CHAPTER 6

CONCLUSIONS AND FUTURE PROSPECTS

6.1 STUDY AIMS AND OBJECTIVES: MOTIVATION AND ACCOMPLISHMENTS

The complexity of the mitochondrion and the diverse functionality of metallothioneins limit our understanding of their associations. The involvement of MTs in scavenging ROS and regulating enzymes and transcription factors involved in energy metabolism have been hypothesized and demonstrated, albeit mostly with *in vitro* models. The problems associated with *in vitro* models, such as the use of non-physiological amounts of MTs and metals, often lead to biased results not portraying the *in vivo* environment. The need to use systems biology technology to elucidate these associations *in vivo* have been recognised (Chapter 2) and have already been introduced in the field of metallothionein research by others. However, in addition to the lack of metabolomics investigations, the focus of the reported transcriptomics and proteomics studies were not orientated to elucidate the involvement of MTs in mitochondrial function. This then augmented the need to use metabolomics technology to investigate this putative association. Furthermore, the limitations of current *in vitro* and *in vivo* models, the limited reports focusing on this association and problems/bias associated with hypothesis-driven and focused investigations on this association (as mentioned in Chapter 3), verified the need for an untargeted metabolomics investigation using *in vivo* models such as MT knockout mouse models. The interplay of metabolites and metabolic pathways related to energy metabolism (which is generally ignored) with more targeted investigations, would then allow the generation of new hypotheses and/or the confirmation of existing theories with the addition of new concepts.

In light of the proposed limitations to existing knowledge and problems associated with MT- and mitochondrion-related research, two sub-studies were designed to get a novel perspective on the proposed association between MTs and mitochondrial function. An untargeted, hypothesis-generating metabolomics approach, using advanced metabolomics technology to fingerprint large areas of the metabolome, was selected to study the impact of MT knockout on the metabolism during unchallenged, challenged and diseased conditions. WT, MT1+2KO and MT3KO mice were

studied by challenging their mitochondrial metabolism with exercise, high-fat intake or both exercise and a high-fat diet. Putting strain on mitochondrial metabolism would thus show differences in respiration and oxidation rates, which could be linked to variance in metal cofactor supply/uptake to/from enzymes and transcription factors, variation in free radical scavenging of ROS produced during these interventions, and variance in cell signalling pathways. The involvement of MTs in mitochondrial function during diseased conditions was also studied by inhibiting complex I of the respiratory chain of WT and MT1+2KO mice. The role of MTs in the consequences associated with mitochondrial disease, e.g. reduced OXPHOS function and the consequential shift in redox state towards increased oxidative stress was thus evaluated.

This study was undertaken in an attempt to answer the following questions:

i. Are there any differences in the metabolism of MT1+2KO, MT3KO and WT mice which are reflected in the metabolome during unchallenged conditions?
ii. Are there any differences in the metabolism of these mice when mitochondrial metabolism is challenged with exercise and/or a high-fat diet?
iii. Are there differences in the metabolism of MT1+2KO and WT mice during mitochondrial disease, when complex I of the ETC is inhibited with rotenone?
iv. Can these differences (if any) emphasise the main regions that are affected by the absence of MTs in the MTKO mice?
v. Can the results of this study give a clearer picture of the involvement of MTs with mitochondrial metabolism, and can these results be used to generate new hypotheses in addition to its primary aim to only emphasise regions of the metabolism mostly affected by the absence of MTs for further investigation?

The primary aim of this study was thus to highlight regions in the (mitochondrial) metabolism that were markedly different between WT and MT knockout mice. As metabolomics is a hypothesis-generating methodology, these differences can be investigated in future studies using more targeted and focused approaches. The secondary aim of this study was to cast more light on the involvement of MTs in mitochondrial metabolism in light of the metabolic results. A few smaller objectives and associated sub-studies were put forth to accomplish the aim(s) of this investigation. The accomplishment of these objectives will be discussed in the following sections. Among these was the development of metabolomics methodology as it has not been fully developed at the Centre for Human Metabonomics, North-West University. Methodology development and

evaluation encompassed a significant part of this thesis. Although method development was not the primary aim of this study, it was essential in order to obtain reliable and reproducible results.

6.1.1 THE ESTABLISHMENT AND STANDARDISATION OF A HIGH-THROUGHPUT AND PRECISE METABOLOMICS DATA GENERATION, DATA PROCESSING AND STATISTICS WORKFLOW.

