. 0.00 µg/ml; $P > 0.05$), hexadecanol (0.001 vs. 0.000 µg/ml; $P > 0.05$), decane (0.009 vs. 0.006 µg/ml; $P > 0.05$), dodecane (0.006 vs. 0.00 µg/ml; $P > 0.05$), hexadecane (0.011 vs. 0.002 µg/ml; $P > 0.05$) and tetracosane (0.018 vs. 0.00 µg/ml; $P > 0.05$), were also significantly elevated in the CMS treated *Mtb* comparatively, when considering their P-values, further supporting this mechanism (Figure 4-3). Octanoic acid and octadecenoic acid, are the well-known substrates for the synthesis of methylated-branched chain fatty acids and mycolic acids, both important components of arabinogalactan (AG) in the cell wall of *Mtb* [44,45]. These methylated branched fatty acids additionally serve as hydrophobic modulators for the host's cellular immune system, and are also considered virulence factors in the microbe [46]. As indicated in Figure 4-3, these methylated cell wall intermediates are synthesised via 3 possible routes: 1. fatty acid methylation via S-adenosylmethionine (SAM) functioning as the methyl donor [45], 2. methylmalonyl-CoA derived polyketide synthase complexes, originating from propionyl-CoA and malonyl-CoA [47] and 3. acetyl-CoA metabolism to butyric acid, which in turn reacts with propionyl-CoA [48].

Additionally, methyladipic acid was found elevated, which is formed from methylhexanoic acid, one of the metabolites in the branched fatty acid synthesis pathways of *Mtb* [49].

Similarly as to what we previously saw for the CS treated *Mtb* [42], glycolysis and its associated pathways are also upregulated in the CMS treated *Mtb*. In our previous metabolomics investigation using CS treated *Mtb*, elevated levels of glucose, acetic acid and oxalic acid were detected [50]. This suggests that the CS treated *Mtb* needed to preferably utilize fatty acids towards cell wall repair, and subsequently these organisms need to resort to glucose (which was freely available in the growth media) as the primary energy substrate [51]. Similarly, in the CMS treated *Mtb* in the current investigation, elevated levels of oxalate were also detected (Table 4:1) in addition to elevated glucose (1.21 vs. 0.88 µg/ml; $P > 0.05$) and acetic acid (0.218 vs. 0.102 µg/ml; $P > 0.05$) when considering significance using the latter two compounds P-values. Additional evidence supporting this and indicated in Table 4:1 and Figure 4-3, where elevated levels of valine, threonine, and pentanediol, which also suggests a shift towards glucose utilization for energy and fatty acid synthesis in the CMS treated *Mtb*. Furthermore, the elevated levels of acetic acid (or acetyl-CoA) can subsequently result in the elevated synthesis of threonine and pentanediol, detected in the CMS treated *Mtb* [52]. Also associated directly with this pathway, is elevated valine synthesis from pyruvate [53], which feeds into the tricarboxylic acid (TCA) cycle via succinate [54]. Another branch chain amino acid metabolic pathway affected by CMS in *Mtb* is that of leucine's catabolism to acetyl-CoA, and the reduced amounts of 3-methylgluturic acid attests to an increased flux in this direction [55] and the subsequent synthesis of cell wall fatty acids or energy [56]. The reduced concentrations of methylmaleic acid [57] and diethylene glycol in the CMS treated *Mtb*, serve as further confirmation for the flux of glucose utilisation for growth and fatty acid synthesis via glycerol and monopalmitin, both of which were elevated in the CMS treated *Mtb* comparatively (Table 4:1 and Figure 4-3).

An interesting observation in the CMS treated *Mtb*, where two metabolite markers associated with the pentose phosphate pathway namely, a reduced arabinose and an elevated erythrose (Table 4:1). As indicated in Figure 4-4, due to the high demand in fatty acid synthesis for cell repair, and an increased demand for this and energy production via glycolysis, the pentose phosphate pathways is most likely additionally utilised during such conditions for generating more intermediates for glycolysis [58], with the reduction in arabitol and elevated levels of erythrose, indicating a metabolic flux towards glyceraldehyde-3-phosphate and fructose-6-phosphate synthesis, something which wasn't previously seen in the CS treated *Mtb* [59]. A study conducted by Henry et al. (2015), indicated differentiation of gene expression following colistin treatment. These results are consistent with that

found in the current study, which also suggests that colistin treatment alters the outer membrane composition and results in subsequent damage to the outer membrane of *Mtb*, as previously described [60].

Additionally, the alterations made by CMS to the *Mtb* cell membrane, results in it becoming less hydrophobic, hence it could be suggested that CMS be used in synergy with other hydrophilic drugs, which previously struggle to cross these bacterial membranes. This has been previously observed by Bax et al. (2015) and van Breda et al (2015). It is possible that disruption of the hydrophobic barrier of *Mtb* by INH (inhibiting mycolic acid synthesis) or CS from CMS could lead to a greater uptake via the self-promoted uptake for CS causing a synergistic effect. In the case of INH, disruption of the hydrophobic barrier can lead to an uptake of hydrophilic INH. According to Nasiruddin, Neyaz, & Das (2017), a promising drug delivery model could be to encapsulate the hydrophilic drugs to be carried over the membrane, however in synergy with CMS, this may no longer be required [61]. Proof for this hypothesis, are the results by Al-Shaer, Nazer, & Kherallah (2014), where a combination therapy approach of rifampicin and CMS was used against MDR *A. baumannii*, which resulted in the successful treatment of 64% of the patients, with very little side effects reported [62]. Unfortunately, the results of these investigations are limited and no effect is given on the clinical outcomes of rifampicin induced - hepatotoxicity.

Table 4:1 The 22 metabolite markers best explaining the variance between the individually cultured *Mtb* samples in the absence (*Mtb*-Controls) and presence (*Mtb*-CMS) of colistin methanesulfonate.

Metabolite name (Chemspider ID)	<i>Mtb</i> controls: Average concentrati on (mg/g cells)	<i>Mtb</i> controls: standard error of the mean	<i>Mtb</i> treated with CMS: Average concentrati on (mg/g cells)	<i>Mtb</i> treated with CMS: standard error of the mean	PCA (Power)	PLS- DA (VIP)	Effect sizes (d-value)	t-test (P-value)	Fold Chan ge (log2)
Tetramethylhexadecanol (92535)	0,005	0,001	0,011	0,003	0,663	1,087	2,095	0,003	1.14
Methyladipic Acid (5367266)	0,159	0,024	0,204	0,042	0,984	1,592	1,063	<0,001	0.36
Arabitol (84971)	0,554	0,133	0,328	0,062	0,989	1,603	1,703	<0,001	-0.59
Diethylene glycol (DEG) (13835180)	0,072	0,020	0,036	0,008	0,925	1,111	1,870	0,002	-1.00
Dotriacontane (10542)	0,070	0,013	0,053	0,013	0,722	1,147	1,279	0,001	-0.40
Erythrose (84990)	0,166	0,030	0,731	0,289	0,806	1,277	1,955	<0,001	2.14
Glycerol (733)	0.007	0.006	0.023	0.014	0,860	1,034	1,852	0,005	1.72
Hentriacontane (11904)	0,605	0,202	1,007	0,483	0,980	1,574	0,834	<0,001	0.74
Methyldodecanoic acid (92948)	0,045	0,011	0,097	0,010	0,987	1,582	4,830	<0,001	1.11
Methylmaleic acid (553689)	0,078	0,026	0,033	0,011	0,926	1,322	1,731	<0,001	-1.24
Methylglutaric acid (11549)	0,071	0,022	0,043	0,007	0,844	1,184	1,287	0,001	-0.72
Methyltetradecanoic acid (90098)	0,317	0,021	0,840	0,302	0,799	1,010	1,731	0,007	1.41
Monopalmitin (110006)	0,165	0,058	0,220	0,063	0,935	1,472	0,873	<0,001	0.42
Octacosane (11902)	0,046	0,017	0,087	0,035	0,921	1,318	1,201	<0,001	0.92
Octadecane (11145)	0,020	0,011	0,077	0,024	0,982	1,571	2,364	0,000	1.95
Octadecenoic acid (393217)	<0,001	<0,001	0,010	0,009	0,925	1,467	1,125	<0,001	3.32
Octanoic acid (370)	0,204	0,192	0,019	0,013	0,903	1,273	0,969	<0,001	-3.43
Oxalate (946)	0,027	0,008	0,113	0,020	0,986	1,572	4,247	<0,001	2.07
Pentanediol (133167)	0,014	0,013	0,028	0,008	0,965	1,580	1,033	<0,001	1.00
Tetradecanol (10714572)	0,016	0,003	0,047	0,013	0,991	1,482	2,380	<0,001	1.56
Threonine (6051)	<0,001	<0,001	0,007	0,008	0,859	1,229	0,891	<0,001	2.81
Valine (6050)	<0,001	<0,001	0,033	0,041	0,872	1,272	0,822	<0,001	5.04

The total amount of variance explained by the first two principal components (PCs) (R2X cum) was 55.9 %, of which PC1 accounted for 43.4 %, and PC2 accounted for 12.5 %. Subsequently, by compliance with all of the following criteria: a PCA modelling power > 0.5 (Buydens, et al., 2009), a PLS-DA VIP value > 1 (Cho et al., 2008), a t-test P-value < 0.05 and an effect size > 0.5 (Smith, 2004), the metabolites that contributed most to this differentiation were selected (Figure 4-2) and listed in Table 4:1. These metabolite markers were mapped on a metabolic chart as indicated in

Figure 4-3 and discussed below. As indicated, the metabolomics investigation of the cultured *Mtb* in the presence and absence of CMS, led to the identification of various significantly altered metabolite markers. Glucose uptake was increased in the CMS treated *Mtb*, as the preferential energy source (as opposed to fatty acids which are now preferentially being used for cell wall repair) to fuel an upregulated glyoxylate cycle, and substrate further cell wall fatty acid synthesis via the glycerolipid metabolic pathway. However, the CMS treated *Mtb*, also showed comparatively elevated metabolites associated with pentose phosphate, valine, threonine, and pentanediol metabolism. These results confirm that CMS disrupts the *Mtb* cell membrane, and that these bacteria attempt to compensate for this via upregulation of various metabolic pathways related to cell wall repair.

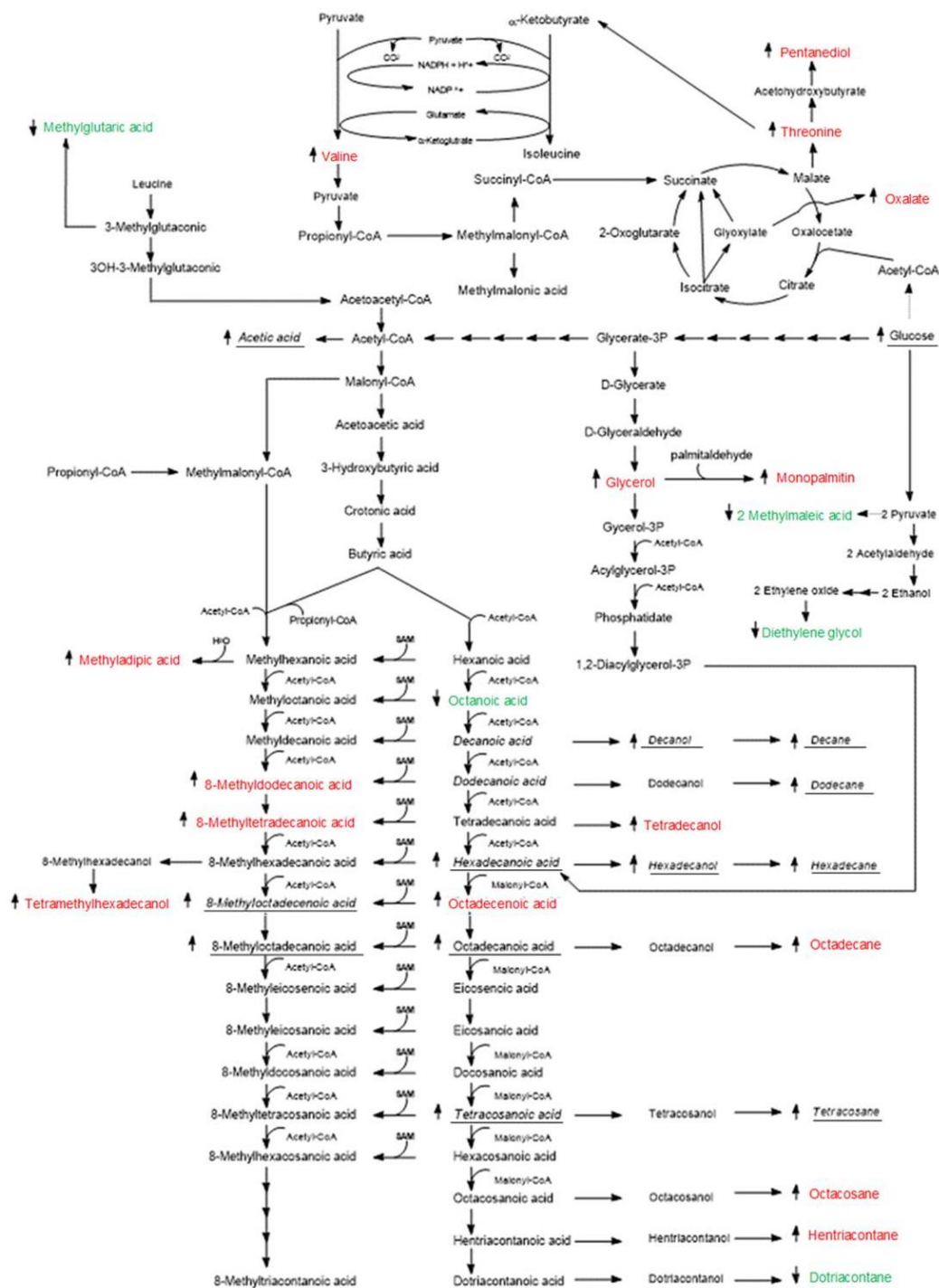


Figure 4-3 Metabolite markers best describing the variation in the metabolome of the CMS treated *Mtb* compared to that of *Mtb* cultured without CMS, are schematically represented in bold and those metabolites which were not necessarily significantly elevated using the statistical procedure selected, but still showed significance via considering their P-values, indicated in italics. Elevated and reduced concentrations of each metabolite marker indicated by either \uparrow or \downarrow respectively.

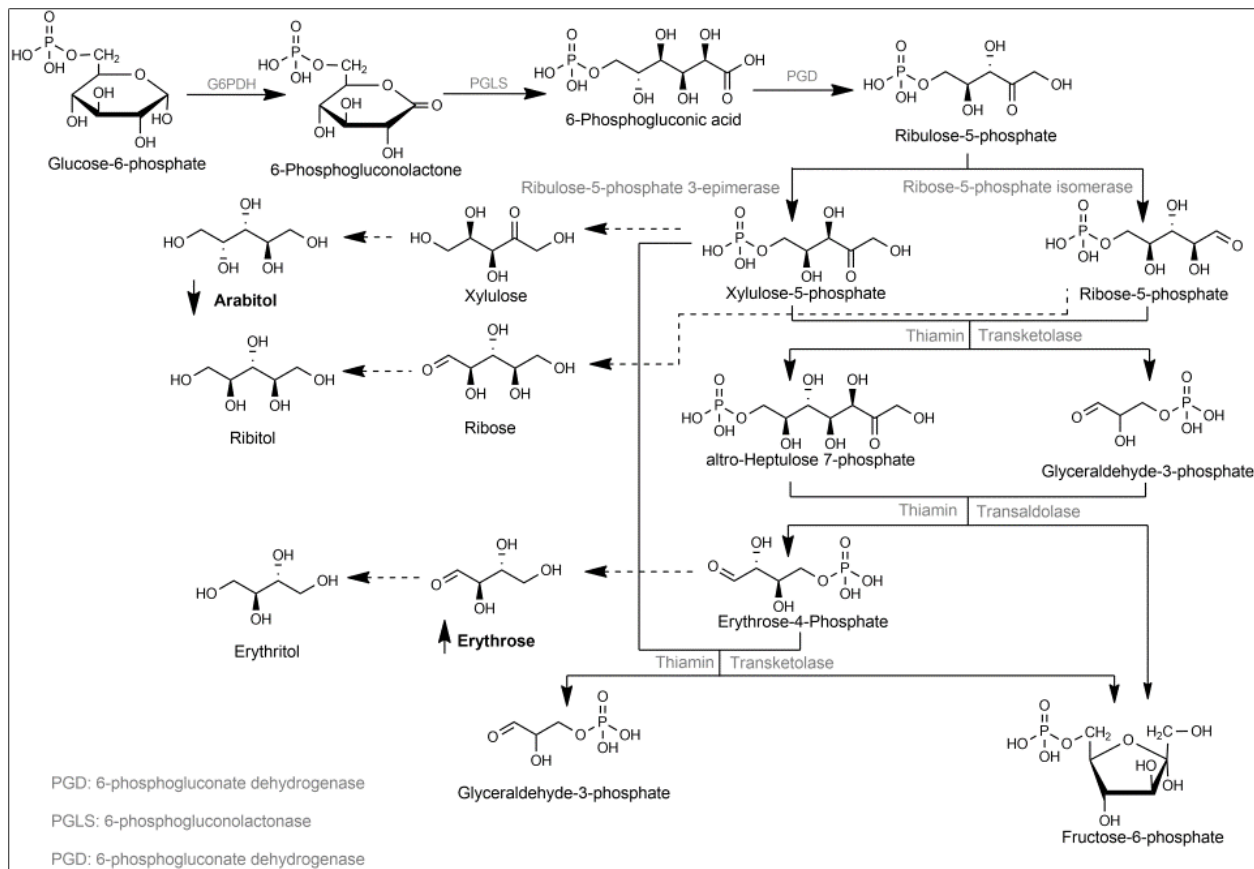


Figure 4-4 Pentose phosphate pathway indicating an elevated flux in the CMS treated *Mtb* towards glyceraldehyde-3-phosphate and fructose-6-phosphate, via the elevated erythrose and reduced arabinose concentrations.

