

# Evaluation of metallothionein involvement in the modulation of mitochondrial respiration in mice

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This dissertation is dedicated to:

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and so easily made proud, and believed I could  
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## ABSTRACT

Metallothioneins (MTs) are small, non-enzymatic proteins that are involved in cellular detoxification and metal homeostasis because of their high cysteine content. MTs have also been identified as one of the vast number of adaptive responses to mitochondrial respiratory chain (RC) deficiencies. Aside from this, numerous other studies have linked MTs to several mitochondrion-linked components, including reactive oxygen species (ROS) and oxidative stress, apoptosis, glutathione, energy metabolism and nuclear- and mitochondrial DNA transcription regulation. However, most of the reports concerning the putative link between MTs and mitochondria are from *in vitro* studies and relatively little supportive *in vivo* evidence has been reported. Information on the involvement of MTs with respiratory chain function is especially limited. It was therefore the aim of this study to investigate the involvement of MTs in mitochondrial respiration and respiratory chain enzyme function by using an MT knockout (MTKO) mouse model, which was treated with the irreversible complex I inhibiting reagent, rotenone. The aim was achieved by implementing three objectives: firstly, the RC function was investigated as a complete working unit; secondly, the functional and structural properties of single units (enzymes) of the RC were investigated utilising enzyme activity assays and BN-PAGE/western blot analysis; and thirdly, the possible effect of MTs on mtDNA copy number was investigated. While some tendencies of variation in RC enzyme activity and expression were identified, no significant effect on the overall mitochondrial respiratory function, or any significant differences in the relative mtDNA copy number of MTKO mice were observed. Thus it is concluded, while MTs have in this study revealed relatively small changes in respiratory chain function, which may still prove to have biological significance *in vivo*, the exact nature of the putative role of MTs in mitochondrial respiration or oxidative phosphorylation remains undefined.

Key words: mitochondrion; metallothionein; oxidative phosphorylation; respiratory chain; mitochondrial disease; MTKO mouse model; complex I deficiency; rotenone; enzyme activity analyses; mitochondrial respiration analyses

# TABLE OF CONTENTS

|                                                          |             |
|----------------------------------------------------------|-------------|
| <b>ACKNOWLEDGEMENTS .....</b>                            | <b>I</b>    |
| <b>ABSTRACT .....</b>                                    | <b>II</b>   |
| <b>TABLE OF CONTENTS .....</b>                           | <b>III</b>  |
| <b>LIST OF FIGURES .....</b>                             | <b>XIII</b> |
| <b>LIST OF TABLES.....</b>                               | <b>X</b>    |
| <b>LIST OF EQUATIONS.....</b>                            | <b>X</b>    |
| <b>LIST OF SYMBOLS AND ABBREVIATIONS .....</b>           | <b>XI</b>   |
| <br>                                                     |             |
| <b>CHAPTER 1 INTRODUCTION .....</b>                      | <b>1</b>    |
| <br>                                                     |             |
| <b>CHAPTER 2 LITERATURE OVERVIEW .....</b>               | <b>3</b>    |
| <br>                                                     |             |
| 2.1 INTRODUCTION.....                                    | 3           |
| <br>                                                     |             |
| 2.2 THE MITOCHONDRION .....                              | 3           |
| 2.2.1 MITOCHONDRIAL STRUCTURE .....                      | 4           |
| 2.2.2 MITOCHONDRIAL RC COMPLEXES .....                   | 5           |
| 2.2.3 MITOCHONDRIAL DISEASES.....                        | 7           |
| 2.2.4 ADAPTIVE RESPONSES TO MITOCHONDRIAL DISEASES ..... | 8           |
| <br>                                                     |             |
| 2.3 METALLOTHIONEINS .....                               | 9           |
| 2.3.1 METALLOTHIONEIN STRUCTURE.....                     | 9           |
| 2.3.2 METALLOTHIONEIN ISOFORMS.....                      | 10          |
| 2.3.3 METALLOTHIONEIN FUNCTION AND EXPRESSION .....      | 10          |

|                                                                                                         |           |
|---------------------------------------------------------------------------------------------------------|-----------|
| 2.4 METALLOTHIONEIN INVOLVEMENT IN MITOCHONDRIAL FUNCTION.....                                          | 11        |
| 2.4.1 CELLULAR LOCALISATION AND PUTATIVE MITOCHONDRION-LINKED<br>INTERACTIONS OF METALLOTHIONEINS ..... | 11        |
| 2.4.2 MITOCHONDRIA-ASSOCIATED FUNCTIONS OF METALLOTHIONEINS .....                                       | 12        |
| 2.5 INVESTIGATION OF METALLOTHIONEIN USING DISEASE MODELS .....                                         | 17        |
| 2.6 PROBLEM STATEMENT .....                                                                             | 18        |
| 2.7 AIM AND STRATEGY .....                                                                              | 19        |
| <br><b>CHAPTER 3 MATERIALS AND METHODS .....</b>                                                        | <b>22</b> |
| 3.1 INTRODUCTION.....                                                                                   | 22        |
| 3.2 THE MTKO MOUSE MODEL.....                                                                           | 23        |
| 3.3 IDENTIFICATION OF MICE .....                                                                        | 24        |
| 3.4 GENOTYPING OF MICE .....                                                                            | 24        |
| 3.4.1 MATERIALS.....                                                                                    | 25        |
| 3.4.2 METHODS .....                                                                                     | 26        |
| 3.4.2.1 AMPLIFICATION OF DNA BY POLYMERASE CHAIN REACTION (PCR) .....                                   | 26        |
| 3.4.2.2 CHARACTERISATION OF DNA WITH AGAROSE GEL ELECTROPHORESIS .....                                  | 28        |
| 3.5 SORTING OF MICE INTO GROUPS.....                                                                    | 29        |
| 3.6 INDUCING A COMPLEX I DEFICIENCY IN EXPERIMENTAL ANIMALS .....                                       | 29        |
| 3.6.1 ROTENONE AS INHIBITOR OF COMPLEX I .....                                                          | 29        |
| 3.6.2 DETERMINATION OF OPTIMAL ROTENONE LOAD.....                                                       | 30        |
| 3.6.3 TREATMENT OF EXPERIMENTAL ANIMALS WITH PBS AND ROTENONE .....                                     | 32        |
| 3.7 EUTHANIZING OF MICE AND SAMPLE COLLECTION .....                                                     | 32        |

|                                                                                             |    |
|---------------------------------------------------------------------------------------------|----|
| 3.8 PREPARATION OF DIFFERENT CELLULAR AND SUB-CELLULAR FRACTIONS FOR SEVERAL ANALYSES ..... | 33 |
| 3.8.1 MATERIALS.....                                                                        | 33 |
| 3.8.2 METHODS .....                                                                         | 34 |
| 3.9 PROTEIN ASSAY .....                                                                     | 37 |
| 3.9.1 MATERIALS.....                                                                        | 37 |
| 3.9.2 METHODS .....                                                                         | 37 |
| 3.10 RESPIRATION ANALYSES .....                                                             | 38 |
| 3.10.1 MATERIALS.....                                                                       | 39 |
| 3.10.2 METHODS .....                                                                        | 40 |
| 3.11 RESPIRATORY CHAIN AND CITRATE SYNTHASE ENZYME ANALYSES.....                            | 43 |
| 3.11.1 MATERIALS.....                                                                       | 45 |
| 3.11.2 METHODS .....                                                                        | 46 |
| 3.11.2.1 CS ACTIVITY .....                                                                  | 46 |
| 3.11.2.2 CI ACTIVITY.....                                                                   | 47 |
| 3.11.2.3 CII ACTIVITY.....                                                                  | 48 |
| 3.11.2.4 CII+III ACTIVITY.....                                                              | 49 |
| 3.11.2.5 CIII ACTIVITY.....                                                                 | 49 |
| 3.11.2.6 CIV ACTIVITY .....                                                                 | 50 |
| 3.12 BN-PAGE/WESTERN BLOT ANALYSIS .....                                                    | 51 |
| 3.12.1 METHODS .....                                                                        | 51 |
| 3.13 RELATIVE $\text{mtDNA}$ COPY NUMBER DETERMINATION USING REAL-TIME PCR.....             | 52 |
| 3.13.1 MATERIALS.....                                                                       | 53 |
| 3.13.2 METHODS .....                                                                        | 54 |
| 3.13.2.1 ISOLATION OF DNA FROM HEART HOMOGENATE .....                                       | 54 |
| 3.13.2.2 QUANTIFICATION OF ISOLATED DNA .....                                               | 55 |
| 3.13.2.3 REAL-TIME PCR.....                                                                 | 55 |
| 3.14 STATISTICAL ANALYSES.....                                                              | 57 |

|                                                                                                                                 |           |
|---------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>CHAPTER 4 RESULTS AND DISCUSSION .....</b>                                                                                   | <b>62</b> |
| 4.1 INTRODUCTION.....                                                                                                           | 62        |
| 4.2 RESPIRATION AND RC ENZYME ACTIVITY ANALYSES IN LIVER TISSUE.....                                                            | 63        |
| 4.2.1 RESPIRATION ANALYSES .....                                                                                                | 63        |
| 4.2.1.1 RESULTS .....                                                                                                           | 63        |
| 4.2.2 RC ENZYME ACTIVITIES.....                                                                                                 | 66        |
| 4.2.2.1 RESULTS .....                                                                                                           | 66        |
| 4.2.3 DISCUSSION.....                                                                                                           | 69        |
| 4.3 RESPIRATION, RC ENZYME ACTIVITY, BN-PAGE/WESTERN BLOT ANALYSES<br>AND MTDNA COPY NUMBER DETERMINATION IN HEART TISSUE ..... | 70        |
| 4.3.1 RESPIRATION ANALYSES .....                                                                                                | 70        |
| 4.3.1.1 RESULTS .....                                                                                                           | 71        |
| 4.3.2 RC ENZYME ACTIVITIES.....                                                                                                 | 72        |
| 4.3.2.1 RESULTS .....                                                                                                           | 72        |
| 4.3.3 BN-PAGE AND WESTERN BLOT ANALYSIS .....                                                                                   | 75        |
| 4.3.3.1 RESULTS .....                                                                                                           | 76        |
| 4.3.4 RELATIVE mtDNA COPY NUMBER DETERMINATION.....                                                                             | 78        |
| 4.3.4.1 RESULTS .....                                                                                                           | 78        |
| 4.3.5 DISCUSSION.....                                                                                                           | 79        |
| 4.4 RC ENZYME ACTIVITY ANALYSES IN SKELETAL MUSCLE TISSUE.....                                                                  | 81        |
| 4.4.1 RC ENZYME ACTIVITIES.....                                                                                                 | 82        |
| 4.4.1.1 RESULTS .....                                                                                                           | 82        |
| 4.4.2 DISCUSSION.....                                                                                                           | 84        |
| 4.5 RC ENZYME ACTIVITY ANALYSES IN BRAIN TISSUE.....                                                                            | 84        |
| 4.5.1 RC ENZYME ACTIVITIES.....                                                                                                 | 85        |
| 4.5.1.1 RESULTS .....                                                                                                           | 85        |
| 4.5.2 DISCUSSION.....                                                                                                           | 87        |

|                                                |                |
|------------------------------------------------|----------------|
| <b>CHAPTER 5 CONCLUSIONS .....</b>             | <b>90</b>      |
| 5.1 INTRODUCTION.....                          | 90             |
| 5.2 PROBLEM STATEMENT, AIM AND OBJECTIVES..... | 90             |
| 5.3 EFFECTIVENESS OF ROTENONE TREATMENT .....  | 91             |
| 5.4 OBJECTIVES.....                            | 94             |
| 5.5 SUMMARY AND DISCUSSION OF RESULTS.....     | 98             |
| 5.5 CONCLUDING REMARKS .....                   | 105            |
| <br><b>REFERENCES .....</b>                    | <br><b>110</b> |
| <b>APPENDIX .....</b>                          | <b>122</b>     |
| APPENDIX A.....                                | 122            |
| PREPARATION OF REDUCED CYTOCHROME C .....      | 122            |
| PREPARATION OF DECYLBUBIQUINOL .....           | 123            |
| MILLI-Q® WATER SYSTEM .....                    | 123            |
| APPENDIX B.....                                | 124            |
| APPENDIX C .....                               | 126            |

## LIST OF FIGURES

|                                                                                                                                                           |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 2.1: Schematic representation of the enzymes of the OXPHOS system. ....                                                                            | 5  |
| Figure 2.2: Graphic representation of MT2A from rat. ....                                                                                                 | 9  |
| Figure 2.3: Schematic representation of the summarised putative interactions of<br>MTs with components of the mitochondrion. ....                         | 12 |
| Figure 2.4: Schematic representation of the strategy used to achieve the aims of<br>the overall investigation, which included five separate studies. .... | 20 |
|                                                                                                                                                           |    |
| Figure 3.1: An illustration of the numbering system used to mark the mice. ....                                                                           | 24 |
| Figure 3.2: Screen capture of software settings illustrating the stages and cycles<br>of PCR used for genotyping. ....                                    | 27 |
| Figure 3.3: Example of results obtained during genotyping of mice. ....                                                                                   | 28 |
| Figure 3.4: CI inhibitory effect of a range of rotenone concentrations administered<br>to mice over a period of three weeks. ....                         | 31 |
| Figure 3.5: Schematic representation of the protocol followed during preparation of<br>cellular and sub-cellular fractions, specific to each tissue. .... | 36 |
| Figure 3.6: Example of a typical respiration trace, using a glutamate+malate (G+M)<br>substrate combination. ....                                         | 41 |
| Figure 3.7: Example of a photographed western blot during analysis with<br>GeneTools software. ....                                                       | 52 |
| Figure 3.8: Screen capture of software settings illustrating the stages and cycles<br>of real-time PCR. ....                                              | 56 |
| Figure 3.9: Schematic representation of the statistical methods followed during<br>analyses of data. ....                                                 | 59 |

|                                                                                                                                                                                                      |         |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|
| Figure 4.1: Box and whisker plots comparing mitochondrial state 3 respiration<br>measured on different days. ....                                                                                    | 64      |
| Figure 4.2: Box and whisker plots comparing liver mitochondrial state 3 respiration<br>of PBS- and rotenone-treated WT and MTKO mice. ....                                                           | 65      |
| Figure 4.3: Box and whisker plots of different OXPHOS enzyme activities for liver. ....                                                                                                              | 68      |
| Figure 4.4: Box and whisker plots comparing heart mitochondrial state 3 respiration<br>of PBS- and rotenone-treated WT and MTKO mice. ....                                                           | 71      |
| Figure 4.5: Box and whisker plots of different OXPHOS enzyme activities for heart. ....                                                                                                              | 74      |
| Figure 4.6: Western blot of OXPHOS complexes after separation using BN-PAGE. ....                                                                                                                    | 76      |
| Figure 4.7: Graphical presentation of enzyme quantities of different genotype and<br>treatment mice, as determined using BN-PAGE and western blot<br>analysis of heart mitochondrial fractions. .... | 77      |
| Figure 4.8: Representation of relative mtDNA copy number in heart mitochondria<br>quantified using qPCR. ....                                                                                        | 79      |
| Figure 4.9: Box and whisker plots of different OXPHOS enzyme activities for<br>skeletal muscle. ....                                                                                                 | 83      |
| Figure 4.10: Box and whisker plots of different OXPHOS enzyme activities for brain. ...                                                                                                              | 86      |
| <br>Figure 5.1: Comparison of RC enzyme activities for each group, organised per<br>RC complex. ....                                                                                                 | <br>101 |

## LIST OF TABLES

|                                                                                                                                      |     |
|--------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 3.1: Sequence of PCR primers.....                                                                                              | 26  |
| Table 3.2: Data obtained from respiration analyses with calculated RCR-values .....                                                  | 42  |
| Table 3.3: List of substrates used and products formed during assays for respiratory<br>chain enzymes and citrate synthase (CS)..... | 44  |
| Table 3.4: Sequence of qPCR primers for amplification of <i>MT-TY/MT-CO1</i> .....                                                   | 55  |
| Table 3.5: Sequence of qPCR primers for amplification of <i>Gapdh</i> .....                                                          | 56  |
| Table 1: Dosing schedule for Day-groups 1 to 6 .....                                                                                 | 124 |
| Table 2: Dosing schedule for Day-groups 7 to 11 .....                                                                                | 125 |
| Table 3: Processed data points of respiration and CI, CII and CII+III<br>activity analyses. ....                                     | 126 |
| Table 4: Processed data points of CIII and CIV activity analyses .....                                                               | 128 |

## LIST OF EQUATIONS

|                                                                    |     |
|--------------------------------------------------------------------|-----|
| Equation 3.1: Calculation of protein content.....                  | 38  |
| Equation 3.2: Calculation of specific activity of CS.....          | 47  |
| Equation 3.3: Calculation of specific activity of CI .....         | 48  |
| Equation 3.4: Calculation of specific activity of CII .....        | 48  |
| Equation 3.5: Calculation of specific activity of CII+III .....    | 49  |
| Equation 3.6: Calculation of specific activity of CIII .....       | 50  |
| Equation 3.7: Calculation of specific activity of CIV.....         | 51  |
| Equation 1: Calculation of reduced cytochrome c concentration..... | 122 |

## LIST OF SYMBOLS AND ABBREVIATIONS

|                  |                                                 |
|------------------|-------------------------------------------------|
| $\alpha$         | alpha                                           |
| $\beta$          | beta                                            |
| $\mu\text{g}$    | microgram                                       |
| $\mu\text{l}$    | microliter                                      |
| $\mu\text{M}$    | micromolar                                      |
| $\mu\text{mole}$ | micromole                                       |
| ©                | copyright                                       |
| ®                | registered                                      |
| °C               | degrees Celcius                                 |
| a                | adenine                                         |
| ADP              | adenosine diphosphate                           |
| ANOVA            | analysis of variance                            |
| ARE              | antioxidant response element                    |
| ATP              | adenosine triphosphate                          |
| BCA              | bicinchoninic acid                              |
| Bkg              | background                                      |
| blk              | blank                                           |
| BN-PAGE          | blue-native polyacrylamide gel electrophoresis  |
| bp               | base pair                                       |
| BSA              | bovine serum albumin                            |
| c                | cytosine                                        |
| CA               | California                                      |
| CH               | Switzerland                                     |
| CI               | complex I: NADH:ubiquinone oxidoreductase       |
| CII              | complex II: succinate:ubiquinone oxidoreductase |

|                                      |                                                              |
|--------------------------------------|--------------------------------------------------------------|
| CIII                                 | complex III: ubiquinol:ferricytochrome c oxidoreductase      |
| CIV                                  | complex IV: ferrocytochrome-c:oxygen oxidoreductase          |
| CO <sub>2</sub>                      | carbon dioxide                                               |
| CoA                                  | co-enzyme A                                                  |
| coQ                                  | co-enzyme Q                                                  |
| CoV                                  | co-efficient of variance                                     |
| COX                                  | cytochrome c oxidase                                         |
| C <sub>T</sub>                       | threshold cycle                                              |
| C-terminal                           | carboxyl-terminal                                            |
| CuSO <sub>4</sub> .5H <sub>2</sub> O | copper (II) sulphate pentahydrate                            |
| CV                                   | complex V: ATP phosphohydrolase                              |
| Cyt c                                | cytochrome c                                                 |
| Da                                   | Daltons                                                      |
| DCIP                                 | 2,6-dichloroindophenol sodium salt hydrate                   |
| DE                                   | Germany                                                      |
| DMSO                                 | dimethyl sulfoxide                                           |
| DNA                                  | deoxyribonucleic acid                                        |
| dNTP                                 | deoxyribonucleotide triphosphate                             |
| dsDNA                                | double stranded DNA                                          |
| DTNB                                 | 5,5'-dithio-bis[-2-nitrobenzoic acid]                        |
| dUTP                                 | deoxyuridine triphosphate                                    |
| e <sup>-</sup>                       | electron                                                     |
| <i>e.g.</i>                          | <i>exempli gratia</i> : Latin abbreviation for “for example” |
| EDTA                                 | ethylene diamine tetraacetic acid                            |
| EGTA                                 | ethylene glycol tetraacetic acid                             |
| ES                                   | Spain                                                        |
| <i>et al.</i>                        | <i>et alii</i> : Latin abbreviation for “and others”         |
| <i>etc.</i>                          | <i>et cetera</i> : Latin abbreviation for “and so forth”     |
| ETC                                  | electron transport chain                                     |

|                                 |                                                  |
|---------------------------------|--------------------------------------------------|
| FAD <sup>+</sup>                | flavin adenine dinucleotide                      |
| FADH <sub>2</sub>               | reduced flavin adenine dinucleotide              |
| Fe <sup>2+</sup>                | iron-ferrous oxidation state                     |
| Fe <sup>3+</sup>                | iron-ferric oxidation state                      |
| FeS                             | iron-sulphur proteins                            |
| <i>g</i>                        | gravitational force                              |
| g                               | gram                                             |
| g                               | guanine                                          |
| G+M                             | glutamate and malate                             |
| GSH                             | reduced glutathione                              |
| GSSG                            | glutathione disulphide                           |
| H <sup>+</sup>                  | hydrogen                                         |
| H <sub>2</sub> O                | water                                            |
| HeLa                            | cervical cancer cell line (from Henrietta Lacks) |
| HEPA                            | High-Efficiency Particulate Air                  |
| IB                              | isolation buffer                                 |
| <i>i.e.</i>                     | <i>id est</i> : Latin abbreviation for “that is” |
| IgG                             | immunoglobulin G                                 |
| IMM                             | inner mitochondrial membrane                     |
| IN                              | Indianapolis                                     |
| K <sub>2</sub> HPO <sub>4</sub> | di-potassium hydrogen phosphate                  |
| kDa                             | kilo Daltons                                     |
| kg                              | kilogram                                         |
| KH <sub>2</sub> PO <sub>4</sub> | potassium dihydrogen orthophosphate              |
| KPi                             | potassium phosphate                              |
| Ltd                             | limited                                          |
| M                               | molar: moles per litre                           |
| MA                              | Miami                                            |
| mg                              | milligram                                        |

|                                 |                                                    |
|---------------------------------|----------------------------------------------------|
| min                             | minute                                             |
| Min-Max                         | range between minimum and maximum values           |
| ml                              | milliliter                                         |
| mM                              | millimolar                                         |
| MNGIE                           | mitochondrial neurogastrointestinal encephalopathy |
| mol                             | mole                                               |
| MOPS                            | 3-(N-morpholino) propanesulfonic acid              |
| MPTP                            | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine       |
| MRE                             | metal response elements                            |
| MT                              | metallothionein                                    |
| MT2A                            | metallothionein 2A isoform                         |
| mtDNA                           | mitochondrial DNA                                  |
| MTKO                            | metallothionein 1 and 2 knockout                   |
| Na <sub>2</sub> SO <sub>3</sub> | sodium sulfite anhydrous                           |
| Na <sub>2</sub> SO <sub>4</sub> | sodium sulfate                                     |
| NAD <sup>+</sup>                | nicotinamide adenine dinucleotide                  |
| NADH                            | reduced nicotinamide adenine dinucleotide          |
| nDNA                            | nuclear DNA                                        |
| NL                              | Netherlands                                        |
| nm                              | nanometer                                          |
| NO                              | nitrogen oxide                                     |
| N-terminal                      | amino-terminal                                     |
| NWU                             | North West University                              |
| O <sub>2</sub>                  | oxygen                                             |
| OAA                             | oxaloacetate                                       |
| OH <sup>·</sup>                 | hydroxyl radicals                                  |
| OMM                             | outer mitochondrial membrane                       |
| OXPHOS                          | oxidative phosphorylation                          |
| pAmp                            | picoampere                                         |

|                |                                                     |
|----------------|-----------------------------------------------------|
| PBS            | phosphate buffered saline                           |
| PCR            | polymerase chain reaction                           |
| PDH            | pyruvate dehydrogenase                              |
| Pi             | inorganic phosphate                                 |
| pmole          | picomole                                            |
| Pty            | proprietary company                                 |
| qPCR           | real-time polymerase chain reaction                 |
| R.A.           | respiration analyses                                |
| R <sup>2</sup> | coefficient of determination of a linear regression |
| RB             | respiration buffer                                  |
| RC             | respiratory chain                                   |
| RCR            | respiratory control ratio                           |
| REST           | Relative Expression Software Tool                   |
| RNA            | ribonucleic acid                                    |
| RNS            | reactive nitrogen species                           |
| ROS            | reactive oxygen species                             |
| SD             | standard deviation                                  |
| SDS            | sodium dodecyl sulfate                              |
| SE             | standard error                                      |
| SH             | thiol group                                         |
| SOD            | superoxide dismutase                                |
| Std dev        | standard deviation                                  |
| Std error      | standard error                                      |
| t              | thymine                                             |
| t              | time                                                |
| TBE            | Tris, boric acid and EDTA buffer                    |
| TCA            | tricarboxylic acid                                  |
| T <sub>m</sub> | melting temperature                                 |
| TNB            | 2-nitro-5-thiobenzoate                              |

|               |                                                                       |
|---------------|-----------------------------------------------------------------------|
| TPP           | thiamine pyrophosphate                                                |
| Tris.HCl      | tris-hydrochloric acid (2-amino-2-hydroxymethyl)-1,3-diol             |
| Triton X-100™ | Triton X-100™: Octylphenolpoly(ethylene-glycoether)n: C <sub>24</sub> |
| ™             | trademark                                                             |
| TW            | Taiwan                                                                |
| Tween® 20     | polyoxyethylene (20) sorbitan monolaurate                             |
| UAE           | United Arab Emirates                                                  |
| UCII          | μmole/min complex II                                                  |
| UCS           | μmole/min citrate synthase                                            |
| UK            | United Kingdom                                                        |
| UV            | ultra violet                                                          |
| vs.           | <i>versus</i> : Latin abbreviation for “against”                      |
| v/v           | volume per total volume                                               |
| VT            | Vermont                                                               |
| w/v           | weight per volume                                                     |
| WT            | wild type                                                             |
| ZA            | South Africa                                                          |

# CHAPTER 1

## INTRODUCTION

As the main source of cellular energy, **the mitochondrion** is at the centre of an extensive energy network that enables the normal functioning of most other cellular processes. This vital function is achieved via five enzyme complexes contained in the mitochondrion, which are responsible for ATP production through the process of oxidative phosphorylation (OXPHOS) (Nicholls and Ferguson, 2002). Mitochondria also play a central role in the metabolism of carbohydrates, lipids and amino acids. The respiratory chain, which includes the first four of the five enzyme complexes of the OXPHOS system, is furthermore considered to be the major source of cellular reactive oxygen species (ROS). Because of this ROS production, mitochondria are therefore also the main cellular bodies that determine the fates of cells, by initiating apoptosis or necrosis (Tanaka, 2010).

Hence it is not surprising that mitochondrial dysfunction has been implicated in a vast range of disorders, such as obesity, metabolic syndrome, type 2 diabetes and atherosclerosis. Through altered ROS production, defective mitochondria have been linked to neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, and more recently, bipolar disorders, depression, schizophrenia and autistic spectrum disorders (Tanaka, 2010).

To cope with deficiencies, various adaptive responses to a mitochondrial deficiency are triggered. Recently, and of particular relevance to this study, **metallothioneins (MTs)** have been identified as one of these adaptive responses to complex I deficiencies (van der Westhuizen *et al.*, 2003; Reinecke *et al.*, 2006). MTs are small, cysteine-rich, non-enzymatic molecules that play an important role in especially copper and zinc homeostasis, heavy metal detoxification and free radical scavenging (Ogra *et al.*, 2006; Carpenne *et al.*, 2007). However, it is thought that a primary function of MTs remains undisclosed.

In an attempt to address this, associations between MTs and several organelles have been investigated. Of particular interest to this study are reports on the association of MTs with components of the mitochondrion or mitochondrion-associated processes, such as ROS and oxidative stress, apoptosis, glutathione, energy metabolism (metal homeostasis and enzyme activity, enzyme inhibition and activation, cytochrome c and co-enzyme Q, the mitochondrial permeability transition pore and nucleotide complex formation), as well as nuclear- and mitochondrial DNA transcription regulation (Lindeque *et al.*, 2010). However, few or no *in vivo* studies have been reported to support *in vitro* evidence of a putative link between MTs and the mitochondrion. Particularly, reports on the involvement of MTs with respiratory chain function are relatively limited.

The aim of this study was to evaluate the possible *in vivo* involvement of MTs in the modulation of mitochondrial respiration. This was achieved by investigating several key parameters of mitochondrial function/dysfunction in a rotenone-induced, complex I deficient MT knockout (MTKO) mouse model. These parameters were the respiratory chain function as a working unit, the respiratory chain function as individual units (individual enzyme complex activities), as well as the relative mtDNA copy number. The addition of data on the proteome (this study) and metabolome (another, linked study) to the current transcriptome-focused published data sets, could facilitate in better understanding of the possible role that MTs play in cellular processes, including those of the mitochondrion.

## CHAPTER 2

### LITERATURE OVERVIEW

#### 2.1 INTRODUCTION

In Chapter 1 the association of mitochondrial function and dysfunction in cellular function, differentiation and death was highlighted. As mitochondrial dysfunction is a primary or secondary cause in the development of pathologies in various diseases (Dröge, 2002), a key focus in the preservation of cellular health would frequently include an increase of mitochondrial-controlled cellular survival through various mechanisms. This chapter gives an overview of the fundamental aspect of mitochondrial function, namely oxidative phosphorylation, as well as metallothioneins and the putative associative role of metallothioneins in mitochondrial function. This overview therefore serves as a rationale and motivation for this study. The specific problem statement, aim and strategy for this study are also presented in this chapter.

#### 2.2 THE MITOCHONDRION

Mitochondria are small, double membrane organelles found in the cytoplasm of most eukaryotic cells. They contain the four enzyme complexes (CI - CIV)<sup>1</sup> of the respiratory chain (RC) as well as ATP synthase (CV), which are together mainly responsible for energy production in the form of ATP. This combined process is known as oxidative phosphorylation (OXPHOS). Mitochondria are found in greater abundance in tissues that have high energy expenditure, such as skeletal muscle, heart and liver (Henze and Martin, 2003; McBride *et al.*, 2006).

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<sup>1</sup> complex I: NADH:ubiquinone oxidoreductase, EC 1.6.5.3; complex II: succinate:ubiquinone oxidoreductase, EC 1.3.5.1; complex III: ubiquinol:ferricytochrome c oxidoreductase, EC 1.10.2.2; complex IV: ferrocycytochrome-c:oxygen oxidoreductase, EC 1.9.3.1; complex V: ATP phosphohydrolase, EC 3.6.1.3.

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### 2.2.1 MITOCHONDRIAL STRUCTURE

The mitochondrion is made up of two membranes, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The IMM is folded elaborately inwards to increase the surface area. These folds are called *cristae*. The membranes divide the mitochondrion into two compartments: the inter-membrane space (IMS), which is situated between the two membranes, and the matrix, which is enclosed by the IMM (Mannella, 2006). Both membranes consist of elastic phospholipid bilayers, but the OMM contains mostly porin proteins, which form large pores, making it permeable to molecules smaller than 10 kDa. The IMM is much more impermeable and will only allow the free movement of water, oxygen and carbon dioxide over it. Special transporter proteins are responsible for the movement of ions (most notably copper, iron and calcium), substrates and fatty acids over the IMM (Herrmann and Neupert, 2000; Nicholls and Ferguson, 2002; Chipuk *et al.*, 2006). The OXPHOS complexes are arranged throughout the IMM, together with two co-enzymes, namely cytochrome *c* and co-enzyme Q (coQ). Recent studies by Dencher and associates (Haus *et al.*, 2005, Lechner *et al.*, 2006; Seelert *et al.*, 2009, Schon and Dencher, 2009; Frenzel *et al.*, 2010) suggest that, unlike the currently favoured textbook “random collision” model, where RC complexes are individually infused in the membrane and the transfer of electrons depends on the random collision of co-enzymes with RC complexes (Figure 2.1), it is more likely that the OXPHOS complexes are arranged into super-complexes, sometimes containing multiple copies of certain complexes. These latter models are referred to as the “solid-state” model, where all five complexes, including multiple copies of some, are arranged into one super-complex, and the “plasticity” model, where complexes occur individually and in super-complexes, and where the different arrangements depend on tissue type and bio-energetic demand. OXPHOS complexes arranged according to the latter two models would be much more energy efficient and better protected against hazardous electron leakage (Schon and Dencher, 2009). It would also influence the manner in which OXPHOS enzymes are affected by deficiencies and their compensational abilities during stressful conditions (Dencher, 2010, private communication).

### 2.2.2 MITOCHONDRIAL RC COMPLEXES

Mitochondria have numerous functions, although the most well-known (and also the focus of this study) is the production of energy. During the final catabolic process of the two main sources of energy, carbohydrates and fatty acids, these molecules are broken down to form electron donors, reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ( $\text{FADH}_2$ ) in the mitochondrion. As illustrated in Figure 2.1, these donors donate electrons to the first two complexes in the RC, respectively.

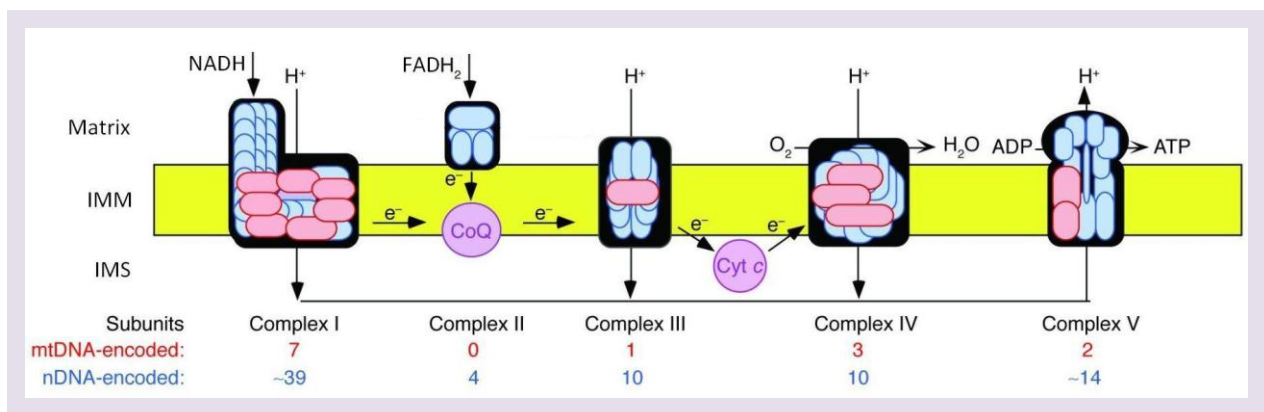


Figure 2.1: Schematic representation of the enzymes of the OXPHOS system. Electrons are delivered to the RC by NADH (at CI) and  $\text{FADH}_2$  (at CII) and are carried to molecular oxygen via RC enzyme complexes and co-enzymes.  $\text{H}^+$  ions are carried across the IMM into the IMS to create a proton gradient. When this gradient is alleviated via CV, ADP is converted to ATP. NADH: reduced nicotinamide adenine dinucleotide;  $\text{FADH}_2$ : reduced flavin adenine dinucleotide; IMM: inner mitochondrial membrane; IMS: inter membrane space; CoQ: co-enzyme Q; Cyt c: cytochrome c; ADP: adenosine diphosphate; ATP: adenosine triphosphate (Adapted from DiMauro, 2004).

