

CHAPTER 8

Discussion and conclusions

1. INTRODUCTION

Due to the fact that an extensive discussion of the results has already been done in each of the previous chapters, Chapter 8 will only summarise the main findings of the study and discuss recommendations regarding the future applications of the developed methodology.

The aim of this study was to develop methodology and investigate the capacity of metabolomics as a tool for better characterising TB. This aim was 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 metabolite marker identification for the purpose of better characterising rifampicin-resistance conferring *rpoB*-mutations in *M. tuberculosis*.
3. The application of the relevant developed methodology in objective 1 to metabolite marker identification for the purpose of better characterising a pulmonary TB disease state in humans using patient collected sputa.

2. SUMMARY OF THE MAIN FINDINGS

As a part of this study, a broad literature review was published addressing the advantages and shortcomings of the currently available TB vaccination, diagnostic and treatment approaches. This review, furthermore, discusses the potential of metabolomics as a tool to better understand the underlying mechanisms of TB and various TB-causing bacteria, which may aid in the development of innovative approaches to control this pandemic.

The first objective of this study led to the analytical refinement of the well-known Bligh-Dyer fatty acid extraction procedure for metabolomics applications. This refinement included the

modification of the method using a vibration mill to enhance the extraction capacity, allowing for a total extraction and derivitisation time of merely 5 hours, which is a vast improvement on the 16 hours required for the completion of the original method. By using this approach, the total solvent volume required for the complete extraction of the fatty acid metabolome was reduced to no more than 1.75 mL, significantly lowering its cost and complexity. In addition, an alternative approach, using a similar extraction principal, however with solvent combinations capable of extracting metabolites belonging to all compound classes including, amongst others; fatty acids, amino acids, alcohols, organic acids, monosaccharides, alkenes, alkanes, purines, pyrimidines etc., was developed. Comparatively, this "total metabolome" extraction procedure has advantages over the fatty acid extraction procedure, previously described, in the sense that it is: 1) simpler, and requires only 1.25 mL of solvent; 2) faster (takes 4 hours); 3) more repeatable, 4) isolates the total metabolome, bettering the chance for identifying more metabolite markers, and; 5) has better detection limit. Both of these extraction methods however, were capable of extracting compounds of biological relevance and were clearly capable of differentiating between various *Mycobacterium* species and *P. aeruginosa* samples groups, using a metabolomics research approach. Although the biological relevance of the most of the metabolite markers identified could be justified from published literature, a number of compounds, never before recognised as markers for these bacterial species, were also identified.

For the completion of objective 2 of this study, we applied the developed methodology (objective 1) to investigate the effect of two different rifampicin-resistance conferring *rpoB* mutations (S522L and S531L) on the fatty acid metabolome of *M. tuberculosis*. Multivariate statistical data analyses of the GC-MS generated metabolite data indicated a natural differentiation between both *rpoB* mutants and the wild-type parent strain on the basis of their differing fatty acid profiles. Subsequently, those metabolites contributing most to the variation between the groups were identified. The interpretation of the identified metabolite markers suggests that mutations to the *rpoB* gene of *M. tuberculosis* result in a reduced synthesis of various 10-methyl branched-chain fatty acids and other cell wall lipids, accompanied by an increased utilisation of the shorter-chain fatty acids and alkanes as carbon sources. Furthermore, the metabolite markers identified in the *rpoB* S531L *M. tuberculosis* mutant, previously reported to occur in well over 50% of all clinically identified rifampicin-resistant strains, suggest a comparatively better capacity for using these alternative energy sources, than the *rpoB* S522L mutant. Therefore, this metabolomics study is the first of its kind to indicate the effects of two *rpoB* mutations, and the role of the β -subunit of RNA-polymerase, on the fatty acid metabolism of rifampicin-resistant *M. tuberculosis*. Furthermore, this study proves the

capacity of the developed metabolomics research approach to identify previously unknown metabolite markers, never before associated with rifampicin-resistance.

