

CHAPTER 7

Characterising TB using patient collected sputum and GCxGC-TOFMS metabolomics

1. INTRODUCTION

In the previous chapter, a simple ethanol homogenisation, prior to extraction and GCxGC-TOFMS analysis, proved to be the best way to process and analyse sputum for metabolite marker identification, using a metabolomics research approach. Using this approach, we were able to differentiate between *M. tuberculosis* spiked and control sputum samples, at a detection limit of only 10 cells per analyses. Those metabolites that best explain the naturally occurring separation between the two sample groups were then identified as potential metabolite markers, characterising *M. tuberculosis* infection. Most of these markers were representative of *M. tuberculosis* cell wall components, proving that this approach is capable of extracting those compounds characteristic of *M. tuberculosis*, and consequently allowing for differentiation of sputum containing this organism, from sputum that doesn't. The methodology described in Chapter 6 does not, however, take the expected variation, which would undoubtedly be present in sputum collected from such a patient cohort, into account. Additionally, the metabolite markers identified in the previous chapter were exclusively detected due to the presence of *M. tuberculosis* in these spiked samples, and although they should be present in TB-positive patient sputum, they may not necessarily represent those markers representative of an altered host metabolome, induced by a *M. tuberculosis* infection. Consequently, we used the previously developed methodology to analyse 95 patient collected sputum samples, in order to determine if this approach could be used to differentiate between TB-positive and TB-negative samples. Furthermore, the characteristic markers of the TB-positive patient sample group were identified and compared to those previously determined in Chapter 6. Using this approach, we were able to confirm which of these markers were detected due to the presence of the *M. tuberculosis* in these patient samples, and which of these markers were detected due to TB-related alterations to the human host metabolome. These metabolite markers may additionally propose "new" mechanisms by which this disease influences its human host.

A number of studies have investigated the viability of various sophisticated chromatographic techniques to differentiate TB-positive from TB-negative patient sputum samples, using a variety of sample preparation methods (Cha *et al.*, 2009; Kaal *et al.*, 2009; Stopforth *et al.*, 2004; Syhre *et al.*, 2009). Most of these studies, however, are targeted analysis of the well-known TB biomarker, tuberculostearic acid (TBSA). To our knowledge, only one research group has applied an untargeted research approach to investigate TB using patient collected sputum samples (Fend *et al.*, 2006). Using an electronic nose, this group was able to differentiate between TB-positive and TB-negative sputum samples with a reported sensitivity of 89% and a specificity of 91%, at a detection limit of 1×10^4 bacteria mL⁻¹. However, as the

nature by which these sensors function is not yet fully understood, the identity and quantities of the differentiating volatiles cannot be determined, which may be seen as a limitation of this sensor array technology

Considering all the above, the current study is the first metabolomics approach to develop an extraction method to comprehensively describe and identify the characteristic TB markers, using GCxGC-TOFMS analysis, from patient collected sputum samples.

Aim: To use the previously developed methodology from Chapters 5 and 6, in order to identify those metabolite markers characterising TB, using patient collected sputum.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

Methoxyamine hydrochloride and 3-phenyl butyric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Pyridine, TMCS and MSTFA were purchased from Merck (Darmstadt, Germany). All organic solvents used were ultra pure Burdick & Jackson brands (Honeywell International Inc., Muskegon, MI, USA).

2.2 Sputum samples

In order to accomplish the aim of this chapter, sputum from 95 patients suspected of having TB, were analyzed using the methodology developed in the previous chapters. Of these samples, 61 were culture proven as TB-negative and 34 were culture proven as TB-positive. The full description of the collection procedures, ethical approval, and clinical data collected, is discussed in Chapter 6 section 2.2.

2.3 Extraction procedure

Each patient collected sputum sample (500 μL) was processed using the ethanol homogenisation method previously described (Chapter 6 section 2.4.4), followed by the addition of 150 μL of 3-phenyl butyric acid (0.525 mg mL^{-1}) as internal standard, prior to extraction using the total metabolome extraction procedure (described in Chapter 5 section 2.4).

2.4 GCxGC-TOFMS parameters

The extracted samples were randomly analysed by injecting 1 μ L of each onto the GCxGC-TOFMS. Chromatographic analyses were done using identical analytical settings and temperature programs as that described in Chapter 6 section 2.5.

2.5 Peak identification and alignment

Mass spectral deconvolution, as well as peak finding, alignment and identification, was done using Leco Corporation ChromaTOF software (version 4.32) as described in Chapter 6 section 2.6, with the exception of deconvolution at a 300 S/N ratio.