During a series of redox reactions, electrons are transported through the RC to the final receiver, oxygen. The energy generated during these thermodynamically favourable redox reactions, is used by CI, CIII and CIV to pump hydrogen ions across the IMM into the IMS. This creates a proton gradient across the IMM. This potential energy is then transformed into free energy during the movement of hydrogen ions through CV back

into the matrix and captured in the high energy bonds of ATP, during the phosphorylation of ADP to ATP (Nicholls and Ferguson, 2002).

Four (CI, CIII, CIV and CV) of the five OXPHOS enzyme complexes are encoded for by genes from both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). All of the RC complexes and co-enzymes like cytochrome *c* contain metals or metal groups that include iron and copper. These metals are also important in the assembly of the RC enzymes (Voet and Voet, 2004). Aberrant concentrations of these metals lead to aversive enzyme assembly. Iron and copper are carried into the mitochondrion by chaperones such as COX17, which carries copper (Lill and Kispal, 2000; Ogra *et al.*, 2006). Deficient transport of copper into the mitochondrion leads to deficient construction of copper containing units of the RC, especially cytochrome *c* oxidase (CIV) and a down regulation of RC complexes occurs. Copper and iron metabolism is tightly controlled by a number of mechanisms. In this control, metallothioneins (MTs) play an important role, especially in copper homeostasis (Ogra *et al.*, 2006).

Mitochondrial respiration can be inhibited in a number of ways. Direct inhibition of RC complexes occurs when substances bind to specific sites on the complex, prohibiting its true substrate from binding (i.e. allosteric regulation). An example of this is the binding of cadmium between the semi-ubiquinone Q<sub>0</sub> site of cytochrome *b* in CIII, prohibiting the binding of semi-ubiquinone to CIII (Wang *et al.*, 2004). Zinc can also bind to CI and CIII with great affinity (Ye *et al.*, 2001; Dineley *et al.*, 2005). Another way that mitochondrial respiration is inhibited is through the disruption of the membrane potential formed by the movement of hydrogen ions across the IMM. Zinc can achieve this by moving into the IMS (Ye *et al.*, 2001). Also, opening of the IMM transition pore causes a flux of ions into the matrix and mitochondrial swelling, resulting in the loss of membrane potential and inhibition of respiration (Simpkins *et al.*, 1998a).

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### 2.2.3 MITOCHONDRIAL DISEASES

Since mitochondria play such an important role in normal cellular function, of which only a part is described here, it is not surprising that the effects are so devastating when mitochondrial function is deficient. Of all the identified inborn errors of metabolism, those associated with the mitochondrion are the most abundant. As defined by Haas *et al.* (2008), primary mitochondrial respiratory chain disease “*is a heterogeneous group of disorders characterized by impaired energy metabolism due to presumed genetically-based OXPHOS dysfunction*”. Mutations in both nDNA and mtDNA can lead to dysfunctional mitochondrial respiration as subunits that make up the OXPHOS enzyme complexes are encoded for by both genomes. These mutations may cause changes in tertiary peptide conformation, leading to anomalous enzyme function and activity. The consequences of any of these dysfunctions are wide-ranging but include disruptions of the nicotinamide adenine dinucleotide (NAD) redox balance, ATP/ADP homeostasis and cellular calcium ( $\text{Ca}^{+}$ ) handling, which is involved in cellular signalling events (Reineke *et al.*, 2009). Mitochondrial disease manifests in a wide variety of clinical and biochemical symptoms ranging from single lesions to complex multisystem syndromes. Definitive confirmation often requires invasive procedures such as tissue biopsies, and multiple diagnostic tests, with enzymatic assays and molecular analyses at the forefront (Haas *et al.*, 2008; McFarland *et al.*, 2010). Of the many mitochondrial disorders identified to date (Online Mendelian Inheritance in Man, <http://www.ncbi.nlm.nih.gov/omim>), successful treatments for only a few of those (MNGIE and coQ deficiencies) have been established (Hirano, 2010). Treatment is always symptomatic as currently, no ethically approved cures have been described (Craven *et al.*, 2010).

The most frequently encountered and thus most thoroughly studied defect of the OXPHOS system is CI deficiency (van der Westhuizen *et al.*, 2003). Pathological mutations have been found in all 14 evolutionary conserved subunits which make up the catalytic core of CI. The primary consequence of isolated CI deficiency is an increased production of superoxide and derived reactive oxygen species (ROS) and nitrogen

species (RNS). While under normal cellular conditions, these molecules play an essential role in signalling mechanisms as messengers, or more directly, as activators of uncoupling proteins for thermogenesis, elevated concentrations of these molecules can have detrimental effects. Studies investigating the damaging effects of ROS and RNS on macromolecules have shown that genetic and functional molecular viability is directly impacted (Dröge, 2002; Jones, 2008). ROS, together with  $\text{Ca}^{+}$ , increases IMM permeability by opening the transition pore, leading to a loss of membrane potential and apoptosis (Simpkins *et al.*, 2008a; Reineke *et al.*, 2009).

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#### 2.2.4 ADAPTIVE RESPONSES TO MITOCHONDRIAL DISEASES

The diversity of cellular adaptive responses during a fundamental deficiency, such as an OXPHOS deficiency, is highly diverse and depends on the type and level of deficiency, tissue or cell line affected and genetic make-up of the organism (Reinecke *et al.*, 2009). Also involved are responses that are initiated directly, such as redox state changes, ROS formation, low ATP production, as well as others including cellular signalling and structural changes (Reinecke *et al.*, 2009). The investigation of mitochondrial diseases and the adaptive responses to it has been a focus area of research at this institute since 2000. In a study done on cultured fibroblast cell lines established from CI deficient patients, van der Westhuizen *et al.* (2003) reported a marked induction of metallothioneins (MTs), using a mitochondrion-targeting microarray. It was postulated that this induction could be ascribed to the increased ROS which occurs in mitochondrial respiratory chain deficiencies. *In vitro* confirmation was provided by Reineke *et al.* (2006), who demonstrated that MT2A, an isoform of MT, expression is induced and offers significant protection against ROS in rotenone-induced CI deficient HeLa cells. As recently evaluated by Lindeque *et al.* (2010), a putative role of MT in mitochondrial function and disease therefore appeared worthwhile to investigate further.

## 2.3 METALLOTHIONEINS

MTs are a group of small non-enzymatic proteins with low molecular weight and are characterised by high cysteine content and no aromatic amino acids. They have a great affinity for metals in the following order: Hg (II) > Ag (I) = Cu (I) > Cd (II) > Zn (II).

### 2.3.1 METALLOTHIONEIN STRUCTURE

When bound to metals, MT consists of two domains, the C-terminal containing  $\alpha$ -domain and the N-terminal containing  $\beta$ -domain (Figure 2.2). The  $\alpha$ -domain contains eleven cysteine amino acids, which bind four divalent metals, while the  $\beta$ -domain contains nine cysteine amino acids, which bind three divalent metals (Carpenè *et al.*, 2007).

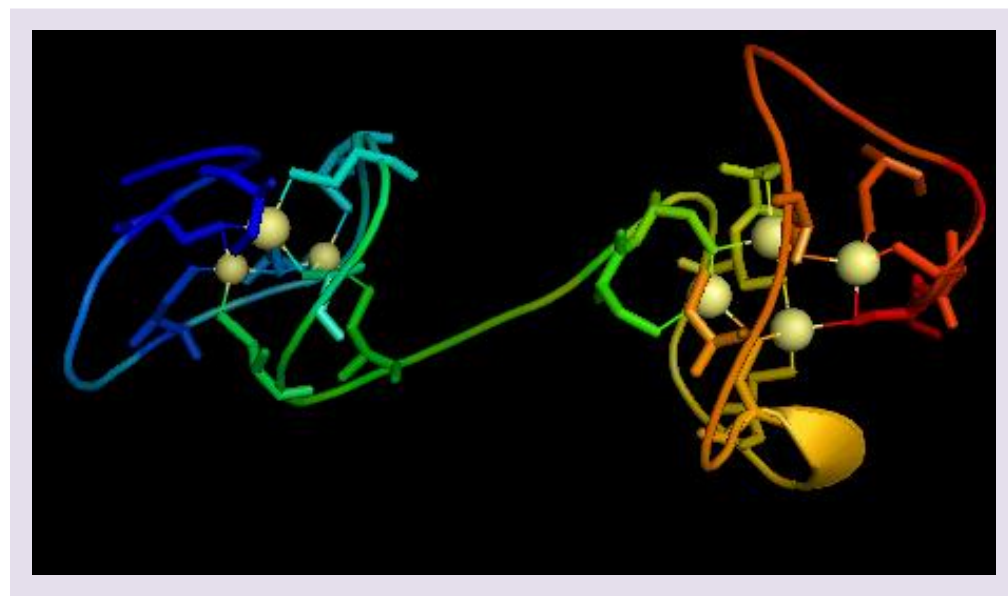


Figure 2.2: Graphic representation of MT2A from rat. The  $\alpha$ -domain is on the right of the figure, bound to four metal ions, while the  $\beta$ -domain is on the left, bound to three metal ions. Cysteine groups are shown in stick format, while metal ions are represented by spheres. Structure PDB id: 4MT2 (Bruan *et al.*, 1992), image generated in PyMOL (Schrödinger, 2010).

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### 2.3.2 METALLOTHIONEIN ISOFORMS

Four major isoforms of MT have been identified, which are MT-1, MT-2, MT-3 and MT-4. MT-1 and MT-2 are expressed in almost all major organs, including the liver and heart. MT-3 is mainly expressed in the brain and MT-4 in differentiating stratified squamous epithelial cells. The isoforms differ from each other by only one or two amino acids (Carpenè *et al.*, 2007).

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### 2.3.3 METALLOTHIONEIN FUNCTION AND EXPRESSION

Many functions have been ascribed to MTs, but the foremost function of this protein has yet to be disclosed. MTs have three primary functions (described as those performed on a micro level). Firstly, they are important for copper and zinc homeostasis, binding these metals and/or delivering them to sites where they are needed (Ogra *et al.*, 2006). Secondly, MTs are involved in metal detoxification as they have a high affinity for metals and have been shown to bind endogenous and exogenous metals. They are also inducible by high cellular metal concentrations (Ogra *et al.*, 2006; Carpenè *et al.*, 2007; Jebali *et al.*, 2008). Thirdly, they act as scavengers of free radicals, scavenging hydroxyl radicals (OH<sup>•</sup>) 300 times more effectively than glutathione. It has also been shown that nitrogen oxide (NO) will displace zinc bound to MTs (Carpenè *et al.*, 2007), and that gene expression of MTs is induced by ROS and, after expression, protects against ROS-mediated cell death (Reinecke *et al.*, 2006).

MT expression is mostly induced indirectly and is regulated via metal responsive elements (MREs) and an antioxidant response element (ARE), that respond to a wide range of effectors (Andrews, 2000; Ghoshal and Jacob, 2000; Haq *et al.*, 2003). MT expression under stress conditions is generally induced by factors which it protects the cell against, and is regulated by negative feedback. Inducing factors include, but are not limited to: metal ions, ROS, hormones, secondary messengers as well as stress-producing conditions like starvation and infection (Kondoh *et al.*, 2003; Carpenè *et al.*, 2007; Jebali *et al.*, 2008).

## 2.4 METALLOTHIONEIN INVOLVEMENT IN MITOCHONDRIAL FUNCTION

In an effort to elucidate the primary function of MTs, recent studies have focused on linking MTs to a variety of specific organelles. Most pertinent to the present study, reports have been made of a putative link between MTs and the mitochondrion (Lindeque *et al.*, 2010).

### 2.4.1 CELLULAR LOCALISATION AND PUTATIVE MITOCHONDRION-LINKED INTERACTIONS OF METALLOTHIONEINS

Despite it not being considered a mitochondrial protein, Ye *et al.* (2000) found that the concentration of MTs localised in the IMS of mitochondria is about 50% of that found in the cytosol, while Sakurai *et al.* (1993) used radioimmunoassays to show that MT concentrations are highest in mitochondria when compared to other organelles. This was also illustrated in HeLa cells using green fluorescent protein tagged MT-1 (Lindeque *et al.*, 2010). Numerous reports have been made on the interaction, be it direct or indirect, of MTs with various components of the mitochondrion itself and those supporting or supplying it. These components include the following as reviewed in detail by Lindeque *et al.* (2010) and are presented in Figure 2.3: ROS and oxidative stress, apoptosis, glutathione, energy metabolism (metal homeostasis and enzyme activity, enzyme inhibition and activation, cytochrome c and coQ, mitochondrial permeability transition pore, nucleotide complex formation) and nuclear- and mitochondrial DNA transcription regulation. Some of these interactions important to this study will be discussed in the next section.

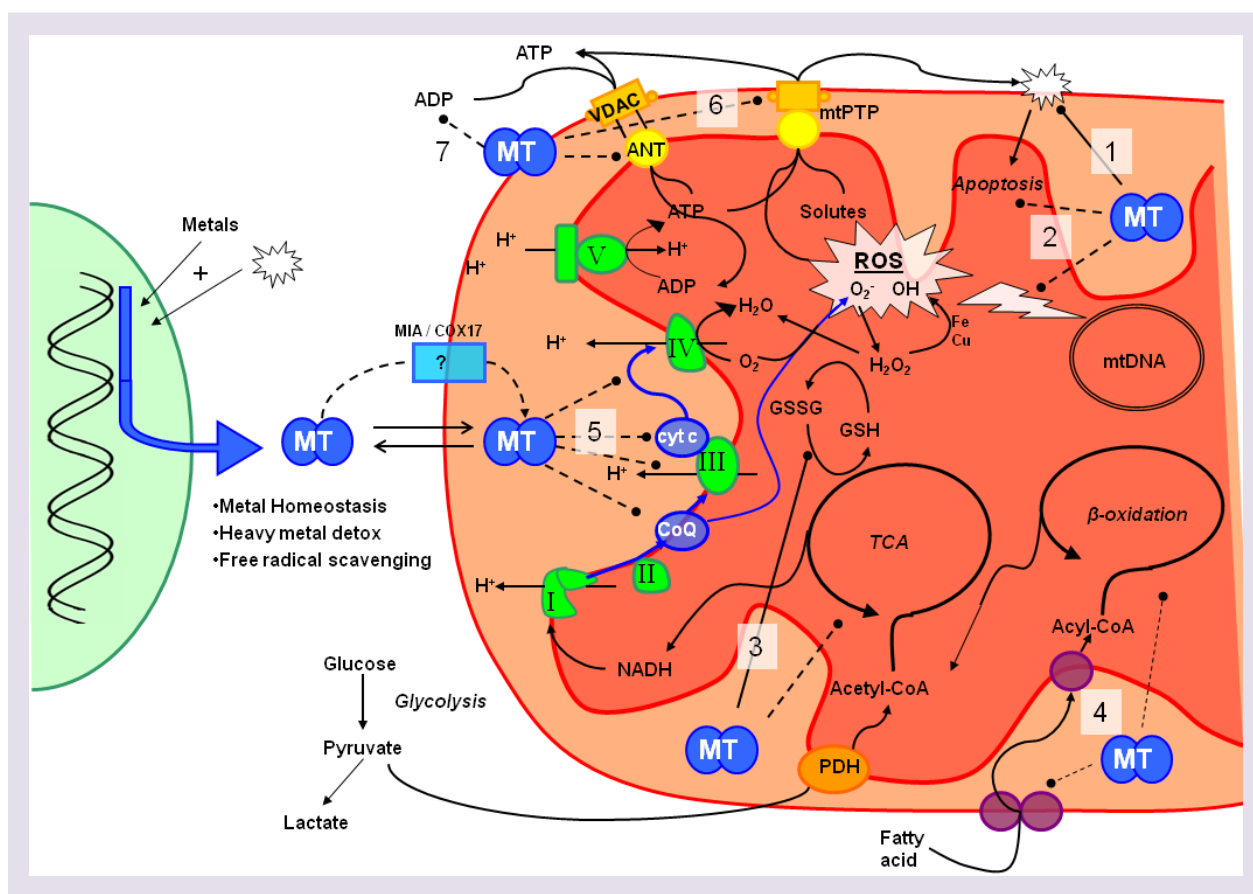


Figure 2.3: Schematic representation of the summarised putative interactions of MTs with components of the mitochondrion. Protection against ROS (1) and apoptosis (2). MT-glutathione cycle and enzyme regulation (3). Involvement in lipid metabolism (4). Interaction of MTs with the RC (5) and the IMM transition pore (6) as well as MT sequestration of ATP (7). Cyt c: cytochrome c; PDH: pyruvate dehydrogenase; TCA: tricarboxylic acid. Stippled lines indicate indirect or uncertain interactions and pathways. (Adapted with permission from J.Z. Lindeque).

#### 2.4.2 MITOCHONDRIA-ASSOCIATED FUNCTIONS OF METALLOTHIONEINS

MTs are predominantly considered stress proteins and consequently mostly studied in disease models. Using a mitochondrion-targeting microarray, van der Westhuizen *et al.* (2003) reported a marked induction of metallothioneins in CI deficient human cell lines. It was postulated that this induction could be ascribed to the increased ROS which occurs in mitochondrial respiratory chain deficiencies. This was confirmed *in vitro* by Reineke *et al.* (2006), who demonstrated that MT2A expression is induced and offers significant protection against ROS in rotenone-induced CI deficient HeLa cells. In an *in*

*vivo* study, Kondoh *et al.* (2001) demonstrated that synthesis of MTs was specifically induced by mitochondrial oxidative stress, when mice were treated with an electron transfer inhibitor (antimycin A) and an uncoupler (2,4-dinitrophenol).

Because of their ability to scavenge ROS and RNS, it was postulated that MTs might be involved in the regulation of **apoptosis**, as ROS is an apoptotic stimulus. In an *in vivo* study, Cai *et al.* (2006) found that by suppressing diabetes-caused mitochondrial oxidative stress and thus suppressing cytochrome c release and caspase 3 activation, MTs inhibit apoptosis in diabetic mouse hearts. Shimoda *et al.* (2003) demonstrated *in vitro* that MTs also protect different cell lines against etoposide-induced apoptosis, which does not make use of ROS, but rather through uncorrected DNA damage. This suggests that MTs might play a generalised role in the inhibition of apoptosis, not only through ROS scavenging but also by other means, such as the release of zinc, which has been shown to strongly inhibit caspase 3.

The regulation of ROS levels in the cell is clearly essential for normal cellular function. Mitochondria are the main sources of ROS production, but also the primary target of oxidative damage (Tanaka, 2010). As mentioned before, MTs protect mitochondria against ROS-related oxidative damage, but it is not the primary free radical scavenger under normal non-pathological conditions. One of the most important components of the cellular antioxidant system is **GSH** (reduced glutathione) (Forman *et al.*, 2009). GSH is distributed throughout the cell, as well as inside mitochondria and is thus readily available to scavenge free radicals. It is only during conditions of high oxidative stress that MTs take over this role, by scavenging free radicals about 300 times more efficiently than GSH (Ding *et al.*, 2002; Cai *et al.*, 2006). But unlike GSH, MTs are not localised throughout the entire cell, and likely also not in the mitochondrial matrix, hence GSH is still needed to protect against ROS where MTs cannot. The preservation of GSH is thus vitally important and the MT-glutathione cycle could play a key role here. GSH is oxidised to GSSG (glutathione disulphide) during free radical scavenging. To restore GSH levels, GSSG is reduced by glutathione reductase, which is localised in the mitochondrial matrix. During conditions of high oxidative stress, MTs might reduce GSSG to restore GSH levels, but this would require a direct interaction between the two

molecules (as with GSSG and glutathione reductase). Brouwer *et al.* (1992) identified *in vitro* a putative binding site for GSH on the N-terminal domain of MT. Although not investigated, a similar interaction with GSSG remains a possibility. Also, Brouwer *et al.* (1992) and Maret (1994) proposed the possibility of metal exchange between MTs and GSH, the direction of the exchange being dependant on the change in redox state of the cell (Jiang *et al.*, 1998). This cooperation might be an important factor in MT function.

One of the primary functions ascribed to MTs is copper and zinc **homeostasis**. Because of this, it is generally accepted that MTs are involved in the **regulation of specific enzymes**. As many as 300 enzymes (including some associated with the mitochondrion) are dependent on metal ions such as copper and zinc as co-factors. In an *in vitro* study using cultured cells established from metallothionein knockout and wild type mice, Ogra *et al.* (2006) found that viability of MT-null cells was decreased compared to wild type cells when treated with bathocuproine sulfonate, a Cu (I) - specific chelator. It was suggested that MTs maintain the activities of cytochrome c oxidase (CIV) and superoxide dismutase 1 (SOD) in Cu depletion, by providing them with Cu via other Cu-chaperones. Therefore, MTs were shown to play a protective role against copper depletion. Beattie *et al.* (1998) also reported decreased liver copper levels in MTKO mice compared to control mice in a study conducted *in vivo*. This implies that the copper ion might be an important link between MTs and **altered metabolism**.

Another MT associating metal that might be a link is zinc. Zinc has been shown in several studies to be involved in **modulation of mitochondrial respiration**. Because zinc, together with copper, is the dominant metal bound to MTs, it is plausible to conclude that MTs play a regulatory role in its modulation. When MT-bound zinc enters the IMS and is released, it inhibits respiration. Zinc has been shown to bind with all four complexes *in vitro*, but it only binds to CI and CIII with high affinity (Ye *et al.*, 2001). It possibly inhibits electron transport at the *bc*<sub>1</sub> subunit of CIII. It has also been shown *in vitro* that MTs directly interact with *m*-aconitase (a TCA-cycle enzyme) in mouse heart mitochondria, by transferring zinc to this enzyme, inhibiting the TCA-cycle and consequently inhibiting energy production (Feng *et al.*, 2005). Another component of the

TCA-cycle,  $\alpha$ -ketoglutarate dehydrogenase complex, was shown to be even more sensitive to zinc mediated inhibition (Brown *et al.*, 2000). The authors go on to suggest that zinc may thus play a regulatory role in mitochondrial energy metabolism. Again, MTs can also be implicated in this regulatory role because of their close association with zinc. Costello *et al.* (2004) also reported *in vitro* evidence to suggest that MTs donate zinc to the putative zinc uptake transporter associated with the IMM and transports zinc into the mitochondrial matrix of prostate and liver mitochondria. It therefore seems likely that MTs may be indirectly involved in the regulation of mitochondrial respiration via zinc. The inhibitory effect of zinc on respiration should however not be considered aversive, but merely as a form of regulation. Dineley *et al.* (2005) also showed *in vitro* that the inhibition of respiration by zinc alleviates succinate-supported ROS, therefore playing a protective role.

Besides the five complexes of the OXPHOS system, two **co-enzymes** also play a vital role in mitochondrial respiration. Cytochrome *c* and coQ are responsible for carrying electrons between RC complexes. Reports on interactions between MTs and these co-enzymes are scarce and inconclusive, but nevertheless, worth mentioning. Simpkins *et al.* (1993) found that cytochrome *c* can be reduced by MT-1 *in vitro*. The transfer of electrons from cytochrome *c* to MT-1 would remove electrons from the RC, hindering proton gradient formation, which could result in decreased respiration, another possible mechanism by which MTs could be involved in the (down)regulation of respiration. While a direct interaction between MTs and coQ is unlikely, since coQ is very hydrophobic and therefore imbedded in the IMM, reports of an indirect relationship have been made by Ebadi *et al.* (1994; 2002; 2005). In an *in vivo* study done on a range of genetically engineered mouse models with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced parkinsonism, striatal coQ levels were significantly reduced in MT-null mice (Ebadi *et al.*, 2005). From this and other studies done by Ebadi *et al.*, it was proposed that MTs may provide neuroprotection by increasing coQ concentrations and thus mitochondrial function, via stimulation of lipoamide dehydrogenase activity.

Another possible component involved in the regulation of mitochondrial respiration is the IMM **transition pore**. The prolonged opening of this pore is associated with mitochondrial swelling and loss of membrane potential, thus inhibiting respiration. A series of *in vitro* studies done by Simpkins *et al.* (1994; 1996; 1998a; 1998b) describe a possible role of MTs in this type of regulation. MTs were found to inhibit ADP stimulated oxygen consumption (Simpkins *et al.*, 1994). This was confirmed in a study on isolated rat liver mitochondria, where it was shown that the addition of MTs caused mitochondrial swelling and depolarisation of the IMM, an action that was counteracted by spermine, leading the investigators to believe that MTs increased the open time of the IMM transition pore (Simpkins *et al.*, 1996). In further investigations, it was found that MTs and calcium synergistically inhibited oxygen consumption, possibly by oxidising SH groups on the IMM, opening the IMM transition pore and thus increasing inner membrane permeability (Simpkins *et al.*, 1998a; Simpkins *et al.*, 1998b). This may seem disadvantageous, but considering that in this case MTs act as an uncoupler and not an inhibitor, thus not increasing ROS levels (Boveris and Chance, 1973), the authors proposed that in high oxidative stress conditions, MTs might prevent further increase in ROS levels, while scavenging free radicals. They concluded that *in vivo*, the inhibitory effect of MTs would be kept in check by spermine and that relative concentrations of MT, spermine and magnesium could possibly regulate the permeability of the IMM (Simpkins *et al.*, 1998b).

It has been shown that MTs can directly interact with zinc-finger transcription factors and supply or remove metal ions, thereby activating or inhibiting them. This leads to the assumption that not only are MTs involved in the various components of the mitochondrion as described above, but possibly also in **transcription regulation** of the nuclear and mitochondrial genes that encode them (Lindeque *et al.*, 2010). Recent transcriptomics studies have found differential gene expression of numerous genes in MTKO mice, including genes involved in energy metabolism, cellular antioxidant defence,  $\beta$ -oxidation of fatty acids, glycolysis, subunits of the mitochondrial electron transport chain, apoptosis and ROS protection. MT involvement with various components involved in mitochondrial function as discussed in the above sections could, however, not only be at transcriptional level, since most of the abovementioned data were obtained from *in vitro* studies, eliminating the role of transcription regulation (Lindeque *et al.*, 2010).

To conclude, it is evident that MTs are involved in various processes of the mitochondrion, as discussed above, but while *in vitro* evidence is plentiful (MT interaction with or in ROS, apoptosis, GSH, enzyme regulation, Zn and Cu homeostasis, cytochrome *c* and the IMM transition pore), supportive and conclusive *in vivo* evidence is either scarce (MT's interaction in or with ROS, apoptosis, Cu homeostasis and coQ) or completely lacking.

## 2.5 INVESTIGATION OF METALLOTHIONEIN USING DISEASE MODELS

As mentioned earlier, MTs are considered stress response proteins and are therefore often studied in disease models where oxidative or other stresses were induced. As this study was focused on mitochondrial respiration, and CI deficiency is the most frequently occurring defect of the mitochondrial respiratory chain, the study was conducted on rotenone-induced CI deficient MTKO mice. As pointed out in the previous section, supportive *in vivo* evidence for *in vitro* studies concerning the putative link between MTs and components of the mitochondrion is scarce. Therefore, an *in vivo* model was chosen for this study. It was also done on mice specifically, since no other MTKO animal models are available. To induce CI deficiency, a number of compounds can be used. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is often used to inhibit CI to study Parkinson's disease, but is classified as a neurotoxin, since it only becomes toxic when metabolised to MPP<sup>+</sup>, after crossing the blood-brain-barrier (Langston, 2002). ADP-ribose has also been shown to compete with NADH binding to CI. It inhibits the forward reaction of CI, but stimulates the reverse action. Since it is an NADH analogue, there is a potential risk that a high concentration of NADH could out-compete ADP-ribose and prevent it from binding to CI (Zharova and Vinogradov, 1997). Although acetogenins and piericidin inhibit CI more efficiently than rotenone, their roles in CI inhibition as well as possible effects on other parts of the metabolism are not as well documented as those of rotenone (Degli *et al.*, 1994; Caboni *et al.*, 2004). Rotenone was also chosen so that the results obtained during this study would be comparable to those of previous studies done at this institute, where rotenone was used to induce CI deficiency (van der Westhuizen *et al.*, 2003; Reineke *et al.*, 2006; van Zweel, 2010).

## 2.6 PROBLEM STATEMENT

Although a number of primary and secondary functions have been attributed to them, the true function of MTs remains intriguingly elusive. This has led to a number of studies involving MTs, in an attempt to better understand this molecule. More recent studies have focused on the interaction of MTs with specific organelles. Quite a number of studies, as recently reviewed by Lindeque *et al.* (2010), have suggested a link between MTs and mitochondria, but efforts to properly define this link have been mired by contradicting reports and a lack of supportive *in vivo* evidence, resulting in uncertainty and speculation. Lindeque *et al.* (2010) go on to suggest that addressing some of these limitations, by supplementing the current data sets published on MTs, which are mainly focused on the transcriptome, with data on the proteome and metabolome, would provide a more accurate overview of the role that MTs play in cellular processes, including those of the mitochondrion. Alleviating some of the confusion surrounding the interaction of MTs with mitochondria might lead to a better understanding of MT involvement in mitochondrial function and disease pathologies, facilitating ongoing efforts to provide treatment for these pathologies. *A more specific problem related to this study is that information on the involvement of MTs with respiratory chain function is limited.* As MTs are associated with the cellular redox state as well as heavy metal housekeeping, they may be directly involved with the OXPHOS enzyme complexes. As mtDNA encodes several of the subunits of these enzymes and as there is also no information available on the effect of MTs on mtDNA copy number, this was an additional focus point of this study.

## 2.7 AIM AND STRATEGY

The aim of this study was therefore *to investigate the involvement of MTs in mitochondrial respiration and respiratory chain enzyme function by using an MT knockout (MTKO) mouse model, which was treated with the CI inhibiting reagent, rotenone*. The effects of the presence or absence of MTs become more pronounced under stressed or diseased conditions (Reinecke *et al.*, 2006; Lindeque *et al.*, 2010), therefore the rotenone treatment was used, as CI deficiency is one of the most prevalent defects in mitochondrial energy production.

The research here presented formed part of a larger study aimed at investigating the involvement of MTs in mitochondrial function and disease. Other parts of this larger study were undertaken by various post-graduate colleagues. The strategy formulated for addressing this overall aim is summarised in Figure 2.4.

As illustrated in the schematic *strategy*, the overall study uses the same animal model, MTKO (homozygous) and wild type mice, on which three main investigations, namely molecular biology, enzymology and metabolism, were conducted. The highlighted part of the figure presents the focus of this study, which is mainly the RC enzymology. In order to evaluate the involvement of MTs in mitochondrial respiration, components involved in oxidative phosphorylation were investigated as an overall working unit using respiration analyses, as *first objective*. The *second objective* was to investigate the RC system by its single units via individual RC enzyme analyses and structurally using blue-native polyacrylamide gel-electrophoresis (BN-PAGE) and western blot analysis. As supportive data for the structure and function of the RC enzyme complexes, as *third objective* the relative mtDNA copy number was also determined using real-time PCR. The detailed methods used to apply this strategy are presented in Chapter 3, whilst the results are presented and discussed in Chapter 4.

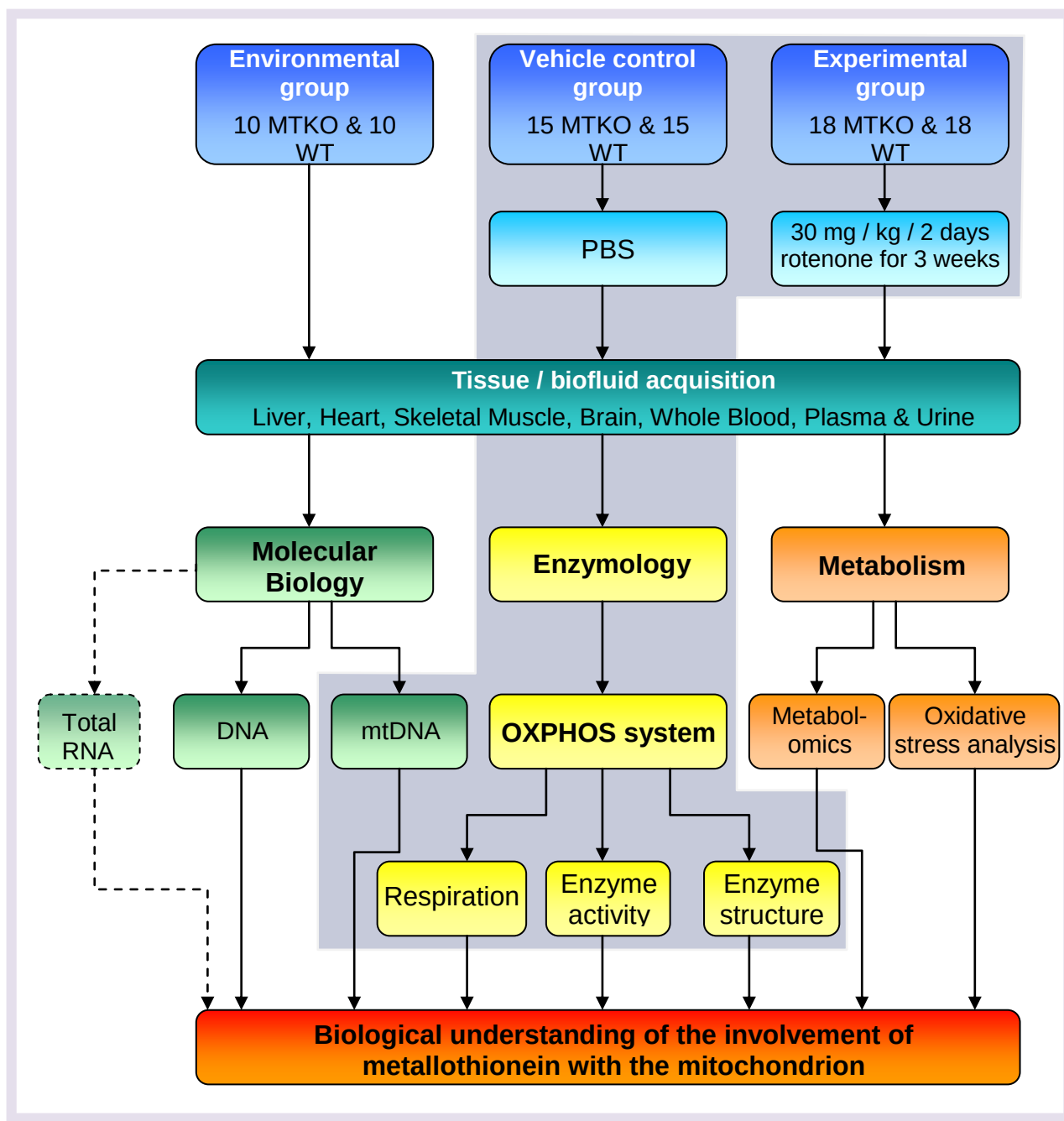


Figure 2.4: Schematic representation of the strategy used to achieve the aims of the overall investigation, which included five separate studies. Highlighted in purple is the part of this strategy used for the present study. Dotted lines refer to uncompleted parts. MTKO: metallothionein 1 and 2 knockout; WT: metallothionein wild type; PBS: phosphate buffered saline; OXPHOS: oxidation phosphorylation.