4.4 Concluding remarks

The most significant metabolite markers identified in this investigation, were the elevated fatty acids indicating a shift towards fatty acid synthesis and cell wall repair in the CMS treated *Mtb*. This is accompanied by an increase in glucose utilisation for energy and an additional flux towards the upregulation of the glyoxylate cycle (a precursor for cell wall fatty acids via the glycerolipid metabolic pathway), similarly to what was previously seen when treating *Mtb* with CS. Further confirmation of this shift of glucose as an energy source, and unique to this investigation is the utilization of the pentose phosphate, valine, threonine, and pentanediol pathways for this purpose. Considering this, it might be possible to use CMS with other first or second line anti-TB drugs (likely only hydrophilic ones). The feasibility, however, to treat both drug sensitive and MDR-TB using lower drug concentrations is subject to clinical trials as it is not known if CMS would be able to successfully target intracellular *Mtb*.

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Chapter 5: Discussion and conclusion

5.1 Introduction

Due to the extensive discussion of the results in each of the previous chapters, Chapter 5 will summarize the most significant findings. The main objectives included: 1) a methodology was developed to optimize the metabolomic profile of colistin-treated *Mtb*; 2) the successful use of this developed methodology towards the identification of metabolite markers, which elucidated CS mechanism of action; 3) the successful application of the developed methodology towards identifying metabolite markers for the purpose of better characterization of CMS mechanism of action. Additional future recommendations emanating from this research investigation will also be discussed.

5.2 Summary of the main findings and future recommendations

Despite the vast amounts of research done to date aimed at the eradication of TB, TB is still considered a worldwide pandemic. This is ascribed to treatment failure, poor patient adherence, lack of new anti-TB drugs, and long treatment duration. Considering this, research into new anti-TB drug candidates is needed, and colistin could serve as a possible treatment option. Metabolomics, the latest additions to the “omics” revolution, allows for the better understanding of not only disease, but also treatment mechanisms, shedding light on the identification of new metabolite markers elucidating significant drug mechanistic pathways. Using a metabolomics research approach, CS and CMS- treated *Mtb* samples were analysed, to identify unique metabolites, characterizing the drug mechanisms.

Before any data was recorded, a repeatability study of the apparatus and the extraction methodology was conducted, in order to validate the generated data for use in the specific metabolomics applications of this study. Thus, for a repeatable and reliable dataset, several statistical approaches are incorporated to ensure the quality of the metabolomics biomarker identification. A metabolomics comparison of cultured *Mtb* in the presence and absence of CS and CMS respectively, led to the identification of various significantly altered metabolite markers. In both the CS and CMS-treated *Mtb*

samples, a metabolic flux towards fatty acid synthesis and cell wall repair was identified. Furthermore, glucose uptake was increased in the treated *Mtb*, indicating this to be the preferential energy source (as opposed to fatty acids which are now preferentially being used for cell wall repair) to fuel an upregulated glyoxylate cycle, and additionally as a precursor for further cell wall fatty acid synthesis via the glycerolipid metabolic pathway. However, CMS also uniquely induced the utilization of the pentose phosphate, valine, threonine, and pentanediol metabolic pathways for energy generation in the treated *Mtb*. These results indicate that both CS and CMS act via disruption of the *Mtb* cell membrane, and that these bacteria attempt to compensate for this via various metabolic pathways related to cell wall repair. Considering this, it might be possible to use CMS and/or CS in combination with other first or second line anti-TB drugs, to successfully treat both drug-susceptible and MDR-TB, using lower drug concentrations, and subsequently cause fewer side effects to the treated patient, since colistin would be expected to disrupt the *Mtb* membranes, making them more susceptible to the other first line anti-TB drugs. Using a metabolomics approach, future studies can be undertaken investigating colistin's performance in synergy with current first or second line anti-TB drugs, for treatment of both drug-susceptible and MDR-TB. Furthermore, the *Mtb* cell wall repair mechanisms identified in this investigation can also potentially be targeted using novel drugs in order to increase colistin's anti-TB drug effects.

This investigation proves the capacity of a metabolomics research approach, towards better drug mechanism characterization. In conclusion, metabolomics is expected to contribute significantly to a new era in drug development and disease research, based on new metabolite marker identification.

Appendix 1

Advances in
PROTEIN CHEMISTRY
and **STRUCTURAL**
BIOLOGY

Personalized Medicine



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VOLUME ONE HUNDRED AND TWO

ADVANCES IN
**PROTEIN CHEMISTRY AND
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VOLUME ONE HUNDRED AND TWO

ADVANCES IN PROTEIN CHEMISTRY AND STRUCTURAL BIOLOGY

Personalized Medicine

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CONTENTS

<i>Contributors</i>	<i>ix</i>
<i>Preface</i>	<i>xi</i>
1. High-Performance Affinity Chromatography: Applications in Drug-Protein Binding Studies and Personalized Medicine	1
Zhao Li, Sandya R. Beeram, Cong Bi, D. Suresh, Xiwei Zheng, and David S. Hage	
1. Introduction	2
2. Frontal Analysis Studies of Drug-Protein Interactions	10
3. Zonal Elution Studies of Drug-Protein Interactions	17
4. Other Methods for Examining Drug-Protein Interactions	22
5. Conclusion	30
Acknowledgments	31
References	31
2. Role of Proteomics in the Development of Personalized Medicine	41
Kewal K. Jain	
1. Introduction	42
2. Protein Biomarkers	43
3. Protein Biochips	45
4. Role of Proteomics-Based Molecular Diagnostics in Personalized Medicine	48
5. Pharmacoproteomics	49
6. Concluding Remarks About Application of Proteomics for Personalized Medicine	50
References	51
3. Metabolomics and Personalized Medicine	53
Nadia Koen, Ilse Du Preez, and Du Toit Loots	
1. Introduction	54
2. The Value of "Omics" Technologies in the Development of Personalized Medicine	55
3. What is Metabolomics?	57
4. The Application of Metabolomics Toward Personalized Medicine	61
5. Concluding Remarks	73
Acknowledgments	74
References	74
	v

4. Clinical Perspectives on Targeting Therapies for Personalized Medicine	79
Donald R.J. Singer and Zoulikha M. Zaïr	
1. Introduction	80
2. What Are Personalized Medicines?	81
3. Rare Diseases	81
4. Evidence for Precision Medicines from Real World Data	82
5. Using "Real World" Data	82
6. Ethical Concerns	83
7. Adaptive Trial Design	84
8. Companion Diagnostics	85
9. Biological Treatments	86
10. Case Studies: Targets for Precision Medicines and Companion Diagnostics	88
11. Network Pharmacology	101
12. Future Developments	103
Acknowledgments	104
References	104
5. Personalized Medicine in Respiratory Disease: Role of Proteomics	115
V.S. Priyadharshini and Luis M. Teran	
1. Introduction	116
2. Personalized Medicine	117
3. Proteomics	118
4. Respiratory Proteomics	120
5. Asthma	121
6. Chronic Obstructive Pulmonary Disease	124
7. Idiopathic Pulmonary Fibrosis	126
8. Aspirin-Exacerbated Respiratory Disease	128
9. Cystic Fibrosis	129
10. Lung Cancer	134
11. Conclusions	138
Acknowledgments	138
References	138
6. Computational Approaches to Accelerating Novel Medicine and Better Patient Care from Bedside to Benchtop	147
Theodore Sakellaropoulos, Junguk Hur, Ioannis N. Melas, Ellen Y. Guo, Leonidas Alexopoulos, Mohammad Bohlooly, and Jane P.F. Bai	
1. Introduction	148
2. Currently Approved Products Targeting Protein Kinases in Oncology	150

3. Tumor Heterogeneity	154
4. Computational Methods for Identifying Driver Mutations	157
5. Literature Mining in Oncology Research	163
6. Precise Genome Editing	166
7. Conclusion	169
Supplementary Material	170
References	170
7. Molecular Dynamics: New Frontier in Personalized Medicine	181
P. Sneha and C. George Priya Doss	
1. Introduction	182
2. Single Nucleotide Polymorphisms in Drug Response	186
3. Drug Discovery	187
4. Advent of Personalized Medicine	188
5. Evolution of Molecular dynamics in the Field of Macromolecule and Binding Interaction Analysis	189
6. Molecular Dynamics—A Boon in Personalized Medicine	191
7. MD Trajectories Analysis	198
8. Application of MD in SNP Analysis Toward Drug Discovery	205
9. Plausible Ways to Overcome the Disadvantages of Molecular Dynamics	207
10. Conclusion	211
Acknowledgments	212
References	212
8. Personalized Pharmacoperones for Lysosomal Storage Disorder: Approach for Next-Generation Treatment	225
S.A. Syed Haneef and C. George Priya Doss	
1. Introduction	226
2. LSDs Classification	228
3. Treatment of LSDs	242
4. Pharmacological Chaperones Versus Chemical Chaperones	250
5. Combination Therapy for LSDs	250
6. How Can PCT Personalized?	251
7. Computational Studies in PCT	253
8. Advantages and Disadvantages of Various Therapies in LSDs	254
9. Future of PCTs	255
10. Conclusion	256
Acknowledgments	256
References	256

9. Investigating the Inhibitory Effect of Wortmannin in the Hotspot Mutation at Codon 1047 of PIK3CA Kinase Domain: A Molecular Docking and Molecular Dynamics Approach	267
D. Thirumal Kumar and C. George Priya Doss	
1. Introduction	268
2. Materials and Methods	271
3. Results	273
4. Discussion	282
5. Conclusion	291
Acknowledgments	291
References	291
10. Role of von Willebrand Factor—A1 Domain Variants P1266L, H1268D, C1272R, and C1272F in VWD: A Molecular Modeling and Simulation Analysis Approach	299
C. George Priya Doss and Shabana Kouser Ali	
1. Introduction	300
2. Material and Methods	305
3. Results	308
4. Discussion	322
5. Conclusion	324
Acknowledgment	325
References	325
<i>Author Index</i>	331
<i>Subject Index</i>	373

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PREFACE

Personalized medicine encompasses the use of biological information for each patient in order to provide customized health care tailored to the individual patient. Such an individualized approach for medical decisions, practices, and treatment became clearly necessary due to a number of observations. For instance, the course of disease differs from one person to another. The effects of drugs used for treatment of a disease also vary largely between different patients. It is now well established that individual differences between patients, such as genetic polymorphisms, have significant effect on the onset of diseases as well as on absorption of drugs and their metabolism in patient's body. Personalized medicine takes such variations into account and tries to take advantage of them. It is believed that such customized therapies will improve patients' response rates to the treatment while reducing significantly any adverse effects the drugs might have.

Personalized medicine is based on the dynamics of systems biology and uses predictive tools to evaluate health risks and to design personalized health plans to help patients to minimize risks, prevent disease, and to treat it with precision when it occurs. Some of the most contemporary and very promising tools employed in personalized treatment of patients are discussed in the first three chapters of this volume. First chapter discusses the general principles of high-performance affinity chromatography and the various approaches that have been used in this technique to examine drug-protein binding and in work related to personalized medicine. This technique is a great asset to personalized medicine because the binding of drugs with proteins and other agents in serum can affect the dosage and action of drugs. The extent of this binding may also vary with a given disease state. Second article in this thematic volume focuses on the advances in proteomic technologies that have made important contribution to the development of personalized medicine by facilitating detection of protein biomarkers, proteomics-based molecular diagnostics as well as protein biochips and pharmacoproteomics. Application of nanobiotechnology in proteomics, nanoproteomics, has further enhanced applications in personalized medicine. Proteomics has already proved to be a good bridge between diagnostics and design of therapeutics. The integration of last two processes shows to be of a great importance for advancing personalized medicine. The third chapter in this volume reviews in detail metabolomics as a tool used in personalized medicine.

Metabolomics is the newest addition to the “omics” domain and the closest to the observed phenotype. It reflects changes occurring at all molecular levels, as well as influences resulting from other internal and external factors. By comparing the metabolite profiles of two or more disease phenotypes, metabolomics can be applied to identify biomarkers related to the perturbation being investigated. These biomarkers can, in turn, be used to develop personalized prognostic, diagnostic, and treatment approaches, and can also be applied to the monitoring of disease progression, treatment efficacy, predisposition to drug-related side effects, and potential relapse.

The fourth chapter discusses the importance of a range of new approaches to developing new and reprofiled medicines to treat common and serious diseases, and rare diseases: new network pharmacology approaches, adaptive trial designs with enriched populations more likely to respond safely to treatment, as assessed by companion diagnostics for response and toxicity risk and use of “real-world” data. Case studies are described of single and multiple protein–drug targets in several important therapeutic areas. These case studies also illustrate the value and complexity in use of selective biomarkers of clinical response and risk of adverse drug effects, either singly or in combination.

Chapters 5 and 6 in this volume give in-depth analyses of applying the tools of personalized medicine in some of the most common diseases. The fifth article in this volume highlights the contributions of proteomics toward the understanding of personalized medicine in respiratory disease and its potential applications in the clinic. The sixth chapter is focused on the challenges of treating different cancer types, which behave like moving targets due to mutation and evolution, and the current state-of-the-art research in this area. A special emphasis is made on the computational approaches to accelerating novel medicine and better personalized patient care from bedside to benchtop.

Chapter 7 in this volume focuses on high-end computational methods, such as molecular dynamics (MD) simulation that has proved to be a constitutive approach for detecting the minor changes associated with single nucleotide polymorphisms (SNPs) in nucleic acids for better understanding of their role in protein structural and functional alterations. MD along with docking analysis can reveal the synergetic effect of an SNP in protein–ligand interaction and provides a foundation for designing a particular drug molecule for an individual. This compelling application of computational power and the advent of other technologies have paved a promising way toward personalized medicine. In the Eighth article of this thematic volume,

authors discuss the available clinical strategies and different methods how pharmacological chaperones can be personalized and used as a next-generation approach to address different lysosomal storage disorders.

The final two chapters in this volume exemplify the applicability of molecular modeling and simulation approaches in personalized medicine by exploring the inhibitory activity of Wortmannin toward oncogenic mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) (Chapter 9), and the importance of mutations in A1-domain of von Willebrand factor (VWD) gene for the structural and functional alterations related to thrombosis, compared to the native VWD protein (Chapter 10).

The aim of this volume is to promote further research and development in the design of new personalized therapeutics and treatments using biological information for each patient via translation of recent genomic, genetic, proteomics, and metabolomics advances into clinical context.

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Metabolomics and Personalized Medicine

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Contents

1. Introduction	54
2. The Value of "Omics" Technologies in the Development of Personalized Medicine	55
3. What is Metabolomics?	57
3.1 Analytical Methods Most Often Used for Metabolomics	58
3.2 Statistical Approaches	59
4. The Application of Metabolomics Toward Personalized Medicine	61
4.1 Identification of Disease Biomarkers for Metabolomics-Based Diagnostics	62
4.2 Pharmacometabonomics	66
5. Concluding Remarks	73
Acknowledgments	74
References	74

Abstract

Current clinical practice strongly relies on the prognosis, diagnosis, and treatment of diseases using methods determined and averaged for the specific diseased cohort/population. Although this approach complies positively with most patients, misdiagnosis, treatment failure, relapse, and adverse drug effects are common occurrences in many individuals, which subsequently hamper the control and eradication of a number of diseases. These incidences can be explained by individual variation in the genome, transcriptome, proteome, and metabolome of a patient. Various "omics" approaches have investigated the influence of these factors on a molecular level, with the intention of developing personalized approaches to disease diagnosis and treatment. Metabolomics, the newest addition to the "omics" domain and the closest to the observed phenotype, reflects changes occurring at all molecular levels, as well as influences resulting from other internal and external factors. By comparing the metabolite profiles of two or more disease phenotypes, metabolomics can be applied to identify biomarkers related to the perturbation being investigated. These biomarkers can, in turn, be used to develop personalized prognostic, diagnostic, and treatment

¹ All authors contributed equally to the writing of this manuscript.

approaches, and can also be applied to the monitoring of disease progression, treatment efficacy, predisposition to drug-related side effects, and potential relapse. In this review, we discuss the contributions that metabolomics has made, and can potentially still make, towards the field of personalized medicine.