As described in Chapter 1, the methodology for metabolomics investigations has, at the start of this study, not been fully developed at the platform where the study was conducted. This is particularly true for tissues, untargeted metabolomics techniques and bioinformatics. For this reason methodology development and evaluation encompassed a significant part of this thesis. An extensive evaluation of current methods and techniques in the field of untargeted metabolomics was made to select appropriate methods and techniques that fit the aim of this study (Annexure A). Three analytical platforms were selected to get a wide coverage of the sample metabolome (as described in Chapter 3). A high-throughput metabolic fingerprinting workflow was successfully established to allow precision and untargeted analysis of as many metabolites as possible using the three selected platforms. Sample preparation methods were selected based on their scope which were validated and standardised for this study to allow repeatable results. Recommended and previously standardised instrument settings were used (Annexure B).

Furthermore, an extensive evaluation of current methods and techniques in metabolomics data mining were made to find appropriate data extraction, processing and statistical methods to accurately mine information from the raw data. From the method validation experiments (Annexure B), it is clear that the different analytical methods performed satisfactorily with regard to repeatability, speed and metabolome coverage. Several data extraction methods and software packages were evaluated and the most reliable and thorough yet high-throughput software and methods were selected. A workflow of data cleanup and normalisation steps relevant to the type of data obtained was incorporated to remove irrelevant variance. Univariate and multivariate methods for data mining were selected based on their focus and advantages (Annexure C).

The successful establishment of this workflow allowed the high-throughput and reliable analysis of numerous muscle, liver, brain, plasma, serum and urine samples to comprehensively investigate the metabolism of the experimental animals.

6.1.2 METABOLIC DIFFERENCES BETWEEN WT, MT1+2KO AND MT3KO MICE DURING UNCHALLENGED CONDITIONS AND WHEN MITOCHONDRIAL METABOLISM WAS SPECIFCALLY CHALLENGED WITH EXERCISE AND/OR HIGH-FAT INTAKE.

This sub-study was designed to answer the question whether there are any differences in the metabolism of MT1+2KO, MT3KO and WT mice as reflected in the metabolome during unchallenged conditions, and when mitochondrial metabolism is challenged with exercise and/or a high-fat diet. The objective of this sub-study was also to identify the pathways most affected by the MT knockout and to specifically attempt to elucidate the role of MTs in mitochondrial function in light of these metabolic changes.

The WT, MT1+2KO and MT3KO mice had clear differences in their gastrocnemius, liver and brain metabolome content which indicates differences in their metabolism during unchallenged conditions, albeit not as prominent as would be expected, as the plasma exometabolome did not reflect these differences very well. Nevertheless, the supposed pathways that were most altered in these mice were identified using pathway analysis and were reported in the respective sections. An overview of these results pointed to possible insulin resistance in the MT knockout mice and a slightly lower respiration rate which resulted in the accumulation of Krebs cycle intermediates, carbohydrates and lipids in the liver and brain when compared to the WT. The direct or indirect involvement of MT-1 and -2 in mitochondrial functions related to energy metabolism is therefore reflected in the metabolome. While the metabolomics data cannot effectively identify the main enzymes involved to clarify the metabolic variation, the role of MTs in metal homeostasis is surely a possibility. This could be through allosteric regulation of enzymes by providing metals to prosthetic groups (e.g. providing copper to complex IV) or to inhibitory sites (releasing zinc to complex III). Furthermore, if it can be reasoned that the available antioxidant systems in the body scavenge ROS sufficiently during unchallenged conditions, then the antioxidant role of MT-1 and -2 in this metabolic variation seems unlikely. It is also hypothesized that the involvement of MT-3 in mitochondrial metabolism in the liver might be indirect through altered hormonal or neuronal signalling. Nonetheless, this also implies a role of this isoform in mitochondrial metabolism.