## CHAPTER 3

# MATERIALS AND METHODS

### 3.1 INTRODUCTION

In this study metallothionein 1 and 2 knockout (MTKO) mice were used as an experimental model to investigate the effect of metallothioneins (MT) on key mitochondrial functions (genotype variable). Using this mouse model, it was further attempted to include a mitochondrial stressor, namely the CI irreversible inhibitor, rotenone (treatment variable). In the experimental approach, a total of 66 male 129S7/SvEvBrd-Mt1<sup>tm1Bri</sup>Mt2<sup>tm1Bri</sup>/J mice, between the ages of 2 and 6 months were used, of which 33 were MTKO and 33 wild type (WT). These mice were randomly distributed into 11 Day-groups each containing six mice which were treated with either 30 mg/kg rotenone, using phosphate buffered saline (PBS) as solvent, or an equivalent volume of PBS every second day for 20 days. They were then euthanized and urine, blood and tissue samples were collected and used immediately or stored according to protocol specifications. A comprehensive range of analyses was conducted in a broader study (including several students) to investigate the possible role that MTs play in mitochondrial function. For the broader study (in addition to this one), these included a metabolomics investigation, ROS analyses, analyses of antioxidant and respiratory chain enzymes, a DNA epigenetics investigation and mtDNA copy number evaluation. In the present study the mitochondrial respiratory chain function and relative mtDNA copy number, which is related to respiratory chain gene expression and function (Reinecke *et al.*, 2009), were investigated which will be described in detail in this chapter. Enzyme activity assays were done on four tissues (liver, heart muscle, skeletal muscle and brain), while respiration analyses were done on two tissues (liver and heart). The results from these analyses were evaluated and consequently, only heart tissue was chosen for further enzyme investigations, *i.e.* BN-PAGE and western blot analysis, as well as mtDNA copy number estimation using real-time PCR.

### 3.2 THE MTKO MOUSE MODEL

The MTKO mouse model was developed in the laboratories of Dr. Richard Palmiter at the University of Washington and Dr. Ralph Brinster at the University of Pennsylvania as described originally by Masters *et al.* (1994) and revised by Palmiter in 2002 (<http://jaxmice.jax.org/strain/002211.html>). Disruption of both the *Mt1* and *Mt2* genes was done simultaneously by inserting in-frame stop codons into these gene exons. While mutant alleles are transcribed, they are not translated (Masters *et al.*, 1994).

Prof. Juan Hidalgo from the Autonomous University of Barcelona, ES, provided 10 breeding pairs of mice that have a single allele disruption in both the *Mt1* and *Mt2* gene loci (heterozygote, MT+/-). For this, an import permit was obtained from the South African Department of Agriculture. These mice were cared for and bred at the pathogen free unit of the Animal Research Centre at the North West University, Potchefstroom Campus. Heterozygotes (MT-/+ ) were used during breeding to produce MT-null (MT-/-), wild type (MT+/+) or heterozygote mice.

Ethical approval was obtained from the Ethics Committee of the North-West University in 2006, approval number 06D07. Animals were fed a standard laboratory diet (Rainbow Farms Pty. Ltd.), had free access to food and water and were kept under the following conditions: temperature was kept at 21 °C ± 1 °C while the relative humidity was 55% ± 5%; a 12 hour light : 12 hour dark cycle was maintained using fluorescent “daylight” tubes that supply full spectrum white light at an intensity of 350 lux, when measured 1 meter off the ground; air exchanges per hour were 16 – 18 times the volume (fresh uncirculated air); air pressure was positive in animal rooms, airtight doors maintain positive air pressure barriers between departments and air quality was maintained using high-efficiency particulate air (HEPA) filters.

### 3.3 IDENTIFICATION OF MICE

Mice were identified by assigning a unique number to each individual. Small holes at different positions were punched into the ears of each mouse. Each position represented a specific number and by using different combinations of these numbers, a mouse could be assigned any number from 1 to 159. Mice were also kept in groups in differently named cages (A to Z), further extending the reach of the numbering system. A mouse was therefore identified as e.g. A26 or F74. The figure below illustrates the numbering system used to mark the mice.

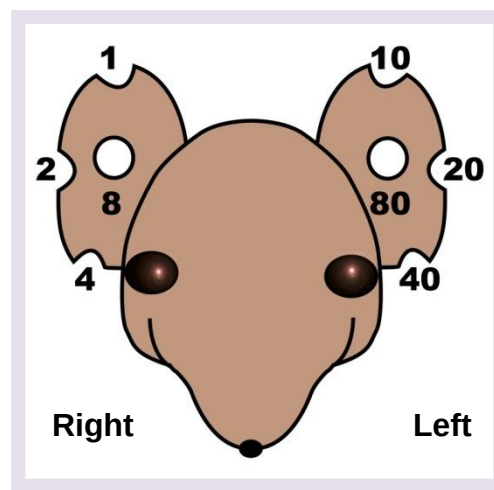


Figure 3.1: An illustration of the numbering system used to mark the mice. Each hole represents a different number. By combining the numbers, any number from 1 to 159 can be represented. For example: if a hole is punched in the centre of the left ear as well as the top of the right ear, the number assigned to the mouse is 81; if three holes are punched around the right ear, the assigned number is 7 ( $1 + 2 + 4$ ).

### 3.4 GENOTYPING OF MICE

Since heterozygotes were used during the breeding process, all mice had to be genotyped before inclusion into the study group. Blood was collected on filter paper by cutting off the tip of the mouse's tail and pressing it against the filter paper. It was then allowed to dry and stored at room temperature in sterile micro-centrifuge tubes until needed.

Because of the inserted oligonucleotides in the *Mt1* and *Mt2* genes in the MTKO mice (Masters *et al.*, 1994), amplified regions (amplicons) of the *Mt2* gene region would be longer than those obtained from WT mouse DNA by means of PCR. This allowed for the identification of MTKO, WT and heterozygote mice, by separating the amplicons utilising agarose gel electrophoresis. Amplicons of the modified/disrupted alleles were larger and thus travelled slower through the gel than WT amplicons. Amplification of the *Mt2* region from heterozygotes using PCR resulted in the production of two amplicon sizes: a longer amplicon (299 bp) from the MTKO allele and a shorter amplicon (282 bp) from the WT allele which could hence be identified by two bands of DNA on the gel. In the same way, amplification of MTKO DNA only produced the longer amplicons, whilst amplification of WT DNA only produced the shorter amplicons.

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#### 3.4.1 MATERIALS

All PCR reactions were done with 2x KAPA Blood PCR Mix A (KAPA Biosystems Pty. Ltd., Cape Town, ZA, KK7004<sup>1</sup>). This PCR-kit is trademarked to KAPA Biosystems Pty. Ltd., and can be used directly on whole blood (full contents are undisclosed). Oligonucleotides (primers) were purchased from Inqaba Biotec™, Pretoria, ZA. Milli-Q® prepared water<sup>2</sup> was used throughout.

Instrumentation<sup>3</sup> used during PCR reactions included: Hybaid MBS 0.2G Gradient Thermal Cycler (Hybaid Limited, Middlesex, UK).

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<sup>1</sup> Numbers in brackets in this chapter indicate the catalogue numbers of reagents purchased from the relevant company.

<sup>2</sup> Milli-Q® prepared water refers to water that has been purified using the Milli-Q® system from Millipore™ (Billerica, MA). A detailed description of the process can be found in Appendix A.

<sup>3</sup> Throughout this study, unless specified otherwise, the following consumables were used: 8-Strip PCR Tubes and Caps (Roche Diagnostics, Randburg, ZA, 11667009001); Eppendorf® Safe-Lock® micro-centrifuge tubes (various volumes, Sigma-Aldrich®); Eppendorf® epT.I.P.S® (various volumes, Sigma-Aldrich®); Costar® 96-Well polystyrene standard microplates (Cole-Parmer® Instrument Company Ltd., London, UK).

For agarose gel electrophoresis the following reagents were purchased: agarose (Conda Micro & Molecular biology, Madrid, ES, 8016); Tris(hydroxymethyl) aminomethane (Melford Laboratories Ltd., Suffolk, UK, B2005); boric acid (Merck Chemicals Pty. Ltd., Modderfontein, ZA, A1223); ethylene diamine tetraacetic acid, dipotassium salt dihydrate (EDTA.K) (Fluka™ Analytical via Sigma-Aldrich® Pty. Ltd., Johannesburg, ZA, 03660); ethidium bromide (Fluka™, 46065); bromophenol blue (BDH® Chemicals, Dubai, UAE, 20015); glycerol (Sigma-Aldrich®, G6279).

Instrumentation used during agarose gel electrophoresis and DNA visualisation included: Horizontal midi-gel kit (C.B.S. Scientific Company Inc., San Diego, CA); Elite 600 electrophoresis power supply (Wealtec Europe, Cambridge, UK); Gel-documentation system (Syngene ChemiGenius Bio-Imaging System, Syngene, Cambridge, UK).

### 3.4.2 METHODS

#### 3.4.2.1 AMPLIFICATION OF DNA BY POLYMERASE CHAIN REACTION (PCR)

Amplification of DNA was done using polymerase chain reaction (PCR), as originally described by Mullis *et al.* (1986). The primers used were synthesised to order by Inqaba Biotec™, the sequences of which can be found in Table 3.1.

Table 3.1: Sequence of PCR primers

| Primer                    | Primer sequence (5' – 3')  | T <sub>m</sub> (°C) | Size (bp) |
|---------------------------|----------------------------|---------------------|-----------|
| <b>oIMR0289 (Forward)</b> | cgc gct cac tga ctg cct tc | 60.15               | 20        |
| <b>oIMR0290 (Reverse)</b> | ctg gga gca ctt cgc aca gc | 60.05               | 20        |

Here, the sequences of the forward and reverse primers used during genotyping of mice are given in 5' to 3' notation, with the melting temperatures and sizes (in base pairs, bp) for each.

The primers specifically target the *Mt2* allele region and are recommended in the JAX® Mice database (<http://jaxmice.jax.org/strain/002211.html>). For genotyping of each mouse, a small piece (~ 2 mm<sup>2</sup>) of filter paper containing dried blood was cut and placed into a PCR-tube. A volume of 25 µl PCR reagent mix (12.5 µl 2x KAPA mix A, 11.5 µl Milli-Q® prepared water, 20 µM forward primer, 20 µM reverse primer) was then added and the PCR tube placed in a thermo cycler. During the initial denaturing step (Stage 1), at 95 °C for 10 minutes, template DNA is released as hematocytes are lysed. This was followed by 35 cycles during which further DNA denaturing (94 °C), primer annealing (60 °C) and DNA extension by KAPA blood DNA polymerase (72 °C) took place (Stage 2). Stage 3 included a prolonged extension step to ensure that all amplicons were completed. The samples were then cooled and held at 4 °C until removed from the thermo-cycler. The software setting for this process is illustrated in Figure 3.2.

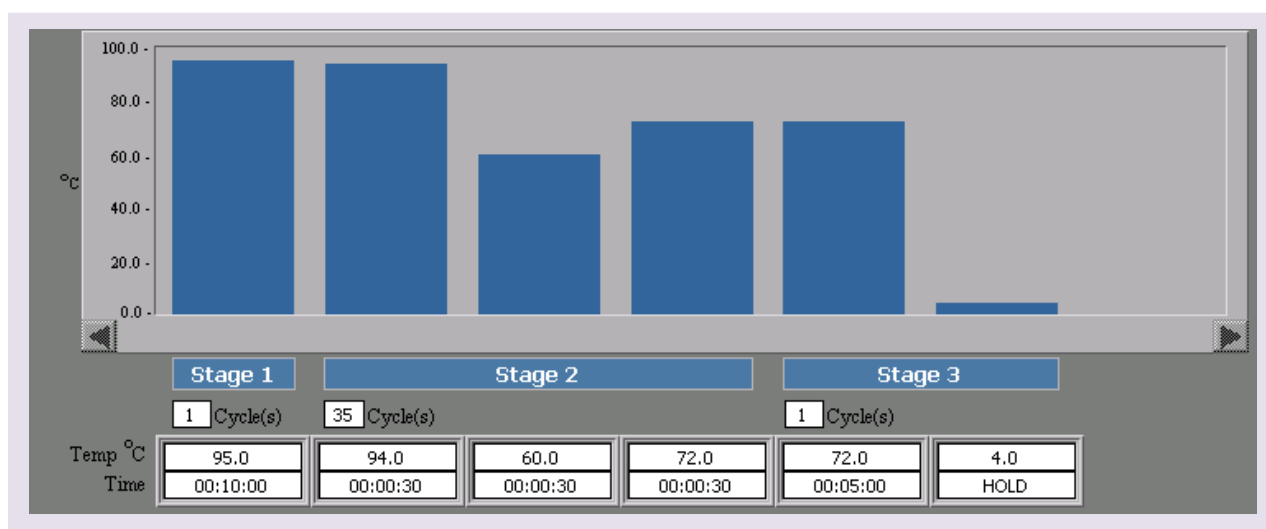


Figure 3.2: Screen capture of software settings illustrating the stages and cycles of PCR used for genotyping. Initial denaturing occurred at 95 °C for 10 min. DNA was denatured at 94 °C for 30 seconds, primers annealed at 60 °C for 30 seconds and extension took place at 72 °C for 30 seconds, after which the cycle was repeated 34 times. This was followed by a prolonged extension step (5 minutes), after which samples were cooled and held at 4 °C until removal from the thermo-cycler.

### 3.4.2.2 CHARACTERISATION OF DNA WITH AGAROSE GEL ELECTROPHORESIS

After amplification, 4  $\mu$ l of a 6 x loading buffer containing bromophenol blue (0.05% w/v) and glycerol (30% v/v) were added to 25  $\mu$ l of PCR product and loaded onto a 2.5% (w/v) agarose gel containing ethidium bromide (0.5  $\mu$ g/ml gel). An electric current of  $\sim 10 \text{ V.cm}^{-1}$  was applied to the holder containing the gel, which was submerged in TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA.K, pH 8.0) buffer. The negatively charged DNA fragments then moved through the gel to the positive pole created by the applied potential difference. This separated DNA essentially according to size. Because of the extra length of DNA (17 bp) inserted into the *Mt2* gene during the knockout process (see Section 3.2), the fragments of MTKO DNA amplified during the PCR reaction were longer and thus travelled through the gel at a slower pace than the shorter WT DNA fragments. This allowed for the discrimination between MTKO, WT and heterozygote samples. A DNA size ladder<sup>4</sup> was used to estimate amplicon sizes. The DNA was then visualised using a UV fluorescence transilluminator at 254 nm. A Syngene gel-documentation system and GeneSnap software (Syngene) were used to photograph the results. An example of such a result can be seen in Figure 3.3.

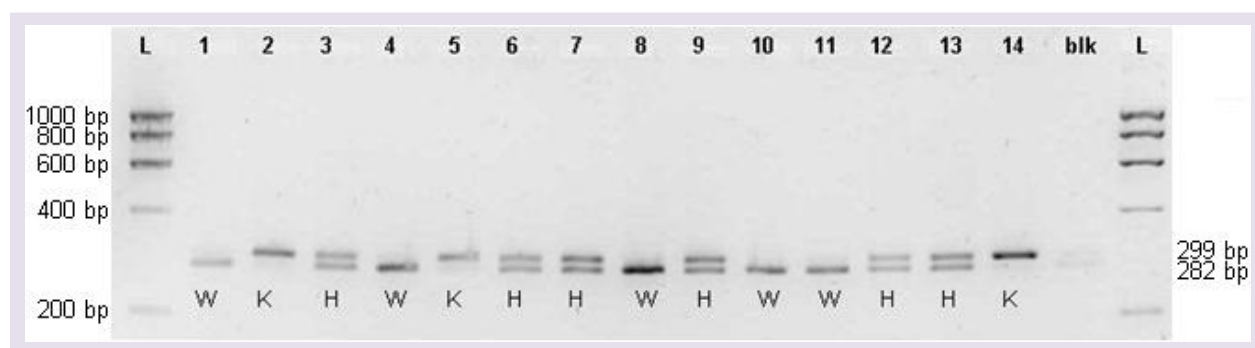


Figure 3.3: Example of results obtained during genotyping of mice. In the first and last lanes a DNA ladder was loaded. The ladder sizes are indicated to the far left, while DNA amplicon sizes are indicated to the far right. The lane marked blk is blank (negative, or no-template, control). The sample DNA was loaded in lanes marked 1 to 14. Two lines indicate heterozygotes (+/-); one higher line at the 299 bp mark indicates an MTKO (-/-) mouse and one lower line at the 282 bp mark indicates a WT (+/+) mouse. Colours are inverted in this figure.

<sup>4</sup> The DNA size ladder was created locally using PCR: primers were specific for a pIRESneo vector acting as a PCR template. Different combinations of the primers produced different lengths of amplicons, as indicated in Figure 3.3.

### 3.5 SORTING OF MICE INTO GROUPS

Respiration analyses had to be done on two tissues of all 66 mice included in this study. However, the available method for respiration analyses is extremely time consuming. Consequently, the mice were divided into 11 Day-groups containing six mice each and separated into different cages. To minimise the influence of day-to-day variation on analyses data, mice of both genotypes and treatments were included in each Day-group. Each Day-group contained three WT/MTKO pairs<sup>5</sup> of mice. Mice in the same Day-group but on different treatments were also separated from each other to eliminate the possibility of cross contamination between the two treatment groups. Because all the mice used during this study were male, violent encounters between them to establish dominance were frequent. To minimise the effect of physical stress on mice, overly aggressive mice were also separated into different cages throughout the treatment period.

### 3.6 INDUCING A COMPLEX I DEFICIENCY IN EXPERIMENTAL ANIMALS

To assess the possible role of MTs in mitochondrial respiration under stressed conditions, a CI deficiency was induced. As motivated in Section 2.5, rotenone was chosen from a range of possible CI inhibitors for this role.

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#### 3.6.1 ROTENONE AS INHIBITOR OF COMPLEX I

Rotenone is a naturally occurring compound found in the roots of several plant species belonging to the genera *Lonchocarpus* or *Derris*. It is frequently used as an insecticide, piscicide and acaricide. Although used for centuries by native South Americans as a fishing aid, it was first isolated in 1895 by Emmanuel Geoffrey and patented for commercial use as an insecticide in 1912 (Ambrose and Haag, 1935; United States environmental protection agency, 2007).

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<sup>5</sup> A WT/MTKO pair consists of one WT mouse and one MTKO mouse.

Rotenone specifically binds between the flavoprotein and iron protein components of CI, blocking CI from binding with and transferring electrons to ubiquinone, and so inhibits CI activity (Teeter *et al.*, 1969; Lambert and Brand, 2004). It has been shown that rotenone is metabolised by cytochrome P450 enzymes via 12a $\beta$  hydroxylation, 2-O-demethylation and oxidation of the isopropenyl substituent, to mono and diol derivatives (Fukami *et al.*, 1969; Caboni *et al.*, 2004). Although rotenone is metabolised to several daughter metabolites, the parent rotenoids seem to be mostly responsible for the CI inhibitory effects associated with rotenone (Caboni *et al.*, 2004). The use of rotenone as a CI inhibitor in rats (Fukami *et al.*, 1969; Bashkatova *et al.*, 2004; Caboni *et al.*, 2004; Swarnkar *et al.*, 2011) and mice (Thiffault *et al.*, 2000; Pan-Montojo *et al.*, 2010) has been widely reported on.

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#### PREPARATION OF ROTENONE AND PBS

For this study, rotenone (Sigma-Aldrich®, R8875) was first suspended in sterile PBS<sup>6</sup> (Invitrogen™, Johannesburg, ZA, 10010-051) and sonicated for 30 minutes using a Soniprep 150 (MSE, London, UK). This was done to break any flakes the rotenone powder might have formed during storage. It was then dried overnight in a sterile flow-cabinet and re-suspended in sterile PBS. The rotenone suspension as well as PBS only, were divided into 2 ml aliquots in amber vials, with polypropylene screw tops containing silicone septa (Agilent Technologies, Johannesburg, ZA).

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#### 3.6.2 DETERMINATION OF OPTIMAL ROTENONE LOAD

Before rotenone treatment of mice could commence, an optimal dosage load had to be established. Two pilot studies were conducted in order to do this. Previous studies conducted on MTKO mice utilising oil as a rotenone vehicle used 6mg/kg/2 days for three weeks, achieving significant levels of CI activity inhibition in liver and heart tissue (van Zweel, 2009). In accordance with this, the first pilot study was conducted (using PBS as rotenone vehicle) with rotenone loads of 0, 4, 6 and 8 mg/kg/2 days for 20 days.

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<sup>6</sup> In this dissertation PBS always refers to 1 x PBS solution (1.06 mM KH<sub>2</sub>PO<sub>4</sub>, 155 mM NaCl, 2.97 mM Na<sub>2</sub>HPO<sub>4</sub>-7H<sub>2</sub>O, pH 7.4).

No fatalities occurred and mice appeared healthy, but CI inhibition levels were below 20% (results not shown). It was postulated that the use of oil as a rotenone vehicle in previous studies resulted in a compounding effect on mice metabolism. A second pilot study then followed using 0, 10, 12.5, 15 and 30 mg/kg/2 days for three weeks in five groups of mice containing three mice each. Again no fatalities occurred and no significant weight loss was observed. Mice that received 30mg/kg/2 days rotenone load for 3 weeks presented inhibition levels of ~39% in liver, ~59% in heart and ~32% in skeletal muscle (Figure 3.4). Therefore, 30mg/kg/2 days was chosen as the maximum rotenone load that could be used safely during the main study.

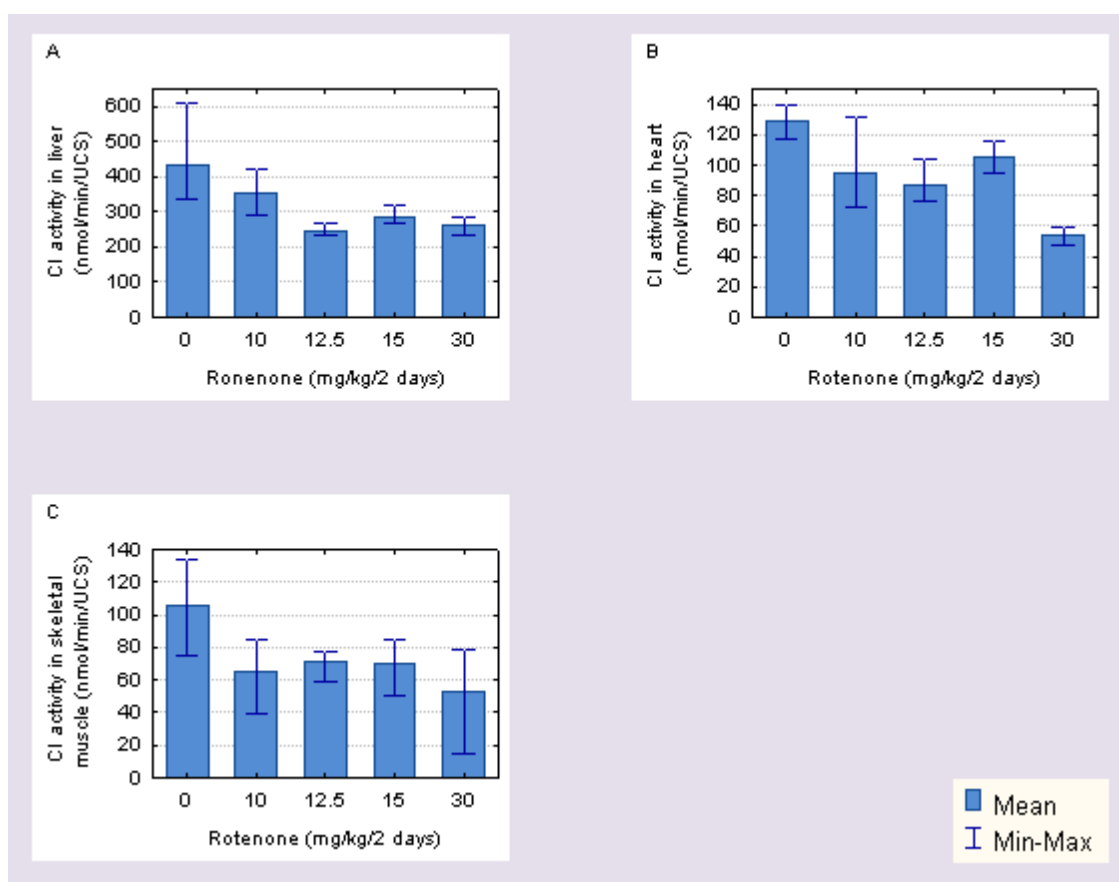


Figure 3.4: CI inhibitory effect of a range of rotenone concentrations administered to mice over a period of three weeks. Graphs show CI inhibition in liver (A), heart (B) and skeletal muscle (C) tissue respectively. Mean CI activity is expressed relative to mitochondrial content represented by the activity ( $\mu$ mole/min, or U) of the mitochondrial matrix enzyme, citrate synthase (CS).

### 3.6.3 TREATMENT OF EXPERIMENTAL ANIMALS WITH PBS AND ROTENONE

Mice were subcutaneously injected with either rotenone (suspended in PBS, 30mg/kg/2days, at volumes of 2 µl/g body weight), or with PBS alone (2 µl/g body weight) using a 1 ml insulin syringe. The PBS-treated mice served as the vehicle control group. There was no environmental control group included in this part of the broader study as such a group would not have contributed more to the evaluation of the two variables under investigation in this study, which were genotype and rotenone treatment. By comparing the rotenone-treated group with the vehicle control group (PBS-treated), rotenone treatment was the only variable, and an environmental control group would not have contained the variable of PBS/dosage procedure. Each Day-group consisted of one WT/MTKO pair on one treatment, and two WT/MTKO pairs on the alternative treatment. For instance, Day-group 1 consisted of two mice on rotenone treatment and four mice on PBS treatment; Day-group 2 consisted of two mice on PBS treatment and four mice on rotenone treatment. This pattern was repeated up to Day-group 10. Day-group 11 mice all received rotenone treatment and served as a back-up group to compensate for any deaths that occurred as a result of rotenone treatment throughout the study. Mice were dosed every second day for 20 days and euthanized on the 21<sup>st</sup> day after the initial dosing. The complete dosing schedule is included in Appendix B.

### 3.7 EUTHANIZING OF MICE AND SAMPLE COLLECTION

Mice were starved for one night prior to being euthanized. The procedures described here were performed by, or with the assistance of, the research animal technician, Mr. Petri Bronkhorst, of the Animal Research Centre at the North West University, Potchefstroom Campus. Mice were anaesthetised with halothane (Safeline Pharmaceuticals Pty. Ltd. via Kyron Laboratories, Johannesburg, ZA, H3017-1) so that blood could be extracted directly from the heart for analyses in studies parallel to this one. After determining that the mice were completely unconscious (by pinching and

observing any reaction) they were euthanized by severing the diaphragm. The heart, liver, brain and skeletal muscles (obtained from the hind legs) were rapidly explanted and immersed in 3 ml of ice-cold isolation buffer, IB (see Section 3.8) using separate 10 ml polypropylene tubes and kept on ice. The subcutaneous space was also inspected for signs of rotenone crystallisation due to subcutaneous injections. This, however, did not occur in any of the cases investigated.

### 3.8 PREPARATION OF DIFFERENT CELLULAR AND SUB-CELLULAR FRACTIONS FOR SEVERAL ANALYSES

After all six mice in a Day-group were euthanized and the different tissues collected, mitochondrial fractions had to be prepared as rapidly as possible so that respiration analyses could commence. This was achieved using differential centrifugation. Conveniently, all other analyses could make use of the cellular or sub-cellular fractions that were obtained during differential centrifugation. Thus, during the preparation process of enriched mitochondrial fractions, parts of the homogenate, 600g/700g supernatants or mitochondrial fractions were collected and stored for later use in several other analyses, as indicated below. Respiration analyses, however, had to be performed on freshly prepared enriched mitochondrial fractions each day.

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#### 3.8.1 MATERIALS

Where possible, reagents of the highest grade were used. The following reagents were purchased from either Sigma-Aldrich®, Fluka™ or Roche Diagnostics, as indicated: sucrose (Sigma-Aldrich®, S7903); Trisma® (Sigma-Aldrich®, T8655); EDTA.K (Fluka™, O3660); ethylene glycol tetraacetic acid (EGTA) (Sigma-Aldrich®, E4378); Tris.HCl (Roche Diagnostics, 10812846001); 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma-Aldrich®, M5789); potassium chloride (KCl) (Sigma-Aldrich®, P9333); fatty acid free bovine serum albumin (BSA) (Roche Diagnostics, 775 835); Milli-Q® prepared water.

The instrumentation used included: 10 ml glass/Teflon® tight-fitting Potter Elvehjem homogenisers (Glas-Col, Terre Haute, IN); Heraeus® Labofuge® 400R benchtop centrifuge (DJB Labcare Ltd., Buckinghamshire, UK); Heraeus® Multifuge® 1 L-R benchtop centrifuge (DJB Labcare Ltd.).

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### 3.8.2 METHODS

Preparation of enriched mitochondrial fractions was essentially done according to the protocol published by Frezza *et al.* (2007). This protocol was chosen as it is specific for the preparation of samples containing functional mitochondria from mouse liver and muscle and, when compared to other protocols in preliminary tests, produced comparatively higher concentrations of intact mitochondria. Based on respiration analyses, these preparations were also viable for longer periods of time. For the preparation of liver mitochondrial fractions an isolation buffer (IB<sub>c</sub>: 200mM sucrose, 10 mM Trisma®/MOPS, 1 mM EGTA/Trisma®, pH 7.4) was used. For the preparation of skeletal muscle, heart muscle and brain mitochondrial fractions a wash buffer (IB<sub>m1</sub>: 67 mM sucrose, 50 mM Tris.HCl, 50 mM KCl, 10 mM EDTA.K , 0.02% w/v BSA, pH 7.4), followed by an isolation buffer (IB<sub>m2</sub>: 250 mM sucrose, 3 mM EGTA/Trisma® , 10 mM Tris.HCl, pH 7.4) was used.

Preparation of mitochondrial fractions from WT and MTKO tissues was done in parallel. Instrumentation and consumables used on tissues from rotenone-treated mice were kept separate to minimise the possibility of rotenone contamination of other tissue samples. All the steps that follow were performed on ice with ice-cold buffers to minimise the activation of damaging phospholipases and proteases. Tissue samples were washed free of blood with IB<sub>c</sub> or a PBS.EDTA buffer (according to tissue specificity) and were then minced into small pieces with a pair of scissors. The IB was replaced with fresh IB<sub>c</sub> or IB<sub>m1</sub> and the mixture was homogenised using 15 strokes of a glass/Teflon® Potter Elvehjem homogeniser. A part of the homogenate was stored for relative mtDNA copy number estimation (discussed in Section 3.13). The rest of the homogenate was then transferred to micro-centrifuge tubes and centrifuged at 600g for liver and 700g for all other tissues for 10 minutes at 4 °C. After a part of the supernatant

was collected and stored for RC and other enzyme activity assays (discussed in Section 3.11), the rest was transferred to clean micro-centrifuge tubes and centrifuged at 7000g for liver and 8000g for all other tissues for 10 minutes at 4 °C. The supernatant was then discarded and the pellet, containing mitochondria, was re-suspended in IB<sub>c</sub> or IB<sub>m2</sub> and again centrifuged at 7000g for liver and 8000g for all other tissues for 10 minutes at 4 °C. Again, the supernatant was discarded and the remaining pellet suspended in as little as possible IB, so as to minimise mitochondrial exposure to oxygen and thus ensuring the retention of so-called “coupled” (inner membrane intact) mitochondria for longer. A part of the mitochondrial fraction was then stored for RC enzyme activity and structural analyses while the rest was used directly for respiration analyses (liver and heart only) after protein concentrations were determined using the BCA method as described in Section 3.9. A schematic representation of this procedure can be found in Figure 3.5.

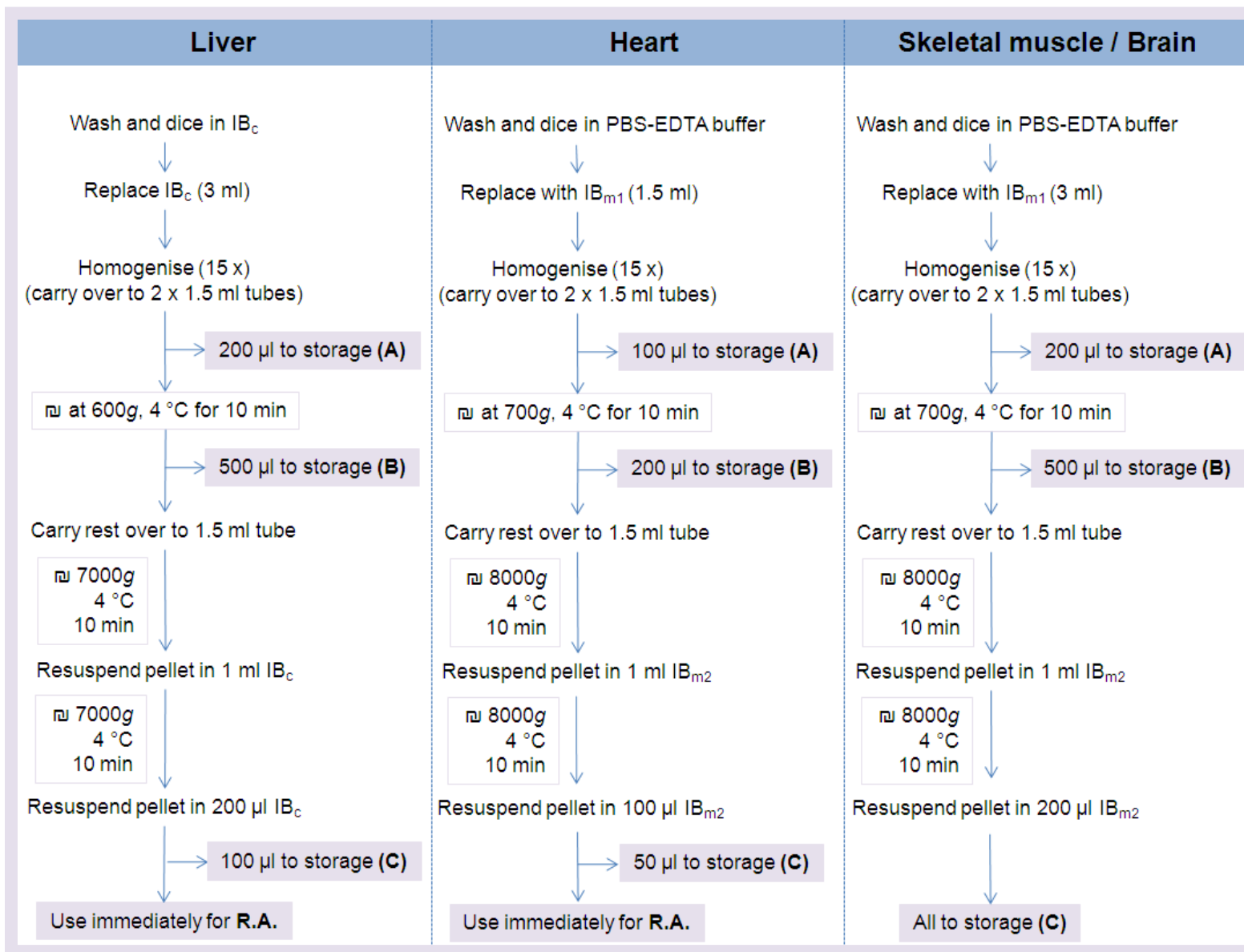


Figure 3.5: Schematic representation of the protocol followed during preparation of cellular and sub-cellular fractions, specific to each tissue. (A) refers to the storage of homogenate fractions for use in relative mtDNA copy number estimation; (B) refers to the storage of 600g or 700g supernatants for RC and other enzyme activity assays; (C) refers to the storage of mitochondrial fractions for use in RC enzyme activity and structural analyses. R.A. refers to respiration analyses; ↻ denotes centrifugation.