1. INTRODUCTION

Conventional disease diagnostics generally entails a physician identifying a disease or abnormality on the basis of a physical examination of the symptomatic patient, with (or without) the additional use of standard diagnostic tests. A positive diagnosis is normally followed by treatment using drugs produced on a large scale and administered at a standardized and universally accepted dosage. These conventional drugs are developed to treat general symptoms or the disease as determined by the mean results obtained over large population groups (Debas, Laxminarayan, & Straus, 2006). However, it is crucial to understand that due to, for example, genetics and a variety of other factors such as individual diet, habits (e.g., smoking), gender etc., not all diseases affect all individuals in the same manner (Jirtle & Skinner, 2007), and neither do all individuals respond to treatment in the same way (Dworkina, McDermottb, Farrarc, O'Connord, & Senn, 2014). This occurrence is clearly reflected by the increasing incidence of treatment failure and relapse, which is especially disturbing when considering their prevalence in life-threatening diseases such as tuberculosis (TB) and acquired immune deficiency syndrome (AIDS).

Although this variation between individuals might not be obvious in the initial clinical presentation of the disease, it is most likely still detectable on a molecular scale. Several researchers groups have subsequently shifted their focus to the development of personalized medicine, which uses the molecular information of an individual, as dictated by his or her genome, transcriptome, proteome, and metabolome (Redekop & Mladsi, 2013) to develop patient-specific diagnostics and drugs. This information can also be used to determine/predict individual treatment response, prior to and during the treatment regimen, in an attempt to lower the incidence of treatment failure or relapse (Salari, 2009), and also to optimize drug dosages, in an attempt to prevent or lessen the severity of the drug-related side effects (Lecea & Rossbach, 2012).

Personalized medicine has undoubtedly been one of the most topical issues over the past decade and several research fields have joined hands

in using this as a tool for optimizing disease management, as it has the potential to transform clinical practice and treatment efficacy.



2. THE VALUE OF “OMICS” TECHNOLOGIES IN THE DEVELOPMENT OF PERSONALIZED MEDICINE

Traditionally, genomics was considered the most important approach for determining individual variation and the development of personalized medicine (Jain, 2009). However, several intermediate processes occur between the genotype and disease phenotype in the “omics” cascade, which may influence disease outcome or treatment response, and includes transcription, translation, and metabolism. Furthermore, various other factors, such as environmental influences and age, may also play a role in the disease phenotype, a phenomenon which genotyping is not able to characterize or explain. The development of personalized medicine requires a holistic view of all molecular variation that may differentiate individuals, and researchers are therefore shifting their focus from using exclusively genetics methodologies for instance, to a systematic/integrative “omics” approach. “Omics” is a general term used to describe the study of all genes (genomics), transcription of these genes (transcriptomics), translation into their respective proteins (proteomics), and all the resulting metabolite changes (metabolomics), and is aimed at acquiring large-scale data sets from a single and/or multiple samples (Wheelock et al., 2013). These “omics” research fields, alone or in combination, have shown to be valuable for the identification of new disease biomarkers for the purpose of elucidating disease mechanisms and the development of personalized diagnostic and treatment regimes. The successful completion of the human genome project was considered a major breakthrough in science, especially for those involved in genomics and personalized medicine, as any variation in a person’s genome at a specific locus, could potentially have a direct or indirect effect on gene expression, thereby influencing disease susceptibility (Ginsburg & Willard, 2009). Genomics has to date identified various genetic risk factors, for a number of chronic diseases, including heart disease, diabetes, and several form of cancers (Ginsburg & Willard, 2009). For example, it is now well known that women with mutations in BRCA1 or BRCA2 are at risk for developing breast and ovarian cancer (Schwartz, Hughes, & Lynch, 2008), and similarly, individuals with a mutation in MLH1 and MSH2 are at risk for developing colon cancer (Wiesner, Slavin, & Barnholtz-Sloan, 2009). In a similar fashion, genomics can be used to predict

drug response, by identifying the effect of drugs on gene expression, as well as the effect of various genes on the variable response of the patient to a drug (Ginsburg & Willard, 2009). One example of this, are the two genes, CYP2C9 and VKORC1, identified using genomics methodologies, which are used to determine/predict the effective maintenance/chronic treatment dose of warfarin, a widely used anticoagulant. CYP2C9 has been shown to be responsible for the metabolic clearance of the *S*-enantiomer of the drug and various genotypic alterations have been associated with a 10-fold variation in *S*-warfarin clearance. It has additionally been estimated that the genotypic variation of CYP2C9, together with that of VKORC1 (which is targeted by warfarin), and other factors including body weight and age, accounts for 35–60% of the variation seen in individual drug response to warfarin (Voora, McLeod, Eby, & Gage, 2005). An additional example of the use of genomics for the purpose of personalized treatment approaches was the development of a blood-based test for the prediction of graft-rejection (Deng, Eisen, & Mehra, 2006). These DNA assessments or “stable” genomics approaches can also be done at birth, to predict a predisposition to a disease or treatment. The other “omics” approaches described above, however, detect “dynamic” disease markers also, which have the capacity to show individual variation due to other factors including diet, individual habits, comedication, coinfection with other pathogens, etc. These biomarkers can therefore be used to characterize diseases and drug action on an even more detailed individualized level (Ginsburg & Willard, 2009).

Several biomarkers identified using transcriptomics approaches have also been validated and used clinically for disease diagnosis and risk assessment. One example of this is the DNA microarray-based diagnostic kit, MammaPrint[®], which can be used for breast cancer prognosis and measures the degree of transcription of 70 specified genes (Hong et al., 2013). OncotypeDX[®] is another example of such an assay, which measures the expression of 21 genes for the prediction of recurrences, death, and response to therapy, in patients with estrogen-positive breast cancer (Hong et al., 2013). Heidecker et al. (2008) additionally investigated the potential use of transcriptomics to identify prognostic biomarkers for heart failure, as several genes were identified to be overexpressed in patients with a good versus a bad prognosis, and when the predictive value of these biomarkers were tested, a 74% sensitivity and 90% specificity were obtained.

In addition to the transcriptome, posttranslational modification of proteins has also been shown to play a major role in various biological processes and disease states. Using proteomics, Ono et al. (2006) identified a

posttranslational modification of alpha-fibrinogen (4-hydroxylated alpha-fibrinogen) in pancreatic cancer patient plasma and subsequently constructed an antibody and validated the assay on the basis of this for the better diagnosis of the disease. The same group also used proteomics to investigate the life-threatening side effects related to gemcitabine monotherapy, a standard treatment used for pancreatic cancer, and subsequently identified haptoglobin as a biomarker for the prediction of this drug's hematological toxicities.

These studies show the value of genomics, transcriptomics, and proteomics, in all fields of personalized medicine. The contribution of metabolomics, the most recent addition to the “omics” revolution, will subsequently be discussed in detail in the remainder of this review.



3. WHAT IS METABOLOMICS?

As per definition, metabolomics is the nonbiased identification and characterization of “all” the small molecular compounds (metabolites) in a biological system, using highly sensitive analytical techniques, in combination with bioinformatics (Dunn, Bailey, & Johnson, 2005). The metabolome, which is a collective term for all the metabolites in a specific biological system/sample, is the ultimate downstream result of genes, transcription, and translation, and will therefore reflect changes to the genome, transcriptome, and proteome, in addition to that caused by a disease state or other environmental factors. The identification of the main differences between the metabolomes of two sample cohorts (disease vs. healthy controls, for example) is a starting point for the discovery of new metabolite biomarkers. Additionally, a comparison of various cohorts with individuals showing variation to disease or response to treatment can also be done in order to identify markers associated with this type of variation, in the context of individualized medicine.

The extraction and analysis of metabolites from a sample or sample group can be done in an untargeted or semitargeted manner. Untargeted metabolomics aims to extract and detect all metabolites (known and unknown, from all metabolite classes), i.e., the total metabolome, as per definition of metabolomics. Semitargeted metabolomics approaches, however, are focused on the analysis of specific fractions of the metabolome or a subclass of metabolites, such as only the lipids or organic acids, for instance. Sample preparation methods for untargeted metabolome analyses are simple, and the generated metabolite profiles can provide researchers with a good general

picture of the effect of the investigated perturbation on the overall metabolome. However, these methods tend to have a lower sensitivity and detection limit when compared to that of the semitargeted approach, which provides simpler metabolite profiles, representing specific metabolic pathways (Wishart, 2010). The choice of the sample preparation method will also depend on the analytical apparatus selected and whether an untargeted or semitargeted approach is required. When using nuclear magnetic resonance (NMR) spectrometry for instance, chemicals such as ethanol and hexane should be avoided, as these solvents are also deuterated and will therefore result in multiple resonances and subsequently interference (Dunn et al., 2005). Currently, there is no single analytical apparatus available with the capacity to identify all the metabolites extracted from a sample, and therefore, when doing untargeted metabolomics, a combination of a number of different analytical approaches is recommended, however, this may not always be a viable option in a particular laboratory as it is dependent on instrument availability. In instances with limited analytical capacity, a lot can still be done in the context of untargeted metabolomics, for instance, derivatization of a sample prior to gas chromatography–mass spectrometry (GC–MS) analysis, in addition to appropriate column selection, can serve well in the detection of a large portion of the metabolome during a single analytical run. Each analytical technique comes with its own set of molecular preferences, advantages, and limitations, as will subsequently be discussed below.

3.1 Analytical Methods Most Often Used for Metabolomics

The most commonly used analytical approaches for metabolome data acquisition include the use of various chromatographic techniques, most commonly gas chromatography (GC), or liquid chromatography (LC), coupled to various different options of mass spectrometry (MS) detectors, and NMR. Without prior separation or derivatization, LC–MS is considered to be the apparatus with the potential to detect the largest variety of metabolites present in a specific sample. However, the derivatization of sample extracts makes GC–MS an even, if not better contender, considering the availability of spectral data for GC–MS compound identification. Furthermore, although LC–MS is ideal for the analysis of polar and ionic compounds, it has a lower chromatographic resolution and higher running costs in comparison. Additionally, a great advancement in GC–MS technology was the development of the GCxGC system, which separates metabolites in two dimensions, on the basis of not only volatility but also polarity,

thereby reducing the amount of coeluting peaks and enhancing the resolution of the eluting metabolites (Marriott & Shellie, 2002). GC–MS analysis also requires smaller sample volumes when compared to that required for LC–MS and NMR, but because these samples undergo metabolite separation and derivatization, they are nonrecoverable after GC analysis. Another downside to GC is the rather long analysis times required for compound separation, and the identification of “unknown” metabolites (those compounds detected with mass fragment patterns not in the commercial libraries) is rather complex.

NMR spectroscopy is based on the principle of detecting metabolites according to the signals produced by their proton content, allowing for straightforward metabolite identification (Bonhommea, Gervaisa, & Laurencin, 2014). This relatively fast method (2–3 min per sample), is mostly used for the detection of polar metabolites and is nondestructive to the sample. NMR instrumentation is, however, rather expensive, requires large sample volumes and has a lower sensitivity when compared to other techniques (Dunn et al., 2005; Halket et al., 2005).

Subsequent to sample analysis, one of the most important steps for generating data, which can be used for metabolomics, is the extraction of reliable data matrixes from the complex chromatographic and mass spectrometric outputs, for subsequent statistical analyses and biomarker selection. This course of action includes peak detection, peak deconvolution, peak alignment, compound quantification, and identification, among various other steps. Most of the analytical methods described above come with their own software packages, specifically designed for this purpose (such as ChromaTOF for the Leco GCxGCTOFMS), whereas other universal software packages, such as MET-IDEA, are also freely available for use for processing data generated from a variety of different commercially available analytical techniques (Broeckling, Reddy, Duran, Zhao, & Sumner, 2006). However, because each of these packages comes with their own advantages and limitations, most researchers prefer to use a combination of software packages, in addition to manual inspection, in order to obtain the optimum data matrix for statistical data analysis and biomarker identification.

3.2 Statistical Approaches

The increasing complexity of the data matrixes obtained from the analytical equipment used in metabolomics studies has led to the use of various multivariate chemometric data analysis methods for biomarker identification/extraction from these data sets. In order to get an overview of the data,

certain unsupervised methods can be used to highlight trends in the data and grouping or differentiation of various sample sets, and to additionally identify potential outlier samples and batch effects. When employing these unsupervised methods, samples are not assigned to specific groups (for example, disease and control) prior to the statistical analysis, allowing the analyst to determine whether or not the samples are naturally differentiated or grouped based on their analyzed metabolite profiles. For this purpose, principle component analysis (PCA) is the method most commonly used. PCA reduces the dimension of the input data matrix by calculating a weighted sum (score) of the compound (metabolite) concentrations detected in each sample and expresses these in terms of principal components (PCs), with PC1 describing the most variation in the data, PC2 the next highest variation, etc. These PCs subsequently serve as coordinates on a scatter plot and provide an overview of the samples and how they relate to each other on the basis of their analyzed metabolomes. Other chemometric methods, such as self-organizing maps, hidden Markov models, and canonical correlation, can also be implemented in this initial, exploratory stage for the same purpose (Madsen, Lundstedt, & Trygg, 2010; Trygg, Holmes, & Lundstedt, 2007). If those samples, belonging to a specific group, do in fact assemble and group together, supervised methods where individual samples are allocated to their respective sample groups before the analyses, can be applied for the purpose of identifying potential biomarkers best describing the variation detected. Partial least squares discriminant analysis (PLS-DA) is one such method, which uses group membership information to build a discrimination model. The variable influence on the projection (VIP) parameter, which is a weighted sum of the squares of the PLS-DA weights, gives an indication of the importance of the metabolite to the prediction model, and can therefore be used to identify those metabolites which are most characteristic of a specific sample group, or those metabolites which vary the most between the specified groups. The metabolites with the highest VIP scores are then ranked and can be used to identify potential biomarkers. Similar supervised classification models also used for biomarker identification, include, but are not limited to, soft independent modeling of class analogy and support vector machines. The technical details of these chemometric methods fall beyond the scope of this review, but the authors suggest the review by Trygg et al. (2007) for a more detailed description of these.

Various software packages and Web-servers, such as MetaboAnalyst (Xia, Psychogios, Young, & Wishart, 2009), have been developed

specifically for researchers with limited statistical knowledge, to perform these essential chemometric analysis on metabolomics data. Although these tools are helpful, most metabolomics research groups still prefer to use qualified biostatisticians, with knowledge of the underlying mathematical programming, for mining the relevant biomarkers from these complex data sets. In these instances, more traditional statistical packages such as Statistica and “R” are used for the analysis of the data in the context of the specific biological question. Identified biomarkers can subsequently be used to explain individual variation in disease and treatment response, by interpreting this as the context of known metabolic pathways, and/or prior genomic, proteomic and transcriptomic data. Furthermore, individual biomarkers or combinations thereof (biosignatures) can be used for diagnostic purposes, the latter of which can be achieved by building a prediction model, such as a classification tree. In the light of this, metabolomics is considered an important tool for personalized medicine.



4. THE APPLICATION OF METABOLOMICS TOWARD PERSONALIZED MEDICINE

The ongoing advancements in technology and subsequent availability of more sensitive analytical equipment for application to metabolomics studies, has made it possible to detect even subtle variations in the metabolomes of even healthy individuals. These variations are now known to be a consequence of a variety of factors, including environment (such as habits, lifestyle differences, and stress), age, genetics (mutations, epigenetics), gender and race ([Hernandez & Blazer, 2006](#)).

By implementing various different GC–MS and LC–MS metabolomics techniques, [Lawton et al. \(2008\)](#) investigated the influence of age, gender and race, on the plasma metabolite profiles of 269 healthy individuals. Of these three variables, age was considered to have the most significant influence on the metabolite concentrations of these individuals, and the identification of elevated oxidative stress markers and changes in protein, energy and lipid metabolism could strongly be associated with age. The proposed antiaging androgen, dehydroepiandrosterone-sulfate, additionally showed inverse correlations with age, whereas certain xenobiotics (e.g., caffeine) positively correlated, potentially indicating decreased activity of cytochrome P450. A significant interaction between age and gender was also identified and a subsequent positive correlation between age and urea concentrations was shown, which is more pronounced in females than in males. When

investigating the metabolome-specific gender-related differences, 35 metabolite markers were detected, indicative of an increased amino acid, energy and nucleotide metabolism, in the males comparatively. When investigating metabolome differences in race, caffeine, paraxanthine and theobromine were detected in far lower concentrations, and hydroxyproline, glycochenodeoxycholate and glycolate were detected in comparatively higher concentrations in African-Americans versus Caucasians. Although variations were also determined when comparing metabolite profiles obtained of Hispanics and African-Americans or Hispanics and Caucasians, the identity of these compounds could not be identified.