The WT, MT1+2KO and MT3KO mice also had clear differences in their gastrocnemius, liver and brain metabolome content after they were challenged with exercise (one hour swim). This indicates more specific differences in energy metabolism and mitochondrial function. The results of the different tissues indicated that the MT1+2KO mice had lower energy producing capabilities

when considering the “accumulation” of several compounds related to the central energy metabolic pathways and fatty acid oxidation. The putative role of MT-1 and -2 in enhancing enzyme activity (activation through the supply of metal cofactors) and concentration (regulating metal dependent transcription factors) evidently resulted in the WT mice having slightly better energy producing capabilities compared to MT knockout mice. However, as strenuous exercise theoretically also results in higher ROS production, which could consequently inhibit enzymes such as α-ketoglutarate dehydrogenase, the antioxidant role of MTs to scavenge these ROS cannot be excluded and could have contributed to the metabolic differences observed. The role of MT-3 in metabolism was again difficult to elucidate even though clear differences in metabolome content were observed in the liver and brain. Seeing that the MT3KO mice brain metabolome was markedly different when compared to the MT1+2KO mice, it would seem that this isoform functions differently compared to the other and is, as expected for this isoform, predominantly involved in the signalling processes of the central nervous system.

The WT, MT1+2KO and MT3KO mice had clear differences in their metabolome and exometabolome content after a long term high-fat diet. As described in detail in Chapter 4, the consequences of a long term high-fat diet is associated with lower respiration and fatty acid oxidation, accumulation of fatty acids in various tissues and blood, and the onset of insulin resistance. The impression from the metabolic data of the MT1+2KO and MT3KO mice is that these mice had moderate insulin resistance and were more susceptible to the changes associated with high-fat intake. Considering the link of these manifestations with the mitochondrion and its function, it was again clear that MTs play a definite role in this organelle even though the mechanisms remain unclear with this untargeted investigation. While the view of higher respiration in the WT mice contradicts the *in vitro* results of Simpkins *et al*. (1998), Molto *et al.* (2007) and Ye *et al.* (2001), who reported that MTs reduce respiration, their functionality *in vivo* (over the long term) seems to be favouring energy metabolism instead of inhibiting it. By controlling metal homeostasis in a more controlled fashion, enzymes or transcription factors important in respiration and fatty acid oxidation in the WT mice resulted in them having slightly higher respiration and the ability to withstand high-fat intake induced changes. Furthermore, the protective role against ROS commonly associated with MTs may prevent the damaging effect of low level oxidative stress on the enzymes of the RC (vicious cycle) over a longer period.

With the combination of exercise and a high-fat diet, the metabolic differences between the WT and MT knockout mice still persisted although some variations were observed. Many of the

varying metabolites indicated lower respiration and energy in the MT knockout mice. However, most of the known patterns of lower respiration and energy were masked by the markedly higher adrenalin detected in the MT knockout mice. Also, while the high-fat diet resulted in the accumulation of certain metabolites such as fatty acids in the tissues, the exercise intervention resulted in the depletion of these metabolites for energy production. Hence, despite the differences seen in the metabolism of these mice, the use of these interventions in tandem made it more difficult to link these changes to energy metabolism.

6.1.3 METABOLIC DIFFERENCES BETWEEN WT AND MT1+2KO MICE DURING UNCHALLENGED CONDITIONS AND WHEN COMPLEX I IS DYSFUNCTIONAL.

This sub-study was designed to shed more light on the differences in the metabolism of MT1+2KO and WT mice (reflected in the metabolome during unchallenged conditions) and answer the question whether there are any differences in the metabolome of the mice when complex I of the ETC is dysfunctional. The objective of this sub-study was also to identify the pathways most affected by the MT knockout and to specifically attempt to elucidate the role of MTs in mitochondrial function and disease in light of these metabolic changes.

Due to the limited systemic information that was obtained in the previous sub-study regarding the metabolic differences between the WT and MT1+2KO mice in unchallenged conditions, it was decided to re-evaluate these differences using serum and urine. The WT and MT1+2KO mice had clear differences in their serum and urine exometabolome content which confirms differences in their metabolism during unchallenged conditions. The supposed pathways that were most altered in these mice were identified for future studies using pathway analysis and were reported in the respective sections. The systemic information obtained from the serum and urine portrayed a combined metabolic state of all the organs and tissues. The main finding was the higher blood glucose and lower Krebs cycle intermediates including lactate in the MT1+2KO mice. These findings imply that respiration in the MT1+2KO mice was higher in comparison to the WT. This result correlates with *in vitro* results as noted before, but contradicts the findings in the previous sub-study. The onset of moderate insulin resistance in these mice could however resolve this contradiction as a lower influx of glucose to the muscles could lead to a lower amount of these intermediates leaking into the circulation. Nevertheless, even if the WT had lower respiration

instead of the MT1+2KO mice, it still indicates a role of MTs in regulating mitochondrial metabolism, specifically OXPHOS, which should be followed up in a more targeted study.