### 3.9 PROTEIN ASSAY

Protein assays were performed throughout this study on various tissue fractions. For this study the bicinchoninic acid (BCA) assay, as originally described by Smith *et al.* (1985) was used, essentially following the instructions provided with the kit.

In this assay, a mixture of BCA and Cu (II) sulphate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in a ratio of 50:1 is added to the sample and incubated. During this incubation  $\text{Cu}^{2+}$  is converted to  $\text{Cu}^+$ , a reaction induced by peptide bonds under alkaline conditions.  $\text{Cu}^+$  formation, which is directly proportional to protein concentration, leads to the reaction of  $\text{Cu}^+$  with BCA to form a purple-coloured chromogen, which can be measured at 562 nm.

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#### 3.9.1 MATERIALS

The reagents used in this assay are contained in the BCA kit (BCA-1) from Sigma-Aldrich®, which includes BCA reagent (B9643),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  solution (2284) and BSA protein standard at 1 µg/µl (P0914).

Instrumentation used included: Synergy™ HT Multi-detection microplate reader (BioTek® Instruments, Inc., Winooski, VT).

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#### 3.9.2 METHODS

The protocol followed was essentially based on the instructions provided with the kit. A BSA protein standard series ranging from 0 µg to 20 µg protein in increments of 4 µg was pipetted into the wells of a 96-well plate. Tissue preparations (homogenates, supernatants or enriched mitochondrial fractions, see Section 3.8) were then diluted 50% (v/v) and 2 µl pipetted in triplicate into the wells of the 96-well plate. Water was added as needed to wells containing protein, so that the end volume of each well was

20 µl. Additionally, 200 µl of a reagent mix, consisting of BCA and CuSO<sub>4</sub>.5H<sub>2</sub>O in a ratio of 50:1, was added to each well and the plate incubated at 30 °C for 35 minutes in a Synergy™ HT Multi-detection microplate reader. This was followed by a measurement of the absorption at 562 nm. The protein content (µg) of each well was calculated by the Gen5™ Data Analysis software (BioTek® Instruments) using linear regression analysis and the standard range values, and was then converted to µg/µl of sample used in the analysis (see Equation 3.1)

$$\frac{\mu\text{g protein (reported by software)}}{\mu\text{l volume (of sample used in assay)}} = \mu\text{g}/\mu\text{l}$$

Equation 3.1

### 3.10 RESPIRATION ANALYSES

Respiration assays had to be performed on freshly prepared enriched mitochondrial fractions, to ensure that inner mitochondrial membranes (IMM) remained intact and the respiratory chain (RC) remained coupled. The protocol used for respiration analyses was based on that of Rossignol *et al.* (2000). As this protocol is specifically for human muscle samples, changes to substrate concentrations were made according to preliminary tests so as to be better suited for use on mouse tissue samples.

The respiration analyses were done using a respirometer system that utilises a Clark-type electrode. This system measures a change in dissolved oxygen concentration in the solution contained in the reaction chamber. Thus, oxygen consumption by intact mitochondria can be measured and the efficacy of mitochondrial respiration determined. The respirometer uses the following principle: a thin O<sub>2</sub>-permeable polypropylene membrane separates the electrode compartment, filled with potassium chloride (KCl) electrolyte, from the reaction chamber. A platinum cathode and silver anode are separated by the salt bridge (formed by the KCl) and connected to an oxygen monitor, which maintains a constant potential difference across the electrodes. Electrons dissipate from the cathode into the solution where it binds with O<sub>2</sub>, which diffuses over

the membrane, and hydrogen to form water. The concentration of O<sub>2</sub> available for the electrons to combine with, is proportional to the rate of electron dissipation from the electrode, inducing an electrical current, which is converted into a voltage by the oxygen monitor (Nicholls and Ferguson, 2002).

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### 3.10.1 MATERIALS

The following reagents were used during respiration analyses: sucrose (Sigma-Aldrich®, S7903); mannitol (Sigma-Aldrich®, M9546); KCl (Sigma-Aldrich®, P9333); Tris.phosphate (Sigma-Aldrich®, T8655); Tris.HCl (Roche Diagnostics, 10812846001); EDTA.K (Fluka™, O3660); malate (Sigma-Aldrich®, M9138); glutamate (Sigma-Aldrich®, G1501); adenosine diphosphate (ADP) (Sigma-Aldrich®, A5285); zinc sulphate (ZnSO<sub>4</sub>) (Sigma-Aldrich®, 3223); Milli-Q® prepared water.

The following instrumentation was used: 10 µl Hamilton 701N syringe (Hamilton Company, Bonaduz, CH, 80300); sterile Acrodisc® syringe filters with HT Tuffryn® membrane (Pall Corporation via Separations, Randburg, ZA, 4454); MT200A respirometer system and software (Strathkelvin Instruments Ltd., Motherwell, UK).

Milli-Q® prepared water and all materials used during the experiments were autoclaved beforehand. Respiration buffers were filtered using a 0.2 µm Acrodisc® syringe filter. This was to ensure that experiments were conducted in conditions as sterile as possible, to avoid interference by bacterial build-up and non-specific respiration.

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### 3.10.2 METHODS

The respirometer was calibrated daily by selecting the appropriate options on the computer software program. When calibrating the zero-point, the sample holder was filled with  $\text{Na}_2\text{SO}_3$  (3 mM) and a reading of 0 pAmp was given. For high-point calibration, the sample holder was filled with oxygen saturated water (vigorously stirred sterile water) and a reading of over 300 pAmp was given. Calibration was done with the magnetic stirrer activated, and at the same temperature (37 °C) at which the experiments were conducted. At these settings the saturated concentration of  $\text{O}_2$  in water is 199  $\mu\text{M}$  (Clark, 1992).

A respiration buffer (2 x RB: 75 mM mannitol, 25 mM sucrose, 100 mM KCl, 50  $\mu\text{M}$  EDTA.K, 10 mM Tris.phosphate and 10 mM Tris.HCl, pH 7.4) was used during experiments. Experiments were conducted at 37 °C in 70  $\mu\text{l}$  volumes. The 2 x RB always occupied 50% of the total volume, hence each analysis was initiated by the addition of 35  $\mu\text{l}$  of 2 x RB to the reaction chamber. The substrates occupied 3  $\mu\text{l}$  of the total volume. Water, and then mitochondrial preparations, were added in such a way that the final protein concentration in the chamber was 0.2  $\mu\text{g}/\mu\text{l}$ . The plug was then inserted and a base line measurement was taken for ~5 seconds. The addition of a glutamate plus malate (3.6 mM glutamate: 3.6 mM malate end concentrations) substrate combination followed by ADP (0.7 mM end concentration) to the reaction chamber, resulted in the production of a respiration trace. An example of such a trace is given in Figure 3.6.

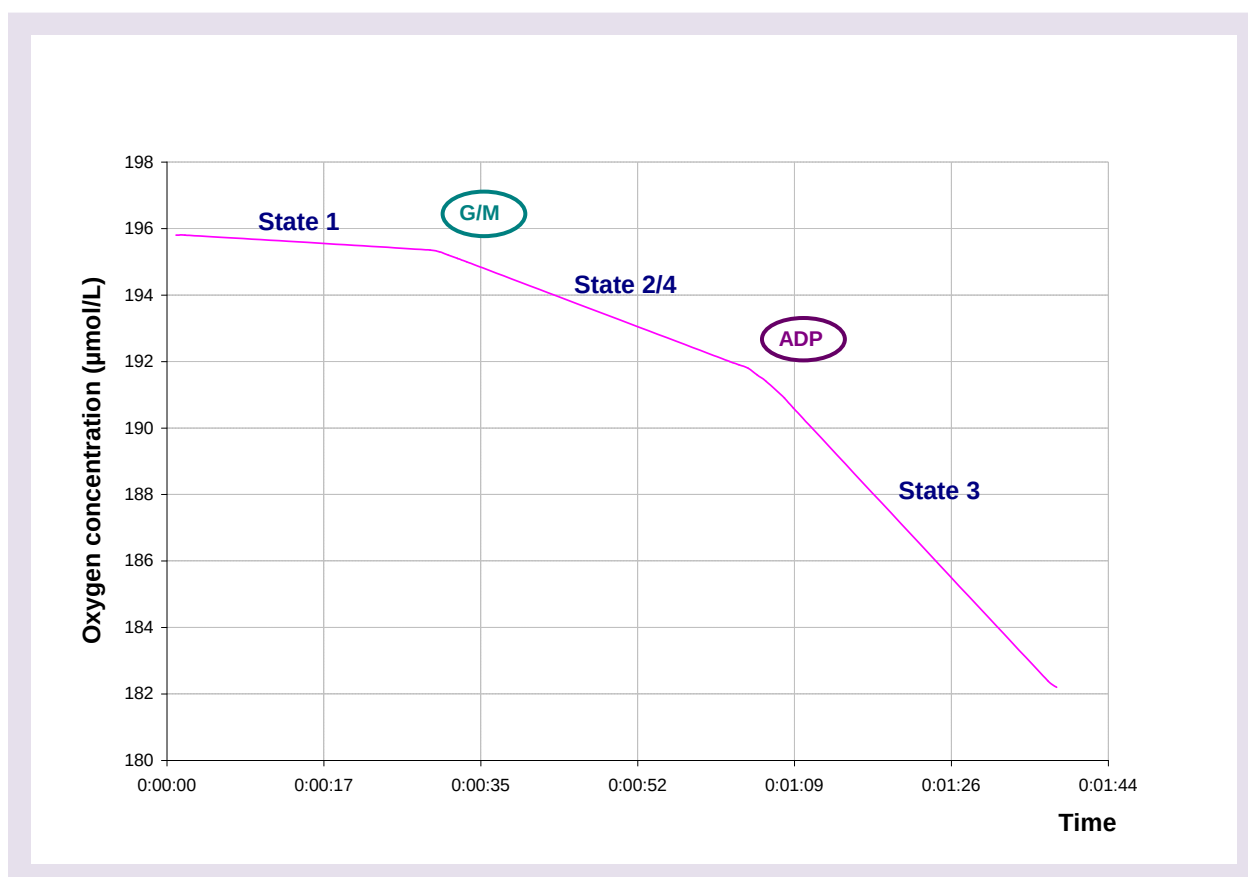


Figure 3.6: Example of a typical respiration trace, using a glutamate+malate (G+M) substrate combination. State 1 represents respiration when only mitochondria are present with no substrate, followed by state 2/4, after G+M is added and finally state 3, after the addition of ADP (Nicholls and Ferguson, 2002).

The trace shown in Figure 3.6 represents an example of the change in dissolved oxygen concentration in the reaction chamber, measured in  $\mu\text{M}$ . During state 1, virtually no respiration took place as the chamber contained only mitochondria. After the glutamate plus malate substrate combination was added, state 2/4 respiration was recorded, which showed a clear increase in respiration as oxygen concentrations decreased rapidly. ADP was then added to push mitochondria into maximum respiration rate during state 3 (ADP-stimulated respiration).

Traces were saved and after each analysis, the reaction chamber was washed more than three times with water to remove any remnants from the previous reaction that could influence the next.

During trace analysis, slopes on the trace for state 2/4 and state 3 respiration were chosen manually, by selecting the most linear area of initial velocity, and the normalised oxygen consumption values were calculated. As the volume settings and protein content were pre-programmed into the parameters, the software calculated and reported the activity in units of  $\mu\text{mole}/\text{min}/\mu\text{g}$ , by dividing the change in dissolved oxygen ( $\mu\text{mole}$ ) over a set period of time selected on a trace (min), by the amount of protein contained in the sample ( $\mu\text{g}$ ).

Values obtained from the respirometer software were converted from  $\mu\text{mole}/\text{min}/\mu\text{g}$  to  $\text{pmole}/\text{min}/\mu\text{g}$  ( $\mu\text{mole}/\text{min}/\mu\text{g} \times 1000\ 000 = \text{pmole}/\text{min}/\mu\text{g}$ ) to make the data more manageable. Data were then compiled into tables and the respiration control ratio (RCR) values were calculated. The RCR is the ratio of state 2/4 respiration to state 3 respiration (State 3 / State 2) and indicates how well mitochondria are coupled. RCR values smaller than 2 can be an indication of inhibited, damaged or uncoupled mitochondria (Rossignol *et al.*, 2002). An example of such a table is given in Table 3.2.

Table 3.2: Data obtained from respiration analyses with calculated RCR-values

| Number | Genotype | Treatment | Tissue | State 2/4<br>( $\text{pmole}/\text{min}/\mu\text{g}$ ) | State 3<br>( $\text{pmole}/\text{min}/\mu\text{g}$ ) | RCR-<br>value |
|--------|----------|-----------|--------|--------------------------------------------------------|------------------------------------------------------|---------------|
| 16     | MTKO     | PBS       | Liver  | 45.0                                                   | 114.3                                                | 2.5           |
| 29     | WT       | PBS       | Liver  | 40.7                                                   | 214.3                                                | 5.3           |
| 31     | WT       | Rotenone  | Heart  | 34.3                                                   | 85.7                                                 | 2.5           |
| 31     | WT       | Rotenone  | Liver  | 28.6                                                   | 92.9                                                 | 3.3           |
| 64     | MTKO     | Rotenone  | Liver  | 42.1                                                   | 128.6                                                | 3.1           |
| 64     | MTKO     | Rotenone  | Heart  | 22.1                                                   | 68.6                                                 | 3.1           |

This table gives examples of original data for mitochondrial respiration for different genotypes, treatments and tissues. The RCR-values are listed in the far right column.

### 3.11 RESPIRATORY CHAIN AND CITRATE SYNTHASE ENZYME ANALYSES

The protocols used for these assays were based on those kinetic spectrophotometric methods previously published (Sheperd and Garland, 1969; Rahman *et al.*, 1996; Janssen *et al.*, 2007; Luo *et al.*, 2008), but modified for routine diagnostic use at the Mitochondria Research Laboratory, Centre for Human Metabonomics, North-West University (du Toit, 2007). These original protocols were specific for use on a BIO-TEK UVIKON XS (BIO-TEK Instruments, Neufarn, DE) spectrophotometer with LabPower Junior software (BIO-TEK Instruments). As the large number of assays done in this study required a higher through-put system, further modifications were made to the original protocols to be better suited to use on a microtiter plate system during this study. This was advantageous not only because a lower volume of sample was needed per reaction, but especially because of the significant reduction in time that was needed to complete a set of reactions. The temperature-controlled microtiter (96-well) plate offered the opportunity to analyse up to 96 samples simultaneously, in contrast to only 10 samples when using a single cuvette spectrophotometer system (when using a 10-cell transporter). Because of this, it was possible to do all experiments in a much shorter time and in triplicate. It was deduced that this would increase sample viability (due to less time in a thawed state) and, considering that less batches are done with repeats for each sample, theoretically improved inter-batch repeatability would be possible. Comparably, a single cuvette spectrophotometer for the same number of single sample assays required in this study (at least 3960) would only allow single analysis over a much longer period and requiring many more controls to correct for inter-batch variations. The assumed loss in sensitivity when using the microtiter plate reader, as opposed to the single cuvette spectrophotometer, is compensated for by this increase in repeats, and combined with the ability to use more accurate and faster pipetting methods such as multi-channel pipettes, increased the overall accuracy of experimental runs, as was seen in the relatively low variation coefficients for the assays (see Section 5.4).

In this study, enzymatic activities for two-substrate reactions were determined using initial velocity (pseudo first-order) calculations as determined from two-substrate kinetic reactions using spectrophotometric assays (Bock and Alberty, 1953). Substrates were added to samples in great excess (several times that of  $K_m$ -values) and reduction or oxidation of these compounds was monitored by measuring the changes in absorbance. The substrates chosen were specific to each RC enzyme complex measured, or their specificity directed by use of enzyme inhibitors. Listed below (Table 3.3) are the substrates and products used and formed during the assays. Highlighted in purple are the critical compounds which absorbed light at the wavelengths indicated.

Table 3.3: List of substrates used and products formed during assays for respiratory chain enzymes and citrate synthase (CS).

| Enzyme complex | Substrates                    |                |                                 | Products             | Wavelength measured (nm) |
|----------------|-------------------------------|----------------|---------------------------------|----------------------|--------------------------|
| <b>CS</b>      | acetyl-CoA                    | oxaloacetate   | citrate                         | CoA ( <b>DTNB*</b> ) | 412                      |
| <b>CI</b>      | decylubiquinone               | NADH           | decylubiquinol ( <b>DCIP*</b> ) | NAD <sup>+</sup>     | 600                      |
| <b>CII</b>     | decylubiquinone               | succinate      | decylubiquinol ( <b>DCIP*</b> ) | fumarate             | 600                      |
| <b>CII+III</b> | cytochrome c (oxidised)       | succinate      | <b>cytochrome c (reduced)</b>   | fumarate             | 550                      |
| <b>CIII</b>    | cytochrome c (oxidised)       | decylubiquinol | <b>cytochrome c (reduced)</b>   | decylubiquinone      | 550                      |
| <b>CIV</b>     | <b>cytochrome c (reduced)</b> | O <sub>2</sub> | cytochrome c (oxidised)         | H <sub>2</sub> O     | 550                      |

Critical reagents measured during absorption are highlighted in purple and the wavelength at which the absorption was measured is shown on the right. Asterisks (\*) indicate where a reaction used a coupled reaction (CS) or an alternative electron acceptor (CI and CII).

### 3.11.1 MATERIALS

The following reagents were used: Trisma® (Sigma-Aldrich®, T8655); 5,5'-dithio-bis[2-nitrobenzoic acid] (DTNB) (Roche Diagnostics, 104 477); Triton® X-100 (Sigma-Aldrich®, T9284); acetyl-CoA, trilithium salt (Roche Diagnostics, 10 101 907 001); oxaloacetate (Aldrich®, 17,125-5); potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (Fluka™, 60218); dipotassium phosphate ( $\text{K}_2\text{HPO}_4$ ) (Fluka™, 60353); NADH, disodium salt (Roche Diagnostics, 10 107 735 001); potassium cyanide (KCN) (BDH® Chemicals, 29603); antimycin A, (Sigma-Aldrich®, A8674); fatty acid free BSA (Roche Diagnostics, 775 835); rotenone (Sigma-Aldrich®, R8875); ethanol (Merck Chemicals, 1.00983.2500); EDTA.K (Fluka™, 03660); ATP, disodium salt (Roche Diagnostics, 10 519 979 001); 2,6-dichloroindophenol, sodium salt hydrate (DCIP) (Sigma-Aldrich®, D1878); decylubiquinone (Sigma-Aldrich®, D7911); dimethyl sulfoxide (DMSO) (Sigma-Aldrich®, C6164); malonate (Sigma-Aldrich®, M1750); succinate (Sigma-Aldrich®, S7903); cytochrome c (Sigma-Aldrich®, C7752); n-dodecylmaltoside (Sigma-Aldrich®, D4641); Tween® 20 (Merck Chemicals, 822184); potassium borohydride ( $\text{KBH}_4$ ) (BDH® Chemicals, 29587); sodium ascorbate (Sigma-Aldrich®, A4034); Milli-Q® prepared water.

Instrumentation used during enzyme activity assays included: Synergy™ HT Multi-detection microplate reader.

### BUFFERS, SOLUTIONS AND PREPARATION OF SPECIFIC REAGENTS

A 0.5 M potassium-phosphate buffer (KPi-buffer) was used throughout the analyses and consisted of 95 mM  $\text{KH}_2\text{PO}_4$  and 405 mM  $\text{K}_2\text{HPO}_4$ , pH 7.4. When indicated this buffer was further diluted to 50 mM with water. DTNB was dissolved in 1 M Trisma®, pH 8.0. Antimycine-A and rotenone were dissolved in ethanol unless indicated otherwise, while KCN and BSA were dissolved in 0.5 M KPi-buffer and dicylubiquonone was dissolved in DMSO unless indicated otherwise. All other reagents were diluted or dissolved in Milli-Q® prepared water. pH values were adjusted using either hydrochloric acid (HCl) or potassium hydroxide (KOH). The preparation of reduced cytochrome c and reduced ubiquinol from the oxidised forms is described in detail in Appendix A.

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### 3.11.2 METHODS

The basic assay conditions used to measure citrate synthase (as mitochondrial marker enzyme) and respiratory chain enzymes are indicated below. The protein content for each assay was optimised to allow for good sensitivity (high activity detection) and protein content-sensitive specific activity.

For the assays, 600g or 700g supernatants (CS; CII; CII+III; CIII and CIV) or enriched mitochondrial preparations (CI and CII) were used. The preparations of these samples were described in Section 3.8. The choice of using supernatants or enriched mitochondrial preparations was made before hand, based on sensitivity and protein content-sensitive specific activity.

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#### 3.11.2.1 CS ACTIVITY

CS was measured essentially as described by Sheperd and Garland (1969). The reaction consisted of acetyl-CoA (60  $\mu$ M), Triton® X-100 (0.04%, v/v), DTNB (0.1 mM), 600g/700g supernatant (see Figure 3.5, 6  $\mu$ l for liver and heart; 2  $\mu$ l skeletal muscle; 4  $\mu$ l brain) and, to initiate the reaction, oxaloacetate (0.5 mM) in a final volume of 200  $\mu$ l, measured at 30 °C. Each sample was analysed in triplicate in 96-well microtiter plates and each plate included a blank (no sample) as well as a control sample to allow for the correction of batch-to-batch variation. DTNB-CoA conjugation (to form TNB) was followed kinetically at 412 nm in 45-second intervals for 5 minutes in a Synergy™ HT Multi-detection microplate reader. By using the Gen5™ Data Analysis software, the initial velocity (linear rate increase) was visually reviewed and, along with the reported  $R^2$ -values (if > 0.98), the period for calculating the initial velocity was selected accordingly. An initial velocity value ( $v_1$ ) was reported for all assays (over the same period) as a mAbs/min value. This value was used to calculate the specific activity, normalised to protein content, as indicated in Equation 3.2. To convert absorbance value to molar value, a molar extinction coefficient of 7465  $\text{mM}^{-1}$  per 200  $\mu$ l, which was determined for the assay using a standard series of product (CoA) and chromogen, was used.

|                              |   |                                                                                |
|------------------------------|---|--------------------------------------------------------------------------------|
| $\mu\text{mol/min/mg (UCS)}$ | = | $(v_1/7465) * 0.2 / (\mu\text{l protein} \times \mu\text{g}/\mu\text{l}/1000)$ |
| $\text{nmol/min/mg}$         | = | $\text{UCS} \times 1000$                                                       |

Equation 3.2

### 3.11.2.2 CI ACTIVITY

CI was measured essentially as described by Janssen *et al.* (2007). The reaction consisted of KPi-buffer (50 mM), antimycin A (1  $\mu\text{M}$ ), decylubiquinone in DMSO (70  $\mu\text{M}$ ), DCIP (60  $\mu\text{M}$ ), BSA (0.35%, w/v), mitochondrial preparation (5  $\mu\text{l}$  for liver and brain; 6  $\mu\text{l}$  heart; 10  $\mu\text{l}$  skeletal muscle) and, to initiate the reaction, NADH (0.2 mM) in a final volume of 200  $\mu\text{l}$ , measured at 30 °C. Each sample was analysed in triplicate, in the presence of either rotenone (2.5  $\mu\text{M}$  in DMSO) or DMSO in 96-well microtiter plates. Each plate included a blank (no sample) as well as a control sample to allow for batch-to-batch variation correction. DCIP oxidation was followed kinetically at 600 nm in 45-second intervals for 5 minutes in a Synergy™ HT Multi-detection microplate reader. By using the Gen5™ Data Analysis software, the initial velocity (linear rate increase) was visually reviewed and, along with the reported  $R^2$ -values (if > 0.98), the period for calculating the initial velocity was selected accordingly. An initial velocity value was reported for all assays (over the same period) as a mAbs/min value in the absence ( $v_1$ ) and presence ( $v_2$ ) of rotenone. This value was used to calculate the rotenone-sensitive specific activity, expressed per units of CII, as indicated in Equation 3.3. A molar extinction coefficient of 12712  $\text{mM}^{-1}$  per 200  $\mu\text{l}$  was used, which was determined for DCIP by using a standard series of DCIP in the assay conditions. To minimise the influence of other NADH utilising enzymes, mitochondrial preparations were used, thereby removing rotenone insensitive enzymes. Also, DCIP was included to serve as proton receiver, as it was observed in advance that the use of DCIP was much more specific for CI activity and, compared to measuring NADH oxidation, resulted in significantly improved rotenone sensitivity. The reason for not using CS as marker was that, during the preparation of enriched mitochondrial samples, some mitochondria may be damaged and thus could leak CS from the matrix. CII however, as a membrane associated protein, would remain intact and was thus used as marker enzyme in this case (Janssen *et al.*, 2007).

|                        |   |                                                                                    |
|------------------------|---|------------------------------------------------------------------------------------|
| $\mu\text{mol/min/mg}$ | = | $[(v_1 - v_2)/12712] * 0.2 / (\mu\text{l protein} * \mu\text{g}/\mu\text{l}/1000)$ |
| $\text{nmol/min/UCII}$ | = | $\mu\text{mol/min/mg} \times 1000/\text{UCII}$                                     |

Equation 3.3

### 3.11.2.3 CII ACTIVITY

CII was measured essentially as described by Janssen *et al.* (2007). The reaction consisted of KPi-buffer (50 mM), antimycin A (1  $\mu\text{M}$ ), decylubiquinone in DMSO (50  $\mu\text{M}$ ), DCIP (80  $\mu\text{M}$ ), BSA (0.1%, w/v), ATP (0.2 mM), EDTA.K (0.08 %, w/v), rotenone (2.5  $\mu\text{M}$ ), mitochondrial preparation (5  $\mu\text{l}$  liver and brain; 6  $\mu\text{l}$  heart; 15  $\mu\text{l}$  skeletal muscle) or 600g/700g supernatant (6  $\mu\text{l}$  liver and brain; 10  $\mu\text{l}$  heart and skeletal muscle). To initiate the reaction, succinate (1.5 mM) was added in a final volume of 200  $\mu\text{l}$ , measured at 30 °C. Each sample was analysed in triplicate in 96-well microtiter plates and each plate included a blank (no sample) as well as a control sample to allow for batch-to-batch variation correction. DCIP oxidation was followed kinetically at 600 nm in 45-second intervals for 5 minutes in a Synergy™ HT Multi-detection microplate reader. By using the Gen5™ Data Analysis software, the initial velocity (linear rate increase) was visually evaluated and, along with the reported  $R^2$ -values (if > 0.98), the period for calculating the initial velocity was selected. An initial velocity value was reported for all assays (over the same period) as a mAbs/min value ( $v_1$ ). CII measured in this way showed a complete sensitivity to malonate (malonate completely stopped CII activity, results not shown) and therefore inclusion of malonate as a control for each reaction was not done as suggested by Janssen *et al.* (2007). This value ( $v_1$ ) was used to calculate the specific activity, normalised to protein content or expressed per units of CS, as indicated in Equation 3.4. The same molar extinction coefficient for DCIP as for CI was used here.

|                        |   |                                                                            |
|------------------------|---|----------------------------------------------------------------------------|
| $\mu\text{mol/min/mg}$ | = | $(v_1/12712) * 0.2 / (\mu\text{l protein} * \mu\text{g}/\mu\text{l}/1000)$ |
| $\text{nmol/min/UCS}$  | = | $\mu\text{mol/min/mg} \times 1000/\text{UCS}$                              |

Equation 3.4

#### 3.11.2.4 CII+III ACTIVITY

CII+III activity was measured essentially as described by Rahman *et al.* (1996). The reaction consisted of KPi buffer (50 mM), succinate (10 mM), NaN<sub>3</sub> (20 mM), EDTA.K (0.08 %, w/v), BSA (0.1 % w/v), rotenone (2.5 µM), ATP (2 mM) and 600g/700g supernatant (see Figure 3.5, 12 µl for all tissues). To initiate the reaction, cytochrome c (oxidised, 0.5 mM) was added to a final volume of 200 µl and measured at 30 °C. Each sample was analysed in triplicate in 96-well microtiter plates, and each plate included a blank (no sample) as well as a control sample to allow for batch-to-batch variation correction. Cytochrome c reduction was followed kinetically at 550 nm in 45-second intervals for 5 minutes in a Synergy™ HT Multi-detection microplate reader. By using the Gen5™ Data Analysis software, the initial velocity (linear rate increase) was visually evaluated and, along with the reported R<sup>2</sup>-values (if > 0.98), the period for calculating the initial velocity was selected accordingly. An initial velocity value (v<sub>1</sub>) was reported for all assays (over the same period) as a mAbs/min value. This value was used to calculate the specific activity, expressed per units of CS as indicated in Equation 3.5. A molar extinction coefficient of 4180 mM<sup>-1</sup> per 200 µl was pre-determined for cytochrome c by using a standard series of cytochrome c in the assay conditions.

$$\begin{aligned}\mu\text{mol/min/mg} &= (v_1/4180) * 0.2 / (\mu\text{l protein} * \mu\text{g}/\mu\text{l}/1000) \\ \text{nmol/min/UCS} &= \mu\text{mol/min/mg} \times 1000/\text{UCS}\end{aligned}$$

Equation 3.5

#### 3.11.2.5 CIII ACTIVITY

CIII was measured essentially as described by Luo *et al.* (2008). The reaction consisted of cytochrome c (oxidised, 50 µM) and 600g/700g supernatant (3.2 µl for liver; 4 µl for heart; 6 µl for skeletal muscle and 5 µl for brain) and, to initiate the reaction, a master mix consisting of KPi buffer (250 mM), NaN<sub>3</sub> (20 mM), BSA (0.05 %, w/v), Tween® 20 (0.25 %, v/v), decylubiquinol (reduced, 0.25 mM) in a final volume of 200 µl, measured at 30 °C. Each sample was analysed in triplicate in 96-well microtiter plates and each plate included a blank (no sample) as well as a control sample to allow for batch-to-batch variation correction. Cytochrome c reduction was followed kinetically at 550 nm in

45-second intervals for 5 minutes in a Synergy™ HT Multi-detection microplate reader. By using the Gen5™ Data Analysis software, the initial velocity (linear rate increase) was visually evaluated and, along with the reported R<sup>2</sup>-values (if > 0.98), the period for calculating the initial velocity was selected. An initial velocity value (v<sub>1</sub>) was reported for all assays (over the same period) as a mAbs/min value. This value was used to calculate the specific activity, expressed per units of CS as indicated in Equation 3.6. The same molar extinction coefficient for cytochrome c as used for CII+III was used here.

|                        |   |                                                                           |
|------------------------|---|---------------------------------------------------------------------------|
| $\mu\text{mol/min/mg}$ | = | $(v_1/4180) * 0.2 / (\mu\text{l protein} * \mu\text{g}/\mu\text{l}/1000)$ |
| $\text{nmol/min/UCS}$  | = | $\mu\text{mol/min/mg} \times 1000/\text{UCS}$                             |

Equation 3.6

### 3.11.2.6 CIV ACTIVITY

CIV was measured essentially as described by Rahman *et al.* (1996), but using a higher cytochrome c concentration. The reaction consisted of Kpi buffer (50 mM), dodecylmaltoside (0.5 mM), cytochrome c (reduced, 25  $\mu\text{M}$ ) and 600g/700g supernatant (10  $\mu\text{l}$  for liver and brain; 6  $\mu\text{l}$  for heart and skeletal muscle, see Figure 3.5) in a final volume of 200  $\mu\text{l}$ , measured at 30 °C. Each sample was analysed in triplicate in 96-well microtiter plates and each plate included a blank (no sample) as well as a control sample to allow for batch-to-batch variation correction. Cytochrome c oxidation was followed kinetically at 550 nm in 45-second intervals for 5 minutes in a Synergy™ HT Multi-detection microplate reader. By using the Gen5™ Data Analysis software, the initial velocity (linear rate increase) was visually evaluated and, along with the reported R<sup>2</sup>-values (if > 0.98), the period for calculating the initial velocity was selected accordingly. An initial velocity value (v<sub>1</sub>) was reported for all assays (over the same period) as a mAbs/min value. This value was used to calculate the specific activity, expressed per units of CS, as indicated in Equation 3.7. The same molar extinction coefficient for cytochrome c as used for the two previous assays was used here.

$$\begin{aligned}\mu\text{mol/min/mg} &= (v_1/4180) * 0.2 / (\mu\text{l protein} * \mu\text{g}/\mu\text{l}/1000) \\ \text{nmol/min/UCS} &= \mu\text{mol/min/mg} \times 1000/\text{UCS}\end{aligned}$$

Equation 3.7

### 3.12 BN-PAGE/WESTERN BLOT ANALYSIS

For these specialised analyses, prepared heart mitochondrial fractions were analysed by Dr. Leo Nijtmans at the Nijmegen Centre for Mitochondrial Disease, Radboud University Medical Centre, NL. These samples were prepared to all contain the same amount of CII, according to the measured activity of CII (see Section 3.11). During BN-PAGE, proteins are not denatured before separation on the gel. The separation pattern can thus indicate structural as well as quantitative differences.

#### 3.12.1 METHODS

These analyses were done according to the standard procedures used at the abovementioned centre. Briefly, one sample of each genotype and treatment combination (e.g. PBS-treated WT mouse) was first run on a non-denaturing, Coomassie Blue containing (*i.e.* blue-native) polyacrylamide gel to separate the different RC complexes and then transferred to a cellulose membrane during western blotting. The individual RC enzyme complexes were then bound by monoclonal mouse anti-complex I-V antibodies and detected using rabbit-anti-mouse IgG horse radish conjugated polyclonal antibodies. This allowed for chemiluminescent detection of the individual intact complexes. A photograph of the western blot was provided and further analysed locally, using GeneTools software (SynGene, Cambrigde, UK). Firstly, the lightest section on the blot was identified to serve as background (magenta Bkg block in Figure 3.7), which was subtracted from all other band intensities. Then, a band representing CII (blue block numbered 1 in Figure 3.7) was selected, to which all other band intensities in that sample were normalised. All other CII band intensities were also

normalised to that of Band 1. The band intensities measured for the PBS-treated WT mouse were each time arbitrarily assigned the value of 1.0, and all changes in band intensities from other mice for a specific RC complex were expressed relative to those of the PBS-treated WT mouse.

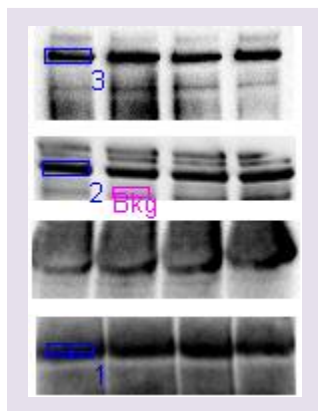


Figure 3.7: Example of a photographed western blot during analysis with GeneTools software. Block 1 was selected as a reference to which other band intensities were normalised. Blocks 2 and 3 identify selected experimental bands. The magenta block (Bkg) identifies a part of the blot that represented background, which was subtracted from all other measured band intensities.