2.6 Statistical data analysis

Statistical analysis of the GCxGC-TOFMS generated data of the extracted patient collected sputum samples, using MetaboAnalyst, involved the following steps:

1. Determining whether a natural differentiation exists between the patient collected TB-positive and TB-negative sample groups on the basis of their extracted metabolite profiles, using PCA;
2. Identifying the characteristic metabolite markers best explaining the variation between the TB-positive and TB-negative sputum samples, using a combination of supervised univariate (*t*-test and fold-change) and multivariate (PLS-DA and random forest) statistical analyses (Madsen *et al.*, 2010). The *t*-test compares the normalised means of the specific compounds between the two sample groups, whereas fold change compares absolute value changes between the two group means. Random forest is a learning algorithm which uses a collection of classification trees, each grown from a random selection of compounds from a bootstrap sample at each branch. Class prediction is then based on the majority vote of the selection. Metabolite markers are then selected based on the classification error when it is permuted. Random forest analyses can also be used to identify outlier samples based on the proximities during tree construction. The principle by which the PLS-DA and the resultant VIP parameter functions, has been described previously (Chapter 3 section 2.6).

3. RESULTS AND DISCUSSION

After spectral deconvolution, peak identification and alignment, and data matrix compilation, a total number of 1055 different compounds from various compound classes, including amongst others; fatty acids, amino acids, alcohols, organic acids, and monosaccharides, were detected using data obtained after GCxGC-TOFMS analyses of all the patient sputum extracts. Due to the larger variation in the metabolite composition of the patient collected samples (as compared to the previously prepared sample groups used for the method development in the Chapters 5 and 6), metabolite marker identification using this sample cohort was not as straight forward as that previously described. The larger variation in the metabolite composition of these samples can be ascribed to variations in diet, social habits (smoking, drinking etc), *M. tuberculosis* load, other co-infections, medication, disease stages, cellular composition of the lesions, immune status, lung region affected etc. (Timm *et al.*, 2003). Consequently, this variation needs to be accounted for and eliminated (by removing the interfering metabolites and / or outlier samples), in order to achieve group separation and identify characteristic metabolite markers. This database cleaning step is especially important when considering that newer research apparatus are continually being developed with an increased capacity to identify a greater number of compounds. Although these analytical improvements increase the possibility of biomarker identification, it also allows the identification of more “noise” or interfering compounds. The procedure we used for database clean-up, prior to metabolite marker identification, will subsequently be described, followed by the interpretation of the markers identified.

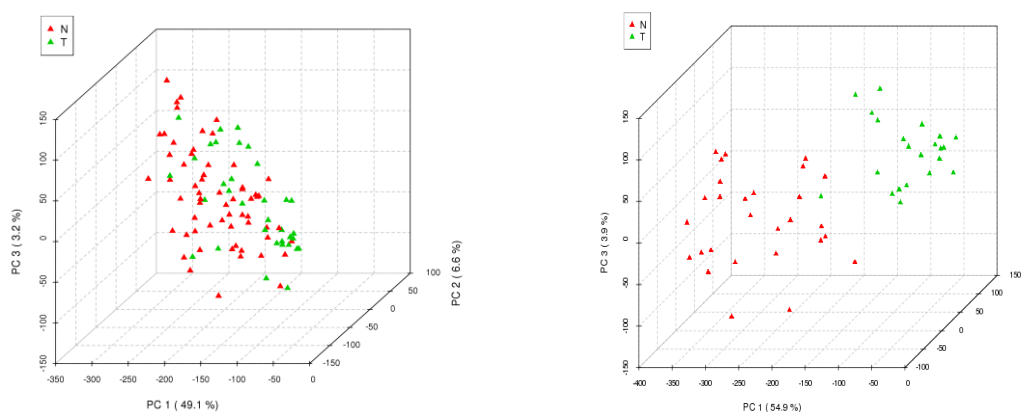
3.1 Differentiation of the patient collected TB-positive and TB-negative sputum samples on the basis of their detected metabolite differences