The WT and MT1+2KO mice had clear differences in their exometabolome content after complex I was inhibited by rotenone treatment. There were notable similarities in the metabolic profiles of the MT1+2KO mice to that of complex I deficient cell cultures (Shaham *et al.,* 2010; Xu *et al.,* 2011) and patients with RC deficiencies (Reinecke *et al.,* 2011), which indicated that the adverse effects of complex I inhibition were more severe in these mice compared to the WT. It was also concluded that the impairment of respiration resulted in more notable insulin resistance as higher blood sugars were present. This is supported by the common observation that glycolysis functions more actively when respiration is impaired. Hence the role of MT-1 and -2 in limiting the effects of complex I deficiency is apparent. The most likely contribution of these MTs is by scavenging ROS produced by the inhibition and to limit the oxidation of complex I that leads to more ROS (Taylor *et al.,* 2003). The protection of other enzymes within the mitochondrion that is affected and/or inhibited by ROS would also allow a slightly faster respiration rate in the WT mice. The role of MT-1 and -2 in providing metal cofactors to enzymes and transcription factors that are part of the adaptation process over the three weeks of rotenone treatment cannot be excluded from this view. This would indicate that the WT mice had better adaptation abilities. These results therefore support *in vitro* results where the involvement of MTs in complex I deficient cells were reported and where it was hypothesized that their expression protects against the effects of dysfunctional respiration and the subsequent consequences (Reinecke *et al.,* 2006; van der Westhuizen *et al.,* 2003).

6.2 NEW CONCEPTS AND HYPOTHESES

The accomplishments of the respective objectives and sub-studies were discussed in the previous section and the metabolomics results concluded with brief reference to the possible role(s) of MTs in the respective results. The putative roles and interactions of MTs with certain elements in the mitochondrion will be discussed in this section by relating the obtained metabolic data to available information in the literature. The results from the different interventions and sub-studies will also be compared to formulate new hypotheses and concepts regarding the MT knockout mice and the role of MTs in the mitochondrion.

6.2.1 HYPOTHETICAL ROLES OF MTs IN MITOCHONDRIAL FUNCTION AND DISEASE

As mentioned before, the metabolomics data of the unchallenged mice indicated that the MT1+2KO mice had possible (moderate) insulin resistance and/or enhanced respiration. Reported *in vitro* studies have shown that the addition of MTs to mitochondria resulted in reduced respiration and oxygen consumption (Molto *et al.,* 2007; Simpkins *et al*., 1998; Ye *et al.,* 2001) which would be in accordance with the metabolic data that point to possible enhanced respiration in the MT1+2KO mice. However, moderate insulin resistance is normally linked to impaired mitochondrial metabolism and obesity which does not fit this view and the concept of reduced respiration in the WT mice in comparison to the MT knockout. Also, in light of the view that MT knockout mice become moderately obese (Beattie *et al.,* 1998; Byun *et al.,* 2011) which is possibly due to a lower metabolic rate (Coyle *et al.,* 2002), it would thus seem that a lower respiration rate fits the profile better despite the controversial results (observed here and reported in the literature). It is also well known that obesity, insulin resistance and diabetes are closely related to impaired respiration which supports this view (Abdul-Ghani & DeFronzo, 2008; Ciapaite *et al.,* 2011; Gaidhu *et al.,* 2010; Kelley *et al.,* 2002; Schrauwen & Hesselink, 2004; Vial *et al.,* 2011).

The hypothesis that the MT1+2KO mice had reduced respiration (metabolic rate) in addition to the possible insulin resistance is supported by the metabolic data obtained after their energy metabolism was challenged with exercise (one hour swim). This intervention specifically challenged energy production via oxidative phosphorylation as explained in Chapter 4. In the accompanying study by Pretorius (2011), who evaluated respiration in the same animals, a slightly lower (~10 %) but not statistically significant state 3 respiration in both heart and liver mitochondria was observed in the MT1+2KO compared to WT mice. The accumulation of substrate and depletion of product (energy) of the energy metabolic pathways indicated (indirectly) lower pathway activity which could have been due to lower activity or concentrations of certain enzyme(s) in the pathway. Furthermore, the hypothesis that the MT1+2KO mice had moderate insulin resistance is also supported by the metabolic data obtained from the mice that were given a high-fat diet for several weeks. This intervention did not only put strain on mitochondrial function (specifically fatty acid oxidation) but also forced the accelerated manifestation of obesity and related implications (as shown in Figure 4.17) which include insulin resistance. The inhibition of complex I with rotenone also enhanced the manifestation of insulin resistance and supports this hypothesis. Moreover, metabolic data from the MT1+2KO mice that received rotenone for three weeks also indicated lower respiration (or more severe inhibition) which can be linked to the putative roles of MTs enhancing respiration (directly or indirectly) in the WT instead of reducing it.