In a similar study, [Urban, Kavvadias, Riedela, Scherera, and Trickerb \(2006\)](#) investigated variations in the metabolite profiles of smokers versus nonsmokers using LC-MS/MS and GC-MS metabolomics. Various acrylamide (a neurotoxin known to be present in tobacco smoke) metabolites (*N*-acetyl-*S*-(2-carbamoyl-ethyl)-*L*-cysteine, glycidamide *N*-(*R/S*)-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-*L*-cysteine, and *N*-2-carbamoyl-ethylvaline) were detected in significantly higher concentrations in the urine and blood samples of the 60 smokers and strongly correlated to the number of cigarettes they smoked daily. These compounds are therefore considered valid biomarkers of acrylamide exposure.

From these and many other related studies, it is evident that metabolomics can be useful for identifying biomarkers explaining individual variation in the metabolome profiles of healthy individuals, related to various “natural” or “habitual” factors. The identification of the metabolite biomarkers related to the variation detected in various disease states and/or the xenobiotic treatment of these diseases, and/or the influence of various other environmental factors on this, can assist in explaining the underlying mechanisms associated with individual variation in disease diagnosis, disease susceptibility, treatment response, and drug-related side effects ([Lawton et al., 2008](#)). These findings will consequently contribute to the development of personalized medicine, a concept which not only involves personalized diagnosis and treatment but also personalized disease susceptibility assessment, health monitoring, and preventative medicine.

4.1 Identification of Disease Biomarkers for Metabolomics-Based Diagnostics

Various metabolomics studies have been performed for the purpose of discovering new diagnostic biomarkers for a variety of diseases. These biomarkers are identified as those metabolites with the highest intergroup

variation after comparing metabolome profiles obtained from healthy or nonspecific-diseased sick individuals with similar symptoms, to those obtained from the specific/investigated diseased patients. One such a study was done with the aim of identifying diagnostic breath biomarkers for pediatric asthma, using NMR metabolomics. By applying linear discriminant analysis and PLS-DA to the metabolite profiles obtained from exhaled breath condensate of 25 children with asthma and 11 healthy controls, [Carraro et al. \(2008\)](#) were able to differentiate between the two groups with a success rate of 86%, which is slightly better when compared to the conventionally used methods (81%). The metabolites contributing most to this differentiation were identified as oxidized and acetylated compounds, indicative of increased oxidative stress and inflammation in the airways of the lungs. Considering this, despite their superior diagnostic ability and capacity to explain disease mechanisms, these compounds are not necessarily specific to asthma, but are rather indicative of a general disease state. For this reason, biomarkers identified in diseases, using healthy subjects as controls, although for the purpose of describing diseased mechanisms, have serious limitations for diagnostic applications. Hence, in order to validate diagnostic markers, comparison to other diseased states with similar symptoms is a true test for potential application in a clinical diagnostic scenario.

A good example of this is a study done by [Sugimoto, Wong, Hirayama, Soga, and Tomita \(2010\)](#), who analyzed saliva metabolites obtained from three different groups of cancer patients (69 individuals with oral cancer, 18 with pancreatic cancer, and 30 with breast cancer), 11 periodontal disease patients, and 87 healthy controls, using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS). Different sets of metabolites were subsequently identified which could be used to differentiate each individual disease state from the healthy controls, with the area under the receiver operating characteristic curves (AUCs) of 0.865 for oral cancer, 0.973 for breast cancer, 0.993 for pancreatic cancer, and 0.969 for periodontal diseases. Although these diseases could not be differentiated from each other using PCA, three metabolites were identified specific for oral cancer (detected in concentrations significantly different to all other groups) and eight compounds specific to pancreatic cancer. No biomarkers could, however, be uniquely identified for breast cancer. The authors hypothesized that the reason for this may be due to the fact that breast cancer has various structurally different forms, dependant on the expression of hormone receptors, and they added that additional factors such as age and menopausal status can

additionally contribute to the diverse metabolite profiles detected in these patients. Considering this, when identifying diagnostic biomarkers for a particular disease, it is crucial to consider all the contributing factors, such as the various stages of the disease, characteristics of the pathogen (drug resistance, pathogen subspecies, active vs. latent infection), and individual variation due to genetic and environmental factors, which could have an influence on the detected metabolite profiles. Various metabolomics studies have investigated the influence of these and other factors with the aim of developing personalized diagnostic approaches.

In a semitargeted metabolomics study, [Zhang et al. \(2006\)](#) investigated the influence of different stages of hepatitis C virus (HCV)-induced liver fibrosis on plasma amino acid profiles. The disease progression, from early to advanced fibroses, correlated with a decrease in branched-chain amino acids and a simultaneous increase in aromatic amino acids. These amino acid profiles were subsequently used to develop a diagnostic classification model which was able to differentiate between early and advanced fibrosis ($\text{AUC} = 0.9 \pm 0.04$) and cirrhosis ($\text{AUC} = 0.99 \pm 0.01$). Similarly, biomarkers related to disease progression have also been identified for colorectal cancer ([Nishiumi et al., 2012](#)) and Parkinson's disease ([Bogdanov et al., 2008](#)), thereby proving that metabolomics not only has the capacity to identify markers specific to a disease but also to identify the specific disease stages. Such approaches can not only be used to identify biomarkers for use in less invasive diagnostic methods (for example, identifying markers in plasma, as opposed to performing a colonoscopy in colorectal cancer patients for instance) but also to assist clinicians in determining the most appropriate treatment protocol, with regards to the patient's individualized disease stage or severity.

When investigating the metabolome of patients with disease resulting from an infectious pathogen, the individual characteristics of the pathogen can also potentially add further interindividual variation to the presentation and treatment response of the patient. [Olivier and Loots \(2012\)](#) used GC-MS metabolomics to identify biomarkers classifying the various *Mycobacterium* species forming part of TB complex, which are known to cause infections with symptoms closely related to that caused by *Mycobacterium tuberculosis* in pulmonary TB patients, but respond differently to standard TB treatment. Based on the lipid metabolite profiles, they were able to differentiate between *M. tuberculosis*, *M. avium*, *M. bovis*, and *M. kansasii*, and they furthermore identified the 12 compounds which best describe the variation between these

disease causing *Mycobacterium* species. These potential biomarkers were then used to build a multivariate diagnostic model, which was able to correctly classify unknown samples with probabilities ranging from 72% to 100%, at an exceedingly better detection limit as compared to current speciation methods. By using the same research approach, this group was additionally able to differentiate various different drug-resistant strains from each other (Du Preez & Loots, 2012; Loots, 2014). In another example of such an application, Denery, Nunes, Hixon, Dickerson, and Janda (2010) used a LC-MS metabolomics approach to differentiate serum and plasma samples collected from patients with active onchocerciasis (*Onchocerca volvulus* positive) and controls (*O. volvulus* negative), and subsequently identified 14 compounds as biomarkers for this infection. They subsequently applied this biomarker set to samples collected from individuals living in onchocerciasis endemic areas, where ivermectin was given as a chronic medication, and were able to distinguish between those subjects with compromised worm viability due to treatment and those still with an active infection, and hence these markers could subsequently be used not only for disease diagnostics but also to determine disease severity and to predict treatment outcome or monitor individual treatment response. These studies subsequently show the capacity of metabolomics for identifying markers which not only accurately diagnose disease but also have the capacity to detect individual variation not necessarily evident from the clinical symptoms, such that an individualized treatment regimen can be given. These markers can additionally be used to monitor treatment response or predict treatment outcome (Redekop & Mladi, 2013).

Lastly, despite the actual metabolome changes induced by a disease or pathogen, individual variation due to genetic and environmental factors can also influence the identified diagnostic biomarkers, in a similar fashion to that previously described in healthy individuals. Szymanska et al. (2012), for example, showed that metabolic biomarkers used for the diagnosis of obesity are strongly influenced by gender. When investigating the plasma and serum metabolite profiles of healthy overweight men and women, central obesity, insulin, cholesterol, VLDL, and certain triacylglycerols, were identified as potential biomarkers for abdominal visceral (VAT) fat distribution in women, whereas in men, the identified VAT-associated biomarkers included different triacylglycerols as compared to women, phosphatidylcholine, and VLDL. These results highlight the fact that individual variation, such as gender, can be detected via metabolomics, and that these variations should be considered when identifying diagnostic biomarkers.

4.2 Pharmacometabonomics

Apart from the contribution that metabolomics has made to the early detection and characterization of a disease state in the context of personalized clinical care, it has also shown promise in the domain of pharmacokinetics, and subsequently to the new research domain termed pharmacometabonomics. This research field investigates the response of a patient to xenobiotics from a metabolism perspective (Baraldi et al., 2009). Xenobiotics include those substances foreign to the biological system, which may originate from the individual's environment, including diet and medication. When these xenobiotic substances are ingested, the first reaction of the body is to eliminate these compounds, by modifying their chemical characteristics by means of any number of enzymatic reactions (xenobiotic metabolism). This response also forms the basis for the activation of various medications, the products of which act to alleviate symptoms or treat the respective disease. Individual variation in xenobiotic metabolism is a concept known for quite some time now and plays an important role in individual response to treatment, and hence treatment outcome, relapse, and side effects (de Villiers & Loots, 2013). It has been shown that various innate factors including race, ethnicity, and gender, in addition to the previously mentioned environmental factors or social habits, such as diet, alcohol consumption, and smoking, can influence individual variation to xenobiotic metabolism and response (Matthews, 1995). Identifying these factors and determining their influence or correlations to individual treatment response, forms the basis of personalized disease treatment and will subsequently be discussed in the following sections, in the context of metabolomics.

4.2.1 Metabolomics and the Elucidation of Drug Mechanisms

Before treatment strategies can be tailored to an individual's unique response to therapy, it is important to understand the general xenobiotic metabolism and underlying mechanisms of the proposed drug. For this purpose, pharmacometabonomics can be applied in a number of ways: (1) the comparison of the metabolite profiles of a patient group receiving the vehicle/placebo versus those receiving the xenobiotic treatment, (2) the comparison of the metabolite profiles of patient groups receiving unlabeled xenobiotic treatment versus stable isotope-labeled xenobiotic treatment, and (3) the comparison of the metabolite profiles of wild-type and genetically modified animals receiving the investigated xenobiotic (Lan & Jia, 2010). These methodologies have been implemented to investigate the metabolism of

various nutrients, drugs, and other xenobiotics, using a variety of analytical equipment and bioinformatics strategies (Lan & Jia, 2010). In addition to drug-derived metabolites originating directly from xenobiotic metabolism, these drug exposure signatures will also include drug-induced alterations to normal metabolism, representing the patient's altered metabolic state in response to the treatment. In one such instance, Rozen et al. (2005) were able to differentiate between patients with motor neuron diseases (MND) and healthy controls, in addition to MND patients with and without Riluzole treatment, using the plasma metabolite profiles of these individuals. Two fatty acid-based compounds were uniquely associated with patients on Riluzole, and although these compounds could not be identified completely, they did not appear to be derivatives of the drug, but rather reflected changes to the patient's normal metabolome. Absolute identification of these compounds might help to elaborate on the still poorly understood drug mechanism of Riluzole and can assist with the deciphering its related side effects.

In a similar fashion, pharmacometabonomics can be implemented to verify or complement drug mechanisms proposed by other omics approaches. Tiziani et al. (2009) applied this approach to investigate the antileukemic activities of the combination of bezafibrate (BEZ) and medroxyprogesterone acetate (MPA), against acute myeloid leukemia (AML). Previous studies have linked the xenobiotic metabolism of these drugs to the production of reactive oxygen species (ROS), and therefore this group proposed that this is responsible for the drug action (Tiziani et al., 2009). In order to prove this hypothesis, and potentially other previously unknown drug mechanisms, the group applied NMR metabolic profiling to three AML cell lines treated with BEZ and MPA. From the generated biosignatures, they were able to identify changes to TCA cycle intermediates (more specifically alterations to the conversion of α -ketoglutarate to succinate), which are consistent with ROS action.

In order to minimize the influence of individual variation on the resultant metabolite profiles, however, many of these studies were done on samples collected from homogeneous patient populations or animal models kept under identical conditions, thereby ensuring that the metabolites emanating as biomarkers are, in fact, related to the xenobiotic metabolism exclusively, with little or no individual variation due to either genetic or environmental factors. Various external factors such as age, stress, diet, gut microbes (microbiome), medication, lifestyle, and disease, in addition to genetic factors including gender, epigenetics, and polymorphisms in genes encoding for

xenobiotic-metabolizing components such as enzymes, transporters, receptors, and ion channels, can also influence xenobiotic metabolism and account for individual variation (Johnson, Patterson, Idle, & Gonzalez, 2012). Clayton, Baker, Lindon, Everett, and Nicholson (2009) demonstrated the application of pharmacometabonomics toward a better understanding of these variables by analyzing pre- and postdose urinary metabolites of patients on acetaminophen (paracetamol), using NMR spectrometry. When analyzing the predose profiles of these individuals, considering the levels of the excreted drug-derived metabolites, they found high levels of predose *p*-cresol sulfate, which correlated with low postdose ratios of acetaminophen sulfate to acetaminophen glucuronide. The ratio of these derivatives, which indicate the extent to which acetaminophen is metabolized through two major phase 2 conjugating processes (O-sulfonation and glucuronidation), is known to be a site and indicator of individual variation in response to paracetamol. The group finally concluded that in patients with high levels of gut microbiome-mediated *p*-cresol generation, competitive *p*-cresol O-sulfonation reduces the capacity to sulfonate acetaminophen, which in turn results in an increased likelihood of drug-induced hepatotoxicity. This study subsequently proves the capacity of metabolomics to identify individual variation in xenobiotic metabolism, related to a variation to individual patient environment.

Pharmacometabonomics can also be implemented as an informative tool, assisting pharmacogenomics in the investigation of genome-related variation in drug metabolism (Johnson et al., 2012). With this goal, Ji et al. (2011) investigated urine metabolites of individuals with major depressive disorder undergoing therapy with selective serotonin reuptake inhibitors (SSRIs), citalopram and escitalopram. On average, 40% of patients generally do not respond to this treatment and previous pharmacogenomic studies failed to identify potential polymorphisms which could be used for the prediction of a SSRI treatment outcome. From the obtained metabolite profiles, elevated glycine levels were identified to be associated with a decreased treatment response, and after subsequent pharmacogenomics studies, polymorphisms in the glycine dehydrogenase gene were identified. These results show that metabolomics can additionally play a significant role in supporting or initiating pharmacogenomics studies, with the intention of identifying genetic factors related to individual variation in drug metabolism.

Considering these and several other studies on the topic (de Carvalho, Darby, Rhee, & Nathan, 2011; Halouska et al., 2007; Lu, Deng, Li, Wang, & Li, 2014; Wang et al., 2013), it is evident that the identification

of drug exposure metabolites can play a significant role in the elucidation of drug mechanisms and the influence of individual variation on these, which in turn, can contribute to the development of more effective drugs, or the positive adjustment of drug dosages and treatment schedules, on the basis of the individual differences detected and explained.

4.2.2 Metabolomics and Understanding Response to Treatment

Despite the elucidation of drug mechanisms, pharmacometabolomics can also be used to investigate and predict an individual's response to treatment. Statins, for example, are commonly prescribed for patients with increased levels of LDL-cholesterol and risk for cardiovascular disease, despite the substantial individual variation in response to this therapy. [Trupp et al. \(2012\)](#) investigated this occurrence using GC-MS metabolomics analyses of patient plasma samples, prior to, and 6 weeks after simvastatin treatment onset. A number of metabolites were identified implicating genetic, gut microbiome and various environmental factors, contribute to the variation in simvastatin response. Additionally, responders and nonresponders to the drug could be differentiated based on their baseline metabolite profiles and the most significant compounds responsible for this differentiation were identified. These metabolite variations could be correlated to different treatment responses and subsequently described the mechanisms related to the individual variation to this therapy. These markers additionally have the potential to be implemented preclinically, to identify those patients who would/would not benefit from simvastatin treatment, prior to commencing treatment. Using the same approach, [Wei et al. \(2013\)](#) built a prediction model, based on four identified serum metabolite biomarkers, in order to predict the outcome of breast cancer neoadjuvant chemotherapy. The model was able to predict complete response (disappearance of all tumor deposits) versus stable disease (tumor reduction less than 50%) with 100% specificity and 80% sensitivity (AUC of 0.95). Despite their prognostic value, the biomarkers identified in these studies also show promise in the development of new, more efficient drugs, and also to subclassify patients during clinical trials.