Considering the PCA results using all 1055 of the GCxGC-TOFMS detected metabolites for all 95 extracted patient sputum samples, no natural discrimination between the TB-positive and TB-negative groups was evident (Figure 7.1a). This overlap is most likely due to the large variation in the study population described above, resulting in variation in the detected metabolites (metabolite “noise”). This noise may result in outliers in both groups, confounding a clear separation of the groups when analysed using PCA. We subsequently used the limited clinical information accompanying the diagnostic samples collected, in order to determine the origin of this noise, but no batch effect related to HIV status, age, or gender occurred (results not shown). This, however, does not rule out the fact that a combination of one or more of the above mentioned or other clinical occurrences, of which we have no information, could

potentially result in this variation. Considering this, it is important to highlight the need for as much as possible clinical information of the patients from which the samples are collected for metabolomics analyses, which could be used as inclusion and exclusion criteria, in order to better define the groups prior to extraction and analysis of the data. Despite the lack of this information, which was beyond our control, and in order to accomplish the primary aim of this metabolomics investigation, which is the identification of potential metabolite markers better characterising TB, a sample sub-cohort of the investigated population was selected using a number of statistical tools, filtering out interfering metabolites and outlier samples.

Using the random forest, unsupervised, multivariate statistical method, 45 out of the 95 analysed samples (47%), was identified as outliers to the specific sample groups (46% of the TB-negative, and 66% of the TB-positive samples). After exclusion of these outliers, the PCA showed a clear differentiation between the two sample groups based on the detected metabolite profiles of each of the selected sub-cohorts (Figure 7.1b), with a 100% individual sample prediction to their respective sample groups, using the random forest analyses.

However, by discarding almost half of the original samples for the selection of this sub-cohort, one may potentially introduce a certain degree of bias, and additionally lose valuable metabolite and contributing patient information.



(a) Total dataset including all analysed samples and all detected variables **(b)** Total dataset comprising of all detected compounds, after the removal of outlier samples identified using random forest analyses

Figure 7.1: PCA scores plots of the GCxGC-TOFMS generated data, showing TB-negative (N) vs. TB-positive (T) patient collected sputum samples before the removal of 'noise' and interfering compounds from the dataset.

Consequently, in order to use the maximum number of samples from the original sample cohort, an alternative statistical approach (Figure 7.2) was used for metabolite noise reduction and sample outlier removal, prior to the identification of the characteristic TB metabolite markers.

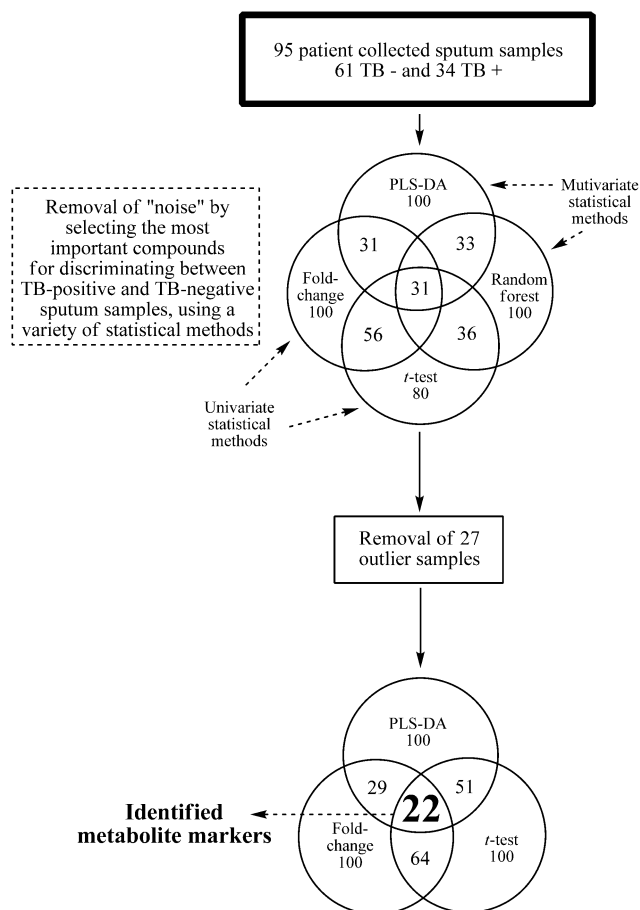
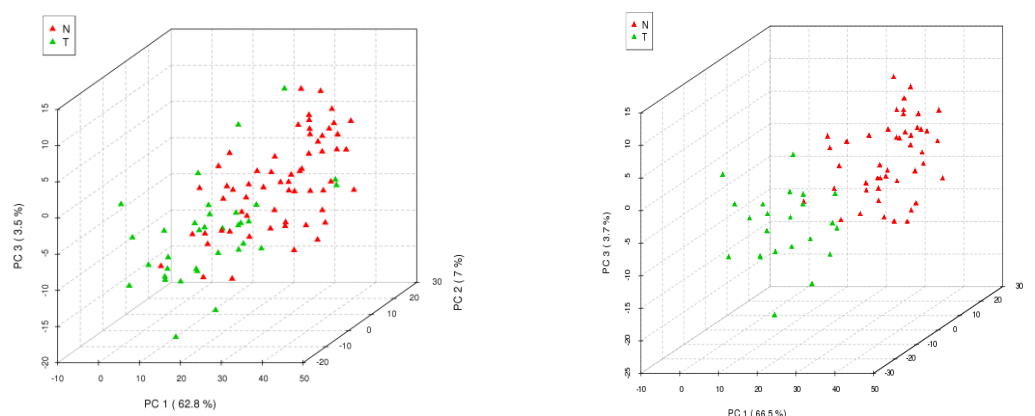