### 3.13 RELATIVE mtDNA COPY NUMBER DETERMINATION USING REAL-TIME PCR

Real-time PCR (qPCR) was utilised in this study to detect changes in mtDNA copy number that might occur in the MTKO mice when compared to WT mice. A relative quantification approach was used, where the change in mtDNA expression in the MTKO mice was expressed relative to that in the WT mice. In this study, SYBR® Green I dye was used to detect PCR products. This method is economical compared to alternative methods such as the Taqman® assay, as a secondary probe is not required. With this method an increase of signal fluorescence is detected as the amplification product increases in length. Unfortunately SYBR® Green binds non-specifically to all double-stranded DNA, which can then produce false positive signals, and contribute to inaccuracies in data (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Kwok and Higuchi, 1989; Livak and Schmittgen, 2001).

The objective of qPCR is to determine the initial relative content of the target template. Amplification occurs exponentially and is measured after each cycle. SYBR® Green I is a DNA binding dye included in the reaction, which binds proportionally to double-stranded DNA as amplicons accumulate. After excitation, the dye emits a fluorescent signal, which can be detected. An amplification plot is produced which can then be used to determine an accurate comparison between the initial level of the templates in each sample. This is useful, as the exact copy number of templates or the use of standard curves is not required. A passive internal reference dye (ROX) serves to normalise the SYBR® Green I fluorescent signal, allowing for the correction of well-to-well variations and fluorescence fluctuations. Besides the target mtDNA gene region (*MT-TY/MT-COI*), in this study a nuclear gene (*Gapdh*) was also amplified and used to normalise data to.

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#### 3.13.1 MATERIALS

For the isolation of whole DNA from tissue homogenates, a 96-well genomic DNA purification kit (Geneaid Biotech Ltd., Sijhih City, TW, GBP04/10) was used.

Instrumentation used during DNA isolation: Heraeus® Labofuge® 400R benchtop centrifuge; modified vacuum manifold.

For quantification of isolated DNA, the following was used: Quant-iT™ PicoGreen® dsDNA assay kit, which included TE buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5) (Invitrogen™, P11496).

Instrumentation used during quantification of isolated DNA: Synergy™ HT Multi-detection microplate reader; Costar® 96-well solid black microplates (Corning® Instrument Company Ltd.)

For qPCR the following were used: QuantiTect™ SYBR® Green PCR kit which included QuantiTect SYBR® Green PCR Buffer, dNTP mix including dUTP, SYBR® Green I, ROX (passive reference dye) and 5 mM MgCl<sub>2</sub>; oligonucleotides (primers) purchased from Inqaba Biotec™; Milli-Q® prepared water.

Instrumentation and consumables included: 7300 Fast Real-Time PCR system (Applied Biosystems™, Johannesburg, ZA); MicroAmp® optical 96-well reaction plates with barcodes (Applied Biosystems™, 4306737).

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### 3.13.2 METHODS

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#### 3.13.2.1 ISOLATION OF DNA FROM HEART HOMOGENATE

A 96-well Genomic DNA purification kit, specifically designed to purify total DNA from blood or animal tissue, was used to isolate genomic DNA according to instructions of the supplier. Of the lyses buffer (GT Buffer, containing a chaotropic salt and SDS) 200 µl as well as 20 µl proteinase K were added to 100 µl thawed heart tissue homogenate (~20 µg protein/µl) and incubated overnight at 60 °C in micro-centrifuge tubes. Of the GB buffer, 200 µl was then added and the samples incubated at 70 °C for 20 minutes. During these steps, cells are lysed and proteins degraded. The samples were centrifuged for 5 minutes at maximum speed (> 13 000g) to precipitate any insoluble material and transferred to a clean 96-well plate. Subsequently, 200 µl of 96% (v/v) ethanol was added to each well and mixed immediately by pipette. The solution was then transferred onto a DNA binding plate placed on top of a vacuum manifold. A vacuum at 25 cm Hg was applied for 30 seconds during which DNA binded to the glass fibre matrix in each well. To remove contaminants, the plate was washed with 300 µl of W1 Buffer, followed by 600 µl of Wash Buffer. After the addition of each buffer a vacuum of 25 cm Hg was applied for 30 seconds. An additional 10 minute vacuum step followed to ensure the removal of any residual ethanol. A collection plate was inserted underneath the DNA binding plate, and the DNA eluted with 100 µl of preheated, low salt Elution Buffer using a vacuum.

### 3.13.2.2 QUANTIFICATION OF ISOLATED DNA

The concentration of the isolated DNA was determined with the Quant-iT™ PicoGreen® dsDNA assay kit. In principle, this assay is designed in a similar way to the protein assay described before, except that dsDNA is detected by intercalation of PicoGreen® that induces fluorescence. The instructions of the kit were followed and a DNA standard curve (0 to 200 ng) was used. DNA-PicoGreen® induced fluorescence was measured using a Synergy™ HT Multi-detection microplate reader with 480 nm as excitation and 520 nm as emission settings. By using the Gen5™ Data Analysis software and linear regression, the amount of DNA (ng) per volume used was calculated.

### 3.13.2.3 REAL-TIME PCR

For this study, a Taq-polymerase was used with SYBR® Green I dye. For amplification of the target mtDNA and nuclear genes, 10 µl of the master mix (2 x SYBR® Green PCR mix, 10 µM forward primer, 10 µM reverse primer, Milli-Q® prepared water) was loaded into each well of a 96-well PCR plate. For each sample, 10 ng of isolated DNA (see Section 3.13.2.1) was added to three separate wells. The primers used were designed by Dr. Oksana Levanets and synthesised to order by Inqaba Biotec™, the sequences of which can be found the Tables 3.4 and 3.5.

Table 3.4: Sequence of qPCR primers for amplification of *MT-TY/MT-CO1*

| Primer                                                 | Primer sequence (5' – 3')  | T <sub>m</sub> (°C) | Size (bp) |
|--------------------------------------------------------|----------------------------|---------------------|-----------|
| <b><i>MT-TY/MT-CO1-mouse-F</i></b><br><b>(Forward)</b> | cag tct aat gct tac tca gc | 49.7                | 20        |
| <b><i>MT-TY/MT-CO1-mouse-R</i></b><br><b>(Reverse)</b> | ggg cag tta cga taa cat tg | 49.7                | 20        |

Here, the sequences of the forward and reverse primers used during amplification of the mtDNA gene region are given in 5' to 3' notation, with the melting temperatures and sizes (in base pairs, bp) for each.

Table 3.5: Sequence of qPCR primers for amplification of *Gapdh*

| Primer                         | Primer sequence (5' – 3')   | T <sub>m</sub> (°C) | Size (bp) |
|--------------------------------|-----------------------------|---------------------|-----------|
| <b>Gapdh-mouse-F (Forward)</b> | ggg aag ccc atc acc atc ttc | 56.3                | 21        |
| <b>Gapdh-mouse-R (Reverse)</b> | aga ggg gcc atc cac agt ct  | 55.9                | 20        |

Here, the sequences of the forward and reverse primers used during amplification of *Gapdh* are given in 5' to 3' notation, with the melting temperatures and sizes (in base pairs, bp) for each.

The qPCR plate was then placed in the 7300 Fast Real-Time PCR system and the amplification run initiated. An initial denaturing step, 3 minutes at 95 °C, was followed by 40 cycles of amplification and detection. A cycle consisted of a denaturing step for 15 seconds at 95 °C, followed by an annealing step for 15 seconds at 53 °C, and an extension step for 27 seconds at 72 °C. During this last step, quantification also took place.

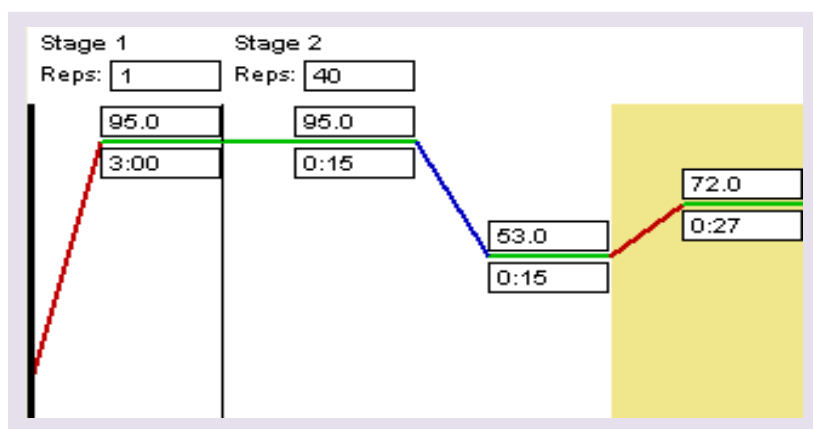


Figure 3.8: Screen capture of software settings illustrating the stages and cycles of real-time PCR. The initial denaturing step took place at 95 °C for 3 min. DNA was denatured at 95 °C for 15 seconds, primers annealed at 53 °C for 15 seconds and extension took place at 72 °C for 27 seconds. Data was captured during the last step. This cycle (Stage 2) was repeated 40 times.

All samples were measured in triplicate. Means were calculated as discussed in Section 3.14. The relative mtDNA copy number, determined by measuring *MT-TY/MT-CO1* gene region copies for each sample was then normalised to *Gapdh*, which represented the nDNA copy number. This was done using the cycle threshold ( $C_T$ ) value of each reaction and the REST (Relative Expression Software Tool) as described by Pfaffl *et al.* (2002). This uses the correction for exact PCR efficiencies (calculated before onset of the study) and the mean  $C_T$ -value deviation between the mice groups (Pfaffl *et al.*, 2002, <http://www.gene-quantification.de>). The expression ratio results of the investigated genes were tested for significance by a Pair Wise Fixed Reallocation Randomization Test<sup>®</sup> and plotted on a graph, using standard error (SE) estimation, via a complex Taylor algorithm (<http://www.gene-quantification.de/rest-384.html>). The PBS-treated WT mice were assigned the arbitrary value of 1, and all changes in other genotype or treatment groups were expressed as relative to that of PBS-treated WT mice.

### 3.14 STATISTICAL ANALYSES

With the exception of BN-PAGE and western blot analysis, all parameters were measured in triplicate. The triplicates were manually examined and obvious outliers (possibly due to experimental errors) were excluded in an attempt to minimise the variation in data. When possible, a co-efficient of variance (CoV) of <10% for repeats was set as a criterion, to ensure good intra-batch repeatability. Thereafter, a mean value for each sample was calculated and used during further analyses.

All statistical analyses were done using Statistica Software, version 10 (StaSoft Inc. Tulsa, OK). Outliers in a specific tissue, for a specific analysis, were then further identified and removed using Tukey's method (Tukey, 1977). This method defines outliers as those data points falling outside the control limits, defined as three interquartile ranges below the 25<sup>th</sup> percentile or above the 75<sup>th</sup> percentile.

A factorial one-way analysis of variance (ANOVA) was performed to determine whether any interaction between genotype and treatment parameters had occurred. No interaction between genotype and treatment was found for any of the measured parameters, and consequently the effect of treatment and genotype could be separately assessed. To assess the day-to-day variation of respiration data, a one way ANOVA was performed, and since differences were found, a post hoc test (Tukey HSD) was used to identify the days responsible for those differences.

Next, the normality of all data sets (a data set defined here as data from a specific analysis, done in a specific tissue) was assessed using Shapiro-Wilk's W-test. A p-value greater than 0.05 indicated normally distributed data, which was then further analysed using parametric statistical methods: statistically significant differences between MTKO and WT mice, or between PBS- and rotenone-treated mice, were identified using Student's t-tests, where a p-value less than 0.05 indicates a significant difference, with 95% certainty. Normally distributed data are presented in Chapter 4 by box-plots with mean-values, and spreads indicated by standard errors, while ranges are indicated by the minimum and maximum data points. If, however, data was deemed skew (p-value < 0.05) when using Shapiro-Wilk's W-tests to determine distribution normality, non-parametric statistical analyses followed: Mann-Whitney U-tests were used to determine statistically significant differences between MTKO and WT mice, or between PBS- and rotenone-treated mice, with a p-value less than 0.05 implying a statistically significant difference. Data not normally distributed are presented in Chapter 4 by box-plots with medians, where spreads are indicated by 25<sup>th</sup> and 75<sup>th</sup> percentiles, and the range by minimum and maximum data points. A schematic summary of the statistical methods followed can be found in Figure 3.9.

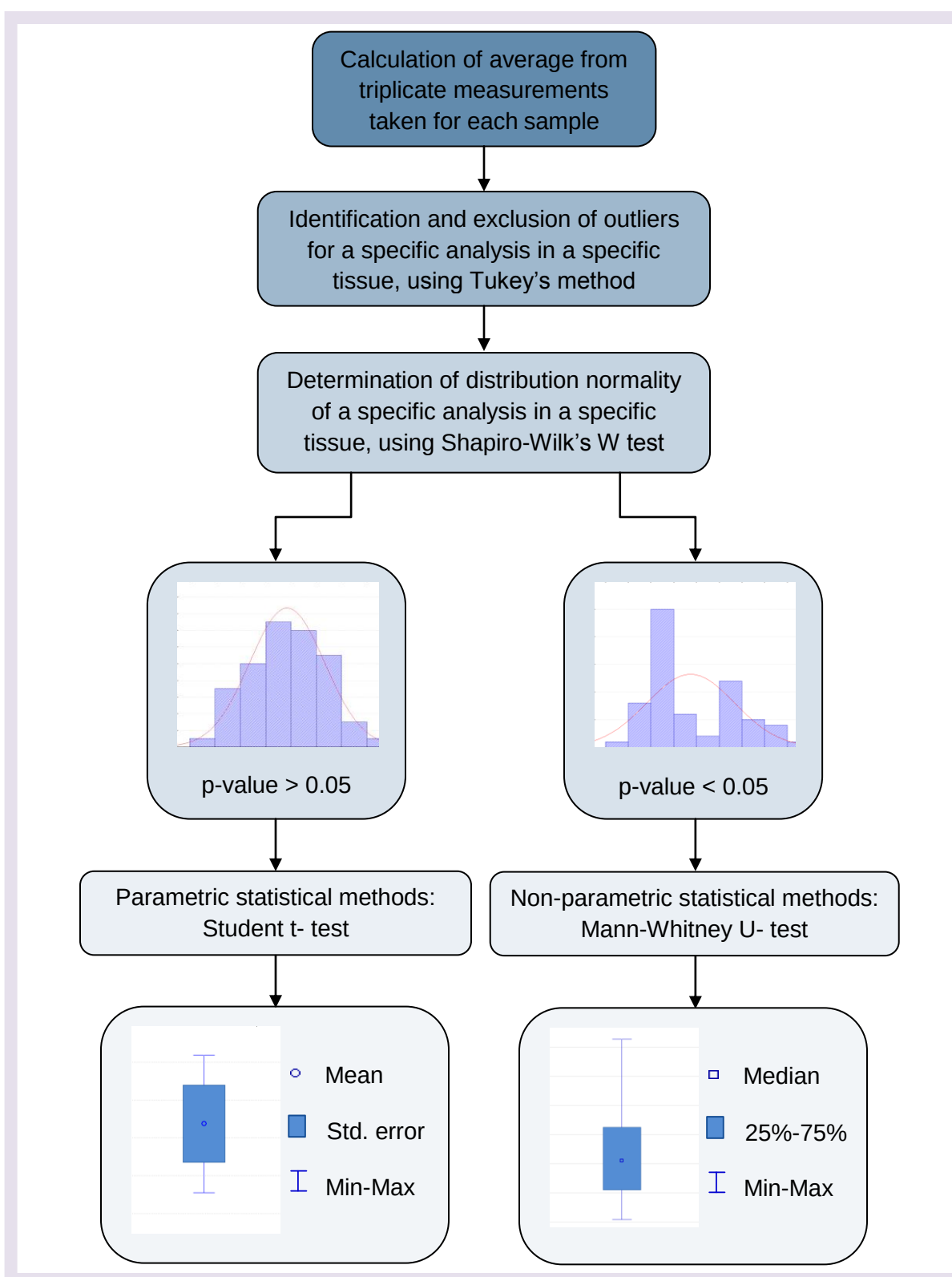


Figure 3.9: Schematic representation of the statistical methods followed during analyses of data. After outliers were excluded, data distribution in a specified group was classed as normal or skewed and followed by parametric and non-parametric statistical methods respectively. Figures given in Chapter 4 (Results) use the graph presentations shown in the bottom part of the scheme.

## CHAPTER 4

# RESULTS AND DISCUSSION

### 4.1 INTRODUCTION

A detailed description of the experimental design and methods used to generate and then analyse data can be found in Chapter 3. The processed data points are given in Appendix C. In this chapter, the final results obtained during this study will be presented and discussed briefly according to the relevant tissue type (liver, heart, skeletal muscle and brain). Further conclusions drawn from the results presented here will be given in Chapter 5.

As discussed in Section 3.5, 33 WT and 33 MTKO mice were randomly distributed into 11 Day-groups containing six mice each. Each Day-group consisted of three WT and three MTKO mice which were treated with either PBS or rotenone, starting on 11 consecutive days and continuing for 20 days. As each Day-group completed 20 days of treatment, the mice were euthanized, tissues were harvested and either used immediately, *i.e.* for respiration analyses, or stored for later analyses, such as RC enzyme assays, BN-PAGE and western blot and mtDNA copy number determination.

Note: In Chapters 4 and 5, the terms “significant” and “statistically significant” refer to the statistical function, indicating p-values < 0.05, determined with various tests as discussed in Section 3.12 (thus statistically significant on the 95% confidence level). All other terms such as “trend”, “tendency”, “correlation” and “ratio” are merely observational and are independent of any statistical functions that might be associated with them.

The term “data points” refers to final mean values calculated for each sample and also represents individual mice in a specific group.

Unless specified otherwise, the terms “data set” and “group” in general refer to the collections of data points for each genotype/treatment combination, and describes either PBS-treated WT mice, rotenone-treated WT mice, PBS-treated MTKO mice or rotenone-treated MTKO mice.

## 4.2 RESPIRATION AND RC ENZYME ACTIVITY ANALYSES IN LIVER TISSUE

Immediately after euthanizing each mouse, the liver was explanted and submerged in ice cold isolation buffer IB. Mitochondrial fractions were prepared using differential centrifugation (as discussed in Section 3.8). A portion of the 600g supernatant (homogenate sans unbroken cells and nuclei) was stored at -80 °C, to be used for enzyme activity assays. Enriched mitochondrial fractions freshly prepared each day were used immediately for respiration analyses.

### 4.2.1 RESPIRATION ANALYSES

Respiration analyses were done using a Clark-type electrode to detect dissolved oxygen levels in the experimental solution, which are inversely related to oxygen consumption, and thus to ATP production (as discussed in detail in Section 3.10). Respiration analyses were used to assess the RC (together with CV) as a single working unit. No conclusions can be made about the activity of individual enzyme complexes of the OXPHOS system from respiration analyses data, but rather, how well the components making up the OXPHOS system work together to achieve a common goal (*i.e.* ATP production). To ensure that electrons enter the RC at CI, respiration analyses were done in the presence of glutamate and malate.

#### 4.2.1.1 RESULTS

The processed data points for the respiration analyses are included in Appendix C. Respiration analyses are time consuming but also have to be completed in a set time frame, ~ four hours after tissue explantation. To allow enough time to analyse samples from two tissues (liver and heart) and each sample in triplicate, a limited number of six mice were euthanized per day after treatment, hence data collection of each group, *e.g.* PBS-treated WT mice, took several days. It was therefore important to estimate the possible inter-day variation, the results of which are given in Figure 4.1. The results are shown as state 3 respiration (pmole/min/μg O<sub>2</sub> consumption) for all mice analysed on a specific day.

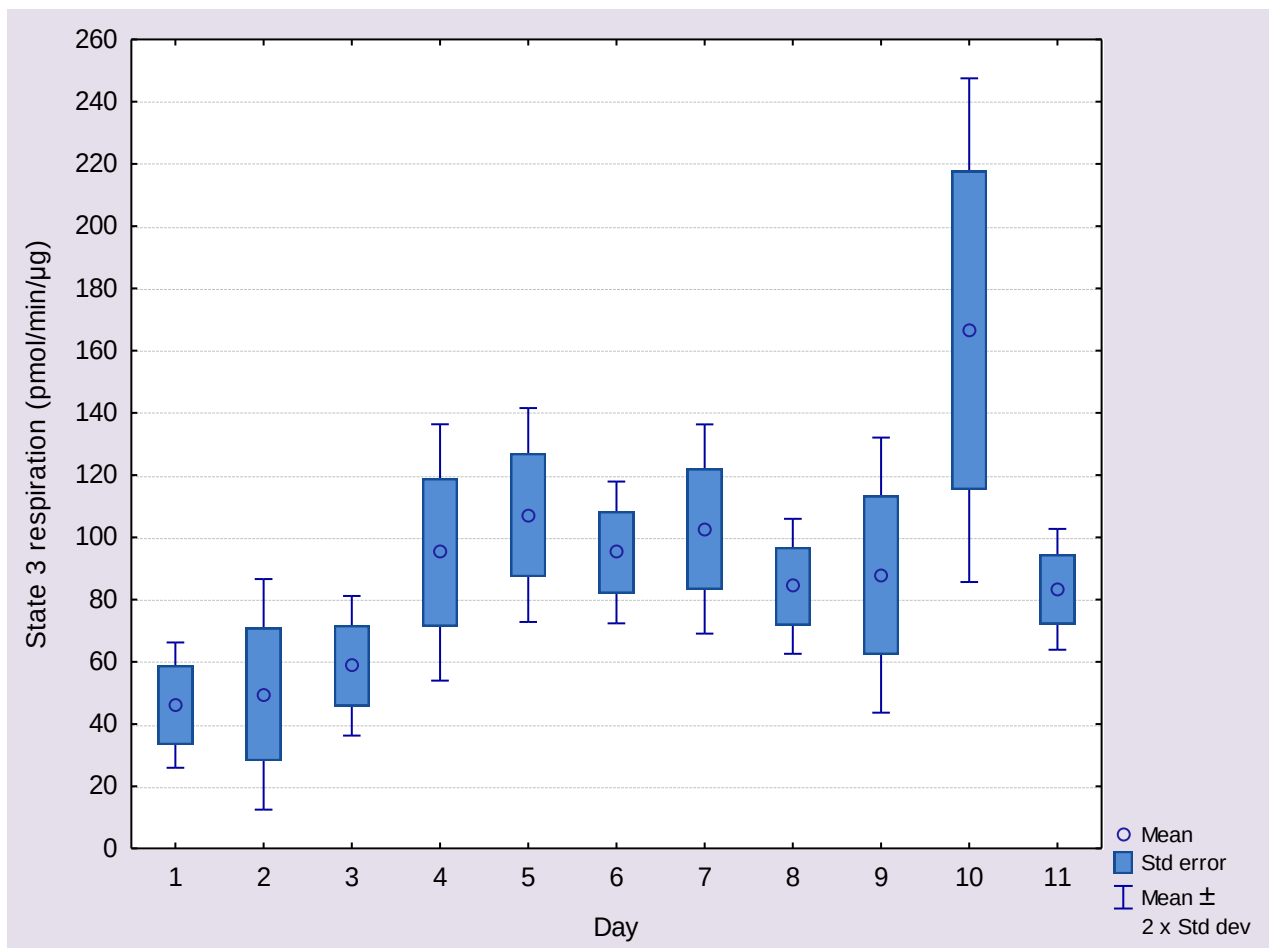


Figure 4.1: Box and whisker plots comparing mitochondrial state 3 respiration measured on different days. A data set for a specific day includes both tissues (liver and heart), both genotypes (WT and MTKO) and both treatment groups (PBS and rotenone). Day 1 to 3 and 10 differed significantly from day 4 to 9 and 11.

When the day-to-day variation was assessed, days 1 to 3 and 10 ( $p < 0.01$  for all groups) significantly differed from days 4 to 9 and 11 (Figure 4.1). This may not be surprising for the following reason: due to the magnitude of the main study, the amount of different tissues collected on a particular day, and the number of people working together, streamlining the whole process took some practice. Because of this, respiration analyses on the first three days commenced several hours after tissue explantation, resulting in less tightly coupled mitochondria and thus decreased mitochondrial respiration. This error in methodology was noted. It is thus reasonable to assume that the variation for the first three days was not biological, but largely technical.

By day ten however, the routine followed to collect and prepare tissues for analyses was being executed much more time efficiently. Hence it can be concluded that technical variation is not the obvious reason for the significantly higher mitochondrial respiration in Day-group 10 mice. A possible explanation is related to the age of the mice. Although mice were randomly distributed into different Day-groups, Day-group 10 acquired only very young mice (~ two months old) by chance. It is possible that the young age of these mice and thus the increased metabolic rate associated with younger, growing organisms (Makarieva *et al.*, 2005) could have affected mitochondrial respiration as well. This group also contained one of the only two mice (both on rotenone treatment) that died during the study, the other being in Day group 11 and also of very young age. This further indicates that the observed variation in this group might be age related. Consequently, the results for Day-groups 1 to 3 and 10 were excluded from the final respiration data set before statistical analyses were performed.

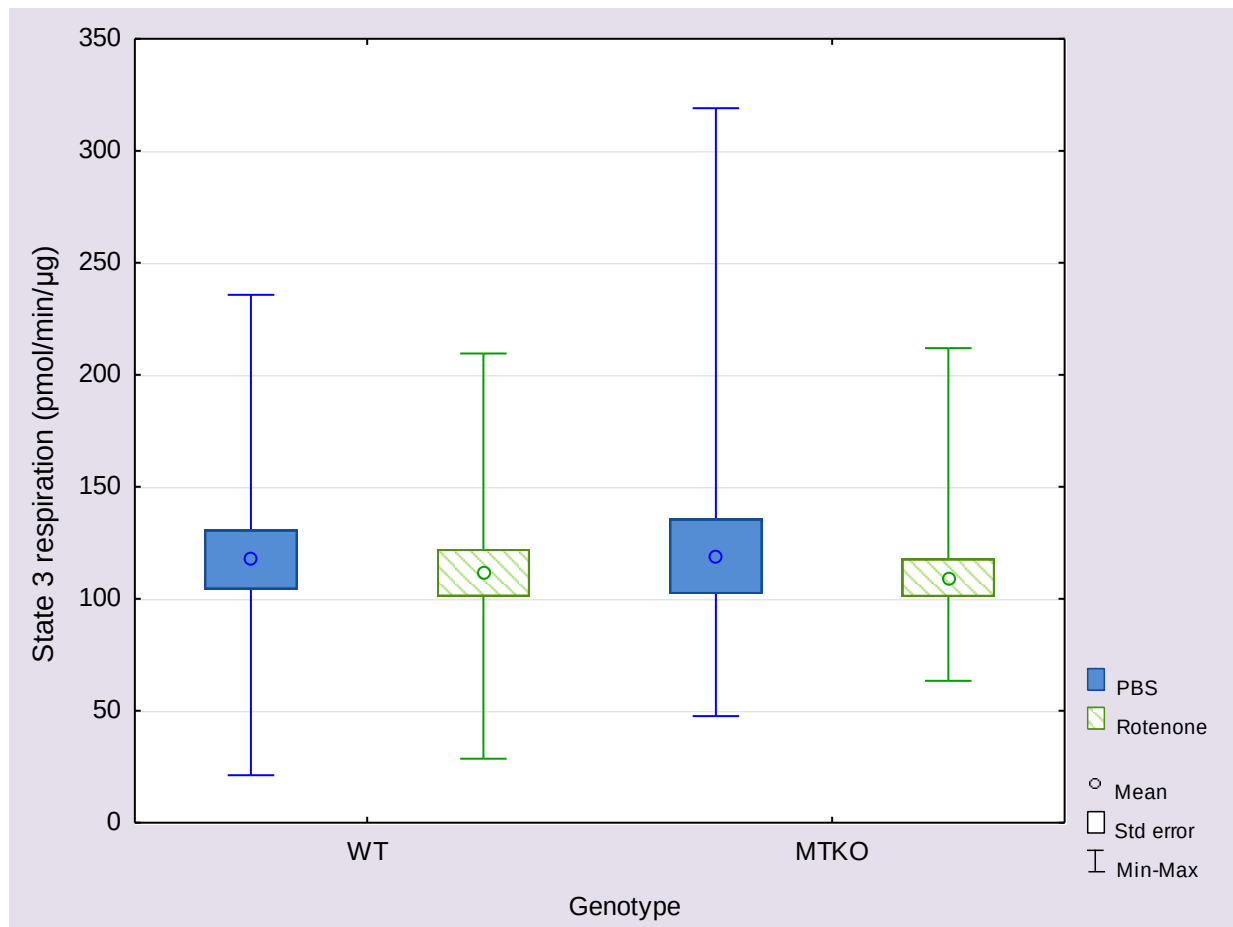


Figure 4.2: Box and whisker plots comparing liver mitochondrial state 3 respiration of PBS- and rotenone-treated WT and MTKO mice. P-values for all comparisons were > 0.05.

The final results for respiration analyses done on liver tissue are illustrated in Figure 4.2. The data of all the groups were normally distributed (results not shown) and therefore parametric statistical methods were applied (Section 3.14). The distribution of data points in all groups was wide and overlapping. It is also evident that mean respiration values of all the groups were between 110 and 120 pmole/min/μg and did not differ significantly when comparing either genotype or treatment.

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#### 4.2.2 RC ENZYME ACTIVITIES

Enzyme activity of the different RC enzyme complexes was measured spectrophotometrically using 600g supernatants or mitochondrial fractions as discussed in detail in Section 3.11. All analyses were done in triplicate and normalised to a reference sample before the mean of each sample was calculated as discussed in Section 3.14. All complex activities were then normalised to CS activity, with the exception of CI, which was normalised to CII activity as described and motivated in Section 3.11.2.2. The distribution of data for each complex was then evaluated and further analysed accordingly, as described in Section 3.14.

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##### 4.2.2.1 RESULTS

As mentioned above, the processed data points are listed in Appendix C. The final results of the different RC complex activities measured in liver are illustrated in Figure 4.3.

For CI activity (normalised to CII) in liver, data points in the groups were not normally distributed and non-parametric statistical methods were applied. From Figure 4.3A it can be observed that the distribution of data point in all groups overlapped considerably, possibly due to the large intra-group variation of the data. MTKO mice showed an apparent, but statistically insignificant, higher CI activity than WT counterparts, irrespective of treatment. More importantly, rotenone treatment resulted in a slightly, but again not significantly, decreased CI activity in both genotypes.

As with CI, CII activity (normalised to CS) data points were not normally distributed and non-parametric statistical methods were applied. CII is generally known to be a highly stable RC complex (Janssen *et al.*, 2007). In accordance with this view, CII activity was not expected to be greatly affected by genotype or treatment and as can be seen in Figure 4.3B, this was indeed the case. No statistically significant differences were observed between any of the groups, although rotenone treatment slightly increased CII activity in both genotypes.

The combined assessment of CII and CIII activity gives an indication of coQ function, as this co-enzyme serves as a mediator between these two complexes. Data points of CII+III activity (normalised to CS) were not normally distributed and consequently non-parametric statistical methods were applied. As illustrated in Figure 4.3C, the intra-group variation of data points was large. Again the effect of genotype was negligible, with no significant changes occurring between WT and MTKO groups. As observed with CII, a tendency of increased CII+III activity was observed with rotenone treatment, especially in WT mice where the activity was significantly increased (22%,  $p = 0.017$ ).

Unlike the previous enzymes, CIII activity data were normally distributed and thus parametric statistical methods were applied. As can be observed in Figure 4.3D, CIII activity was not significantly affected by either genotype or treatment, although there was a tendency towards increased activity induced by rotenone treatment.

CIV activity data were also normally distributed and again parametric statistical methods were applied. As illustrated in Figure 4.3E the slight increase in rotenone-treated mice was observed in CIV activity as well. In MTKO mice, however, this increase (~23%) was significant ( $p = 0.029$ ) and brought MTKO CIV activity *on par* with that of both treatment-groups from WT mice, showing that MTKO CIV activity was somewhat decreased compared to that of WT CIV activity. This difference might have been statistically significant with less variance in data sets.

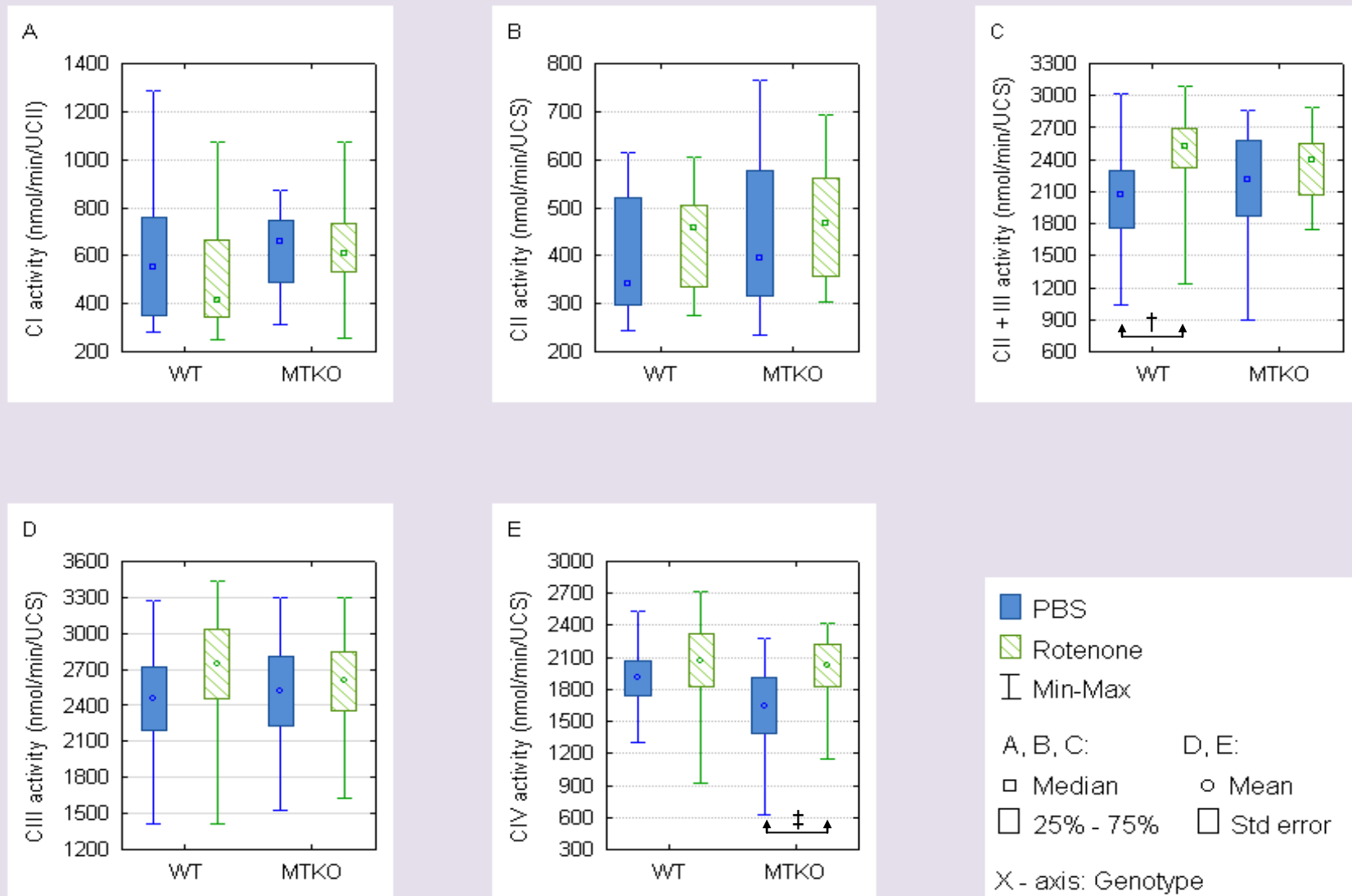


Figure 4.3: Box and whisker plots of different OXPHOS enzyme activities for liver. Statistically significant differences ( $p < 0.05$ ) between different groups are indicated as follows: † p-value = 0.017; ‡ p-value = 0.026. UCII =  $\mu\text{mole/min}$  complex II; UCS =  $\mu\text{mole/min}$  citrate synthase.