The same approach has been used to explain and predict variation to treatment response in patients diagnosed with various infectious diseases. [Das et al. \(2015\)](#) investigated the influence of anti-TB drug treatment on the urine metabolic profiles of TB patients at various treatment intervals. A clear treatment-dependent trend could be seen on the PCA, as the metabolite profiles of each consecutive treatment interval shifted closer to that of healthy controls, with profiles of clinically cured patients very closely

resembling that of the control group. With these profiles, one might be able to build a prediction model for treatment outcome, provided that profiles of patients with failed treatment outcomes are also incorporated. These profiles may additionally give clues as to why certain patients fail to respond to TB treatment within the recommended 6-month therapy regime, and by using a similar approach, metabolite biomarkers predicting treatment failure or relapse might also be identified.

When investigating infectious diseases in this manner, it is important to bear in mind that individual variation to drug treatment outcomes can be a result of the host's variable response to the drug, or the pathogen's resistance to the drug, and therefore, both factors should be considered when identifying biomarkers reflecting treatment response. In a GC-MS metabolomics study, [Du Preez and Loots \(2012\)](#) investigated rifampicin resistance in pulmonary TB by comparing the fatty acid metabolomes of two *M. tuberculosis* strains, with resistance conferring mutations on different locations on the *rpoB* gene, to a fully susceptible wild-type parent strain. All three groups showed a clear differentiation when doing PCA, and a number of metabolites indicating a decreased synthesis of various 10-methyl branched-chain fatty acids and cell wall lipids, and an increased use of the shorter-chain fatty acids as carbon sources, were identified as markers in the drug-resistant strains. In addition, the *rpoB* S531L mutant, previously reported to occur in well over 70% of all clinical rifampicin-resistant *M. tuberculosis* strains, showed a better capacity for using these alternative energy sources, compared to the less frequently detected *rpoB* S522L mutant. This study therefore shows that pharmacometabonomics has the power to not only detect metabolome changes related to pathogen-induced drug resistance, but it can also differentiate between the various genotypes leading to the observed phenotype. The clinical use of these identified markers can significantly contribute to the development of improved treatment approaches, thereby bettering treatment outcomes in patients with drug-resistant TB. When developing such personalized therapeutic approaches, it is also important to realize that not all individuals will respond to an infection in a similar fashion, and although infected with identical pathogen strains, treatment outcomes may vary, and through metabolomics, markers can be identified in order to explain and predict this phenomenon.

4.2.3 Metabolomics and Understanding Drug Toxicology

Several metabolomics studies have identified universal, nonspecific biomarkers, related to general toxicity of various different drugs, which are

unrelated to the specific drug type, drug mechanism, or site of action. These metabolites, including decreased concentrations of TCA cycle intermediates and hippurate, are a reflection of secondary side effects related to the ingestion of these drugs and include general changes to energy demand or energy metabolism, and changes to gut microbiota (Keun, 2006). Additionally, many metabolomics studies have also identified general markers related to hepatotoxicity (Holmes et al., 1992; Robertson et al., 2000; Schnackenberg, Dragan, Reily, Robertson, & Beger, 2007) and nephrotoxicity (Garrod et al., 2005; Lenz, Nicholson, Wilson, & Timbrell, 2000). These markers are especially important in the preclinical drug development phase, considering that liver and kidney damage are the two major reasons for drug withdrawal from the market. Although knowledge of these general toxicity markers is of importance, metabolomics also has the capacity to identify markers related to specific drug mechanisms and side effects to vital organs also, and by using blood and urine for biomarker detection, eliminates the need for intrusive procedures for monitoring these outcomes, such as tissue biopsy for instance. Sumner, Burgess, Snyder, Popp, and Fennell (2010) detected urinary markers related to abnormalities in inositol, carbohydrate, glycerolipid, and glyoxylate metabolism, correlating to hepatic microvesicular lipid accumulation (MVLA), a histopathological side effect related to the treatment of TB with isoniazid. They propose that, if validated, these metabolite changes can be used to develop a noninvasive method for the early detection of MVLA.

A study entitled Consortium for Metabonomic Toxicology (COMET) was one of the most extensive pharmacometabolomics investigations conducted to date. The aim of COMET was to build models for the prediction of organ toxicity (mainly liver and kidney), from NMR spectra obtained from rodent urine and serum, from multiple toxicity studies (Lindon et al., 2005, 2003). A validation of these markers indicated that these methods could predict liver and kidney toxicity with specificities of 100% and 77% and sensitivities of 41% and 67%, respectively (Ebbels et al., 2007). These models are now being implemented by the six pharmaceutical companies involved in the consortium, in preclinical studies.

Another important general drug-related adverse effect to be considered is the change in the patient's intestinal microbiota composition, in response to antibiotic treatment. Pharmacometabonomics has also contributed significantly to this intensely studied research area, indicating that 87% of all metabolites in the intestinal metabolome are influenced by antibiotic treatment. Furthermore, a disturbance in a number of metabolic pathways,

including bile acid, eicosanoid, and steroid hormone synthesis, were identified subsequent to antibiotic treatment (Antunes et al., 2011).

In addition to better describing the molecular mechanisms resulting in general adverse effects such as hepatotoxicity, nephrotoxicity, and intestinal microbiota response to antibiotic treatment, metabolomics has also been used to investigate the mechanisms of more specific drug-related side effects associated with specific treatment regimens. To this end, Loots, Wiid, Page, Mienie, and Helden (2005) evaluated the effects of the combined anti-TB drug, Rifater, on the metabolic profiles of Sprague-Dawley rats. The identified biomarkers indicated elevated oxidative stress levels in the animal models receiving treatment, and the metabolite profiles closely resembled that of human patients suffering from a multiple acyl-CoA dehydrogenase deficiency (MADD). These findings indicated that Rifater treatment could be linked to an inhibition of the electron transport chain flavoproteins, and the group subsequently indicated that this MADD metabolite profile, and hence the associated drug-related side effects, could be corrected for by the coadministration of melatonin. From these and other studies, it is evident that metabolomics can assist in a better description and understanding of the general and specific side effects related to various drugs, which may contribute to the development of safer treatment approaches, or the expansion of therapeutic strategies for the prevention or early management of these occurrences. Once again, it is important to consider all factors which may influence these metabolite changes, including the previously mentioned environmental and genetic factors, when identifying those biomarkers related to toxic insult (Beger, Sun, & Schnackenberg, 2010). Although adverse side effects are not uncommon for many prescription drugs, especially those targeting life-threatening disease states such as cancer for instance, it can also occur in drugs which are widely recognized as safe and are normally well tolerated by most patients. As discussed earlier, the largest contributor to the variation in individual patient xenobiotic metabolism, and hence their toxicology phenotypes, are polymorphisms in genes encoding for xenobiotic-metabolizing enzymes. However, various other factors have been identified to contribute to this, including the patient's health status (hepatic dysfunction, inflammation, infection, and cancer), drug-drug interactions, and exposure to contaminants such as heavy metals, all of which can influence the activities of these drug-metabolizing enzymes (Chen, Gonzalez, & Idle, 2007). Although pharmacogenomics is the primary approach used for the investigation of such variation, metabolomics studies have also shown to be useful in the elucidation of the mechanisms

leading to these phenotypes. For example, a number of genetics studies have linked a polymorphism in the CYP2D6 with an excessive hypotensive response to the antihypertensive drug; debrisoquine. When investigating this phenomenon using LC–MS metabolomics, metabolite profiles from urine could differentiate the treatment response phenotypes (poor metabolizers vs. extensive metabolizers). As expected, debrisoquine was significantly higher in the profiles of poor responders, whereas the products of drug metabolism: 4-hydroxy-debrisoquine, and two-open ring products of debrisoquine: 2-(guanidinoethyl)benzoic acid (I) and 2-(guanidinomethyl) phenyl acetic acid (II), were detected in higher levels in the profiles of the good responders. The two CYP2D6 genotypes could thus be identified using biomarkers determined by metabolomics, proving that pharmacometabonomics has the capacity to identify individual variation in drug-related side effects, originating from these polymorphisms (Zhen et al., 2006). In a similar fashion, metabolomics can also be implemented to investigate other factors leading to this variation, thereby paving the way for the development of more effective, personalized medicines, with a lowered incidence of drug-induced side effects.



5. CONCLUDING REMARKS

The leap in technology over the past few decades has significantly contributed to our knowledge of biological systems on a molecular level, and the newly gained information has subsequently shed light on the variation that exists in the genetic, protein, and metabolite compositions of different individuals. The application of this knowledge to the development and implementation of personalized medicine is currently a hot topic among clinical researchers, and as described above, metabolomics has been a valuable contributor to this. However, before the identified metabolite biomarkers (or any other marker for that matter, identified via genomic, proteomic, or transcriptomic approaches) can be applied toward personalized clinical care, they should be validated using large sample cohorts, with all influencing variables, such as age, gender, habits, and diet considered. It should additionally be noted that current metabolomics, genomics, transcriptomics, and proteomics techniques require highly trained technical competence and infrastructure, and hence, is regarded to be rather expensive to perform, especially if these methods are being considered for use directly for clinical application to diagnostics. For this reason, various research efforts have been made toward using these newly identified diagnostic and prognostic markers for clinical application, through the development of simple, cheap tests,

targeted for identifying these specific compounds/biomarkers previously identified, and validated using these “omics” approaches. The use of genomics, transcriptomics, proteomics, and also more recently, metabolomics data, in large databases such as DrugBank (Knox et al., 2011) (which is a comprehensive collection of drug mechanism and drug target information), proves that despite challenges in these fields, the contribution of all of these technologies to the development of personalized medicine is invaluable.

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Tuberculosis

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Elucidating the antimicrobial mechanisms of colistin sulfate on *Mycobacterium tuberculosis* using metabolomics

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ABSTRACT

Considering the disadvantages of first line anti-tuberculosis (TB) drugs, including poor patient adherence, drug side effects, the long treatment duration and rapidly increasing microbe resistance, alternative treatment strategies are needed. Colistin sulfate (CS), a polymyxin antibiotic considered a last-resort antibiotics for treating multidrug-resistant *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter*, has antimicrobial activity towards mycobacteria, and could serve as a possible anti-TB drug.

Using GCxGC-TOFMS metabolomics, we compared the metabolic profiles of *Mycobacterium tuberculosis* (*Mtb*) cultured in the presence and absence of CS, to elucidate the mechanisms by which this drug may exert its antimicrobial effects.

The principal component analysis of the metabolite data indicated significant variation in the underlying metabolite profiles of the groups. Those metabolites best explaining this differentiation, were acetic acid, and cell wall associated methylated and unmethylated fatty acids, and their alcohol and alkane derivatives. The elevated glucose levels, and various glyoxylate and glycerolipid metabolic intermediates, indicates an elevated flux in these metabolic pathways.

Since all the metabolites identified in the colistin treated *Mtb* indicates an increase in fatty acid synthesis and cell wall repair, it can be concluded that CS acts by disrupting the cell wall in *Mtb*, confirming a similar drug action to other organisms.

1. Introduction

Tuberculosis (TB) is an infectious bacterial disease caused by the organism *Mycobacterium tuberculosis* (*Mtb*) and usually affects the lungs [1]. The World Health Organisation (WHO) reports TB to be one of the world's deadliest communicable diseases, resulting in the death of up to 2 million people per annum. Furthermore, TB is considered the leading cause of death among people living with HIV [2]. TB is currently treated using the 6 month "directly observed treatment short-course" (DOTS) regimen, consisting of the four first-line drugs: rifampicin, isoniazid, ethambutol and pyrazinamide [3]. In patients with drug-susceptible TB, this regimen reportedly has a 1–4% failure rate, and 7% of the patients with a successful treatment outcome, reportedly relapse within 24 months [4]. The WHO has additionally reported 5% of all TB cases have multidrug-resistant TB (MDR-TB) [2], which requires treatment using second-line anti-TB drugs [5]. These second line drugs are not only more expensive, but also have severe side effects, and an even longer treatment duration (approximately 2 years) [6]. These

complexities, in addition to the fact that current anti-TB drugs have cross-reactions and interactions with HIV-antiretroviral therapy, emphasise the need for researching and developing new anti-TB drugs or alternative therapeutic approaches.

Colistin sulfate (CS), a polymyxin antibiotic discovered in the 1940s, is a cyclic peptide with a hydrophobic tail, and was one of the first antibiotics with significant activity against gram-negative bacteria [7], in particular *Pseudomonas aeruginosa* [8], *Acinetobacter baumannii* [9] and *Klebsiella pneumoniae* [10]. Colistin sulfate was proposed to function by binding electrostatically to the lipopolysaccharides and phospholipids on the outer cell membrane of these gram negative bacteria, and subsequently displace the membrane cations (magnesium and calcium) from the phosphate groups of these membrane lipids, creating pores, and subsequently causing cell death [11]. Using *M. aurum*, Rastogi et al. [12], additionally indicated that colistin sulfate has an effect on the cytoplasmic membrane of mycobacteria, and indicated a resultant cell leakage in experiments using *M. avium* [12], *M. aurum*, *M. xenopi* and *M. smegmatis* [13,14], as a consequence of cell

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wall disruption. Considering this evidence, colistin sulfate would also be expected to have similar effects on *Mtb*. Korycka-Machala et al. [15] subsequently showed an increased cell wall permeability in *Mtb* following colistin sulfate treatment, and Van Breda et al. [16] and Bax et al. [17,18], that colistin sulfate also allows for elevated first line TB drug uptake in *Mtb*, as a result of this. Since most of this evidence pertaining to the anti-bacterial mechanisms of colistin sulfate in mycobacteria has been done from a histological and genomics research perspective, research using other “omics” disciplines are also required to understand this drug better, and its possible application to treating TB. Metabolomics is one of the latest additions to the “omics” technologies, and defined as an unbiased identification and quantification of all metabolites present in a sample (disease or treatment related), using advanced analytical techniques, and statistical analysis and bioinformatics, to identify the most important biomarkers for describing a perturbation [19]. We used a two dimensional gas chromatography coupled time-of-flight mass spectrometry (GCxGC-TOF/MS) metabolomics approach, to identify those metabolite markers best differentiating *Mtb* cultured in the presence and absence of colistin sulfate, for the purpose of confirming or elucidating its mechanism of action against *Mtb*.

2. Materials and methods

2.1. Cell culture

The cell cultures were prepared in the presence and absence of colistin sulfate, as described by van Breda et al., [16]; with slight modifications. Briefly, *Mtb* H37Ra ATCC 25177 (obtained from Ampath Pathology Laboratory Support Services, Centurion, Gauteng, South Africa) was swabbed onto Middlebrook 7H10 agar (Becton Dickinson, Woodmead, Gauteng, South Africa), supplemented with 0.5% v/v glycerol (Saarchem, Krugersdorp, Gauteng, South Africa), and enriched with 10% v/v oleic acid, albumin, dextrose, catalase (OADC) (Becton Dickinson). Our reasons for selecting a H37Ra strain in this experiment was due to the fact that the original description of the effects of polymyxins by Rastogi et al. [13,14], also used H37Ra, and a recent publication by Bax et al. [17,18], described similar results using H37Rv as to what van Breda et al., [16] described for H37Ra.

The stock culture was prepared after three weeks of incubation at 37 °C, by suspending the cells in 1 x phosphate buffered saline (PBS) (Sigma Aldrich, Kempton Park, Gauteng, South Africa) containing 0.05% v/v Tween 80 (Saarchem) to a McFarland standard of 3. Aliquots of 1 mL were stored at –80 °C in cryovials, containing 20% v/v glycerol (Saarchem). By using the TB Ag MPT64 Device (KAT Medical, Roodepoort, Gauteng, South Africa), the presence of *Mtb* was confirmed, and the purity was determined by swabbing 100 µL of culture media onto tryptic soy agar (Merck, Darmstadt, Germany) and incubating at 37 °C for 48 h. Before experimental investigations, a cryovial of the stored aliquots was allowed to thaw to room temperature, vortexed and swabbed onto Middlebrook 7H10 agar. Plates were sealed in Ziploc bags and incubated at 37 °C until mid-log growth was reached (approximately 10–14 days).

The mid-log growth culture was suspended to a McFarland standard of 1 (using Sauton media [16]); approximately 1×10^7 colony-forming units (CFU)/mL. The cell suspension (195 µL) was then added to each well in a 96 well microtiter plate (Eppendorf). The antimicrobials were added to final concentrations of 0 µg/mL and 32 µg/mL colistin sulfate respectively, and the plate was sealed using sterile ziploc bags, and incubated at 37 °C for 24 h. The mixture in each well was subsequently transferred to Eppendorf tubes up to a volume of 1 mL. The 10 samples containing 32 µg/mL colistin sulfate and 7 samples containing no colistin sulfate, were centrifuged at 10000 x g for 1 min and showed no difference in the amounts of viable CFU/mL. The supernatant was removed and pellet rinsed and resuspended in 1 x PBS (without Tween 80) and then stored at –80 °C.