Figure 7.2: Statistical approach used for metabolite “noise” reduction and removal of outlier samples, in order to identify metabolite markers best explaining the variation between TB-positive and TB-negative sputum samples.

As shown in figure 7.2, a number of supervised univariate (*t*-test and fold-change) and multivariate (PLS-DA and random forest) statistical data analyses methods were initially used to select those metabolites which differed the most (considering their novelty and relative concentrations) when comparing the GCxGC-TOFMS data from the extracted TB-positive and TB-negative sputum samples. The top 100 ranked metabolites identified by each of these methods (with the exception of the *t*-test, in which case only those compounds with a *p*-value below 0.05 were selected) were compared, and those common to all four methods (31 compounds), were subsequently selected. Using only these 31 metabolites detected in all 95 of

the analysed samples in the original sample cohort, partial separation between the TB-positive and TB-negative sample groups were achieved (Figure 7.3a).



(a) Data set comprising of all analysed samples after the removal of “noise” compounds.

(b) Dataset comprising of all detected compounds, after the removal of outlier samples identified using random forest analyses

Figure 7.3: PCA scoresplots of the GCxGC-TOFMS generated data, showing TB-negative (N) vs. TB-positive (T) patient collected sputum samples after the removal of ‘noise’ compounds from the dataset.

Random forest analysis, using this noise reduced data set, identified 27 outlier samples (28% of the total sample group, comprising of only 26% of the TB-negative and 32% of the TB-positive samples). The removal of these outliers resulted in a clear separation of the TB-positive and TB-negative patient sample groups using PCA, with a 100% individual sample prediction to each of their respective sample groups using the random forest analyses (Figure 7.3b). This approach served only as a means to reduce the noise in order to better identify and remove the outlier samples from the original sample cohort. Once the outliers were removed, all 1055 metabolites detected for the remaining 68 sample patient cohort, were processed using the previously mentioned supervised univariate (*t*-test and fold-change) and multivariate (PLS-DA) data analyses methods, in order to select those compounds best describing the variation between the two sputum sample groups (Table 7.1). Since random forest analysis was used to eliminate the outlier samples for the selection of the currently used sub-cohort, this method was not used again to identify potential metabolite markers.

3.2 Potential TB metabolite markers

Using the data collected up to this point, considering the results from all the previous chapters (Chapter 3, 4, 5 and 6), the 22 metabolite markers identified in the TB-positive patient collected sputum samples, using this metabolomics approach, could be classified according to their various origins including those detected due to: 1) the physical presence of *M. tuberculosis* in these sputum samples; 2) *in vivo* growth conditions of *M. tuberculosis*; and 3) markers related to the host's response to infection (Table 7.1). It should be noted that all conclusions made are based on hypotheses, which should be further investigated in future, using a number of targeted research approaches.

As previously indicated from the experiments using the *M. tuberculosis* spiked sputum in Chapter 6, it can be concluded that the D-glucosamine, N-acetylglucosamine, nonadecanoic acid (C19:0), oleic acid (C18:1 ω 9c), 2-deoxy-d-erythro-pentitol, D-glucopyranose, D-glucopyranoside, D-galactose and most likely also the D-mannopyranoside, L-mannopyranose and C17:1 ω 7c, detected in elevated concentrations in TB-positive sputum samples, are due the actual presence of *M. tuberculosis* in these samples. The roles that these metabolites play in various metabolic processes, including the synthesis of vital and characteristic cell wall components of these organisms, are comprehensively discussed in Chapter 6. An interesting observation is that the almost universally detected TB biomarker, TBSA, was not identified as a metabolite marker for the TB-positive sputum samples. Although this compound was detected, its concentration was not sufficient to be selected as a differentiating marker using the statistical methods described.