Another aspect that is related and could support the hypothesis regarding possible insulin resistance and lower metabolic rate is the probability of increased oxidative stress levels in the MT1+2KO mice. While oxidative stress was not specifically measured, several putative markers and indirect measures did point to this possibility. Firstly, numerous hydroxy-fatty acids which are believed to be the stable end products of lipid peroxidation and oxidative damage (Borchman & Sinha, 2002; Porter *et al.,* 1979) were found to be higher in the MT1+2KO mice. Secondly, the biosynthesis of spermine (which was also commonly found to be higher in the MT1+2KO mice) is also activated by the same stimuli (e.g. ROS) that activate MT expression (Simpkins *et al.,* 1998b). Lastly, the detection of increased oxidized glutathione in some tissues and the detection of increased glutathione (reduced) in others might indicate increased oxidative stress (in specific tissues) which leads to activated glutathione synthesis and increased oxidation. Hence, the possible high oxidative stress levels in the MT1+2KO mice could also have contributed to the hypothetical insulin resistance and impaired respiration. ROS are known to result in insulin resistance by hindering the insulin signal cascade inside the cell (Abdul-Ghani & DeFronzo, 2008; Dumas *et al.,* 2009). The oxidation and/or inhibition of enzymes involved in energy metabolism by ROS could also lead to lower activity in the MT1+2KO mice. The inhibition of α-ketoglutarate dehydrogenase and oxidation of complex I by ROS have been reported to result in reduced activity (Brown *et al.,* 2000; Taylor *et al.,* 2003).

In light of the connections between the different metabolic information obtained and literature reports, it is hypothesized that *the MT1+2KO mice have irregularities in insulin secretion and/or action which is related to lower respiration and/or increased oxidative stress*.

Appending this hypothesis are existing and new concepts on the involvement of MTs in mitochondrial function. Figure 6.1 summarises hypothetical interactions of MTs in certain mitochondrial elements which could have contributed to the higher respiration (energy metabolism), normal insulin signalling and action, and lower oxidative stress in the WT when compared to the MT1+2KO mice.

The theoretical cascade of events, leading to insulin secretion in the pancreatic β-cell when blood glucose rises, is shown and its subsequent activation of glucose and amino acid uptake by insulin-dependent tissues. Insulin secretion is influenced by mitochondrial function (respiration) while insulin action is influenced by oxidative stress along with mitochondrial function (fatty acid oxidation and respiration). Impaired mitochondrial function in MT1+2KO mice (as hypothesized)

would lead to both impaired insulin secretion and insulin action as accumulating fatty acids and ROS inhibit insulin signalling. This would lead to higher circulating blood glucose levels and lower Krebs cycle intermediates in (insulin-dependent) tissues where replenishment of this cycle is greatly hindered by the absence of substrate (carbohydrates and amino acids) and/or impaired fatty acid oxidation. These theoretical metabolic profiles were observed in the MT1+2KO mice which led to this hypothesis. This theoretical cascade of events was prevented by the presence of MT-1 and -2 in the WT mice. The putative roles of these MTs in the mitochondrion which led to the prevention of moderate insulin resistance and reduced respiration will be described with reference to the numbers in the illustration (Figure 6.1).