In the current investigation it is important to note, that the reason Sauton media was used, is because other media, such as Middlebrook 7H9 for instance, contains the following components which antagonize the effects of polymyxins: 1. BSA (forms complexes with polymyxins) [20], 2. Mg^{2+} and Ca^{2+} [21,22], and hence it was important to use media where physiological concentrations of these divalent cations can be controlled, i.e., cation-adjusted to 10–12.5 mg Mg^{2+} /L and 20–25 mg Ca^{2+} /L [23,24], 3. Na^+ [25,26], and 4. Catalase, since the latter is an antioxidant which would inhibit polymyxin induced Fenton reaction mechanisms [27]. Furthermore, it was important to substitute glycerol with 0.2% w/v glucose, since lower than normal MICs have been previously observed for *Mtb* when glycerol was used as the only carbon source [28], and with 0.05% v/v Tween 80, since *Mtb* requires the fatty acids present within Tween 80 for growth [29,30].

2.2. Whole metabolome extraction procedure and derivatization

Prior to GCxGC-TOFMS analysis, 0.5 mg of each of the individually cultured *Mtb* sample pellets described above were weighed into an Eppendorf tube, followed by the addition of 50 µL 3-phenylbutyric acid (0.0175 µg/mL) (Sigma-Aldrich (St. Louis, MO, USA)) as internal standard. Chloroform, methanol (Burdick and Jackson brands (Honeywell International Inc., Muskegon, MI, USA)) and water were added in a ratio 1:3:1, vortexed for 1 min and then placed in a vibration mill (Retsch, Haan, Germany) with a 3 mm carbide tungsten bead (Retsch) for 5 min at 30 Hz/s. Each sample was then centrifuged for 10 min at 10 000 x g and the supernatants transferred to a GC sample vial, and subsequently dried under a nitrogen stream. Each extract was derivatized using 20 µL methoxyamine hydrochloride-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Sigma-Aldrich (Darmstadt, Germany)) (containing 15 mg/mL pyridine) at 50 °C for 90 min, followed by silylation using 40 µL MSTFA with 1% trimethylchlorosilane (TMCS) at 50 °C for 60 min. These extracts were then transferred to a 0.1 mL insert in a clean GC sample vial and capped, prior to GCxGC-TOFMS analysis [31].

2.3. GCxGC-TOFMS analyses

The samples (1 µL) were analysed in random sequence, using a Pegasus 4D GCxGC-TOFMS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA), TOFMS (LECO Africa) and Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany), in a splitless ratio. The necessary quality control (QC) samples were also analysed at regular intervals in order to correct for any batch effects and also monitor the performance of the analysis over time. A Rxi-5Sil MS primary capillary column (30 m, 0.25 µm film thickness and 250 µm internal diameter), and a Rxi-17 secondary capillary column (1.2 m, 0.25 µm film thickness and 250 µm internal diameter) where used for GC compound separation. Helium was used as a carrier gas at a flow of 1 mL/min with the injector temperature held constant at 270 °C for the entire run. The primary column temperature was set at 70 °C for 2 min, and then increased at a rate of 4 °C/min to a final temperature of 300 °C, at which it was maintained for a further 2 min. The temperature of the secondary oven was programmed at 85 °C for 2 min, then increased at a rate of 4 °C/min to final temperature of 305 °C, at which it was maintained for a further 4.5 min. The acquisition voltage of the detector was 1700 V and the filament bias –70 eV. A mass range of 50–800 *m/z* was used for the mass spectra, at an acquisition rate of 200 spectra/s.

2.4. Data processing, clean-up and statistics

Mass spectral deconvolution (at a signal to noise ratio of 20), peak alignment and peak identification, were done on the collected mass spectra using ChromaTOF software (version 4.32). Identical mass

spectra of the compounds in each of the samples were aligned, if they displayed similar retention times. Compounds were identified by comparison of their mass fragment patterns and retention times, to that of libraries compiled from previously injected standards.

Following the data processing steps described above, a standardized metabolomics data clean-up procedure was conducted [32]. Normalization of each of the detected compounds was done using the total useful MS signal (TUS) [33] and by calculating the relative concentration of each compound, using the internal standard as a reference. A 50% filter was applied in order to remove those compounds showing more than 50% zero values within both groups [34] and the QC samples used to correct for any batch effects, using quantile equating [35]. Additionally, a 50% QC coefficient of variation (CV) filter was applied [36], and all zero-values were replaced by a value determined as half of the smallest concentration (i.e. the detection limit) detected in the entire data set, as these may be due to low abundance rather than being absent [37].

The data were subsequently analysed using a variety of multi- and univariate statistical methods, using a web based software package supported by the Metabolomics Society: MetaboAnalyst (based on the statistical package “R”; version 2.10.0), and included principal components analysis (PCA) [38], partial least squares–discriminant analysis (PLS–DA) [39], a *t*-test and effect size calculations [40].

3. Results

Fig. 1 shows clear PCA differentiation between the individually cultured *Mtb* samples in the presence and absence of colistin sulfate. This natural differentiation of the samples of each of the sample groups can be ascribed to the variation in the total metabolite profiles of each, as determined by GCxGC-TOFMS. The total variance explained by the first two principal components (PCs) (R²X cum) was 48.4% of which PC1 contributed to 37.5% and PC2 10.9%, respectively.

Subsequently, those metabolites that contributed most to this differentiation were selected on the basis of complying with all of the

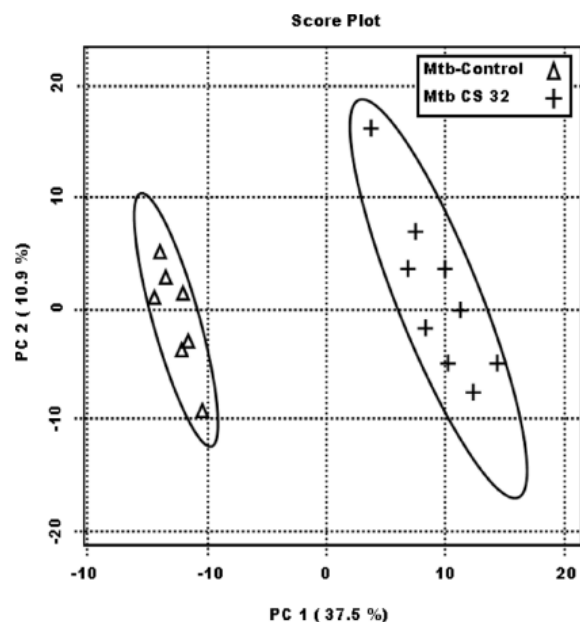


Fig. 1. PCA differentiation using the GCxGC-TOFMS whole metabolome analysed data of the individually cultured *Mtb* in the absence (*Mtb*-Control) and presence (*Mtb*-CS) of colistin sulfate (32 µg/mL). The variances accounted for are indicated in parenthesis.

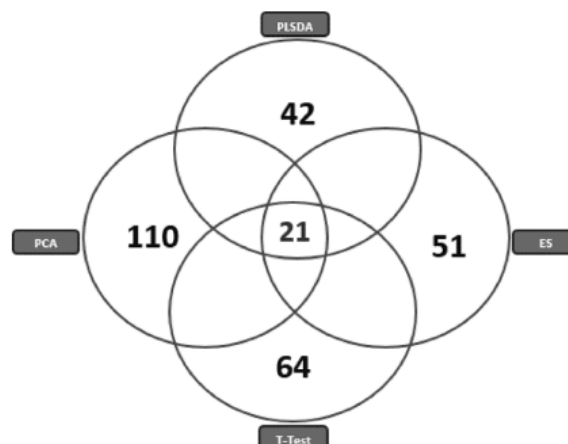


Fig. 2. Venn diagram illustrating a multi-statistical approach for selecting the 21 metabolite markers best describing the variation detected between the individually cultured *Mtb* samples in the presence and absence of colistin sulfate.

following criteria: a PCA modelling power > 0.5 [38], a PLS-DA VIP value > 1 [39], a *t*-test *P*-value < 0.05 and an effect size > 0.5 [40].

Fig. 2 is a summary of the number of metabolite markers selected by each of the univariate and multivariate statistical approaches described above, as well as the selection of the 21 metabolites listed in Table 1, considered most important for explaining the variation detected.

4. Discussion

As previously mentioned, treatment with colistin sulfate results in a structural disruption of the cell wall in *Mtb* [17,18]. The metabolite markers detected in the colistin sulfate treated *Mtb* in the current metabolomics investigation confirms this, and additionally indicates that *Mtb* attempts to rectify this by upregulation of its fatty acid synthesis pathways for subsequent cell wall repair. Accompanying this is an up-regulation of glycolysis which will be described in detail below and summarized in Fig. 3.

In Table 1, 15 out of the 21 metabolite markers identified by the statistics described above, are directly linked to elevated fatty acid biosynthesis and subsequently also cell wall synthesis. These included hexadecanoic acid, octadecanoic acid, eicosanoic acid and hexacosanoic acid, all of which are known to form methyl-branched chain fatty acids and ultimately the mycolic acids [41], an important component of arabinogalactan (AG) in the cell wall core of *Mtb* [42]. Additionally, although not detected as part of the 21 metabolite markers, decanoic acid (0.100 vs. 0.219 µg/mL; *P* < 0.05), dodecanoic acid (0.421 vs. 0.592 µg/mL; *P* < 0.05) and octadecanoic acid (0.491 vs. 0.888 µg/mL; *P* > 0.05) were also found to be elevated, further supporting this. Another important observation was the elevated levels of the methylated branched fatty acids (methyldecanoic acid, methylundecanoic acid and methyltridecanoic acid), in the colistin sulfate treated group comparatively, which in turn not only serve as substrates for mycolic acid synthesis, but also function as hydrophobic modulators of the host's cellular immune function, and various virulence factors in the microbe [43]. These methylated fatty acids are proposed to be formed by 3 possible routes: 1. fatty acid methylation via *S*-adenosylmethionine (SAM) functioning as the methyl donor [41], 2. methylmalonyl-CoA derived polyketide synthase complexes, originating from propionyl-CoA and malonyl-CoA [44] and 3. acetyl-CoA metabolism to butyric acid, which in turn reacts with propionyl-CoA ([45].

Further substantiating these results, are the presence of various alcohols (tetradecanol, nonanol and 2-ethyl-2-methyltridecanol) and alkanes (hexadecane, octadecane, octacosane and methyltridecane)

Table 1

The 21 metabolite markers that best explain the variance between the individually cultured *Mtb* samples in the absence (*Mtb*-Controls) and presence (*Mtb*-CS) of colistin sulfate.

Metabolite name (Chempid ID)	<i>Mtb</i> controls: Average concentration (mg/g cells) (standard error of the mean)	<i>Mtb</i> treated with colistin sulfate Average concentration (mg/g cells) (standard error of the mean)	PCA (Power)	PLS-DA (VIP)	Effect sizes (d-value)	t-test (P-value)
Octanoic acid (NSC 5024 [DBID])	0.205 (0.192)	0.019 (0.010)	0.787	1.423	0.966	> 0.001
Hexadecanoic acid (NSC 5030 [DBID])	0.860 (0.176)	1.259 (0.198)	0.948	1.224	2.022	0.001
Octadecenoic acid (NSC931 [DBID])	0 (0)	0.021 (0.005)	0.861	1.165	4.580	0.003
Eicosenoic acid (4445895)	0 (0)	0.006 (0.007)	0.842	1.181	0.930	0.002
Hexacosanoic acid (NSC 4205 [DBID])	0 (0)	0.059 (0.038)	0.804	1.244	1.541	0.001
Methyldecanoic acid (LMFA01020090)	0.013 (0.004)	0.017 (0.004)	0.822	1.238	0.994	0.001
Methyldodecanoic acid (4445769)	0.045 (0.011)	0.115 (0.025)	0.855	1.123	2.794	0.004
Methyltetradecanoic acid (NSC 189699 [DBID])	0.317 (0.021)	0.789 (0.261)	0.893	1.073	1.810	0.007
Sebacic acid (NSC 19492 [DBID])	0.001 (0.001)	0.004 (0.003)	0.898	1.151	1.278	0.003
Tetradecanol (NSC 4194 [DBID])	0.016 (0.003)	0.059 (0.019)	0.982	1.516	2.251	> 0.001
2-Ethyl-2-Methyl-Tridecanol (921600091)	0.006 (0.001)	0.044 (0.033)	0.809	1.245	1.159	0.001
5-Nonanol (NSC4552 [DBID])	0.001 (0.001)	0.023 (0.004)	0.965	1.054	6.168	0.001
Hexadecane (NSC 172781 [DBID])	0.002 (0.003)	0.031 (0.029)	0.978	1.555	1.024	> 0.001
Octadecane (NSC 172781 [DBID])	0.020 (0.011)	0.115 (0.028)	0.878	1.197	3.446	0.002
Octacosane (NSC 5549 [DBID])	0 (0)	0.016 (0.016)	0.993	1.611	0.979	> 0.001
Methyltetradecane (NSC 172781 [DBID])	0.005 (0.004)	0.044 (0.012)	0.664	1.090	3.186	0.006
Glucose (NSC4552 [DBID])	0.195 (0.187)	0.366 (0.170)	0.819	1.470	0.913	> 0.001
Oxalate (c0017 [DBID])	0 (0)	0.006 (0.006)	0.863	1.266	0.954	0.001
Glycerol (NSC 9230 [DBID])	0.063 (0.012)	0.082 (0.011)	0.891	1.173	1.469	0.002
Monopalmitin (110006)	0.165 (0.058)	0.216 (0.056)	0.987	1.614	0.874	> 0.001
Propyl myristate (A13-31609 [DBID])	0.014 (0.003)	0.023 (0.006)	0.991	1.626	1.328	> 0.001

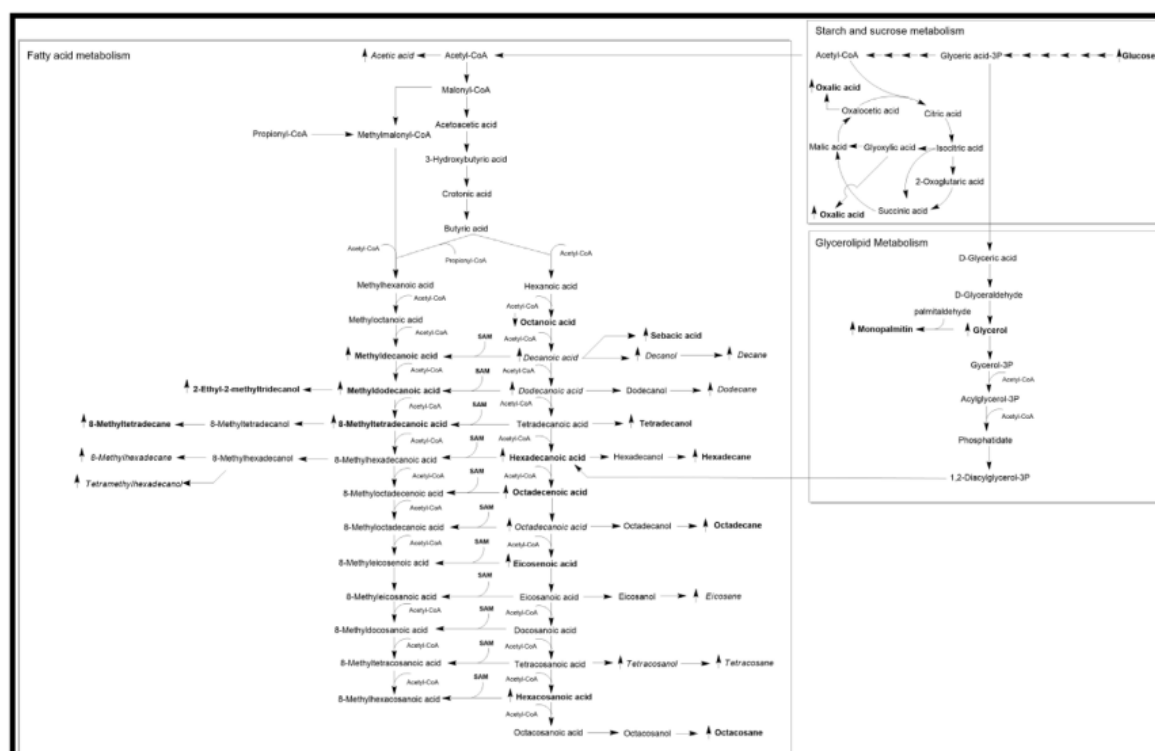


Fig. 3. Altered *Mtb* metabolome induced by treatment with colistin sulfate. The schematic representation indicates the 21 metabolite markers in bold and the confirmatory metabolites which were also elevated, but not necessarily significantly so, indicated in italics. Increase and decrease in the metabolite markers are indicated by ↑↓ respectively.

corresponding to the aforementioned fatty acids [46]. Additionally, although not detected using the marker selection process described above, decanol (0 vs. 0.018 µg/ml; $P < 0.05$), decane (0.006 vs. 0.012 µg/ml; $P > 0.05$), dodecane (0 vs. 0.007 µg/ml; $P > 0.05$), eicosane (0.083 vs. 0.172 µg/ml; $P < 0.05$), tetracosanol (0.001 vs. 0.006 µg/ml; $P > 0.05$), tetracosane (0 vs. 0.01 µg/ml; $P < 0.05$), methylhexacosane (0.132 vs. 0.611 µg/ml; $P > 0.05$), tetramethylhexadecanol (0.001 vs. 0.023 µg/ml; $P < 0.05$) and methylhexadecane (0 vs. 0.01 µg/ml; $P > 0.05$), were also seen to occur in elevated amounts, further confirming these mechanisms.