Table 7.1: Metabolite markers best describing the variation between TB-positive and TB-negative sputum samples.

Compound	TB-negative		TB-positive	
	Relative concentration ($\mu\text{g mL}^{-1}$ sputum) (standard deviation in parenthesis)	No. of samples in which marker detected (total n=45) (% samples in parenthesis)	Relative concentration ($\mu\text{g mL}^{-1}$ sputum) (standard deviation in parenthesis)	No. of samples in which marker detected (total n=23) (% samples in parenthesis)
<i>M. tuberculosis</i> components				
à-D-glucopyranose-2-acetylamino-2-deoxy	0.121 (0.120)	45 (100)	1.29 (1.85)	23 (100)
à-D-glucopyranoside	0.444 (1.129)	26 (57)	1.297 (3.081)	19 (82)
à-L-mannopyranose	0.049 (0.085)	15 (33)	0.120 (0.111)	17 (73)
à-D-mannopyranoside	0.275 (1.236)	23 (51)	0.396 (0.639)	21 (91)
D-galactose-6-deoxy	35.44 (52.48)	44 (97)	158.1 (145.2)	23 (100)
à-D-galactopyranose	0.308 (0.690)	25 (55)	0.488 (0.328)	22 (95)
D-glucosamine	0.054 (0.112)	12 (26)	1.230 (3.336)	15 (65)
N-acetyl-glucosamine	1.976 (2.341)	44 (97)	22.95 (16.89)	23 (100)
Methyl-17-methyl-octadecanoic acid	0.058 (0.171)	15 (33)	0.094 (0.107)	17 (73)
C17:1 ω7c	0.062 (0.087)	20 (44)	0.249 (0.221)	18 (78)
C18:1 ω9c	0.038 (0.112)	12 (26)	0.173 (0.435)	13 (56)
C19:0	0.024 (0.062)	7 (15)	0.127 (0.124)	15 (65)
2-deoxy-d-erythro-pentitol	0.021 (0.055)	7 (15)	0.204 (0.374)	15 (65)
In vivo growth <i>M. tuberculosis</i> markers				
d-citramalic acid	0.028 (0.070)	17 (37)	0.071 (0.099)	18 (78)
Host response markers				
d-gluconic acid δ-lactone	0.054 (0.090)	20 (44)	0.143 (0.155)	19 (82)
Glutaric acid	0.052 (0.136)	15 (33)	0.078 (0.069)	17 (73)
Sebacic acid	0.005 (0.004)	12 (26)	0.133 (0.191)	14 (60)
Ethane	0.031 (0.060)	16 (35)	0.365 (0.998)	14 (60)
Butanal	0.048 (0.110)	20 (44)	0.228 (0.234)	20 (86)
γ-aminobutyric acid (GABA) (also part of bacterial metabolism)	0.715 (3.050)	14 (31)	0.814 (0.834)	17 (73)
3,4-dihydroxybutanoic acid	0.024 (0.032)	25 (55)	0.077 (0.069)	19 (82)
Normetanephine	0.966 (2.765)	31 (68)	4.712 (7.126)	19 (82)

The increased concentrations of citramalic acid (an analog of malic acid) detected in the TB-positive sample group in this metabolomics study, supports a hypothesis postulated by Savvi (2008). In this hypothesis, it was proposed that the citramalate cycle, previously identified in various other organisms (Ivanovsky *et al.*, 1997; Herter *et al.*, 2002; Textor *et al.*, 1997), is also present in *M. tuberculosis*. Considering this, the increased citramalic acid detected in the TB-positive patient sputum, is most likely explained by an increased synthesis of glyoxylate, due to an upregulated glyoxylate cycle in *M. tuberculosis* during pulmonary infection (Shi *et al.*, 2010). This increased glyoxylate, in turn, together with propionyl-CoA, serves as substrates for the citramalate cycle (Meister *et al.*, 2005), explaining the increased concentrations of this in the TB-positive patient sample group, relative to that of the TB-negative patient sample group (refer to Figure 7.4).