Objective 3 of this study investigated the capacity of the developed methodology for applications to metabolomics analyses of patient collected TB-positive and TB-negative sputum samples, which are typically collected for the clinical diagnosis of this disease. Due to the inconsistent composition and fluidity of these samples, four sputum pre-extraction preparation methods, including three standard *Mycobacterium* cell isolation procedures (Sputolysin, NALC-NaOH, and NaOH) and a fourth, applying only a simple ethanol homogenisation step, prior to direct sputum extraction, were compared. This comparison was done to improve the quality of the sample aliquots, prior to extraction and metabolomics analysis, in order to attain repeatable results. When comparing the three cell isolation methods, the NALC-NaOH method allowed for the best comparative extraction efficiency, repeatability, and differentiation capacity, when used in combination with the previously developed metabolomics methods. The ethanol homogenisation sputum pre-extraction preparation method, however, outperformed all the investigated cell isolation methods, based on the aforementioned criteria. The compounds contributing most to the differentiation between the *M. tuberculosis* spiked and control sputum pool samples were, once again, identified, showing increased amounts of various characteristic mycobacterial cell wall components in the *M. tuberculosis* spiked sputum samples. The MACPs and fatty acid markers detected using this methodology, further validates the capacity of this metabolomics approach for identifying *M. tuberculosis* metabolites, despite potential background "noise" caused by those compounds extracted from the sputum matrix.

Subsequently, the developed metabolomics methodology was applied to identify those metabolite markers characterising active TB in humans, using patient collected TB-positive and TB-negative sputum samples. Due to the large variation in the individual patients' sputum metabolite profiles, an innovative approach, using various multivariate and univariate statistical methods, was employed in order to eliminate underlying metabolite "noise" and outlier patient samples from the data set. Those variables best describing the variation between the TB-positive and TB-negative patient sputum samples were subsequently identified. The metabolite markers characterising the TB-positive patient sputum group included: 1) those detected for the isolated *M. tuberculosis* cultures in the previous chapters, confirming these results; 2) *M. tuberculosis* markers related to *in vivo* growth and; 3) metabolite markers suggesting alterations to the human host metabolome due to the active TB disease state. The interpretation of these new metabolite markers led to a number of scientific contributions, including: 1) confirmation of the previously proposed citramalate cycle in *M. tuberculosis*; 2) the interaction of this cycle with

an up-regulated glyoxylate cycle during pulmonary *M. tuberculosis* infection, 3) the increased utilisation of fatty acids and glutamate as alternative carbon sources in *M. tuberculosis* during pulmonary infection; 4) an alternative mechanism by which the host produces hydrogen peroxide via glucose oxidation, in order to eliminate the bacterial infection; 5) inhibition of the ETC due to pronounced oxidative stress during an active TB disease state, resulting in increased concentrations of various neurotransmitters and other metabolites previously associated with an inborn error of metabolism (MADD / GA type II); and 6) elevated concentrations of neurotransmitters potentially explaining a number of symptoms associated with TB.

3. FINAL REMARKS AND FUTURE RECOMMENDATIONS

This study contributes significantly to the application of metabolomics via metabolite marker identification for better characterising and understanding various aspects related to TB. These aspects included: 1) a better characterisation of various *Mycobacterium* species causing pulmonary infections in humans; 2) a better understanding of rifampicin-resistance conferring *rpoB* mutations and its impact on the fatty acid metabolome of *M. tuberculosis*, which subsequently generated a variety of new hypotheses; 3) confirmation of the existence of previously suggested metabolic pathways in *M. tuberculosis*, and; 4) the identification of an array of oxidative stress related metabolite markers and associated mechanisms, never before described or proposed to exist within TB infected humans. Using this approach, one could similarly investigate various other aspects of TB, TB causing bacteria or drug-resistant TB, using either cells isolated from culture, sputum, or any other related biological sample, including blood, urine and tissue. We were additionally able to use the identified metabolite markers to differentiate between a number of TB related and unrelated infectious bacterial species, and to discriminate between TB-positive and TB-negative patient collected sputum samples. This study, therefore, proves the capacity of a metabolomics research approach, not only for characterising various aspects of a TB disease state, but also for potential diagnostic applications using the identified markers, alone, or in combination. Considering this, metabolomics may open the door to a new era in TB research, diagnostics and drug development, based on new metabolite marker identification. Alternatively, other disease states could also be investigated in this manner.