Another important observation supporting the unanimous metabolic flux observed in this study towards fatty acid biosynthesis and cell wall repair, is the significantly elevated concentrations of glucose, glycerol and monopalmitic acid. According to de Carvalho et al. [47]; *Mtb*'s central carbon metabolism is able to co-catabolise multiple carbon sources for energy [47]. Considering the colistin sulfate treated *Mtb*'s need to preferably utilize fatty acids towards cell wall repair, one would expect that this organism would subsequently resort to glucose, which was freely available in the growth media, as the primary energy substrate, in conjunction with an upregulated glyoxylate cycle [48], substantiated in this investigation by the elevated glucose and oxalic acid detected [49]. Furthermore, as shown in Fig. 3, various intermediates of glycolysis, can additionally serve as substrates for fatty acid biosynthesis, including acetyl-CoA, as previously mentioned, and glycer-aldehyde-3-phosphate (G-3-P) via glycerol [19], the latter of which is supported by elevations in monopalmitic acid and the glycerol present in the growth media.

5. Concluding remarks

This study, is the first of its kind to use a metabolomics research approach in order to identify biomarkers explaining the antibacterial mechanisms of colistin sulfate against *Mtb*, and additionally shows the capacity of metabolomics for identifying metabolite markers which can be used to better understand or confirm drug action. The fatty acid metabolite markers identified in the colistin sulfate treated *Mtb*, shows a metabolic flux towards fatty acid synthesis and cell wall repair. Furthermore, glucose uptake is increased, serving as the preferential energy source (as opposed to fatty acids which are now preferentially being used for cell wall repair) to fuel an upregulated glyoxylate cycle, and additionally as a precursor for further cell wall fatty acid synthesis via the glycerolipid metabolic pathway. Considering this, it can be concluded that colistin sulfate acts by disrupting the cell wall in *M. tuberculosis*, confirming a similar drug action as that seen in other organisms.

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Transparency declarations

None to declare.

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journal homepage: www.elsevier.com/locate/tubeMetabolomics of colistin methanesulfonate treated *Mycobacterium tuberculosis*Nadia Koen^{a,1}, Shane Vontelin van Breda^b, Du Toit Loots^{a,*}^a Human Metabolomics, North-West University, Private Bag x6001, Box 269, Potchefstroom, 2531, South Africa^b Department of Internal Medicine, Division of Infectious Diseases, University of Pretoria, Pretoria, 0002, South Africa

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ABSTRACT

Over the past 5 years, there has been a renewed interest in finding new compounds with anti-TB action. Colistin methanesulfonate or polymyxin E, is a possible anti-TB drug candidate, which may in future be used either alone or in combination to the current 6 month “directly observed treatment short-course” (DOTS) regimen. However its mechanism of action has to date not yet been fully explored, and only described from a histological and genomics perspective. Considering this, we used a GCxGC-TOFMS metabolomics approach and identified those metabolite markers characterising *Mycobacterium tuberculosis* (*Mtb*) cultured in the presence of colistin methanesulfonate, in order to better understand or confirm its mechanism of action. The metabolite markers identified indicated a flux in the metabolism of the colistin methanesulfonate treated *Mtb* towards fatty acid synthesis and cell wall repair, confirming previous reports that colistin acts by disrupting the cell wall of mycobacteria. Accompanying this, is a subsequently elevated glucose uptake, since the latter now serves as the primary energy substrate for the upregulated glyoxylate cycle, and additionally as a precursor for further fatty acid synthesis via the glycerolipid metabolic pathway. Furthermore, the elevated concentrations of those metabolites associated with pentose phosphate, valine, threonine, and pentanediol metabolism, also confirms a shift towards glucose utilization for energy production, in the colistin methanesulfonate treated *Mtb*.

1. Introduction

In 2015, an approximated 10.5 million new cases of tuberculosis (TB) were reported globally, which subsequently contributed to 1.4 million deaths [1]. Tuberculosis is caused by the infectious organism *Mycobacterium tuberculosis* (*Mtb*), a mycobacteria bacillus which mainly targets the lungs [2]. Currently, the WHO approved treatment approach entails a 6 months combination treatment approach which is called the “directly observed treatment short-course” (DOTS) regimen [3]. According to the annual WHO report, a significant improvement to current treatment strategies is going to be a challenge, however the identification of new anti-TB drug candidates and or alternative treatment regimens, might be a plausible option for speeding up treatment duration and subsequently lowering the TB prevalence globally [4,5]. Although there are currently a number of new potential anti-TB drugs undergoing phase II and III preclinical trials, delamanid and bedaquiline are the only two new anti-TB drugs to have been approved over the last 50 years. These drugs, however, are currently only used for treating adults with MDR-TB, and considered as last option medications, when no other alternatives prove to be effective [6]. Considering this, there is

still urgent need for new TB drugs and alternative TB treatment approaches.

Another possible anti-TB drug candidate is the antibiotic colistin methanesulfonate (CMS), an inactive prodrug of colistin sulfate (CS), also known as polymyxin E [7]. CMS has previously been shown to have high anti-bacterial activities against *P. aeruginosa*, *A. baumannii*, and *Klebsiella pneumoniae*, and additionally shown to be resistant to these organisms developing drug tolerance [8]. CMS is produced via a reaction from commercially synthesised CS with formaldehyde and sodium bisulphite, resulting in the subsequent addition of a sulfomethylated group to the primary amine groups of CS [9]. The original reason for modifying CS in this manner is that the resulting CMS is considered less toxic when administered parenterally [10]. When administered, a hydrolysis reaction occurs, where CMS in an aqueous solution forms both CS and various partially sulfomethylated derivatives of CS [11]. Apart from the varying toxicity characteristics of CS and CMS, these two forms of the drug show different pharmacokinetic characteristics [12–14]. A study conducted by Plachouras et al. (2009), indicated that colistin concentrations increase gradually after the administration of CMS in critically ill patients, reaching a steady state after 2 days,

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suggesting benefits of treatment commencement with a loading dose [15]. Various colistin derivatives have also been proposed to promote first line anti-TB drug uptake, by creating pores in the outer membrane of *Mtb*, after binding electrostatically to the outer cell membrane lipopolysaccharides and phospholipids [16]. Very little data however exists describing the antimicrobial action of CMS against *Mtb*, and that which has been described to date, was attained solely from a histological or genomics approach. Metabolomics, the latest addition to the “omics” family, is defined as an unbiased identification and quantification of all metabolites present in a sample, using highly specialised analytical procedures and a statistical analysis/bioinformatics, by which the most important metabolites characterising a perturbation (or drug) can be identified [17]. In this investigation, we extracted the intracellular metabolome of *Mtb* cultured in the presence and absence of 32 µg/ml CMS, and analysed these extracts using a 2 dimensional gas chromatography time of flight mass spectrometry (GCxGC-TOFMS) metabolomics approach, for the purpose of identifying those metabolite markers best characterising the changes to the *Mtb* metabolome induced by CMS.

2. Materials and methods

2.1. Cell culture

As described by van Breda et al. (2015), the cell cultures were prepared in the presence and absence of CMS, with slight modifications. Briefly, *Mtb* H37Ra ATCC 25177 (obtained from Ampath Pathology Laboratory Support Services, Centurion, Gauteng, South Africa) was swabbed onto Middlebrook 7H10 agar (Becton Dickinson, Woodmead, Gauteng, South Africa), supplemented with 0.5% v/v glycerol (Saarchem, Krugersdorp, Gauteng, South Africa), and enriched with 10% v/v oleic acid, albumin, dextrose, catalase (OADC) (Becton Dickinson). The reasons for selecting a H37Ra strain in this experiment, was due to the fact that the original description of the effects of polymyxins by Rastogi et al. (1986), used H37Ra, and a recent publication by Bax et al. (2015), described similar results using H37Rv as to what van Breda et al. (2015) described for H37Ra [16,18,19].

The stock culture was prepared after three weeks of incubation at 37 °C, by suspending the cells in 1 × phosphate buffered saline (PBS) (Sigma Aldrich, Kempton Park, Gauteng, South Africa) containing 0.05% v/v Tween 80 (Saarchem) to a McFarland standard of 3. Aliquots of 1 ml were stored at –80 °C in cryovials, containing 20% v/v glycerol (Saarchem). By using the TB Ag MPT64 Device (KAT Medical, Roodepoort, Gauteng, South Africa), the presence of *Mtb* was confirmed, and the purity was determined by swabbing 100 µL of culture media onto tryptic soy agar (Merck, Darmstadt, Germany) and incubating at 37 °C for 48 h. Before experimental investigations, a cryovial of the stored aliquots was allowed to thaw to room temperature, vortexed and swabbed onto Middlebrook 7H10 agar. Plates were sealed in Ziploc bags and incubated at 37 °C until mid-log growth was reached (approximately 10–14 days).

The mid-log growth culture was suspended to a McFarland standard of 1 (using Sauton media [16]); approximately 1×10^7 colony-forming units (CFU)/mL. The cell suspension (195 µL) was then added to each well in a 96 well microtiter plate (Eppendorf). The antimicrobials were added to final concentrations of 0 µg/mL and 32 µg/mL CMS respectively, and the plate was sealed using sterile ziploc bags, and incubated at 37 °C for 24 h. The mixture in each well was subsequently transferred to Eppendorf tubes up to a volume of 1 mL. The 10 samples containing 32 µg/mL CMS and 7 samples containing no CMS, were centrifuged at $10000 \times g$ for 1 min and showed no difference in the amounts of viable CFU/mL. The supernatant was removed and pellet rinsed and resuspended in 1 × PBS (without Tween 80) and then stored at –80 °C.

In the current investigation it is important to note, that the reason Sauton media was used, is because other media, such as Middlebrook 7H9 for instance, contains the following components which antagonize

the effects of polymyxins 1. BSA (forms complexes with polymyxins) [20], 2. Mg²⁺ and Ca²⁺ [21,22], and hence it was important to use media where physiological concentrations of these divalent cations can be controlled, i.e., cation-adjusted to 10–12.5 mg Mg²⁺/L and 20–25 mg Ca²⁺/L [23,24], 3. Na⁺ [25,26], and 4. Catalase, since the latter is an antioxidant which would inhibit polymyxin induced Fenton reaction mechanisms [27]. Furthermore, it was important to substitute glycerol with 0.2% w/v glucose, since lower than normal MICs have been previously observed for *Mtb* when glycerol was used as the only carbon source [28], and with 0.05% v/v Tween 80, since *Mtb* requires the fatty acids present within Tween 80 for growth [29,30].

2.2. Whole metabolome extraction procedure and derivatization

Prior to GCxGC-TOFMS analysis, 0.5 mg of each of the individually cultured *Mtb* sample pellets described above were weighed into an Eppendorf tube, followed by the addition of 50 µL 3-phenylbutyric acid (0.0175 µg/mL) (Sigma-Aldrich (St. Louis, MO, USA)) as internal standard. Chloroform, methanol (Burdick and Jackson brands (Honeywell International Inc., Muskegon, MI, USA)) and water were added in a ratio 1:3:1, vortexed for 1 min and then placed in a vibration mill (Retsch, Haan, Germany) with a 3 mm carbide tungsten bead (Retsch) for 5 min at 30 Hz/s. Each sample was then centrifuged for 10 min at $10\,000 \times g$ and the supernatants transferred to a GC sample vial, and subsequently dried under a nitrogen stream. Each extract was derivatized using 20 µL methoxyamine hydrochloride-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Sigma-Aldrich (Darmstadt, Germany)) (containing 15 mg/mL pyridine) at 50 °C for 90 min, followed by silylation using 40 µL MSTFA with 1% trimethylchlorosilane (TMCS) at 50 °C for 60 min. These extracts were then transferred to a 0.1 mL insert in a clean GC sample vial and capped, prior to GCxGC-TOFMS analysis [31].

2.3. GCxGC-TOFMS analyses

The samples (1 µL) were analysed in random sequence, using a Pegasus 4D GCxGC-TOFMS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA), TOFMS (LECO Africa) and Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany), in a splitless ratio. The necessary quality control (QC) samples were also analyzed at regular intervals in order to correct for any batch effects and also monitor the performance of the analysis over time. A Rxi-5Sil MS primary capillary column (30 m, 0.25 µm film thickness and 250 µm internal diameter), and a Rxi-17 secondary capillary column (1.2 m, 0.25 µm film thickness and 250 µm internal diameter) were used for GC compound separation. Helium was used as a carrier gas at a flow of 1 mL/min with the injector temperature held constant at 270 °C for the entire run. The primary column temperature was set at 70 °C for 2 min, and then increased at a rate of 4 °C/min to a final temperature of 300 °C, at which it was maintained for a further 2 min. The temperature of the secondary oven was programmed at 85 °C for 2 min, then increased at a rate of 4 °C/min to final temperature of 305 °C, at which it was maintained for a further 4.5 min. The acquisition voltage of the detector was 1700 V and the filament bias –70 eV. A mass range of 50–800 *m/z* was used for the mass spectra, at an acquisition rate of 200 spectra/s.

2.4. Data processing, clean-up and statistics

Mass spectral deconvolution (at a signal to noise ratio of 20), peak alignment and peak identification, were done on the collected mass spectra using ChromaTOF software (version 4.32). Identical mass spectra of the compounds in each of the samples were aligned, if they displayed similar retention times. Compounds were identified by comparison of their mass fragment patterns and retention times, to that

of libraries compiled from previously injected standards.

Following the data processing steps described above, a standardized metabolomics data clean-up procedure was conducted [32]. Normalization of each of the detected compounds was done using the total useful MS signal (TUS) [33] and by calculating the relative concentration of each compound, using the internal standard as a reference. A 50% filter was applied in order to remove those compounds showing more than 50% zero values within both groups [34] and the QC samples used to correct for any batch effects, using quantile equating [35]. Additionally, a 50% QC coefficient of variation (CV) filter was applied [36], and all zero-values were replaced by a value determined as half of the smallest concentration (i.e. the detection limit) detected in the entire data set, as these may be due to low abundance rather than being absent [37].

The data were subsequently analysed using a variety of multi- and univariate statistical methods, using a web based software package supported by the Metabolomics Society: MetaboAnalyst (based on the statistical package “R”; version 2.10.0), and included principal components analysis (PCA) [38], partial least squares–discriminant analysis (PLS–DA) [39], a *t*-test and effect size calculations [40].

3. Results and discussion

Fig. 1 shows clear PCA differentiation of the individually cultured *Mtb* samples in the presence and absence of CMS, using the collected GCxGC-TOFMS metabolomics data. The total amount of variance explained by the first two principal components (PCs) (R2X cum) was 55.9%, of which PC1 accounted for 43.4%, and PC2 accounted for 12.5%. Subsequently, by compliance with all of the following criteria: a PCA modelling power > 0.5 [38], a PLS-DA VIP value > 1 [39], a *t*-test P-value < 0.05 and an effect size > 0.5 [40], the metabolites that contributed most to this differentiation were selected (Fig. 2) and listed in Table 1. These metabolite markers were mapped on a metabolic chart as indicated in Fig. 3 and discussed below. As indicated, the metabolomics investigation of the cultured *Mtb* in the presence and absence of CMS, led to the identification of various significantly altered metabolite markers. Glucose uptake was increased in the CMS treated

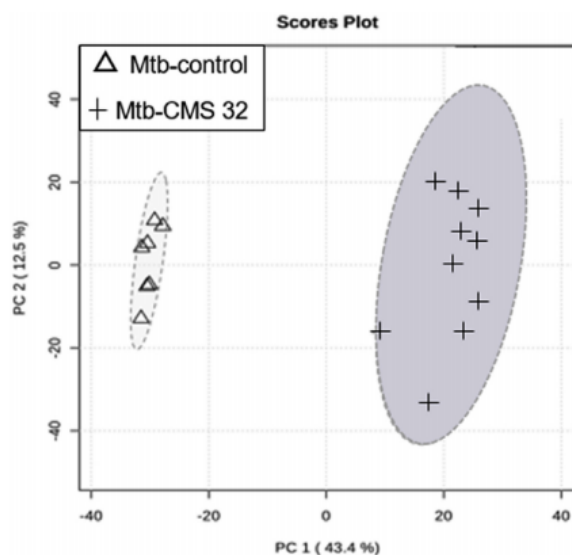


Fig. 1. PCA differentiation of individually cultured *Mtb* in the absence (*Mtb*-control) and presence (*Mtb*-CMS) of colistin methanesulfonate (32 µg/mL) and analysed via GCxGC-TOFMS. The variances accounted for are indicated in parenthesis.