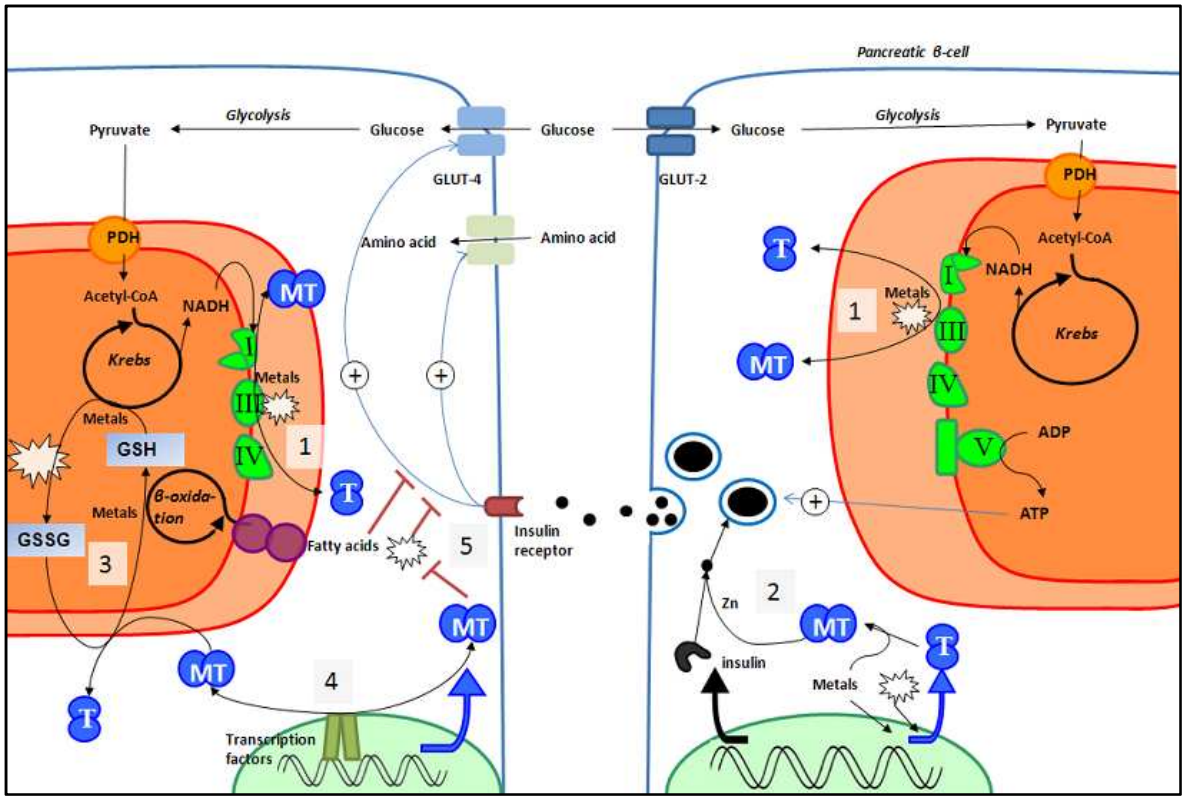

Figure 6.1: Schematic presentation illustrating the hypothetical roles of MTs in mitochondrial and insulin function. MTs supply metal cofactors to and take inhibitory metals from complexes of the respiratory chain, while also protecting them from oxidative damage (1). MTs supply zinc to insulin's structure (2). The MT-glutathione cycle which allows MTs to deliver metals and scavenge free radical indirectly in the mitochondrial matrix (3). MTs supply metals to and regulate transcription factors that require zinc (4). MTs protect against insulin resistance by scavenging ROS and promoting respiration and fatty acid oxidation (5).

1) MTs could favour respiration by releasing metal cofactors such as copper to complexes of the respiratory chain; or by removing metals such as zinc from inhibitory sites in complex I and III; or by protecting these complexes (especially complex I) from oxidative damage and reduced oxidized sites. Unimpaired respiration would thus lead to unimpaired insulin secretion and action. These actions (especially the latter) would also be advantageous during mitochondrial disease when the over-expression of MTs is induced.
2) Furthermore, MTs might also have a putative role in supplying insulin with zinc which is part of its structure.
3) Similar to the putative diverse involvement of MTs with the respiratory chain, the putative and indirect release of metal cofactors to enzymes in the mitochondrial matrix via glutathione, as well as their indirect protection against oxidative damage would ensure unimpaired respiration and fatty acid oxidation.
4) In addition to the putative role of MTs in activating or inhibiting enzyme activity, their regulation of transcription factors via metal ions could potentially also result in increased expression of specific enzymes leading to higher concentrations.
5) By scavenging ROS and enhancing respiration and fatty acid oxidation, MTs putatively promote insulin action.