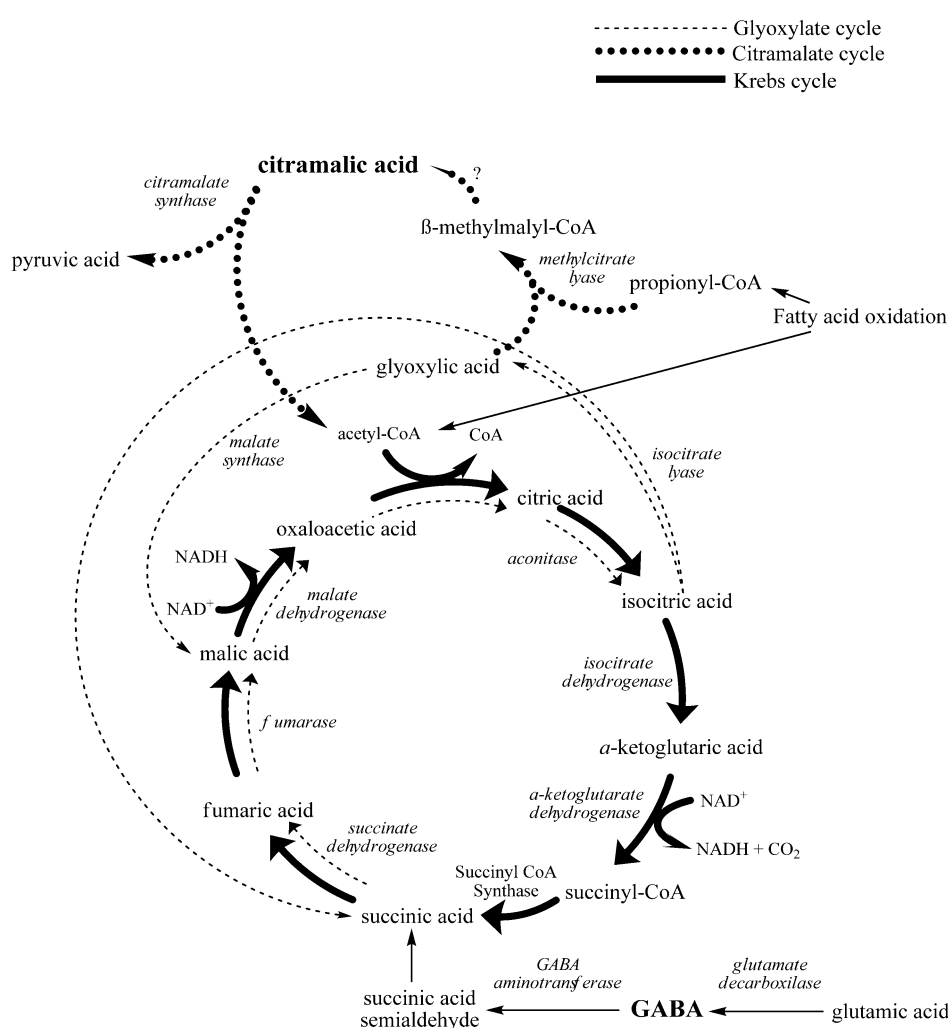


Figure 7.4: Schematic representation of the interaction between the glyoxylate, citramalate and Krebs cycles of *M. tuberculosis*. During pulmonary infection, both fatty acid oxidation and the glyoxylate cycle are up regulated in *M. tuberculosis*, leading to increased citramalic acid, through the up-regulated citramalate cycle, and GABA for the purpose of fuelling the up-regulated glyoxylate cycle.

Additionally, the increased glyoxalate cycle activity of *M. tuberculosis* previously mentioned to occur during pulmonary infection (Shi *et al.*, 2010), and the role by which glutamate serves to fuel this cycle through GABA, as an intermediate to succinic acid synthesis (Paley & Karp, 2006), could also explain the elevated concentrations of GABA in the TB positive patient sputum (Figure 7.4). Apart from being converted to malate, succinate can also be converted to propionyl-CoA via (R)-methylmalonyl-CoA, using the enzyme methylmalonyl-CoA mutase in *M. tuberculosis* (Paley & Karp, 2006), fuelling the citramalate cycle previously described.

The remainder of the metabolites identified most likely represent those which were detected due to an altered host metabolism, induced by the TB disease state, or metabolites excreted by the infectious organisms, altering the normal human metabolism. These include: d-gluconic acid δ -lactone; glutaric acid; sebacic acid; ethane; butanal; γ -aminobutyric acid (GABA); 3,4-dihydroxybutanoic acid, and; normetanephrine, which to date have never been identified or associated with TB.