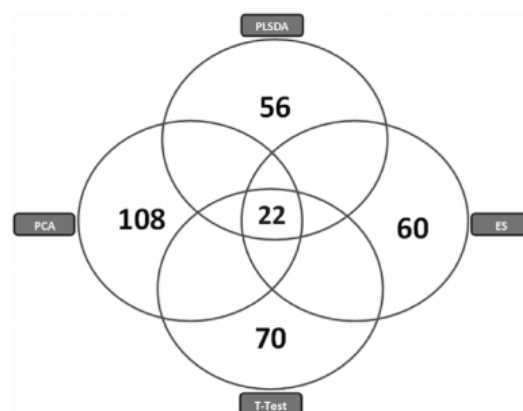


Fig. 2. Venn diagram illustrating the multi-statistical selection criteria of the 22 metabolite markers best describing the variation between the individually cultured *Mtb* sample groups in the presence and absence of CMS.

Mtb, as the preferential energy source (as opposed to fatty acids which are now preferentially being used for cell wall repair) to fuel an up-regulated glyoxylate cycle, and substrate further cell wall fatty acid synthesis via the glycerolipid metabolic pathway. However, the CMS treated *Mtb*, also showed comparatively elevated metabolites associated with pentose phosphate, valine, threonine, and pentanediol metabolism. These results confirm that CMS disrupts the *Mtb* cell membrane, and that these bacteria attempt to compensate for this via upregulation of various metabolic pathways related to cell wall repair.

Colistin has been previously reported to have an antimicrobial activity, which function by binding electrostatically to the lipopolysaccharides and phospholipids on the outer cell membrane of these gram negative bacteria, subsequently displacing the membrane cations (magnesium and calcium) from the phosphate groups of their membrane lipids, subsequently creating pores, which results in cell death [41]. This was supported by our previous metabolomics work on the topic, which showed that elevated fatty acid synthesis and cell wall repair mechanisms are activated in the CS treated *Mtb* [42]. As previously described by Bax et al. (2015) and van Breda et al. (2015), the CMS used in the current investigation, also forms colistin once administered, and hence, would also be expected to result in a structural disruption of the *Mtb* cell wall via the same mechanism as to when CS is administered. This is supported by the elevated levels of the cell wall associated with methylated and unmethylated fatty acids (methyladipic acid, methyldecanoic acid, methyltridecanoic, octadecenoic acid) and their fatty acid associated alcohols and alkanes [43] (tetramethylhexanediol, octacosane, octadecane, tetradecanol, and hentriacontane (Table 1)). Additionally, although not selected using the markers selection statistics approach defined in the methods section, methyldecanoic acid (0.49 vs. 0.42 µg/mL; *P* > 0.05), hexadecanoic acid (0.859 vs. 0.857 µg/mL; *P* > 0.05), octadecanoic acid (22.45 vs. 8.07 µg/mL; *P* > 0.05), tetracosanoic acid (24.54 vs. 14.15 µg/mL; *P* > 0.05), decanol (0.02 vs. 0.00 µg/mL; *P* > 0.05), hexadecanol (0.001 vs. 0.000 µg/mL; *P* > 0.05), decane (0.009 vs. 0.006 µg/mL; *P* > 0.05), dodecane (0.006 vs. 0.00 µg/mL; *P* > 0.05), hexadecane (0.011 vs. 0.002 µg/mL; *P* > 0.05) and tetracosane (0.018 vs. 0.00 µg/mL; *P* > 0.05), were also significantly elevated in the CMS treated *Mtb* comparatively, when considering their *P*-values, further supporting this mechanism (Fig. 3). Octanoic acid and octadecenoic acid, are the well-known substrates for the synthesis of methylated-branched chain fatty acids and mycolic acids, both important components of arabinogalactan (AG) in the cell wall of *Mtb* [44,45]. These methylated branched fatty acids additionally serve as hydrophobic modulators for the host's cellular immune system, and are also

Table 1

The 22 metabolite markers best explaining the variance between the individually cultured *Mtb* samples in the absence (*Mtb*-Controls) and presence (*Mtb*-CMS) of colistin methanesulfonate.

Metabolite name (Chempid ID)	<i>Mtb</i> controls: Average concentration (mg/g cells)	<i>Mtb</i> controls: standard error of the mean	<i>Mtb</i> treated with CMS: Average concentration (mg/g cells)	<i>Mtb</i> treated with CMS: standard error of the mean	PCA (Power)	PLS-DA (VIP)	Effect sizes (d-value)	t-test (P-value)	Fold Change (log2)
Tetramethylhexadecanol (92535)	0,005	0001	0,011	0003	0,663	1087	2095	0,003	1.14
Methyladipic Acid (5367266)	0,159	0024	0,204	0042	0,984	1592	1063	< 0,001	0.36
Arabitol (84971)	0,554	0133	0,328	0062	0,989	1603	1703	< 0,001	−0.59
Diethylene glycol (DEG) (13835180)	0,072	0020	0,036	0008	0,925	1111	1870	0,002	−1.00
Dotriacontane (10542)	0,070	0013	0,053	0013	0,722	1147	1279	0,001	−0.40
Erythrose (84990)	0,166	0030	0,731	0289	0,806	1277	1955	< 0,001	2.14
Glycerol (733)	0,007	0,006	0,023	0,014	0,860	1034	1852	0,005	1.72
Hentriacontane (11904)	0,605	0202	1007	0,483	0980	1574	0,834	< 0,001	0.74
Methyldodecanoic acid (92948)	0,045	0011	0,097	0010	0,987	1582	4830	< 0,001	1.11
Methylmaleic acid (553689)	0,078	0026	0,033	0011	0,926	1322	1731	< 0,001	−1.24
Methylglutaric acid (11549)	0,071	0022	0,043	0007	0,844	1184	1287	0,001	−0.72
Methyltetradecanoic acid (90098)	0,317	0021	0,840	0302	0,799	1010	1731	0,007	1.41
Monopalmitin (110006)	0,165	0058	0,220	0063	0,935	1472	0,873	< 0,001	0.42
Octacosane (11902)	0,046	0017	0,087	0035	0,921	1318	1201	< 0,001	0.92
Octadecane (11145)	0,020	0011	0,077	0024	0,982	1571	2364	0,000	1.95
Octadecenoic acid (393217)	< 0,001	< 0,001	0010	0,009	0925	1467	1125	< 0,001	3.32
Octanoic acid (370)	0,204	0192	0,019	0013	0,903	1273	0,969	< 0,001	−3.43
Oxalate (946)	0,027	0008	0,113	0020	0,986	1572	4247	< 0,001	2.07
Pentanediol (133167)	0,014	0013	0,028	0008	0,965	1580	1033	< 0,001	1.00
Tetradecanol (10714572)	0,016	0003	0,047	0013	0,991	1482	2380	< 0,001	1.56
Threonine (6051)	< 0,001	< 0,001	0007	0,008	0859	1229	0,891	< 0,001	2.81
Valine (6050)	< 0,001	< 0,001	0033	0,041	0872	1272	0,822	< 0,001	5.04

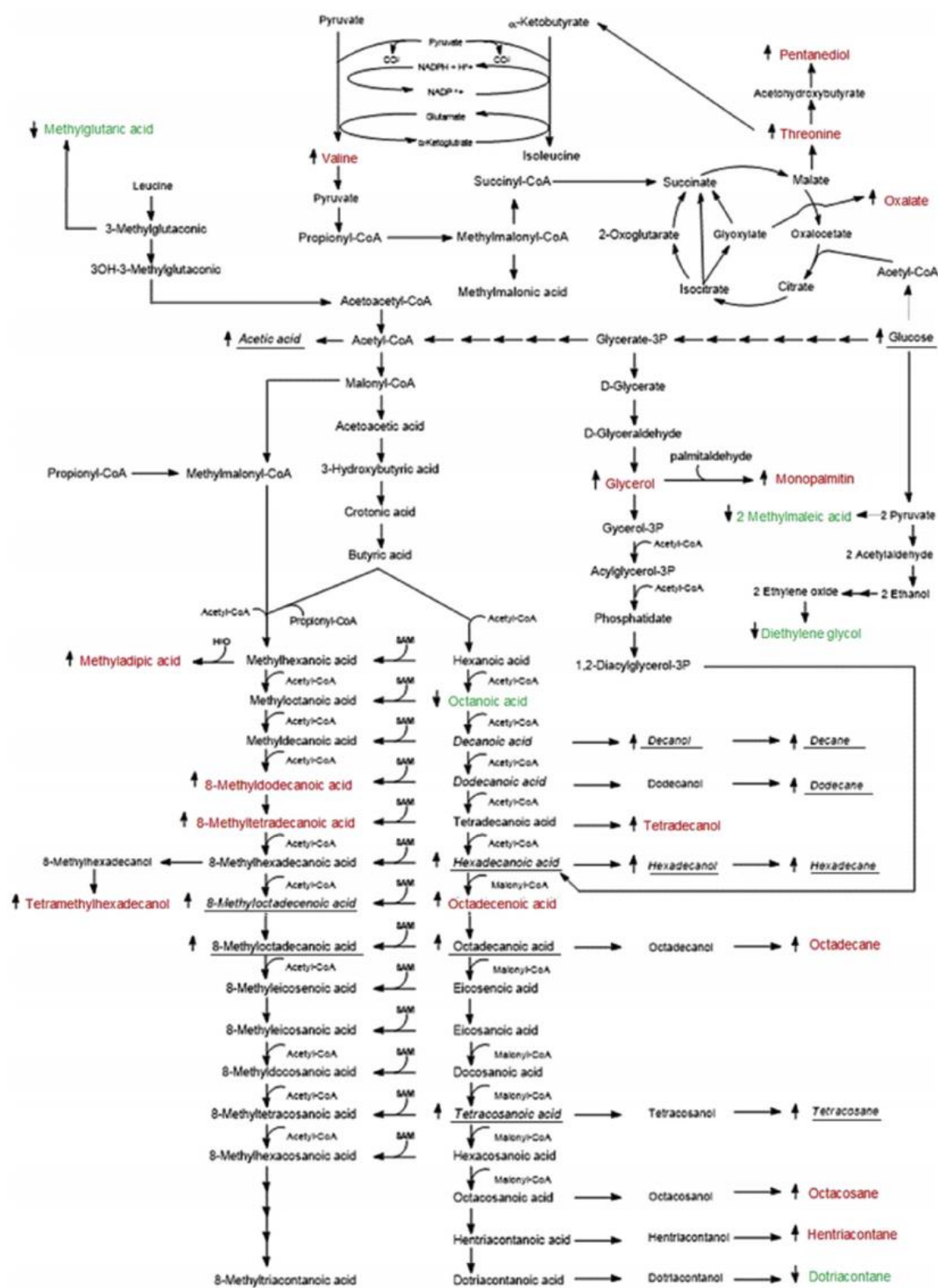
considered virulence factors in the microbe [46]. As indicated in Fig. 3, these methylated cell wall intermediates are synthesised via 3 possible routes: 1. fatty acid methylation via S-adenosylmethionine (SAM) functioning as the methyl donor [45], 2. methylmalonyl-CoA derived polyketide synthase complexes, originating from propionyl-CoA and malonyl-CoA [47] and 3. acetyl-CoA metabolism to butyric acid, which in turn reacts with propionyl-CoA [48]. Additionally, methyladipic acid was found elevated, which is formed from methylhexanoic acid, one of the metabolites in the branched fatty acid synthesis pathways of *Mtb* [49].

Similarly as to what we previously saw for the CS treated *Mtb* [42], glycolysis and its associated pathways are also upregulated in the CMS treated *Mtb*. In our previous metabolomics investigation using CS treated *Mtb*, elevated levels of glucose, acetic acid and oxalic acid were detected [50]. This suggests that the CS treated *Mtb* needed to preferably utilize fatty acids towards cell wall repair, and subsequently these organisms need to resort to glucose (which was freely available in the growth media) as the primary energy substrate [51]. Similarly, in the CMS treated *Mtb* in the current investigation, elevated levels of oxalate were also detected (Table 1) in addition to elevated glucose (1.21 vs. 0.88 µg/ml; $P > 0.05$) and acetic acid (0.218 vs. 0.102 µg/ml; $P > 0.05$) when considering significance using the latter two compounds P-values. Additional evidence supporting this and indicated in Table 1 and Fig. 3, where elevated levels of valine, threonine, and pentanediol, which also suggests a shift towards glucose utilization for energy and fatty acid synthesis in the CMS treated *Mtb*. Furthermore, the elevated levels of acetic acid (or acetyl-CoA) can subsequently result in the elevated synthesis of threonine and pentanediol, detected in the CMS treated *Mtb* [52]. Also associated directly with this pathway, is elevated valine synthesis from pyruvate [53], which feeds into the tricarboxylic acid (TCA) cycle via succinate [54]. Another branch chain amino acid metabolic pathway affected by CMS in *Mtb* is that of leucine's catabolism to acetyl-CoA, and the reduced amounts of 3-methylglutaric acid attests to an increased flux in this direction [55] and the subsequent synthesis of cell wall fatty acids or energy [56]. The reduced concentrations of methylmaleic acid [57] and diethylene

glycol in the CMS treated *Mtb*, serve as further confirmation for the flux of glucose utilisation for growth and fatty acid synthesis via glycerol and monopalmitin, both of which were elevated in the CMS treated *Mtb* comparatively (Table 1 and Fig. 3).

An interesting observation in the CMS treated *Mtb*, where two metabolite markers associated with the pentose phosphate pathway namely, the reduced arabinose and the elevated erythrose (Table 1). As indicated in the supplementary figures, due to the high demand in fatty acid synthesis for cell repair, and an increased demand for this and energy production via glycolysis, the pentose phosphate pathways is most likely additionally utilised during such conditions for generating more intermediates for glycolysis [58], with the reduction in arabitol and subsequent increase of erythrose, indicating a metabolic flux towards glyceraldehyde-3-phosphate and fructose-6-phosphate synthesis, something which wasn't previously seen in the CS treated *Mtb* [59]. A study conducted by Henry et al. (2015), indicated differentiation of gene expression following colistin treatment. These results are consistent with that found in the current study, which also suggests that colistin treatment alters the outer membrane composition and results in subsequent damage to the outer membrane of *Mtb*, as previously described [60].

Additionally, the alterations made by CMS to the *Mtb* cell membrane, results in it becoming less hydrophobic, hence it could be suggested that CMS could be used in synergy with other hydrophilic drugs, which when administered alone, usually struggle to cross these bacterial membranes. This has been previously observed by Bax et al. (2015) and van Breda et al. (2015). It is possible that disruption of the hydrophobic barrier of *Mtb* by INH (inhibiting mycolic acid synthesis) or CS from CMS, could lead to a greater uptake via the self-promoted uptake for CS causing a synergistic effect. In the case of INH, disruption of the hydrophobic barrier can lead to an uptake of hydrophilic INH. According to Nasiruddin, Neyaz, & Das (2017), a promising drug delivery model could be to encapsulate the hydrophilic drugs to be carried over the membrane, however in synergy with CMS, this may no longer be required [61]. Proof for this hypothesis, are the results by Al-Shaer, Nazer, & Kherallah (2014), where a combination therapy approach of



(caption on next page)

Fig. 3. Metabolite markers best describing the variation in the metabolome of the CMS treated *Mtb* compared to that of *Mtb* cultured without CMS, are schematically represented in bold and those metabolites which were not necessarily significantly elevated using the statistical procedure selected, but still showed significance via considering their P-values, indicated in italics and underlined. Elevated and reduced concentrations of each metabolite marker indicated by either ↑ or ↓ and red and green respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

rifampicin and CMS was used against MDR *A. baumannii*, which resulted in the successful treatment of 64% of the patients, with very few side effects [62]. Unfortunately, the results of these investigations are limited and no effect is given on the clinical outcomes of rifampicin induced - hepatotoxicity.

4. Concluding remarks

The most significant metabolite markers identified in this investigation, were the elevated concentrations of various fatty acids indicating a shift towards fatty acid synthesis and cell wall repair in the CMS treated *Mtb*. This is accompanied by an increase in glucose utilisation for energy and an additional flux towards the upregulation of the glyoxylate cycle (a precursor for cell wall fatty acids via the glycerolipid metabolic pathway), similarly to what was previously seen when treating *Mtb* with CS. Further confirmation of this shift towards glucose as an energy source, and unique to this investigation, is the utilisation of the pentose phosphate, valine, threonine, and pentanediol pathways for this purpose also. Considering this, it might be possible to use CMS with other first or second line anti-TB drugs (likely only hydrophilic ones). The feasibility, however, to treat both drug sensitive and MDR-TB using lower drug concentrations is subject to further clinical trials as it is not known if CMS would be able to successfully target intracellular *Mtb*.

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Transparency declarations

None to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.tube.2018.06.008>.

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