The above hypothesis and related concepts are specifically focused on MT-1 and -2 but not MT-3. The involvement of this isoform of the MT-family (which is normally expressed in the central nervous system and brain), with mitochondrial metabolism was not evidently seen in the metabolic results. The variation in the metabolic data and small numbers of MT3KO mice limits the understanding of the role this isoform plays in energy metabolism. MT-3's involvement in tissues where it is not readily expressed implies an indirect link of this isoform to metabolism, most probably through the signalling events of the central nervous system and accompanied neuronal activity. The recognised involvement of this isoform in zinc homeostasis, zinc signalling and growth inhibition in the brain and specifically the hypothalamus, which is the regulatory centre responsible for energy homeostasis, might result in indirect effects on the (mitochondrial) metabolism of tissues where this isoform is not expressed (Byun *et al.,* 2011). Moreover, since it was reported that MT3KO mice also consume less oxygen, which implies lower respiration (Byun *et al.,* 2011), it could thus still be involved in mitochondrial function within the central nervous system. However, since these mice still contain MT-1 and -2, it is difficult to highlight the separate role of MT-3 in energy metabolism, even within the brain.

6.3 CRITICAL ASSESSMENT OF THIS STUDY

The importance and need of this investigation was recognised and steered the design to specifically cast more light on the involvement of MTs in mitochondrial function (energy metabolism) and disease. However, this study and selected design had several limitations (as indicated below) which were taken into consideration when the final conclusions were drawn.

- While the use of five to sixteen experimental animals per experimental group is acceptable due to the controlled conditions and consequent low variance between them (Erban *et al.,* 2007), the small experimental groups in this study remain a disadvantage. This was especially the case with the investigation of the metabolic differences between the WT, MT1+2KO and MT3KO mice during unchallenged and challenged conditions (Chapter 4). The findings of this sub-study are therefore not as conclusive as when larger groups would have been compared. The small number of MT3KO mice in the experimental groups is particularly a shortcoming to accurately understand the role of MT-3 in the metabolism.

- Adding to the above problem is the differences between the genders (Chapter 4). Not only did gender differences result in small experimental groups when the liver metabolomes were compared, but also unnecessary variance in the data of the other tissues/bio-fluid, which masked the influence of the MT knockout in many ways. Hence, the metabolic differences between the strains could have been more pertinent if only male mice were used in the study when the metabolic differences between the WT, MT1+2KO and MT3KO mice were studied during challenged and unchallenged conditions.

- The putative influence of hormones and systemic signalling mechanisms and other indirectly induced perturbations hindered attempts to unmask the sole role of MTs in mitochondrial function. Despite the comprehensive evaluation (in terms of all the tissues screened), the interplay and differences between the studied tissues and bio-fluids complicated matters and often led to more questions than answers. The hypothesized but putative variation in hormonal control (action), signalling and secretion induced by MTs might have played a more dominant role in the results seen instead of their direct involvement in enzyme regulation. Hence, many of the metabolic differences observed between the WT and MT knockout mice may be due to differences in hormonal action (e.g. insulin resistance). This made it difficult to pinpoint the contribution of the respective

enzymes or pathways to the metabolome composition and therefore metallothionein's involvement in enzyme and transcription factor regulation through metal ions.

- The use of rotenone to induce complex I dysfunction in order to study the involvement of MTs in mitochondrial disease was not the ideal mitochondrial disease intervention. Firstly, although several studies have been undertaken in recent years with rotenone models, the problems associated with administration of this inhibitor to test animals, and especially mice, remain unresolved. The solubility of rotenone in water-based solutions is poor. However, the use of other media, such as previously used peanut oil, is similar to a high-fat diet giving secondary effects not associated with complex I inhibition. Although the suspension of rotenone in PBS was not optimal, it was at the time of the study considered to be the most effective way to administer this inhibitor. Furthermore, the absorption, transport and distribution of rotenone in the mice was clearly heterogeneous as seen in the immensely different levels of inhibition observed in different tissues of treated mice (Annexure F).

- It is clear that the experimental setup and design of this study was not without its shortcomings. Added to this is the limitation in metabolomics technology and the related statistical methods. Firstly, because of the complexity of the metabolome in terms of compound classes and dynamic concentration ranges, it is (currently) not possible to screen the complete metabolome. Information on specific compartmentalisation of metabolites in cells and fluxes are not covered with most metabolomics methods and also adds to the incomplete assessment of metabolic alterations. Secondly, due to the design of this study (by using tissue material reflecting steady state conditions), it was not possible to perform a time-based study. Only a snapshot of the metabolism was taken which means that time-related alterations in the metabolism were ignored. The same applies to the 14 hour urine sample which, although it gave a more prolonged snapshot, was not collected and analysed in time segments. Furthermore, metabolomics investigations make use of relative metabolite quantities as the comparison of control and test groups are important. However, the inability to compare metabolite concentrations to references values in literature or between labs makes it difficult to comprehend the margin by which interventions or diseases influence the metabolism.