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#### 4.2.3 DISCUSSION

Neither genotype nor treatment had any significant effect on mitochondrial respiration in liver. It has been reported in several studies, reviewed by Rossignol *et al.* (2002) that the activity of individual OXPHOS complexes can be inhibited to a great degree before a biochemical threshold is reached and changes in mitochondrial respiration are observed. The degree of inhibition that can be tolerated is tissue and organism specific, and differs between complexes, but is always at least above 50% (Rossignol *et al.*, 2002), much more than the degree of inhibition achieved during this study. Even though the activity of individual complexes was affected by rotenone treatment, it was not necessarily enough to affect overall mitochondrial respiration. In liver mitochondria, with the exception of CI, rotenone treatment tended to increase complex activity. The rotenone induced decrease in CI activity is perfectly reasonable and expected, since rotenone specifically inhibits CI by binding between the flavoprotein and iron protein components of CI, thus blocking CI from binding with and transferring electrons to ubiquinone (Teeter *et al.*, 1969; Lambert and Brand, 2004). This rotenone-induced decrease in CI activity might also have led to the increased activity of the other RC complexes in liver. The inability of CI to accept electrons would have forced electrons to enter the RC at CII, possibly inducing a compensatory up-regulation of CII assembly/activation as well as the other enzymes that follow in the RC. Because of their smaller size and increased mobility, co-enzymes like coQ and cytochrome c are among the first components of the RC to be up-regulated to compensate for dysfunctions elsewhere in the RC (Rossignol *et al.*, 2002). This may explain the significantly increased CII+III activity observed in rotenone treated WT mice.

CIV activity of PBS-treated MTKO mice was decreased when compared to PBS and rotenone treated WT mice, albeit not significantly. MT's involvement in copper homeostasis might have played a role here. Ogra *et al.* (2006) demonstrated that MTs maintained CIV and SOD activity during Cu-depletion in fibroblast cells cultured from MTKO and WT mice, and went on to suggest that MTs might play a role in the delivery of Cu to CIV, a metal essential to the catalytic site of CIV (Lill and Kispal, 2000). In MTKO mice, the decreased CIV activity observed could be the result of aberrant CIV assembly, due to irregular delivery of Cu to CIV in the absence of MTs. This decreased

activity of CIV in PBS treated MTKO mice had no effect on overall respiration rate, so up-regulation of its activity was probably not necessary. The capacity to increase CIV activity in MTKO mice is certainly present, as can be seen by the significant increase in CIV activity in rotenone treated MTKO mice. The absence of MTs therefore seems not to have had a detrimental effect on CIV function.

#### 4.3 RESPIRATION, RC ENZYME ACTIVITY, BN-PAGE/WESTERN BLOT ANALYSES AND mTDNA COPY NUMBER DETERMINATION IN HEART TISSUE

Although cardiac cells do contain MTs, it is currently believed that MTs, or at least known MT isoforms, are not found in heart mitochondria (Lindeque *et al.*, 2010). Because of this it might be expected that the absence of MTs would have a lesser effect on heart tissue, but this expectation assumes that the possible interaction between MTs and enzymes of the OXPHOS system is of a direct nature. Previous studies at this institution on MTKO mice indicated significant changes in MTKO heart mitochondria (van Zweel, 2010), therefore heart tissue was included in this study.

After euthanizing of mice, hearts were explanted immediately and submerged in ice-cold isolation buffer IB. As with liver tissue, heart mitochondrial fractions were prepared using differential centrifugation. Portions of the 700g supernatant and the enriched mitochondrial fraction were stored at -80 °C for later use in enzyme activity assays and BN-PAGE/western blot analysis (Section 3.8). Respiration analyses were done immediately on enriched mitochondrial fractions.

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##### 4.3.1 RESPIRATION ANALYSES

Respiration analyses were performed each day on freshly prepared heart mitochondrial fractions. As with respiration analyses results from liver mitochondria, data from Day-groups 1 to 3 and 10 from heart respiration analyses were excluded for reasons described in Section 4.2.1.1.

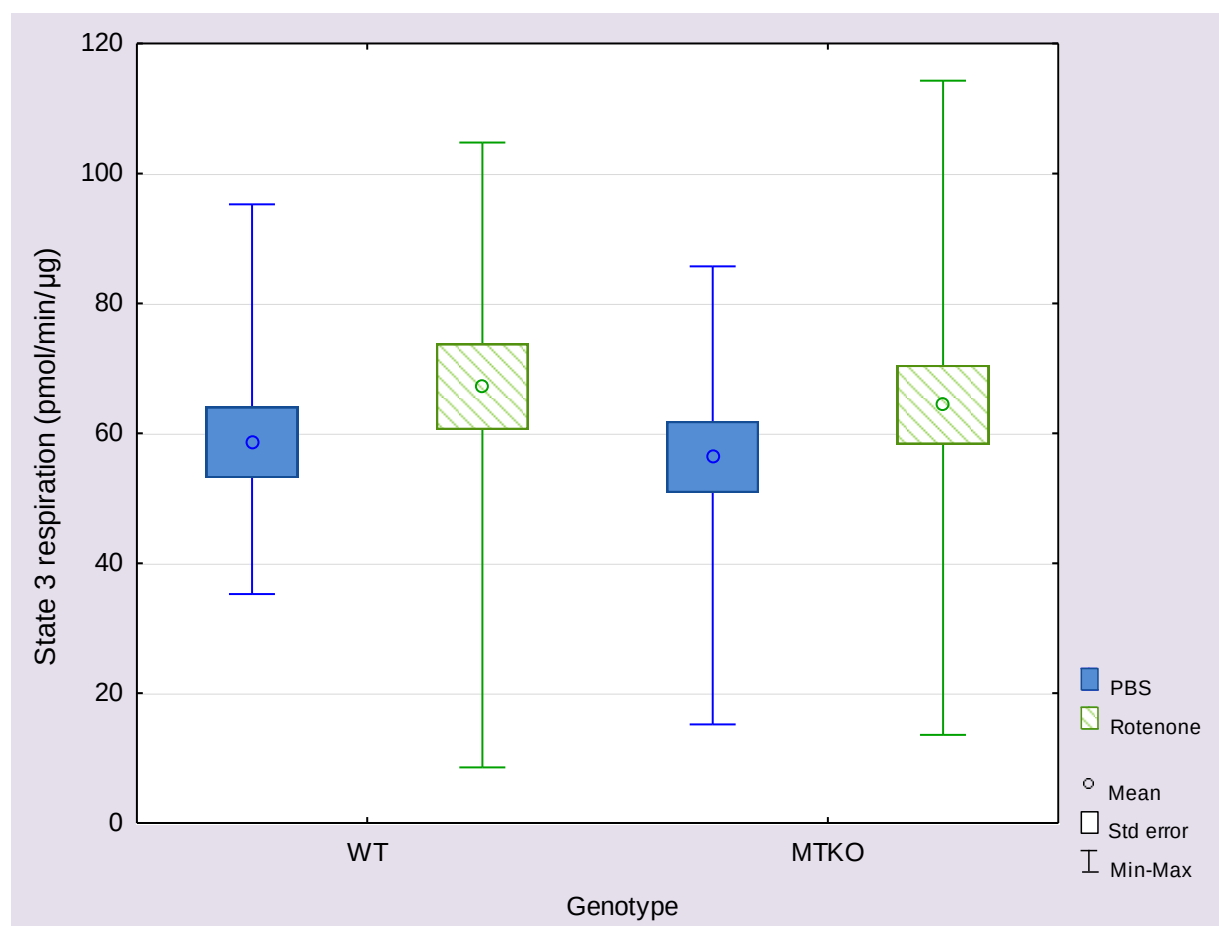


Figure 4.4: Box and whisker plots comparing heart mitochondrial state 3 respiration of PBS- and rotenone-treated WT and MTKO mice. P-values for all comparisons were > 0.05.

#### 4.3.1.1 RESULTS

The final results for respiration analyses done on heart tissue are given in Figure 4.4. As with data obtained from respiration analyses done in liver, respiration analyses data for the heart was normally distributed (results not shown) and consequently parametric statistical methods were applied. As can be observed in Figure 4.4, rotenone treatment slightly increased the intra-group variation of data points in both genotypes. Mean respiration values in all the groups were between 55 and 65 pmole/min/μg and no statistically significant differences were observed when comparing either genotype or treatment.

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### 4.3.2 RC ENZYME ACTIVITIES

The activities of the different RC enzymes in heart mitochondria were measured spectrophotometrically using 700g supernatants or mitochondrial fractions (Section 3.11). A mean value for each sample was calculated from triplicate measurements as described in Section 3.14. CI activity was normalised to CII, while all other complex activities were normalised to CS (Section 3.11.2). The distribution of data for each complex was then evaluated and accordingly analysed further as described in Section 3.14.

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#### 4.3.2.1 RESULTS

The processed data points are given in Appendix C. The final results of the different RC complex activities measured in heart are illustrated in Figure 4.5.

In heart, CI activity (normalised to CII) data were not normally distributed and thus non-parametric statistical methods were applied. Notably, the intra-group variation of data points in rotenone-treated groups was much less than that of PBS-treated groups. As can be observed in Figure 4.5A, CI activity was significantly inhibited in WT mice (~27%,  $p < 0.001$ ) and in MTKO mice (~8%,  $p = 0.02$ ) by rotenone treatment. However, genotype had no statistically significant effect on the activities.

CII activity data were normally distributed and parametric statistical methods were therefore applied. As can be seen in Figure 4.5B, mean values ranged between 61 and 67 nmole/min/UCS, indicating remarkably little variance between groups. CII activity was therefore not significantly affected by either genotype or treatment.

CII+III activity data were also normally distributed and again parametric statistical methods were applied. As with CII activity, CII+III activity showed relatively little variation between groups, with mean values ranging from 175 to 197 nmole/min/UCS (Figure 4.5C). No statistical differences between the groups were observed, indicating that neither genotype nor treatment played a significant role in CII+III activity.

As with CII and CII+III data, CIII activity data were normally distributed and parametric statistical methods were utilised. As can be observed in Figure 4.5D, intra-group variation of CIII activity data points were relatively small, with mean values ranging from 257 to 287 nmole/min/UCS. Despite this, PBS-treated MTKO mice showed significantly increased CIII activity (11%) when compared to PBS-treated WT mice ( $p = 0.02$ ). Rotenone treatment had no significant effect on CIII activity in heart tissue.

CIV activity data, however, was not normally distributed and therefore non-parametric statistical methods were applied. As can be seen in Figure 4.5E, intra-group variation of data points from all groups was large, and more pronounced in rotenone-treated than in PBS-treated groups. CIV activity was not significantly affected by either genotype or treatment, but a slight tendency towards increased CIV activity was observed in rotenone-treated groups of both genotypes.

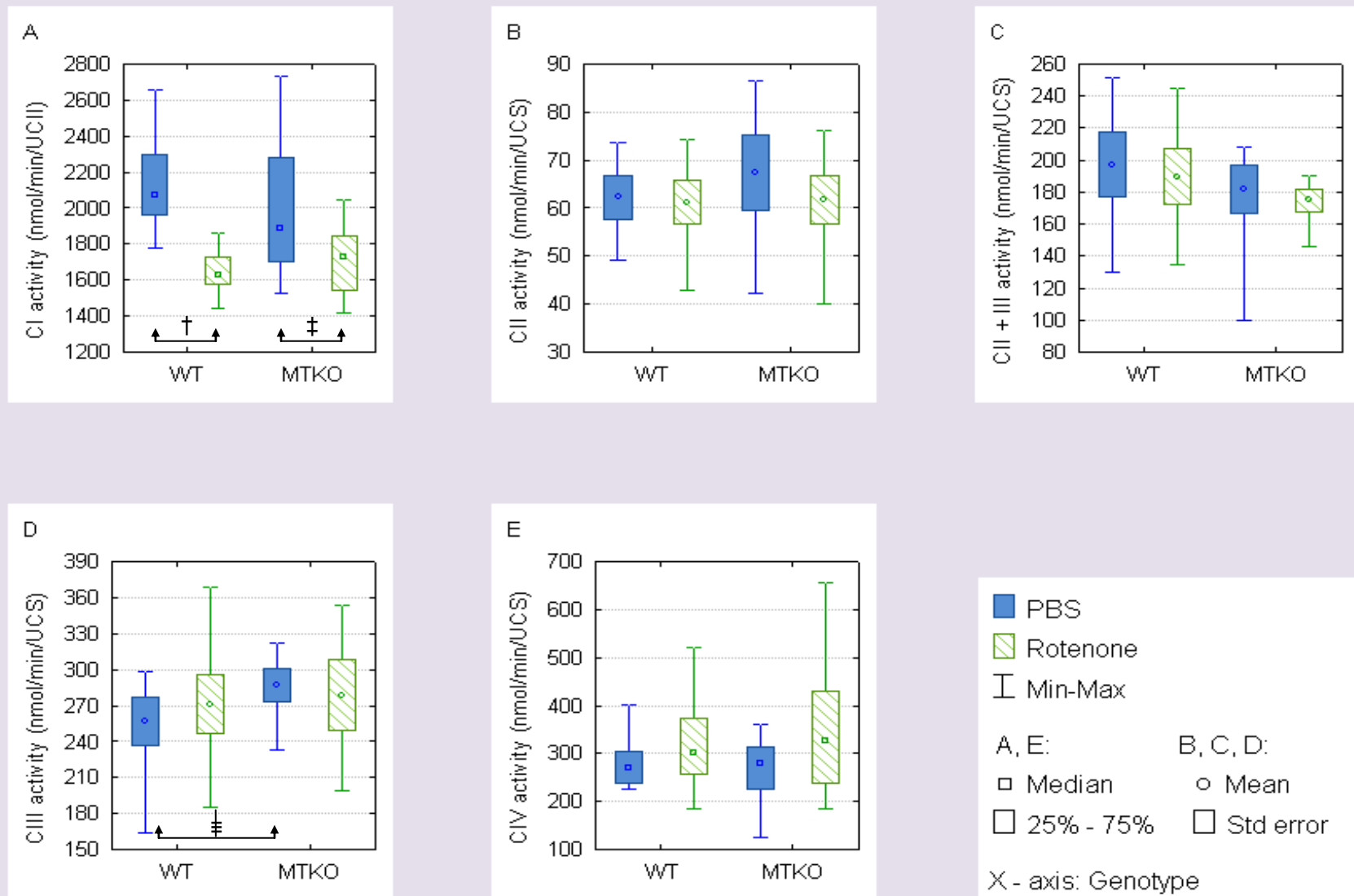


Figure 4.5: Box and whisker plots of different OXPHOS enzyme activities for heart. Statistically significant differences ( $p < 0.05$ ) between different groups are indicated as follows: † p-value < 0.001; ‡ p-value = 0.020; ‡ p-value = 0.023.

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#### 4.3.3 BN-PAGE AND WESTERN BLOT ANALYSIS

Because of the observations made while analysing RC enzyme activity results, it was decided to analyse heart mitochondrial RC enzymes further using blue-native polyacrylamide gel-electrophoresis (BN-PAGE) and immune-stained western blot analysis. For these specialised analyses, prepared heart mitochondrial samples were sent to Dr. Leo Nijtmans at the Nijmegen Centre for Mitochondrial Disease. When using BN-PAGE, non-denatured protein separation patterns can indicate structural and quantitative differences. Four samples (one from each group) were run on a non-denaturing, blue-native polyacrylamide gel to separate the different RC complexes, and then transferred to a cellulose membrane during western blotting. The individual RC enzyme complexes were bound to monoclonal mouse anti-complex I-IV antibodies and then detected using rabbit-anti-mouse horse radish conjugated polyclonal antibodies. This allowed for chemilluminiscent detection of the individual intact complexes. The band intensities of CI, CIII and CIV were all then quantified relative to the CII band intensity (Section 3.12). PBS-treated WT samples of each complex were then arbitrarily assigned the value of 1.0 and all changes in other sample quantities expressed relative to PBS-treated WT samples.

#### 4.3.3.1 RESULTS

A photograph of the western blot showing the different RC complexes from heart samples can be seen in Figure 4.6, while Figure 4.7 represents the final analysed results obtained from this blot.

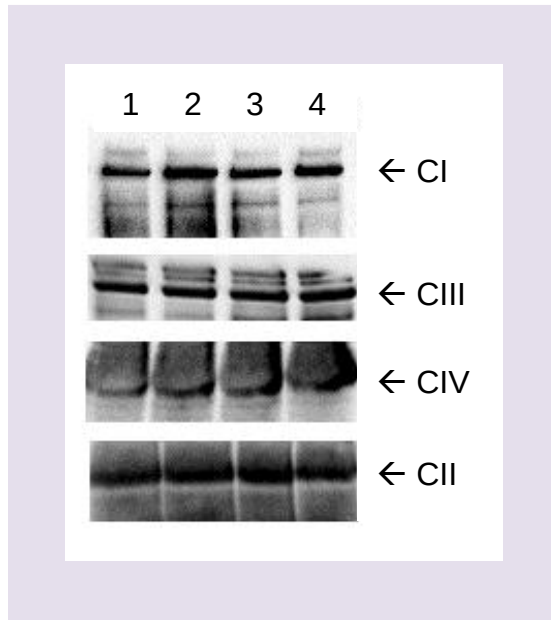


Figure 4.6: Western blot of OXPHOS complexes after separation using BN-PAGE. From the left, the lanes contain complexes from: 1) PBS-treated WT mice, 2) rotenone-treated WT mice, 3) PBS-treated MTKO mice, 4) rotenone-treated MTKO mice. From the top, bands represent concentrations of native structures of CI, CIII, CIV and CII after immuno-detection using antibodies against a specific subunit of each complex.

Samples were prepared according to CII activity. As can be observed in Figure 4.6, CII quantities in all four samples appeared to be relatively equal. CI quantities seemed slightly increased in rotenone treated samples, whilst CIII quantities appeared equal in all four samples. However CIV quantity seems to have progressively increased when observed from the PBS-treated WT mouse to the rotenone-treated MTKO mouse. It is difficult to assess the exact degree of changes by a simple observation of a blot. Therefore, computer software (GeneTools) was used to analyse the blot by measuring the intensities of the bands (Section 3.12). These results are presented in Figure 4.7.

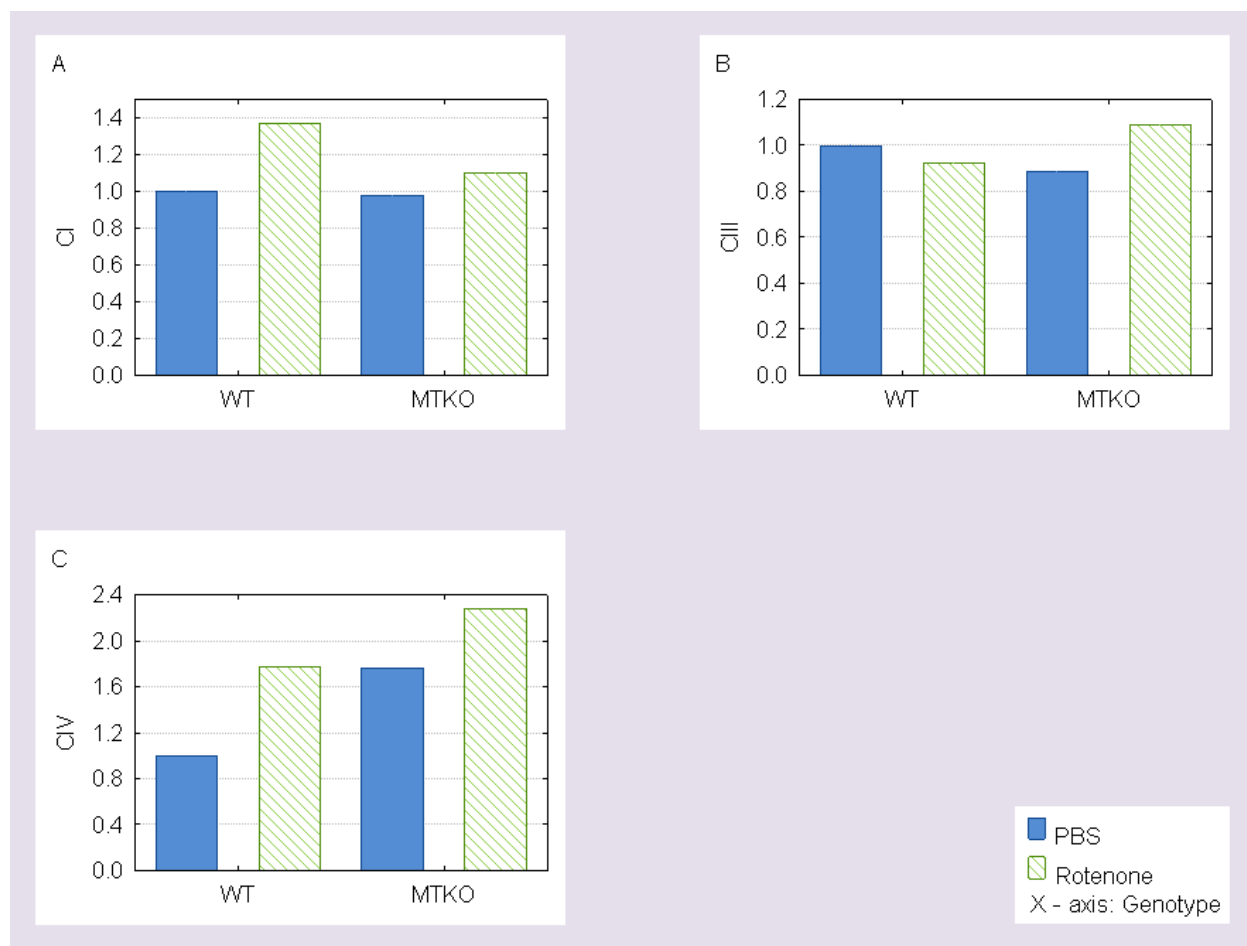


Figure 4.7: Graphical presentation of enzyme quantities of different genotype and treatment mice, as determined using BN-PAGE and western blot analysis of heart mitochondrial fractions. CI, CIII and CIV were normalised to CII (Section 3.12). All changes in enzyme quantity are shown as relative to those of the PBS-treated WT sample, to which were assigned the arbitrary value of 1.0.

From Figure 4.7A it can be seen that rotenone treatment increased CI expression by 38% in the WT sample, but only by 10% in the MTKO sample. Virtually no change in CI expression was observed in the PBS-treated MTKO mouse when compared to the PBS-treated WT mouse.

CIII expression was decreased by 8% in the rotenone-treated WT mouse and by 11% in the PBS-treated MTKO mouse when compared to the PBS-treated WT mouse, as illustrated in Figure 4.7B. However, in the rotenone-treated MTKO mouse, CIII expression was increased by 22% when compared to the PBS-treated MTKO mouse.

CIV expression was increased by 80% in the rotenone-treated WT mouse when compared to the PBS-treated WT mouse, as can be seen in Figure 4.7C. This is comparable to the 80% increase in CIV expression observed in the PBS-treated MTKO mouse when compared to the PBS-treated WT mouse. Rotenone treatment lead to a further 27% increase in CIV expression in the MTKO mouse when compared to the PBS-treated MTKO mouse.

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#### 4.3.4 RELATIVE mtDNA COPY NUMBER DETERMINATION

Relative mtDNA quantification was also done on DNA isolated from heart tissue, using realtime-PCR (qPCR). While qPCR can give an indication of the relative copy number of mtDNA in a sample, it cannot determine the exact number of mtDNA copies per cell or per mitochondrion. The *MT-TY/MT-CO1* gene was used as the target gene in mtDNA and was normalised to the nuclear *Gapdh* gene. Mean values were calculated from triplicates as discussed in Section 3.14. A relative quantification approach was used, where changes in mtDNA expression in rotenone-treated WT mice and both treatment groups of MTKO mice were expressed relative to those in PBS-treated WT mice, which were assigned the value of 1.0, as discussed in detail in Section 3.13.

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##### 4.3.4.1 RESULTS

The final results for the relative mtDNA copy number are given in Figure 4.8. Data from all groups were normally distributed and parametric statistical methods were therefore applied. When comparing mean values of each group, the relative mtDNA copy number in rotenone-treated WT mice was decreased by 18% when compared to PBS-treated WT mice. Virtually no change in mtDNA expression was observed in PBS-treated MTKO mice, while a 10% decrease in mtDNA expression can be observed in rotenone-treated MTKO mice when compared to PBS-treated WT mice. None of the changes were of statistical significance when comparing either genotype or treatment groups, possibly due to the large intra-group variation of data.

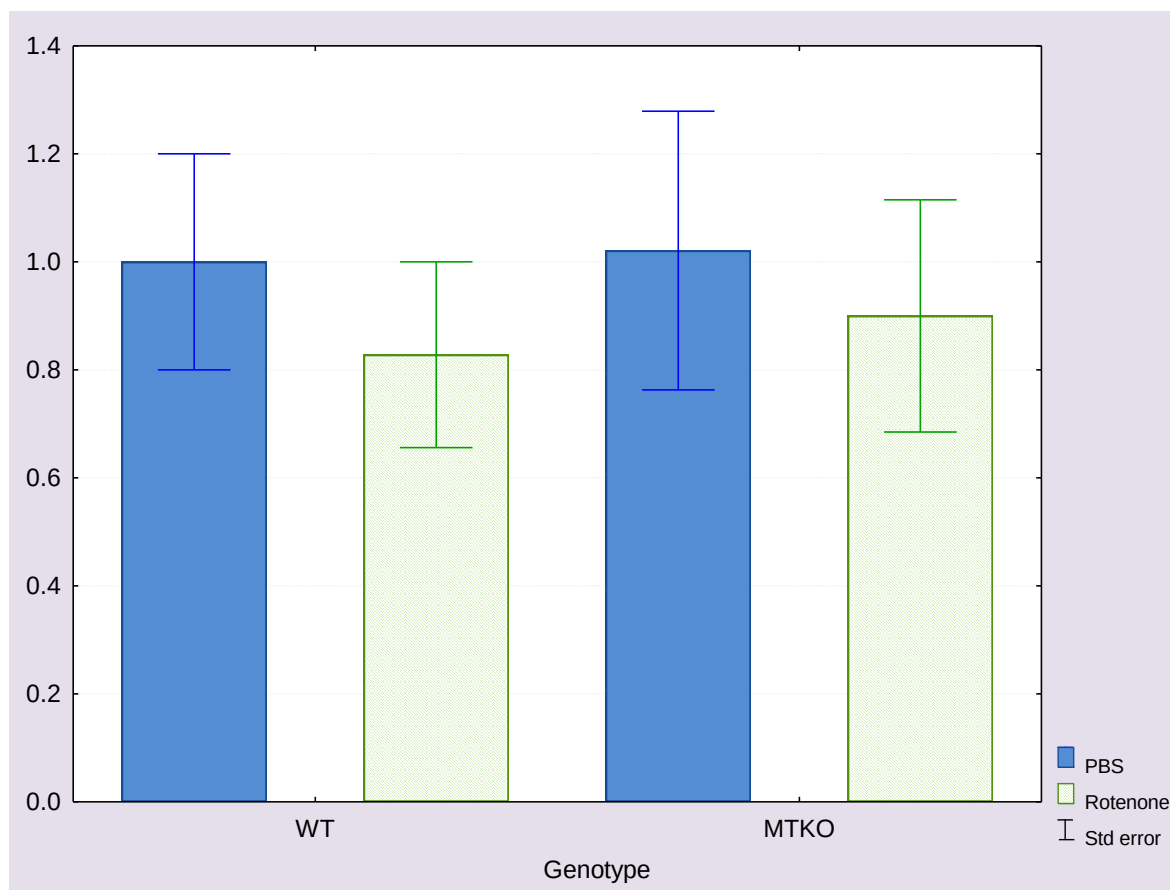


Figure 4.8: Representation of relative mtDNA copy number in heart mitochondria quantified using qPCR. PBS treated WT mice were arbitrarily assigned the value of 1.0 and all values for groups are expressed as relative to PBS treated WT mice. Bars indicate mean values.

#### 4.3.5 DISCUSSION

In heart tissue, CI activity was significantly decreased in rotenone-treated mice. As with liver tissue, this decrease is expected as rotenone specifically inhibits CI, but it appears that heart tissue was more sensitive to rotenone treatment than any other tissue. CIII activity in heart mitochondria was the only instance where genotype had a definitive effect on complex activity with an increase of ~10% in MTKO mice ( $p = 0.02$ ). Because of this observed change, BN-PAGE and western blot analysis as well as mtDNA copy number determination were performed on heart tissue samples. From enzyme activity assay results for heart tissue, it was expected that the amount of CIII units might be increased in MTKO mice. Based on the BN-PAGE data, this was not the case, as can be observed in Figures 4.6 and 4.7. Only CIII activity was significantly affected by

genotype, but not the quantity thereof. It is thought that as a protective mechanism, all enzymes function at a rate much lower than their maximum potential ( $V_{max}$ ) during favourable conditions (Rossignol *et al.*, 2002), a mechanism that would require several regulatory processes. Zinc delivered by MTs is known to bind to CIII with great affinity, leading to inhibition of this complex (Ye *et al.*, 2001). This would imply a possible regulatory role that MTs might play in OXPHOS enzyme activity. In the absence of MTs, as is the case in MTKO mice, this putative down regulatory role of zinc might have been aberrantly affected, leading to the increased CIII activity observed in PBS-treated MTKO mice. While MTs are not thought to be located in the IMS of heart mitochondria, the delivery of Zn to the IMS by MTs is not yet well understood and the possibility of an indirect interaction is still plausible.

The variation in CIV quantity, as can be seen in Figure 4.7C, was not expected. Rotenone treatment of the WT mouse increased CIV expression with ~80%, an increase comparable to that of CIV in the PBS-treated MTKO mouse when compared to the PBS-treated WT mouse. CIV quantity in the rotenone-treated MTKO mouse was more than 200% that of the PBS-treated WT mouse and 27% more than that of the PBS-treated MTKO mouse. These drastic changes in CIV quantity are not reflected in CIV activity data. The up-regulation of CIV expression in the rotenone-treated WT mouse and PBS-treated MTKO mouse might be of the same degree, but the induction pathways should have been vastly different. Rotenone treatment of the WT mouse might have lead to the up-regulation of CIV to compensate for the inhibition of CI, but this could not have been the case in the PBS-treated MTKO mouse. In an *in vivo* study done on cardiac-specific MT over-expressing mice, the expression of CIV subunit Va was slightly down-regulated when compared to its expression in WT mice (Merten *et al.*, 2005). Since BN-PAGE is non-denaturing, the indicated up-regulated expression of CIV in the MTKO mouse could possibly mean that subunit Va expression was up-regulated as well, when taking into account the results presented in the Merten *et al.* (2005) study. Subunit Va of CIV is thought not to be essential for catalytic action of this enzyme, but rather to play a regulatory role in CIV activity, possibly as a binding site during ATP-induced inhibition of CIV activity (Arnold *et al.*, 1998; Kadenbach, 2003). Thus, if subunit

Va was up-regulated in the MTKO mouse, it might have lead to irregular inhibition of CIV via ATP binding to subunit Va. This could explain the lack of increase in CIV activity of PBS-treated MTKO mice when compared to PBS-treated WT mice, despite the drastic up-regulation of CIV expression in the MTKO mouse.

Interestingly, CI expression in the rotenone-treated WT mouse was increased by ~40%, while rotenone treatment of the MTKO mouse increased CI expression by only ~10%. As discussed before, CI activity in rotenone treated mice was significantly decreased in both genotypes, but CI quantity was increased. It is possible that in an effort to cope with the biochemical stress induced by rotenone treatment, CI expression was up-regulated. Despite the large up-regulation of CI expression in the rotenone treated-WT mouse, inhibition was not successfully counteracted. Also in rotenone-treated MTKO mice inhibition was not successfully hindered, but the degree of both activity inhibition and expression up-regulation of CI in this group was much less.

#### 4.4 RC ENZYME ACTIVITY ANALYSES IN SKELETAL MUSCLE TISSUE

When investigating mitochondrial diseases in humans, skeletal muscle is a vital tissue to analyse (Haas *et al.*, 2008). The mice used in this study however, were largely sedentary and skeletal muscle activity was minimal. For this reason, RC enzyme activities from skeletal muscle were not expected to present the same degree of differences than, for instance, heart muscle.

After euthanizing mice and explanting livers, hearts and brains, skeletal muscle tissue was removed from the hind legs and submerged in ice cold isolation buffer IB. 700g supernatants and mitochondrial fractions were prepared as discussed in Section 3.8 and stored at -80 °C, to be used for enzyme activity assays.

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#### 4.4.1 RC ENZYME ACTIVITIES

Enzyme activities were measured spectrophotometrically using 700g supernatants or mitochondrial fractions as discussed in detail in Section 3.11. Samples were analysed in triplicate and normalised to a reference sample, after which mean values for each sample were calculated as described in Section 3.14. All complex activities were then normalised to CS activity, with the exception of CI, which was normalised to CII activity (Section 3.11.2). Data distribution for each complex was then evaluated and accordingly analysed further as described in Section 3.14.

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##### 4.4.1.1 RESULTS

The processed data points are given in Appendix C. The final results of the different RC complex activities measured in skeletal muscle are given in Figure 4.9.

For CI activity (normalised to CII) in skeletal muscle, data were distributed normally and parametric statistical methods were applied. As can be observed in Figure 4.9A, CI activity was significantly inhibited by rotenone treatment in WT (~34%,  $p = 0.01$ ) as well as MTKO (~20%,  $p = 0.02$ ) mice. CI activity in MTKO mice was slightly, but not significantly, less than in WT mice.

As with CI, CII activity (normalised to CS) data were normally distributed and thus parametric statistical methods were applied. The mean values of all the groups ranged between 96 and 105 nmole/min/UCS, again indicating that CII is a highly stable complex and was therefore not significantly affected by genotype or treatment as can be seen in Figure 4.9B.

Data points of CII+III activity were not normally distributed and consequently non-parametric statistical methods were applied. As shown in Figure 4.9C, intra-group variation of data points was relatively small, and median values of the different groups only ranged between 351 and 382 nmole/min/UCS. No significant differences were observed when comparing either genotype or treatment groups.