The majority of the metabolites identified as potential TB markers in this group are related to oxidative stress. This increased oxidative stress is mainly a result of the production of predominantly hydrogen peroxide, and other reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), by the *M. tuberculosis* engulfing macrophages, as the primary mechanism by which the host attempts to eliminate the infection (Stenger and Modlin, 1999; Chan *et al.*, 2001). Hydrogen peroxide synthesis and the resultant oxidative stress, although functioning as a host defence mechanism against infective organisms, may also lead to host cellular and tissue damage by inducing lipid peroxidation and DNA damage, amongst other complications (Maxwell, 1995; Yu, 1994). These marker metabolites will subsequently be discussed with reference to their synthesis in the host in response to the TB disease, as graphically indicated in Figure 7.5.

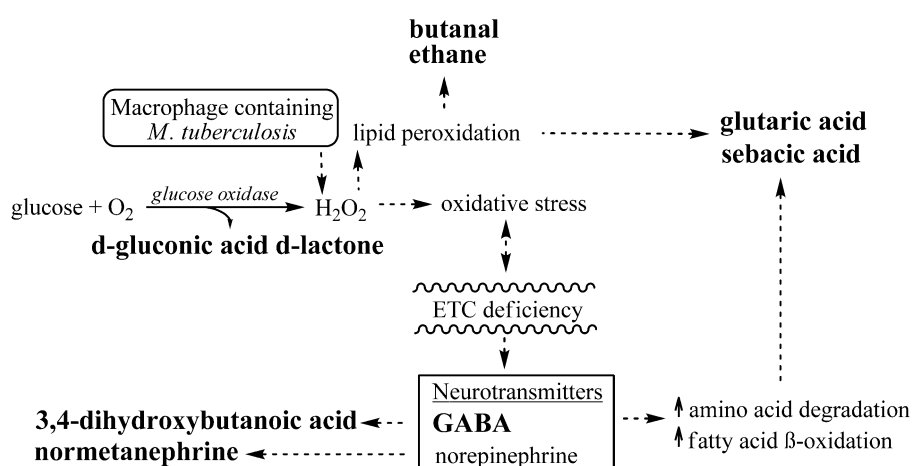


Figure 7.5: Proposed changes to the metabolism of the human host due to pulmonary *M. tuberculosis* infection. This diagram indicates elevated concentrations of various metabolites (those in bold), as a result of an increased production of H_2O_2 via glucose oxidation and direct synthesis from the infected macrophage, as a defence mechanism, resulting in increased oxidative stress and lipid peroxidation.

The presence of d-gluconic acid δ -lactone in elevated concentrations in the TB-positive sputum, leads to a number of speculations regarding alternative host induced mechanisms for eliminating these infectious organisms, or an additionally adding to the TB induced oxidative stress. D-gluconic acid δ -lactone is synthesised by the enzyme glucose oxidase, from glucose, in the presence of oxygen. Large amounts of hydrogen peroxide are also formed as a by product of this reaction (Ravid *et al.*, 2002). D-gluconic acid δ -lactone has also previously been detected in patients with diabetes (Lindsay *et al.*, 1997), thought to be formed due to the elevated blood glucose levels associated with this disease, with the resulting hydrogen peroxide synthesised, thought to contribute to the oxidative stress characteristics of this disease (Kawamura *et al.*, 1994). Considering this, the increased d-gluconic acid δ -lactone detected in the TB-positive sputum samples may be an additional means for synthesising hydrogen peroxide by the host, in order to eliminate the bacteria, or alternatively, may be induced by the elevated glucose levels, previously associated with tuberculosis (Oluboyo & Erasmus, 1990). This elevated glucose level may further be explained by the elevated normetanephrine levels detected in this metabolomics investigation, as will be discussed later.

Furthermore, hydrogen peroxide, and the associated ROS, is well known for its capacity to oxidise lipids, resulting in the formation of lipid hydroperoxides and damage to cell membranes in particular (Repine *et al.*, 1997). These lipid peroxidation products have previously been associated with a number of lung diseases including lung cancer, emphysema (Petruzzelli, *et*

al., 1990) asthma (Barnes, 1990), and chronic obstructive pulmonary disease (Repine *et al.*, 1997). Examples of these lipid peroxidation products are saturated dicarboxylic acids, formed as a result of the oxidation of *cis*-polyunsaturated fatty acids, as well as phospholipids and triacylglycerols containing *cis*-polyunsaturated fatty acids (Passi, *et al.*, 1993). These dicarboxylic acids have previously been identified as markers for increased oxidative stress in the urine of diabetic patients (Inouye *et al.*, 2000). Considering this, the two dicarboxylic acids, glutaric and sebacic acid, detected in elevated concentrations in the sputum of the TB-positive patients, are most likely formed as a result of the oxidative stress induced lipid peroxidation.