- Since the aim of metabolomics investigations is to comprehensively understand and report alterations in the metabolism, it seems inappropriate to use classical (univariate) statistical

methods such as ANOVA and t-tests. The use of multivariate methods has therefore become more popular as it focuses more on the covariance of metabolites. However, seeing that this covariance cannot be controlled, it often leads to complex or irrelevant results as not all metabolites co-vary in the biological system (such as two compounds in completely different pathways). Techniques such as pathway analysis try to overcome this flaw but are also limited by our knowledge of metabolic pathways and disability to cover the entire metabolome. Pathway analysis and manual interpretation are also limited by our ability to identify detected compounds with great accuracy and confidence which is especially the case with LC-MS data.

6.4 FINAL CONCLUSION AND FUTURE PROSPECTS

Considering the inherent limitations of this study (Section 6.3) it is thus clear that there remain certain shortcomings in our knowledge regarding the involvement of MTs with mitochondrial function and disease. These limitations in our knowledge also reveal opportunities for future studies on this matter.

One of the main concerns of this study was the small experimental groups, especially in the investigation of the metabolic differences between the WT, MT1+2KO and MT3KO mice during unchallenged and challenged conditions. While the controlled environment and consequent lower variation between experimental animals validates the use of experimental groups containing only five to ten mice, the outcome could have been be more conclusive if larger experimental groups were used. This could have allowed the statistical methods to provide greater confidence in the true variance. Hence, it seems necessary to repeat certain parts of this investigation with more mice per experimental group (especially the MT3KO mice). Considering that this study was indeed able to highlight metabolic differences that are influenced by MTs, any repeated investigations (or parts thereof) can be accompanied with a more focused (targeted) metabolomics investigation. Moreover, as mentioned in Annexure A, it is also mandatory to validate important findings of this study with more targeted metabolomics methods, not only to obtain quality data but also absolute quantification (as defined in Annexure A) to compare the findings with reference values in the literature and other research entities. This would give a much deeper insight into the role MTs play in certain parts of the metabolism.

The influence of hormones and organ specific metabolite transport/uptake in the mice masked the exclusive role of MTs in mitochondrial metabolism. It was noticed that the metabolome composition of the MT1+2KO (especially) were possibly influenced by hormone action and other related factors. This may have resulted in the accumulation of metabolites in one organ and the depletion of similar metabolites in another, making it difficult to pinpoint the contribution of the respective enzymes or pathways involved. It is therefore necessary to perform a similar study on (MT knockout) cell cultures where hormone action and nutrient availability does not influence the metabolome content as significantly. The addition of high amounts of lipids or free fatty acids to the culture medium, which simulates high levels of fatty acids in the plasma from a high-fat diet, would challenge mitochondrial function and give more specific results. The transfection of such cells with a vector expressing a MT isoform as a control would also give more conclusive results, if any. The use of cell cultures to study the involvement of MTs in mitochondrial disease (using metabolomics) would also be advantageous as the uptake, transport and distribution of respiratory chain inhibitors (such as rotenone) would be more controlled and homogenous.

In addition to the larger experimental groups and use of cell cultures it would also be informative to incorporate certain changes in the metabolomics methodology to clarify the involvement of MTs in mitochondrial metabolism. The use of more targeted approaches has been mentioned. However, the combination of targeted metabolite profiling over time would cast more light on time-related changes in the metabolism which could be determined by the expression and turnover of MTs. The influence of substrate fluxes would then be better resolved. The use of cell cultures or mouse urine (to eliminate stress) in such a time-series study would give valuable insight on the role MTs play in mitochondrial metabolism.

In conclusion, an opening in the understanding of the association between MTs and mitochondrial function and disease, as well as the absence of metabolomics data for the MT knockout mouse strains were identified after thoroughly investigating available literature. A study was formulated to meet the requirements for the degree *Philosophiae Doctor* (Biochemistry) using advanced system biology research methodologies and technologies to take one step closer to elucidate the role of MTs and in particular their involvement in mitochondrial energy metabolism. The metabolic findings confirmed an association of metallothioneins with mitochondrial function during unchallenged, challenged and diseased conditions which hypothetically results in enhanced functioning of energy metabolism and the prevention of irregularities in insulin function.