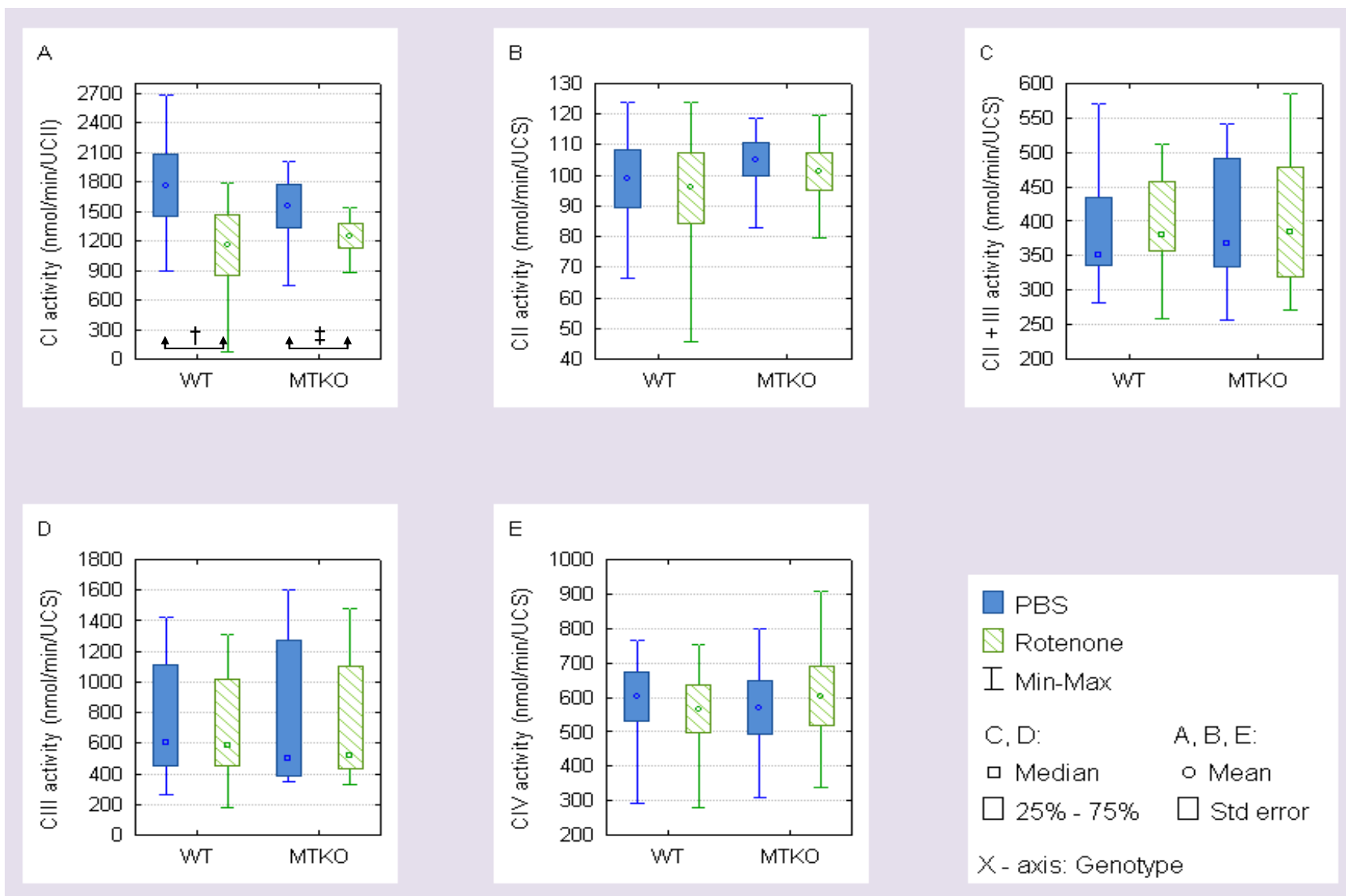


Figure 4.9: Box and whisker plots of different OXPHOS enzyme activities for skeletal muscle. Statistically significant differences ( $p < 0.05$ ) between different groups are indicated as follows: † p-value = 0.011; ‡ p-value = 0.023

CIII activity data, like that of CII+III, was also not normally distributed and thus non-parametric statistical methods were applied. As can be observed in Figure 4.9D, the intra-group variation of CIII activity data was relatively large, ranging between 180 and 1600 nmole/min/UCS. Despite this large variation in data sets, the medians of the different groups only ranged between 496 and 606 nmole/min/UCS, and no statistically significant differences between the groups were observed.

CIV activity data were normally distributed and parametric statistical methods were applied. As shown in Figure 4.9E, mean values differed only marginally between groups, and the distribution of data points in all groups overlapped considerably. Therefore neither genotype nor treatment had any significant effect on CIV activity in skeletal muscle.

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#### 4.4.2 DISCUSSION

CI activity of skeletal muscle was significantly inhibited by rotenone treatment in both WT and MTKO mice but no significant differences between WT and MTKO CI activity were observed. CII activity was not significantly influenced by either genotype or treatment, neither that of CII+III, CIII or CIV. Considering that the mice used in this study were largely sedentary, these results are not surprising.

#### 4.5 RC ENZYME ACTIVITY ANALYSES IN BRAIN TISSUE

Although MT1 and MT2 are completely knocked out in the MTKO mice, MT3 is still present. MT3 is mainly found in the brain and is the only MT that has a specific function as a neuronal growth inhibitory factor (Coyle *et al.*, 2002). Aside from this function, other MT functions are still ascribed to MT3. Brain tissue was therefore included in this study as a control tissue and no differences in RC complex activities were expected.

After euthanizing a mouse and removing the heart and liver, the head was severed, the brain removed and then submerged in ice cold isolation buffer IB. 700g supernatants and mitochondrial fractions were prepared as discussed in Section 3.8 and stored at -80 °C, to be used for enzyme activity assays.

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#### 4.5.1 RC ENZYME ACTIVITIES

The activities of the RC enzymes in brain were measured spectrophotometrically using 700g supernatants or mitochondrial fractions (Section 3.11). All analyses were done in triplicate and normalised to a reference sample contained on each 96-well plate, before the mean values of each sample was calculated as described in Section 3.14. CI activity was normalised to CII, while all other complex activities were normalised to CS (Section 3.11.2). After the normality of data distribution for each complex was evaluated, appropriate statistical analyses were applied as described in detail in Section 3.14.

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##### 4.5.1.1 RESULTS

As mentioned above, the processed data points are given in Appendix C. The final results of the different RC complex activities measured in brain tissue are given in Figure 4.10.

In brain, CI activity (normalised to CII) data were normally distributed, hence parametric statistical methods were applied. As illustrated in Figure 4.10A, CI activity was not significantly inhibited by rotenone treatment, although CI activity inhibition in PBS-treated WT mice was almost significant on the 95% level ( $p = 0.06$ ) when compared to rotenone-treated WT mice and to both treatment groups of MTKO mice.

CII activity data were not normally distributed and non-parametric statistical methods were therefore applied. As can be seen in Figure 4.10B, intra-groups variation of data points in PBS-treated groups was notably less than that of rotenone-treated groups, although median values only ranged between 64 and 70 nmole/min/UCS. CII activity was therefore not significantly affected by either genotype or treatment.

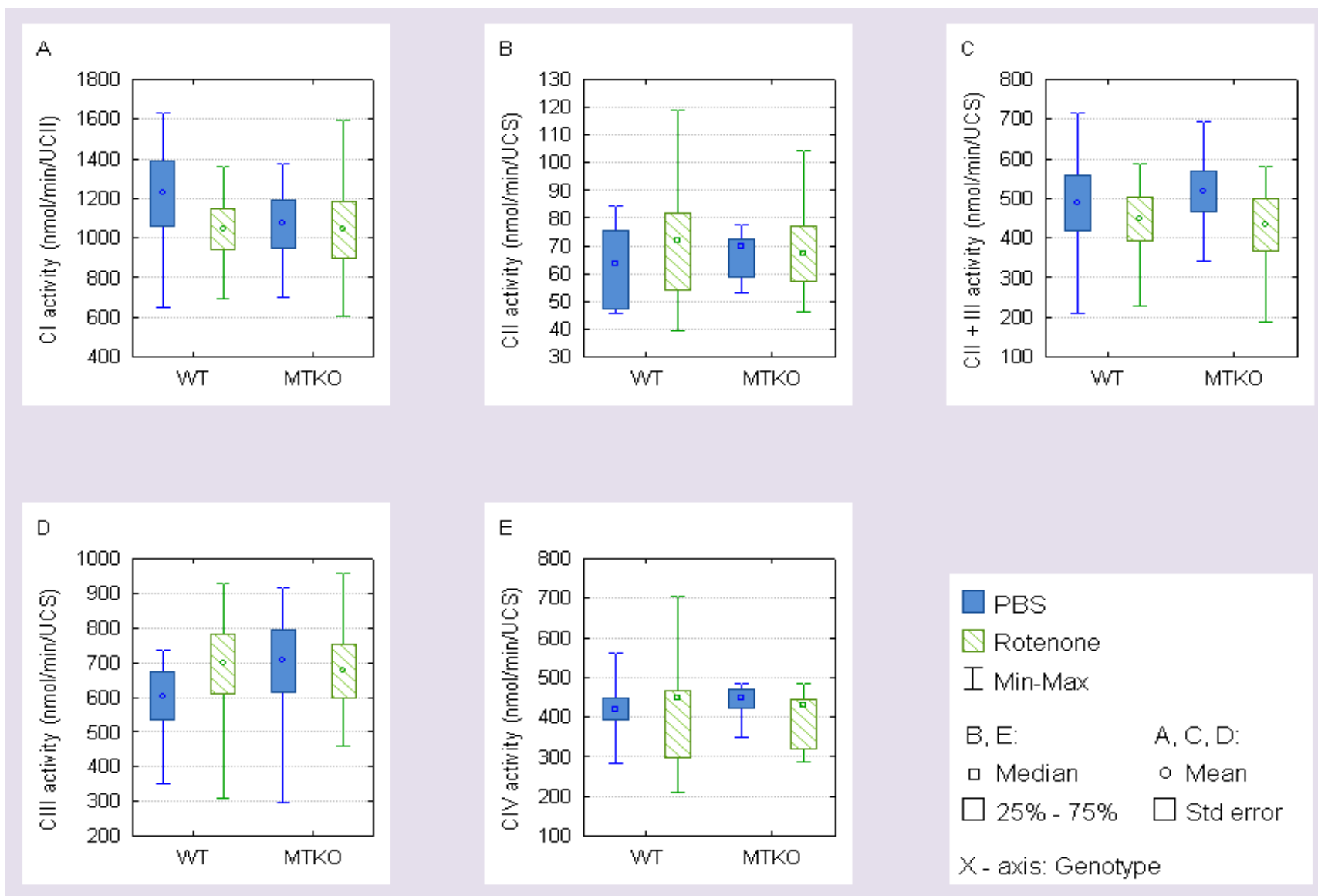


Figure 4.10: Box and whisker plots of different OXPHOS enzyme activities for brain. All differences between the groups had p-values greater than 0.05.

CII+III activity data were also normally distributed and again parametric statistical methods were applied. As can be observed in Figure 4.10C, rotenone treatment decreased CII+III activity, albeit not significantly. Thus neither genotype nor treatment played a significant role in CII+III activity.

As with CII and CII+III data, CIII activity data were normally distributed and parametric statistical methods followed. As can be observed in Figure 4.10D, CIII activity in PBS treated WT mice was marginally lower when compared to that of the other groups, but like all other differences in mean values from brain, this change was not significant. Therefore neither genotype nor treatment played a statistically significant role in CIII activity.

CIV activity data were not normally distributed and therefore non-parametric statistical methods were applied. As can be seen in Figure 4.10E, intra-group variation in CIV activity data from rotenone-treated WT mice were exceptionally larger than in other groups. Medians from different groups ranged narrowly between 418 and 450 nmole/min/UCS, thus no statistically significant changes were observed when comparing either genotype or treatment groups.

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#### 4.5.2 DISCUSSION

Genotype did not affect any RC complex activities significantly, as was expected, since MT3 is still present in the brain. CI activity was not greatly affected by rotenone treatment, despite the fact that rotenone is lipophylic and can cross the blood-brain-barrier. All other complex activities were also unaffected by rotenone treatment. It is possible that rotenone was metabolised in the liver, before any damage causing concentrations could reach the brain. The animal model, the vehicle and mode of administration used in this study, are all factors that could have influenced the delivery of rotenone to the brain.

# CHAPTER 5

## CONCLUSIONS

### 5.1 INTRODUCTION

In Chapter 2 a literature overview of the fundamental aspects of mitochondrial function, specifically oxidative phosphorylation and MTs was presented. Indications of a putative association of MTs with the mitochondrion were highlighted and served as a rationale and motivation for this study. A problem statement, aim and strategy were also presented in Chapter 2. Detailed methods used to apply this strategy were presented in Chapter 3. Final results were presented and discussed to a certain degree in Chapter 4. In this chapter, the approach and methods used, and the results obtained during this study are thoroughly evaluated and interpreted in order to reach reliable conclusions and propose viable future prospects.

### 5.2 PROBLEM STATEMENT, AIM AND OBJECTIVES

Although several primary and secondary functions have been attributed to them, the true function of MTs remains intriguingly elusive. Recent studies have focused on the interaction of MTs with specific organelles. Quite a few studies, as recently reviewed by Lindeque *et al.* (2010), have suggested a link between MTs and mitochondria, but efforts to properly define this link have been challenged by contradicting reports and a lack of supportive *in vivo* evidence, resulting in uncertainty and speculation. *Information on the involvement of MTs with respiratory chain function is limited, which served as the problem statement of this study.* As MTs are associated with the cellular redox state as well as heavy metal housekeeping, they may have a direct involvement with the RC enzyme complexes.

The aim of this study was therefore *to investigate the involvement of MTs in mitochondrial respiration and respiratory chain enzyme function by using an MT knockout (MTKO) mouse model, which was treated with the complex I (CI) inhibiting reagent, rotenone*. In order to evaluate the involvement of MTs in the mitochondrial RC, substrate driven (coupled) respiration was evaluated as the *first objective*. The *second objective* was to investigate the RC system by its single units via individual RC enzyme analyses and structurally using blue-native polyacrylamide gel-electrophoresis (BN-PAGE) and western blot analysis. As supportive data for the structure and function of the RC enzyme complexes, as a *third objective* the relative mtDNA copy number was also determined using real-time PCR.

### 5.3 EFFECTIVENESS OF ROTENONE TREATMENT

An important milestone in reaching the aim of this study was to create a disease model for mitochondrial dysfunction. As this study already used a MTKO mouse line, this mitochondrial intervention had to be done using the same mouse genotype. In this study, rotenone was used to induce a CI deficiency in both WT and MTKO genotype groups. However, as this is a drug-induced intervention, it is necessary to consider the effectiveness of rotenone treatment during this study:

In **liver**, CI activity in WT mice was inhibited with rotenone treatment by ~ 25% and in MTKO mice by ~ 8%, neither of which were statistically significant. Excluding brain tissue, liver tissue was the least sensitive to rotenone treatment. Since rotenone is metabolised in the liver, the main site of detoxification, it is possible that liver tissue is better equipped than other tissues to handle toxins while allowing the minimum amount of damage to intracellular structures.

In **heart** muscle, CI activity was significantly inhibited in both WT and MTKO mice (p-values of <0.01 and 0.02 respectively). CI was inhibited by ~27% in WT mice and ~8% in MTKO mice.

In **skeletal muscle**, CI activity was inhibited significantly by rotenone treatment in both WT and MTKO mice (p-values: 0.01 and 0.02 respectively). CI activity in WT mice was inhibited by ~ 34% and in MTKO mice by ~20%. Skeletal muscle therefore seemed most sensitive to rotenone treatment, much more so than liver tissue.

In **brain**, CI activity was not significantly inhibited by rotenone treatment, even though rotenone can pass over the blood-brain-barrier, as it is lipophilic. Considering that rotenone is frequently used to induce parkinsonism in animal models, this result seems surprising. However, the treatment vehicle, the method of administration of rotenone and the animal model used, seem to be of great importance here: rotenone is usually dissolved in either DMSO (Drolet *et al.*, 2009; Swarnkar *et al.*, 2011) or natural oil (Alam and Schmidt, 2002; Bashkatova *et al.*, 2004) to mediate tissue absorption, and administered to male Sprague-Dawley or Lewis rats by intra-peritoneal injection (Alam and Schmidt, 2002; Bashkatova *et al.*, 2004), in the jugular vein or directly into the brain (Swarnkar *et al.*, 2011). These methods of administration in rat models are effective enough to induce parkinsonism or PD-like symptoms in animals with doses much lower (6mg/kg/2 days) compared to the dose used during this study (30mg/kg/2 days). In this study, a mouse model was used. Rotenone was suspended in PBS and injected subcutaneously, which might have had an impact on rotenone absorption in the brain. In an *in vivo* study done on C57BL/6J mice, chronic intra-gastric administration of low doses of rotenone (5 mg/kg for five days a week) dissolved in carboxymethylcellulose and chloroform for six weeks, resulted in PD-like symptoms in mice, but no rotenone could be detected in the systemic blood or central nervous system and no systemic CI inhibition could be detected (Pan-Montojo *et al.*, 2010). In an *in vivo* study by Thiffault *et al.* (2000), the subcutaneous administration of rotenone dissolved in oil, to C57BL/6 mice did not lead to overt neurotoxicity. It would therefore seem that rat models are selectively more sensitive to neurological effects of rotenone treatment than mouse models.

In the present study, while CI inhibition of between 40% and 60% was achieved during the pilot studies (Section 3.6.2), the same rotenone load did not produce the same level of inhibition during the main study (maximum CI inhibition was 34% in skeletal muscle). The reason for this notable difference in inhibition levels might be due to the fact that pilot studies only included three mice per group, which made it difficult to determine the statistical significance of the observed inhibition levels. Also, the mice used during the pilot studies were much older than the mice used in the main study, which could have affected their ability to handle rotenone loads. Hence the results obtained during determination of the optimal rotenone dose might have been misleading.

In conclusion, rotenone treatment using PBS as a vehicle to induce a CI deficiency in mice was not a very effective intervention. This study had to use the mouse model for reasons mentioned before, and in combination with a chemical inducer of mitochondrial deficiency, which had clear inefficiencies. To overcome these difficulties in the future, it would be advisable to use a more reliable disease model. More successful inhibition of CI has been achieved in past studies by using a different rotenone delivery vehicle, such as peanut oil. However, the much lower rotenone load required, a high mortality rate, as well as the notable difference between environmental and vehicle control groups (van Zweel, 2009), suggest a possible compounding effect of oil together with rotenone. The same can be said for organic vehicles like DMSO or ethanol. Changes observed can therefore not only be attributed to the inhibition of CI, but the possible effects of these vehicles on other parts of the metabolism also have to be taken into account. Changing the rotenone vehicle therefore does not seem to be an option that would ensure success. Of course, a completely different CI inhibitor, such as acetogenins or piericidin could be considered, but again, possible compounding effects of vehicles, as well as non-specific effects of inhibitors, on other parts of the metabolism could prove problematic. To ensure a guaranteed CI deficiency in future studies, a possible solution would be to cross-breed the MTKO model with a CI knockout mouse model, which was recently developed by Kruse *et al.* (2008).

## 5.4 OBJECTIVES

To evaluate the involvement of MTs in mitochondrial respiration, three objectives were formulated:

**First objective:** *to investigate components involved in oxidative phosphorylation as an overall working unit using respiration analyses.*

The detailed methodology used for respiration analyses was discussed in Section 3.10 and the results presented and discussed in Sections 4.2 and 4.3. Respiration analyses posed two time related challenges: firstly, it is time consuming. To overcome this challenge, the number of mice to be analysed on one day were limited to six. Therefore the 66 mice used in this study were divided into 11 Day-groups containing six mice each. This meant that respiration analyses were conducted on 11 different days, resulting in possible day-to-day variation (Figure 4.1). To minimise the influence of this non-biological variation on genotype and treatment comparisons, all 11 Day-groups contained mice from both genotypes and both treatments. Secondly, over time, freshly isolated mitochondria become less tightly coupled, resulting in decreased respiration rates of the same samples at a later time of the day. To overcome this challenge, samples from different mice were analysed in a staggered fashion, that is to say, a first assessment was completed for all samples, then a first repeat for all samples and then a second repeat for all samples.

By applying these precautionary measures, the influence of non-biological variation on respiration analyses results was minimised. This, together with analysing each sample in triplicate, greatly contributed to the reliability of results. A disadvantage of respiration analyses done with the methods described for this study, is the necessity to manually add all the different solutions to the sample chamber at different time intervals. Slopes of the respiration traces were also selected manually. Although as much care as possible was taken to be consistent throughout these procedures, human errors will always be more prevalent in manual systems than in automated systems. The resulting coefficient of variance (CoV) average was 14%, which was acceptable (in this study) for respiration analyses done with these types of instruments (Section 3.10), but not

comparable to the accuracy of more advanced instruments like the Seahorse XF analyser from Bioscience. This system measures the extracellular flux of analytes and can much more accurately compile a bioenergetics profile by revealing basal respiration rate, ATP-linked respiration, proton leak and reserve capacity. It also utilises a convenient microplate format (Ferrick *et al.*, 2010), increasing the number of measurements that can be done at one time and therefore also the number of repeats possible for one sample, which greatly improves inter-batch variation. As a future prospect for respiration analyses, this type of system should be seriously considered. Notably, variation between different samples, i.e. individual mice, in a specific group, was also large. Halothane, the compound used to anaesthetise the mice, has been shown to inhibit mitochondrial respiration at CI and disrupt the ETC (Harris *et al.*, 1970; Grist and Baum, 1974). Although RCR values for all samples were well above 2, thus indicating tightly coupled mitochondria, the unspecified amount of halothane inhaled by each mouse might have contributed to this variation, as well as the varying ages (between 2 and 6 months) and consequent differential metabolic rates between mice in the same group (Makarieva *et al.*, 2005).

***Second objective:*** *to investigate the RC system by its single units via individual RC enzyme analyses and structurally using blue-native polyacrylamide gel electrophoresis (BN-PAGE) and western blot analysis.*

The detailed methodologies used for these analyses were discussed in Section 3.11 and the results presented and discussed throughout Chapter 4. The protocols used during the RC enzyme activities analyses were well defined and used routinely at this institute, but were designed for use in a conventional spectrophotometer using standard size cuvettes and sample throughput. When using such a conventional setup as was available for this study, only 10 samples could be analysed at the same time and excessive manual pipetting and mixing was required. Because of the vast number of assays to be completed (66 mice x 4 tissues x 5 enzyme assays x 3 repeats), the existing protocols were adapted for use in a 96-well plate reader. This greatly reduced the time requirements for these analyses and enabled the completion of analyses of a specific complex in a specific tissue on the same day, ruling out the possibility of day-to-day variation in a specific complex. Being able to assess 96 samples at the same time,

in the same conditions (temperature, exact buffer concentrations etc.) and with the use of multi-channel pipetting techniques, also attributed greatly to the improvement of intra- and inter-batch variation. To further improve inter-batch variation, each plate contained a control sample, which was used for normalisation. Triplicates were also inspected manually to identify obvious outliers, which could have been caused by technical errors, and if possible, a CoV of <10% was set as a criterion for individual repeats to be included in the final mean value calculated for each sample. After the implementation of all these precautions, the combined average CoV for all RC enzyme analyses done in all complexes was 6.5%.

All these precautions contributed to more reliable RC enzyme activity results and therefore the results were considered to be of relatively high quality compared to the conventional low-throughput approach.

BN-PAGE and western blot analyses were done on four samples (one sample of each group) by Dr. Leo Nijtmans at the Nijmegen Centre for Mitochondrial Disease. The resultant blot was further analysed locally. Only one sample from each group (genotype/treatment combination) was used for RC complex quantification, therefore the variance between different mice from a single group could not be estimated and the significance of the differences observed in Figure 4.7 could not be determined with statistical methods. The smears visible on the blot also attested of possible damage to, or denaturing of, complexes during the separation run, which undermined the quality of the results. Although these results can serve as an indication of possible observations in the groups, they are in no way conclusive and should not be treated as such. To obtain more reliable results in the future, more samples should be included for each group. It would also be advantageous to add a second dimension SDS-PAGE separation after BN-PAGE, to investigate subunits of the different RC complexes (Calvaruso *et al.*, 2008). This would allow one to rely less on a *quantitative* measurement, which is problematic when evaluating small changes using western blot techniques, and more on *qualitative* evaluation of the RC structures. These adjustments could greatly enhance the contributions that this possibly invaluable analysis could make, to better understand RC complex form and function in WT vs. MTKO mice.

**Third objective:** *to determine the relative mtDNA copy number using real-time PCR, as supportive data for the structure and function of the RC enzyme complexes.*

The detailed methodology used for relative mtDNA copy number determination was discussed in Section 3.13 and the results presented and discussed in Section 4.3.4.

Because of limited resources, relative mtDNA copy number determination was only done for heart tissue. When doing real-time PCR, several different assays can be chosen. The Taqman® assay is known to be very specific and sensitive, but is costly. With a limited budget, using Taqman® would have reduced the number of repeats possible per sample. In comparison with Taqman®, the SYBR® Green I assay is less sensitive because it non-specifically binds to all double stranded DNA, but is much more cost effective and still very reliable (Pfaffl *et al.*, 2002). The use of SYBR® Green in this study was motivated by the fact that its lower cost allowed the analysis of samples in triplicate, as well as the reanalysis of outlier samples. Plates contained a control sample for normalisation purposes. Means were calculated for each sample as discussed in Section 3.14. The reliability of these results was therefore mostly limited only by the limitations of the SYBR® Green I assay, and the results were still considered trustworthy. While qPCR can give an indication of the relative copy number of mtDNA in a sample, it cannot determine the amount of mtDNA copy numbers per cell or per mitochondrion, since tissues that have a high energy demand contain more mitochondria per cell and each mitochondrion can contain several copies of mtDNA. If there were differences in the arrangement of mtDNA copies throughout the heart when comparing WT and MTKO mice, it would not have been detected by this method.

In conclusion, with the exception of BN-PAGE and western blot analysis, much effort was put into overcoming the challenges and limitations set by the methods used to reach the proposed objectives of this study. It is important to note that the sometimes large variation that can be observed in a group for a specific parameter reflects the variation between individual mice in that group, and not variation between measurements of the same sample. It can thus be largely considered as biological variation and to a lesser extent as technical variation. The provisions made to address the abovementioned issues greatly contributed to the quality of the results and the attainment of the abovementioned objectives.

## 5.5 SUMMARY AND DISCUSSION OF RESULTS

Respiration analyses done on liver and heart mitochondria revealed no significant changes in mitochondrial respiration, irrespective of genotype or treatment, and despite several significant changes in OXPHOS enzyme activities and expression.

While CI was inhibited by rotenone **treatment** to a greater or lesser extent in all tissues, only heart and skeletal muscle CI activity was significantly decreased. Liver CI was inhibited, but was not as sensitive to rotenone treatment as the muscle tissues, whilst brain CI was least sensitive. Only in liver tissue could a tendency of increased activity after rotenone treatment (excluding CI) be observed, possibly a compensatory response to CI inhibition, but this tendency or any other consistent pattern did not immerge in the other tissues. Interestingly, in heart tissue, rotenone treatment caused a 40% increase in CI expression in WT mice but only a 10% increase in CI expression in MTKO mice. As discussed in Section 4.3, in heart, CI activity in rotenone-treated mice was significantly decreased in both genotypes, but CI quantity was increased. It is possible that in an effort to cope with the biochemical stress induced by rotenone treatment, CI expression was up-regulated. Despite the large up-regulation of CI in rotenone-treated WT mice, inhibition was not successfully counteracted. Also in rotenone-treated MTKO mice, inhibition was not successfully hindered, but the degree of both inhibition and up-regulation of CI was much less in this group.

Two conclusions can be drawn from this:

- 1.) The capacity of WT mice to up-regulate CI expression under stress conditions was greater than that of MTKO mice. It is reasonable to argue that, had CI been up-regulated to the same degree in MTKO mice as in WT mice, rotenone-induced inhibition could easily have been overcome completely. MTs have never been associated with the expression of CI subunits, but this does not exclude the possibility that MTs might by some unknown mechanism be involved in post-translational processes of CI subunits or the assembly thereof.

2.) MTKO mice were less affected by rotenone treatment than WT mice. In fact, when taking into account CI inhibition data from all tissues, this becomes even more apparent, since in each case, rotenone-induced CI inhibition in MTKO mice was consistently less than in WT mice. A possible explanation could be that the absence of MTs affected the transport or metabolism of rotenone in some way, so that its delivery to the mitochondria was not as efficient as in the presence of MTs. The metabolism of rotenone is not well understood, but in a study done with human cytochrome P450 supersomes, rotenone was shown to be metabolised by 3A4 and 2C19, while five other P450 enzymes investigated were not involved (Caboni *et al.*, 2004). In another study done on MT-null mice, differential expression of genes for P450 7b1 and 2a4 was reported (Merten *et al.*, 2005). The latter two P450 enzymes were not investigated during the Caboni study, therefore their involvement in rotenone metabolism is unknown. However, the differential expression of P450 enzymes in MTKO mice and the involvement of P450 enzymes in rotenone metabolism could offer a possible link between MTs and rotenone metabolism that could account for the difference in CI sensitivity to rotenone when comparing MTKO to WT mice. Other than CI activity, rotenone treatment played a statistically significant role in liver only (CII+III in WT mice and CIV in MTKO mice).

Throughout all tissues, **genotype** played almost no significant role in the measured RC enzyme activity. In liver, genotype had little to no significant effect on the ability of mitochondria to handle rotenone treatment. Comet assays performed on these liver samples indicated an impaired global DNA repair capacity in MTKO mice, irrespective of treatment (Steenkamp, 2011). Consequently, it would seem reasonable to expect downstream impairments in protein expression and enzyme assembly, but this expectation was not reflected in RC enzyme activity data, nor did it correlate with results from other liver enzyme activity assays done on SOD, catalase, glutathione peroxidase and glutathione reductase, all of which also revealed no statistically significant differences between genotype groups. Only CIII activity in heart mitochondria was affected by genotype: MTKO CIII activity was significantly increased when compared to WT CIII activity. If MTs play a regulatory role in mitochondrial respiration via the regulation of Zn (which inhibits respiration), an absence of MTs, as is the case in MTKO

mice, could lead to increased CIII activity as Zn cannot be delivered to the IMS to inhibit respiration. While Zn can bind to all complexes, it binds to CI and CIII with a higher affinity. In heart, CI activity was already greatly affected by rotenone treatment, which is a possible reason why this loss of regulation by Zn release was only observable in CIII activity. While not significantly so, all other tissues also presented an increase in CIII activity in MTKO mice when compared to WT counterparts, adding to the plausibility of this explanation.

Both genotype and treatment had a possible effect on RC enzyme complex expression, as indicated by the changes in CI and CIV expression in heart mitochondria. The up-regulated expression of CIV in heart was however not reflected in the enzyme activity data. As discussed earlier, the allosteric inhibition of the Va subunit by ATP might be involved. Another possibility is related to the organisation of OXPHOS complexes in the IMM. Studies done by Dencher and colleagues (Haus *et al.*, 2005, Lechner *et al.*, 2006; Seelert *et al.*, 2009, Schon and Dencher, 2009; Frenzel *et al.*, 2010) suggest that the OXPHOS complexes are arranged in super-complexes, and this arrangement could have an effect on the activity status of the different complexes. Dencher is of the opinion that so-called “reserve pools” (Rossignol *et al.*, 2002) of inactive OXPHOS complexes are present in the mitochondrion, which are then only activated when arranged in a super-complex with other OXPHOS complexes (Dencher, 2010, private communication). If this is true, it could be possible that although more units of CIV were present in heart tissue, some might not have been arranged in super-complexes and thus inactive. This could indicate a role of MTs in the assembly of super-complexes, thought to be held together by cardiolipids (Dencher, 2010). If the structural differences between an active and inactive form of CIV is small enough, discrimination between the two forms would not be demonstrated during one dimensional BN-PAGE and western blot analyses, but certainly by enzyme activity analyses, since the activity of the inactive enzyme would not be measured. The reliability of the BN-PAGE and western blot data from this study is limited however, for reasons discussed in Section 5.4. Again, further second dimensional investigation of these complexes with the help of SDS-PAGE would be invaluable to assess this conjecture.

Despite all the changes observed in RC complex activity and expression in heart when comparing WT and MTKO mice, no differences in relative mtDNA copy number could be detected. If the changes observed in heart tissue are indeed realistic and MTs are somehow involved, the lack of differences in relative mtDNA copy number would suggest that this involvement is either post-translational or nuclear DNA linked, but not via mtDNA alteration.

It is important to point out that while all other complex activities were measured in homogenate and normalised with CS activity (*i.e.* per units of CS), CI activity was measured in enriched mitochondrial fractions and therefore had to be normalised to CII (*i.e.* per units of CII) as discussed in Section 3.11.2. For this reason, the ratio of absolute CI activity values compared to that of other complexes cannot be commented on. However, the comparison of individual complexes in different tissues as shown in Figure 5.1 may still be useful.

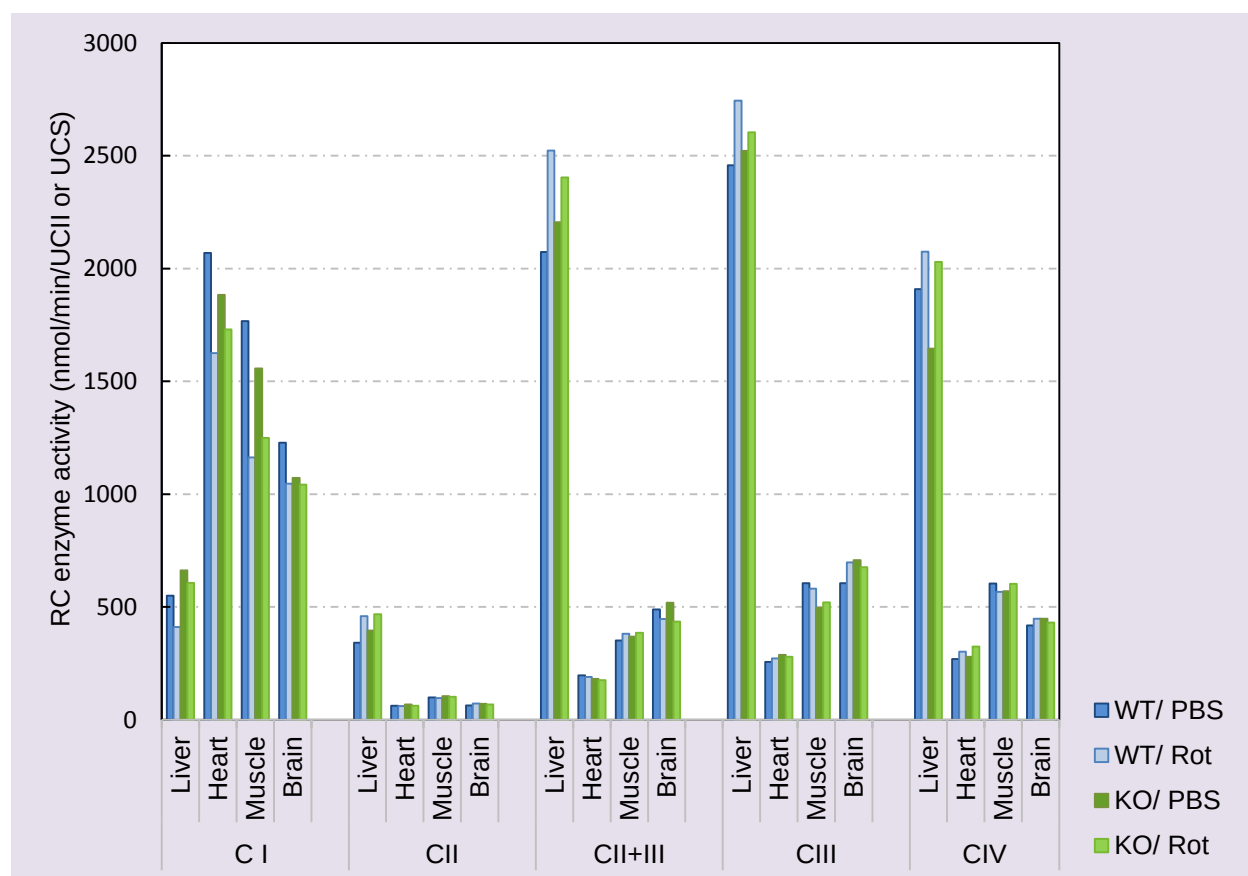


Figure 5.1: Comparison of RC enzyme activities for each group, organised per RC complex. CI activity is presented per units of CII, all other activities per units of CS (Section 3.11.2).