The aforementioned lipid hydroperoxides are also known to form a variety of by-products during their decomposition, including, amongst others, various hydrocarbons (Hageman *et al.*, 1992) and carbonyls (De Zwart *et al.*, 1997), explaining the increased detection of the ethane and butanal metabolite markers in the TB-positive sputum samples. Elevated hydrogen peroxide is additionally known to result in impaired Cl^- gradients and a reduced efficiency of the GABA_A receptor (Sah & Schwartz-Bloom, 1999), consequently resulting in the extracellular / systemic accumulation of GABA, and potentially its β -oxidation product 3,4-dihydroxybutanoic acid (Jakobs *et al.*, 1990), subsequently explaining the elevated concentrations of these two metabolites detected in the TB-positive sputum.

Normetanephrine, a derivative of norepinephrine, was also detected in almost 5 times higher concentrations in the TB-positive sputum samples, comparative to the TB-negative samples. In a study done by Alaniz *et al.* (1999), norepinephrine was shown to play a significant role in T cell-mediated immunity modulation during pulmonary *M. tuberculosis* and other lung infection in rats. The results of our investigations possibly confirm this occurrence in TB infected humans.

Furthermore, it is well known that ROS induces inhibition of the electron transport chain (ETC) (Loots *et al.*, 2004) which, in turn, reduces ATP production in the mitochondria. It has also been proposed that during ETC inhibition, the levels of various neurotransmitters increase as a compensatory mechanism, stimulating ATP production via the upregulation of carbohydrate, protein, and triacylglycerol catabolism (Reinecke *et al.*, in press). Complementing these findings, are the increased concentrations of the neurotransmitters, normetanephrine and GABA, which were detected in the TB-positive patients in this investigation. Inhibition of the ETC has also previously been associated with an increased production of glutaric and sebacic acid, mimicking a metabolic profile previously associated with an inborn error of metabolism, multiple acyl-CoA dehydrogenase deficiency (MADD) also known as glutaric acidemia type II (GA type II) (Loots *et al.*, 2004, Reinecke *et al.*, 2011).

Additionally, if the almost 5 times higher concentrations of the norepinephrine derivative, normetanephrine, in the TB-positive sputum is an indication of what is potentially occurring

systemically, it could also explain the weight loss, insomnia, glucose intolerance (additionally contributing to the increased d-gluconic acid δ -lactone detected) and night sweats (Lobue & Catanzaro, 1997) experienced by these *M. tuberculosis* infected individuals. Additional side effects associated with systemic norepinephrine include increased blood pressure and increased heart rate, and the association of these side effects in TB patients could also potentially be investigated.

4. CONCLUSIONS

This study highlights the variation in the individual metabolome information obtained when analysing patient sputum samples, which naturally exists due to a variety of external factors, and which cannot necessarily be compensated for beforehand. Despite this however, the capacity of a metabolomics research approach and the various statistical tools available for processing the data, allows for the removal or minimising of this variation by eliminating the underlying metabolite “noise” and possible outliers, in order to identify those markers which better characterise the perturbation being studied, as was accomplished in this investigation.

Subsequently, the markers identified in this study could be directly linked to: 1) the presence of the *M. tuberculosis* in these samples; 2) changes in the bacterial metabolome due to *in vivo* growth conditions and; 3) changes in the human metabolome due to pulmonary *M. tuberculosis* infection. The novelty of those markers identified in this study, which are directly linked to the presence of *M. tuberculosis* in these samples, lies in the fact that this untargeted metabolomics approach led to the simultaneous identification of a variety of cell wall components including fatty acids, mycolic acids and carbohydrates. This contribution becomes even more evident when one considers that previous research has only really contributed to this topic by the identification of TBSA and various mycolic acids. A further major contribution of this study was the identification of elevated citramalic acid and GABA, which confirms previous clues for the presence of a citramalate cycle in *M. tuberculosis*. The interactions of the citramalate cycle with the glyoxylate cycle, and the increased utilisation of fatty acids and glutamate as alternative carbon sources during pulmonary infection, is further suggested by the markers identified in this metabolomics investigation. Finally, considering those metabolites associated with a host response to TB infection, these compounds not only shed light on potential alternative mechanisms for eliminating the bacteria by the host through the production of hydrogen peroxide via glucose oxidation, but also suggest an inhibition of the ETC, leading to the increased concentrations of the neurotransmitters observed in the TB-positive samples. This increase in the neurotransmitters, especially norepinephrine, explains a number of symptoms associated with TB.

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