CI activity was much lower in liver than in any other tissue, while all other complex activities were greatly increased in liver when compared to other tissues. In fact, with the exception of brain, a decreased CI activity lead to increased activities of the other RC enzymes when comparing individual activities in different tissues. Because this pattern was observable even in PBS treated WT mice, the cause of this pattern cannot be attributed to any influence of genotype or treatment. This, along with the observation that overall respiration was not affected despite significant changes in individual complexes, lends weight to the theory that the OXPHOS enzymes exist and function in a delicate balance, where higher activities of some complexes compensate for lower activities of other complexes and thus an optimum output can still be maintained even in the presence of deficiency (Rossignol *et al.*, 2002). The overall output of the OXPHOS system would then only be impaired if a deficiency is so severe that this balance cannot be maintained any more – the so-called “threshold effect”.

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#### THE INFLUENCE OF INTRA- AND INTER-GROUP VARIANCES

At first glance, when considering the few significant changes that could be detected between groups in the results from this study, it is tempting to consider even those few changes to be anomalies. However, considering the relatively low levels of CI inhibition using rotenone, it appears more likely that the rotenone treatment failed to induce a severe enough stress on mitochondria to expose the role that MTs might play in mitochondrial respiration. More importantly, the metabolomic profiles of these mice do indicate significant differences between WT and MTKO mice, with MTKO mice profiles even mirroring those of mitochondrial diseased patients (Lindeque, 2011, Louw *et al.*, 2011). In short, the metabolite profiles of rotenone-treated MTKO mice suggested that energy production had shifted from the primary pathway (TCA-cycle and OXPHOS) to alternative pathways (e.g.  $\beta$ -oxidation of fatty acids). That would imply that MTs play a very important role in mitochondrial function and respiration. Since metabolomic assays are much more sensitive than the methods used in this study, a more likely explanation for the lack of evidence for MT's involvement in mitochondrial respiration would be that the intra-group variation in each of the methods utilised in the present study was greater than the inter-group variation and, in fact, could mask it. When considering this, the presence or absence of differences between genotype groups in each method makes

more sense: metabolomic assays are very sensitive, as metabolites are most often much more stable and are measured directly, and therefore can identify even subtle differences. Enzymatic assays are often much less sensitive and intra-day variation is much more likely to have an effect, therefore fewer differences were identified, whilst respiration analyses are very crude and likely only to identify very severe changes. For this reason it is still practical to consider the differences identified with enzymatic assays as *bona fide* and thus they were discussed as such. Another indication that these differences were of real importance is the fact that tendencies that reflected some of the statistically significant differences were present and might even prove to be statistically significant if the intra-group variation could be decreased by increasing group sizes. Another important point to remember is simply the meaning of “statistically significant” and that it is a purely mathematical function, which does not always accurately reflect the true biological significance of results, since no evidence outside the specific parameter being tested at that time, are taken into consideration (Schervish, 1996; Goodman, 1999; Sterne and Smith, 2001).

A potentially important shortcoming of this study is revealed when again considering metabolomic data obtained on these same mice in a parallel study (Lindeque, 2011): the practice of normalising data with other seemingly stable cell components. When analysing data obtained from OXPHOS enzyme assays, it is standard practice to normalise values to CS or CII activity (Janssen *et al.*, 2007), while complex quantities are compared per CII quantities in BN-PAGE and western blot analyses. When investigating isolated cases of mitochondrial defects, these methods of normalisation could still be justified, but MTs are involved in a vast number of cellular processes and the reliability of these usually stable components are drawn into doubt. In fact, metabolomic data obtained on these mice indicated that CII might have been affected in MTKO mice (Lindeque, 2011). The data suggested that CII levels in MTKO mice were decreased. If these changes are taken into account, the results obtained in this study could reveal very different results. An example of this can be seen when reconsidering BN-PAGE and western blot results. CI, CIII and CIV quantities are determined as per CII. When CII was considered to be stable throughout all groups, the results showed that CI and CIII slightly differed between groups while CIV expression drastically

changed in MTKO samples. If the metabolomics data is now taken into account and CII is considered to be increased in MTKO mice, it would render the difference in CIV even more pronounced and possibly even reveal an increased amount of CI and CIII in MTKO mice. The biggest flaw in this argument however, is that metabolomic studies were done on urine and are therefore a systemic evaluation, while all analyses done in this study, including RC enzyme activity assays and BN-PAGE and western blot analysis were tissue specific. The results are therefore not completely reconcilable, but can still be taken into consideration for the purpose of comparison. In contrast, Miura and Koizumi (2005) found that CII expression was increased in MTKO mice, which would have the opposite effect on normalised data. Either way, it stands to reason that the choice of seemingly stable cellular components for normalisation purposes when assessing the effects of multi-functional compounds such as MTs, should be carefully considered. Performing metabolomic analyses on specific tissues in support of enzymatic studies would also be invaluable and serve to help assess the effect of the variables under investigation on these components.

In conclusion, the aim of this study was *to investigate the involvement of MTs in mitochondrial respiration and respiratory chain enzyme function by using an MTKO mouse model, which was treated with the CI inhibiting reagent, rotenone.*

When considering the results obtained during this study *only*, the following could be deduced: these results strongly suggested that loss of MT1+2 affects CIII activity, at least in heart tissue. While evidence of MTs involvement in RC enzyme expression in heart tissue was inconclusive, it provided an important indication of the possible differential RC enzyme expression that could be present in MTKO mice. This could serve as a focus point for future studies aiming to further investigate MT's involvement in RC enzymes. Despite the presence of changes in RC enzyme function and expression when comparing WT and MTKO mice, none of the changes were severe enough to have a pronounced effect on mitochondrial respiration. If MTs do play a role in mitochondrial respiration, it is probably in conjunction with other mechanisms that regulate the same functions, so that the loss of this putative involvement of MTs alone is not deleterious to the mitochondrion.

## 5.5 CONCLUDING REMARKS

After a thorough study of the current literature the following problem was identified: while a link between MTs and mitochondria seems structurally and functionally possible, efforts to properly define this link have been mired by contradicting reports and a lack of supportive *in vivo* evidence, resulting in uncertainty and speculation. To address this problem, a large overall study was formulated to investigate the involvement of MTs in mitochondrial function in an *in vivo* model. The study presented in this dissertation formed part of this overall study, with the focus being on mitochondrial respiration. The specific problem statement for this study was thus *that information on the involvement of MTs with respiratory chain function is limited*. The aim consequently formulated for this study was *to investigate the involvement of MTs in mitochondrial respiration and respiratory chain enzyme function by using an MTKO mouse model, which was treated with the CI inhibiting reagent, rotenone*. To reach this aim, a strategy was formulated consisting of three objectives. As discussed earlier (Section 5.4), these objectives were successfully accomplished. The aim of this study was partially reached, as rotenone-induced inhibition of CI proved to be less effective than was hoped for. Despite this, reliable results were obtained that suggested the possible involvement of MTs in mitochondrial respiration and RC enzyme function. However, the shortcomings identified in this chapter made it extremely difficult to identify a clear involvement. Even so, throughout Chapters 4 and 5, instances were identified where the results of this study could provide *in vivo* support for certain *in vitro* observations discussed in Chapter 2. In the same way, certain *in vitro* observations were contradicted by these results. An example of this is the involvement of MTs in the opening of the IMM transition pore as reported in *in vitro* studies by Simpkins *et al* (1998a; 1998b). Although IMM transition pore parameters were not directly measured, the lack of changes in overall mitochondrial respiration in the presence or absence of MTs suggested that this involvement was not as pronounced *in vivo* as *in vitro* and that other regulatory mechanisms also play a role. Thus, possible *in vivo* evidence to support or oppose *in vitro* observation was provided, addressing the problem statement of this study.

It is important to remember that this study formed part of a larger study with an overall aim of *investigating the involvement of MTs in mitochondrial function*. Therefore, while the results from this study alone could not provide conclusive evidence, they were crucial in reaching the aim and addressing the problem statement of the overall study.

Finally, this study also made two other unrelated contributions:

1.) The protocols used in this study to assess RC enzyme activities have contributed to the development of standard operating procedures now used routinely at this institute. The optimisation of these protocols for use in a 96-well plate reader not only allows for more reliable results in a shorter period of time, but also significantly reduces the amount of sample and other, sometimes costly, chemicals needed for each assay.

2.) Together with the results obtained from other parts of the overall study, it helped to emphasise the importance of integrating different platforms when investigating a ubiquitously involved protein such as MT. All of the different studies that formed part of the overall study were supportive of each other and none could have been excluded.

As was discussed in the previous section, it sometimes even served to point out limitations in certain methods that would otherwise not have been considered. An example of this is the possible changes in CII levels identified by metabolomics data, an enzyme routinely used for normalisation in different assays. These types of examples highlight the importance of including some type of *omics* studies in future project. The exponential advancements made in bioscience technologies every day and the increasing availability thereof to smaller research facilities, together with the recent shift in paradigm that dictates a more holistic approach to solving biological problems (Wallace, 2010), should also help to encourage the assemblage of these comprehensive, multi-platformed studies to become standard practice, rather than exceptional cases.

Parts of this study were included in poster or oral presentations at the following conferences, presented by the author listed first in each case:

Pretorius M, Lindeque JZ, Steenkamp A, Dreyer W, Bothma K, Crous A, Pretorius PJ, Louw R, van der Westhuizen FH. The investigation of metallothionein involvement in mitochondrial function and disease. 7th Annual Meeting of ASMRM and 10th J-mit in Fukuoka, Japan, Dec 15-18, 2010. Poster presentation.

Louw R, Lindeque JZ, Pretorius M, Dreyer W, Bothma K, Crous A, van der Westhuizen FH. The involvement of metallothioneins in mitochondrial function and disease. 8<sup>th</sup> Euromit Conference, Zaragoza, Spain, 20-23 June, 2011. Oral presentation.

van der Westhuizen FH, Lindeque JZ, Hidalgo J, Pretorius M, Louw R. The involvement of metallothioneins in mitochondrial function and disease. 8th Conference on Mitochondrial Physiology (MiP2011), Bordeaux, France, 5-8 September, 2011. Oral presentation.

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## APPENDIX

### APPENDIX A

The preparation of reduced cytochrome *c*, decylubiquinol and Milli-Q® water are essentially as described by Du Toit (2007) and Luo *et al.* (2008) and are routinely used at the Mitochondrial Research Laboratory, NWU.

#### PREPARATION OF REDUCED CYTOCHROME *c*

Reduced cytochrome *c* (500 µM) is prepared from 2 mM cytochrome *c* as follows:

- add 10 mM sodium ascorbate (Sigma-Aldrich®, A4034), Mr: 198 g/mole thus 2 mg/ml, to 2 mM cytochrome *c*
- incubate at room temperature for 5 min (dark red colour change to light red)
- remove ascorbate by dialysing (Pierce Slide-A-Lyzer Mini Dialysis unit, 69570) for 5 x 2 hrs in 200 ml 5 mM KPi buffer at 4 °C
- change buffer at each interval.

To check reduction, pipette 5 µl to 9995 µl KPi buffer (50 mM) into a cuvette. Scan 500-600 nm. If  $A_{550}/A_{565} > 8$  it is sufficiently reduced.

To calculate the reduced cytochrome *c* concentration the following equation is used:

$$\text{Reduced cytochrome } c \text{ (mM)} = \frac{A_{550}}{29.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}} \times 200 \text{ (dilution)}$$

Equation 1

Dilute the reduced cytochrome *c* with KPi (50 mM) buffer to 500 µM, aliquot 200 µl volumes into micro-centrifuge tubes and store at -20 °C.

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## PREPARATION OF DECYLUBIQUINOL

Decylubiquinol, *i.e.* reduced decyubiquinone, (9.26 mM) was prepared as follows:

Prepare the following reagents:

- 10 mM decylubiquinone (DB) (Sigma-Aldrich®, D7911), Mr: 322.4 g/mole thus 3.224 mg/ml ethanol.
- 0.1N HCl in ethanol.
- 0.1M potassiumphosphate buffer, pH7.4, and 0.25 M sucrose. Add 2 ml 0.5 M KPi buffer to 0.8558 g sucrose and top up to 10 ml.
  - i. To 100 µl DB add 6 ml of buffer /sucrose mix.
  - ii. Add 1 ml cyclohexane and 0.7 g of solid sodium dithionite, vortex until colourless.
  - iii. Transfer upper cyclohexane layer to separate vial and repeat step ii without dithionite.
  - iv. Combine the organic phases and evaporate *in vacuo* until only a light-yellow syrup is left.
  - v. Dissolve this in 90 µl ethanol and 10 µl of 0.1 mol/l HCl, and store in aliquots at -80 °C.

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## MILLI-Q® WATER SYSTEM

A Progard pre-treatment pack initially removes particles and free chlorine from tap water to prevent mineral scaling. Next, the water is purified by reverse osmosis and then passed through an electrode-ionisation module. This final purification stage reduces levels of organic and mineral contaminants. Organic compounds are oxidised and bacteria killed by exposing the water to UV light at both 185 and 254 nm wavelengths. Trace ions and oxidation by-products produced during this last step, are removed by the quantum cartridge. Purified water then passes through an ultra filtration module, which acts as a barrier to colloids, particles and organic molecules with a molecular weight greater than 5000 Da. When water is tapped from the system, it finally moves through a 0.22 µm membrane, which removes particles and bacteria greater than 0.22 µm in size, and prevents recontamination of the system.

## APPENDIX B

Table 1: Dosing schedule for Day-groups 1 to 6

|       | 4 x PBS/ 2 x rot |           | 4 x ROT/ 2 x pbs |           | 4 x PBS/ 2 x rot |           | 4 x ROT/ 2 x pbs |           | 4 x PBS/ 2 x rot |           | 4 x ROT/ 2 x pbs |           |       |
|-------|------------------|-----------|------------------|-----------|------------------|-----------|------------------|-----------|------------------|-----------|------------------|-----------|-------|
| Day   | Group 1          |           | Group 2          |           | Group 3          |           | Group 4          |           | Group 5          |           | Group 6          |           | Total |
|       | 3 x WT           | 3 x MTKO  | 3 x WT           | 3 x MTKO  | 3 x WT           | 3 x MTKO  | 3 x WT           | 3 x MTKO  | 3 x WT           | 3 x MTKO  | 3 x WT           | 3 x MTKO  |       |
| Mon   | Dose             | Dose      |                  |           |                  |           |                  |           |                  |           |                  |           | 6     |
| Tues  |                  |           | Dose             | Dose      |                  |           |                  |           |                  |           |                  |           | 6     |
| Wed   | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           |                  |           |                  |           | 12    |
| Thurs |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           |                  |           | 12    |
| Fri   | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           |                  |           |                  |           | 12    |
| Sat   |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           |                  |           | 12    |
| Sun   | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           |                  |           |                  |           | 12    |
| Mon   |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           |                  |           | 12    |
| Tues  | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | 18    |
| Wed   |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      | 18    |
| Thurs | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | 24    |
| Fri   |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      | 18    |
| Sat   | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | 24    |
| Sun   |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      | 18    |
| Mon   | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | 30    |
| Tues  |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      | 24    |
| Wed   | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | 36    |
| Thurs |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      | 30    |
| Fri   | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | 36    |
| Sat   |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      | 30    |
| Sun   | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | 36    |
| Mon   | Sacrifice        | Sacrifice | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      | 30    |
| Tues  |                  |           | Sacrifice        | Sacrifice | Dose             | Dose      |                  |           | Dose             | Dose      |                  |           | 30    |
| Wed   |                  |           |                  |           | Sacrifice        | Sacrifice | Dose             | Dose      |                  |           | Dose             | Dose      | 24    |
| Thurs |                  |           |                  |           |                  |           | Sacrifice        | Sacrifice | Dose             | Dose      |                  |           | 24    |
| Fri   |                  |           |                  |           |                  |           |                  |           |                  |           | Dose             | Dose      | 18    |
| Sat   |                  |           |                  |           |                  |           |                  |           | Dose             | Dose      |                  |           | 24    |
| Sun   |                  |           |                  |           |                  |           |                  |           |                  |           | Dose             | Dose      | 18    |
| Mon   |                  |           |                  |           |                  |           |                  |           | Dose             | Dose      |                  |           | 24    |
| Tues  |                  |           |                  |           |                  |           |                  |           | Sacrifice        | Sacrifice | Dose             | Dose      | 18    |
| Wed   |                  |           |                  |           |                  |           |                  |           |                  |           | Sacrifice        | Sacrifice | 18    |

Table 2: Dosing schedule for Day-groups 7 to 11

|       | 4 x PBS/ 2 x rot |           | 4 x ROT/ 2 x pbs |           | 4 x PBS/ 2 x rot |           | 4 x ROT/ 2 x pbs |           | 6 x ROT   |           |       |
|-------|------------------|-----------|------------------|-----------|------------------|-----------|------------------|-----------|-----------|-----------|-------|
| Day   | Group 7          |           | Group 8          |           | Group 9          |           | Group 10         |           | Group 11  |           | Total |
|       | 3 x WT           | 3 x MTKO  | 3 x WT           | 3 x MTKO  | 3 x WT           | 3 x MTKO  | 3 x WT           | 3 x MTKO  | 3 x WT    | 3 x MTKO  |       |
| Thurs | Dose             | Dose      |                  |           |                  |           |                  |           |           |           | 24    |
| Fri   |                  |           |                  |           |                  |           |                  |           |           |           | 18    |
| Sat   | Dose             | Dose      |                  |           |                  |           |                  |           |           |           | 24    |
| Sun   |                  |           |                  |           |                  |           |                  |           |           |           | 18    |
| Mon   | Dose             | Dose      | Dose             | Dose      |                  |           |                  |           |           |           | 30    |
| Tues  |                  |           |                  |           | Dose             | Dose      |                  |           |           |           | 24    |
| Wed   | Dose             | Dose      | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 36    |
| Thurs |                  |           |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 30    |
| Fri   | Dose             | Dose      | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 36    |
| Sat   |                  |           |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 30    |
| Sun   | Dose             | Dose      | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 36    |
| Mon   |                  |           |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 30    |
| Tues  | Dose             | Dose      | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 30    |
| Wed   |                  |           |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 24    |
| Thurs | Dose             | Dose      | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 24    |
| Fri   |                  |           |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 18    |
| Sat   | Dose             | Dose      | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 24    |
| Sun   |                  |           |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 18    |
| Mon   | Dose             | Dose      | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 24    |
| Tues  |                  |           |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 18    |
| Wed   | Dose             | Dose      | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 18    |
| Thurs | Sacrifice        | Sacrifice |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 12    |
| Fri   |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 12    |
| Sat   |                  |           |                  |           | Dose             | Dose      |                  |           | Dose      | Dose      | 12    |
| Sun   |                  |           | Dose             | Dose      |                  |           | Dose             | Dose      |           |           | 12    |
| Mon   |                  |           | Sacrifice        | Sacrifice | Dose             | Dose      |                  |           | Dose      | Dose      | 12    |
| Tues  |                  |           |                  |           | Sacrifice        | Sacrifice | Dose             | Dose      |           |           | 6     |
| Wed   |                  |           |                  |           |                  |           | Sacrifice        | Sacrifice | Dose      | Dose      | 6     |
| Thurs |                  |           |                  |           |                  |           |                  |           | Sacrifice | Sacrifice |       |

In Tables 1 and 2, the days on which dosing of mice with either rotenone or PBS are indicated. Groups indicate different Day-groups (six mice each). The number of mice in each Day-group dosed with either rotenone or PBS is indicated in the first row. The total number of mice dosed on a single day is indicated in the far right column.

## APPENDIX C

Table 3: Processed data points of respiration and CI, CII and CII+III activity analyses

| Assigned number | Genotype | Treatment | State 3 Respiration (pmon/min/μg) |       | complex I activity (nmol/min/UCII) |         |         |         | complex II activity (nmol/min/UCS) |       |        |        | complex II+III activity (nmol/min/UCS) |        |        |        |
|-----------------|----------|-----------|-----------------------------------|-------|------------------------------------|---------|---------|---------|------------------------------------|-------|--------|--------|----------------------------------------|--------|--------|--------|
|                 |          |           | Liver                             | Heart | Liver                              | Heart   | Muscle  | Brain   | Liver                              | Heart | Muscle | Brain  | Liver                                  | Heart  | Muscle | Brain  |
| 1               | MTKO     | PBS       | 65.0                              | 39.3  | 407.93                             | 1820.39 | 1452.63 | 1335.88 | 367.28                             | 59.51 | 87.16  | 74.56  | 1998.01                                | 179.88 | 541.38 | 438.41 |
| 2               | MTKO     | PBS       | 83.3                              | 67.9  | 679.96                             | 1592.63 | 1953.44 | 1145.22 | 611.07                             | 50.98 | 112.63 | 72.50  | 2153.31                                | 159.34 | 457.92 | 403.19 |
| 3               | WT       | PBS       | 50.3                              | 36.8  | 550.59                             | 2044.21 | 1217.18 | 1077.10 | 294.04                             | 63.26 | 95.56  | 58.64  | 1952.22                                | 164.53 | 440.23 | 411.99 |
| 4               | WT       | PBS       | 21.2                              | 35.2  | 360.18                             | 1962.67 | 1204.11 | 1448.68 | 537.79                             | 53.19 | 73.29  | 63.69  | 1260.46                                | 180.73 | 351.63 | 420.35 |
| 5               | WT       | Rotenone  | 28.6                              | 48.3  | 343.08                             | 1619.29 | 1882.34 | 1282.71 | 464.64                             | 60.12 | 130.43 | 50.54  | 2653.97                                | 176.27 | 372.59 | 400.81 |
| 6               | MTKO     | Rotenone  | 97.6                              | 13.6  | 977.81                             | 1733.64 | 1227.86 | 1138.42 | 496.85                             | 49.60 | 89.14  | 60.52  | 2088.48                                | 171.01 | 502.75 | 413.08 |
| 7               | MTKO     | Rotenone  | 63.3                              | 15.9  | 670.01                             | 2048.97 | 1021.93 | 1680.03 | 510.52                             | 53.44 | 101.09 | 58.99  | 2638.32                                | 168.90 | 496.37 | 436.24 |
| 8               | MTKO     | PBS       | 104.8                             | 15.2  | 1872.84                            | 2542.49 | 787.18  | 1444.41 | 423.23                             | 44.40 | 115.31 | 72.01  | 2280.49                                | 190.85 | 491.46 | 500.92 |
| 9               | WT       | Rotenone  | 75.2                              | 8.6   | 662.11                             | 1831.09 | 465.54  | 774.38  | 330.62                             | 58.93 | 123.32 | 53.97  | 2687.20                                | 155.91 | 463.22 | 431.21 |
| 10              | WT       | Rotenone  | 71.2                              | 15.2  | 666.25                             | 1697.48 | 911.57  | 1039.01 | 279.57                             | 63.14 | 79.94  | 84.67  | 2525.47                                | 182.11 | 339.98 | 732.14 |
| 11              | WT       | PBS       | 73.8                              | 42.3  | 759.51                             | 3306.45 | 935.36  | 1108.97 | 243.80                             | 51.60 | 91.75  | 84.31  | 2297.37                                | 166.30 | 289.33 | 683.03 |
| 12              | MTKO     | PBS       | 47.6                              | 36.0  | 745.11                             | 1883.72 | 938.80  | 1000.86 | 316.29                             | 91.03 | 112.99 | 77.74  | 2212.22                                | 105.16 | 368.29 | 731.80 |
| 13              | MTKO     | PBS       | 48.6                              | 44.1  | 871.88                             | 1699.34 | 1429.89 | 852.83  | 340.63                             | 84.35 | 92.10  | 114.95 | 1797.08                                | 159.20 | 334.93 | 469.53 |
| 14              | MTKO     | Rotenone  | 68.3                              | 61.0  | 733.21                             | 1516.50 | 1452.59 | 912.67  | 352.72                             | 80.07 | 105.88 | 104.11 | 2076.94                                | 130.91 | 471.50 | 577.50 |
| 15              | WT       | PBS       | 111.9                             | 36.7  | 647.14                             | 1963.71 | 1791.98 | 681.99  | 341.18                             | 67.88 | 86.88  | 50.83  | 1757.23                                | 65.98  | 357.57 | 541.33 |
| 16              | WT       | PBS       | 69.3                              | 36.0  | 1285.37                            | 1781.73 | 1563.34 | 873.28  | 279.29                             | 74.78 | 69.84  | 66.91  | 1667.43                                | 161.54 | 282.35 | 470.18 |
| 17              | WT       | Rotenone  | 88.1                              | 51.4  | 410.98                             | 2084.06 | 1878.22 | 1222.53 | 287.76                             | 55.21 | 88.02  | 119.05 | 2010.07                                | 178.97 | 643.35 | 618.18 |
| 18              | MTKO     | Rotenone  | 102.4                             | 49.8  | 534.68                             | 1863.59 | 1181.82 | 747.54  | 354.80                             | 66.38 | 100.00 | 72.81  | 1750.38                                | 172.84 | 584.91 | 610.00 |
| 19              | MTKO     | Rotenone  | 104.8                             | 74.8  | 606.20                             | 1823.40 | 1113.15 | 1194.35 | 356.87                             | 66.36 | 98.53  | 75.37  | 1852.68                                | 194.87 | 487.33 | 518.22 |
| 20              | MTKO     | PBS       | 142.9                             | 82.1  | 616.12                             | 2117.81 | 1992.50 | 982.67  | 235.84                             | 57.88 | 104.87 | 61.32  | 1440.21                                | 163.79 | 515.70 | 525.38 |
| 21              | WT       | Rotenone  | 131.0                             | 51.5  | 328.72                             | 1860.67 | 1357.58 | 851.26  | 339.09                             | 78.15 | 97.03  | 45.59  | 1249.41                                | 154.97 | 419.75 | 552.37 |
| 22              | WT       | Rotenone  | 109.5                             | 40.7  | 529.07                             | 1858.57 | 1050.68 | 852.42  | 335.72                             | 63.86 | 98.06  | 54.59  | 1707.56                                | 156.89 | 497.27 | 519.03 |
| 23              | WT       | PBS       | 171.4                             | 95.2  | 344.41                             | 2299.71 | 2331.32 | 1131.72 | 297.02                             | 63.59 | 130.32 | 70.89  | 1812.86                                | 202.56 | 571.15 | 578.90 |
| 24              | MTKO     | PBS       | 131.0                             | 65.7  | 491.04                             | 2731.17 | 2120.92 | 813.71  | 343.92                             | 66.94 | 110.00 | 70.40  | 1873.02                                | 189.49 | 477.66 | 525.46 |

| Assigned number | Genotype | Treatment | State 3 Respiration (pmon/min/μg) |       | complex I activity (nmol/min/UCII) |         |         |         | complex II activity (nmol/min/UCS) |       |        |        | complex II+III activity (nmol/min/UCS) |        |        |        |
|-----------------|----------|-----------|-----------------------------------|-------|------------------------------------|---------|---------|---------|------------------------------------|-------|--------|--------|----------------------------------------|--------|--------|--------|
| 25              | MTKO     | PBS       | 150.0                             | 74.8  | 716.14                             | 2058.29 | 1558.83 | 1140.80 | 477.09                             | 74.90 | 108.25 | 70.10  | 2007.62                                | 190.94 | 256.99 | 556.35 |
| 26              | MTKO     | Rotenone  | 131.0                             | 87.1  | 977.57                             | 1645.62 | 1262.65 | 1276.41 | 302.40                             | 55.03 | 94.65  | 55.29  | 2037.16                                | 299.36 | 270.59 | 472.56 |
| 27              | WT       | PBS       | 140.5                             | 54.5  | 883.33                             | 2425.13 | 1732.35 | 980.62  | 495.09                             | 69.77 | 104.72 | 45.99  | 3013.96                                | 215.19 | 386.47 | 550.10 |
| 28              | WT       | PBS       | 126.2                             | 78.7  | 1010.05                            | 2548.09 | 1586.25 | 1097.55 | 318.63                             | 68.21 | 99.75  | 47.01  | 2097.41                                | 211.28 | 345.96 | 552.94 |
| 29              | WT       | Rotenone  | 126.2                             | 90.2  | 528.15                             | 1562.19 | 1649.57 | 1039.45 | 276.22                             | 52.84 | 107.91 | 55.78  | 2474.86                                | 232.69 | 280.47 | 511.36 |
| 30              | MTKO     | PBS       | 116.7                             | 85.7  | 311.53                             | 2289.32 | 1689.52 | 1159.98 | 301.71                             | 53.10 | 93.08  | 53.06  | 2240.97                                | 218.66 | 365.38 | 605.03 |
| 31              | MTKO     | Rotenone  | 131.0                             | 72.9  | 678.94                             | 1836.98 | 1624.33 | 1172.02 | 317.51                             | 67.32 | 119.49 | 46.38  | 2402.95                                | 177.76 | 374.01 | 523.97 |
| 32              | MTKO     | Rotenone  | 109.5                             | 73.2  | 563.37                             | 1859.28 | 1393.33 | 831.18  | 437.38                             | 42.24 | 117.27 | 55.42  | 2516.55                                | 161.30 | 334.47 | 520.84 |
| 33              | WT       | PBS       | 133.3                             | 90.5  | 306.72                             | 2653.56 | 1691.19 | 1560.01 | 302.48                             | 56.87 | 106.80 | 67.26  | 1887.39                                | 265.24 | 442.22 | 576.20 |
| 34              | WT       | Rotenone  | 88.1                              | 73.3  | 391.12                             | 1728.00 | 1060.99 | 1290.00 | 513.34                             | 73.39 | 118.44 | 81.77  | 2324.93                                | 235.19 | 380.81 | 560.08 |
| 35              | WT       | Rotenone  | 97.6                              | 85.4  | 452.48                             | 1611.70 | 1195.60 | 1234.62 | 381.55                             | 65.00 | 108.72 | 103.97 | 2444.73                                | 186.73 | 370.49 | 435.97 |
| 36              | MTKO     | PBS       | 116.7                             | 60.6  | 748.63                             | 1701.39 | 1422.99 | 735.29  | 577.15                             | 82.58 | 119.22 | 60.24  | 2205.13                                | 176.38 | 351.86 | 534.24 |
| 37              | MTKO     | PBS       | 128.6                             | 53.8  | 338.92                             | 1784.98 | 1854.27 | 1031.79 | 637.95                             | 67.66 | 124.76 | 110.61 | 2774.26                                | 212.16 | 329.34 | 606.93 |
| 38              | MTKO     | Rotenone  | 119.0                             | 79.3  | 309.41                             | 1417.58 | 1511.99 | 1132.15 | 606.18                             | 49.69 | 95.21  | 78.47  | 2532.05                                | 200.56 | 428.66 | 584.75 |
| 39              | WT       | PBS       | 109.5                             | 85.5  | 281.32                             | 2119.49 | 2466.56 | 1302.84 | 510.97                             | 61.82 | 115.06 | 78.78  | 2433.95                                | 217.71 | 351.60 | 753.48 |
| 40              | WT       | PBS       | 138.1                             | 55.0  | 403.69                             | 2066.75 | 1854.71 | 1694.17 | 521.94                             | 56.87 | 125.96 | 46.50  | 2496.81                                | 171.47 | 335.44 | 372.95 |
| 41              | WT       | Rotenone  | 159.5                             | 104.8 | 1071.92                            | 1631.75 |         | 989.56  | 478.64                             | 47.65 | 101.78 | 54.83  | 2663.99                                | 257.92 | 511.68 | 424.74 |
| 42              | MTKO     | PBS       | 77.4                              | 59.0  | 829.03                             | 2200.87 | 28.90   | 756.87  | 766.10                             | 56.38 | 99.04  | 58.88  | 2700.22                                | 212.71 | 351.61 | 434.01 |
| 43              | WT       | PBS       | 131.0                             | 62.6  | 549.31                             | 2070.75 |         | 1719.84 | 615.99                             | 53.81 | 157.41 | 47.46  | 2253.75                                | 245.69 | 597.69 | 405.29 |
| 44              | MTKO     | Rotenone  | 109.5                             | 58.0  | 259.69                             | 1727.26 | 12.29   | 1454.26 | 693.23                             | 67.05 | 83.64  | 63.47  | 2544.37                                | 177.61 | 376.45 | 457.11 |
| 45              | WT       | Rotenone  | 95.2                              | 80.4  | 384.95                             | 1721.84 |         | 728.08  | 605.28                             | 71.85 | 118.54 | 70.61  | 2926.75                                | 224.62 | 457.92 | 481.34 |
| 46              | MTKO     | Rotenone  | 88.1                              | 59.0  | 658.07                             | 1433.43 |         | 1167.28 | 633.32                             | 70.89 | 108.07 | 61.10  | 2422.09                                | 180.21 | 393.87 | 491.56 |
| 47              | WT       | Rotenone  | 78.8                              | 97.6  | 896.90                             | 1609.54 |         | 1432.22 | 815.57                             | 66.85 | 124.60 | 51.26  | 3091.96                                | 223.05 | 357.36 | 470.12 |
| 48              | MTKO     | PBS       | 109.5                             | 29.0  | 643.80                             | 1525.39 | 1485.14 | 1365.45 | 515.75                             | 88.85 | 97.25  | 53.76  | 2855.68                                | 199.69 | 502.73 | 361.73 |
| 49              | MTKO     | PBS       | 142.9                             | 46.2  | 515.84                             | 1817.44 | 4043.51 | 971.42  | 1646.82                            | 79.86 | 104.00 | 76.40  | 2585.60                                | 164.43 | 371.93 | 191.77 |
| 50              | MTKO     | Rotenone  | 128.6                             | 68.5  | 595.68                             | 1589.58 |         | 968.75  | 670.91                             | 56.46 | 98.49  | 47.17  | 2890.18                                | 153.30 | 316.22 | 247.19 |
| 51              | WT       | PBS       | 133.3                             | 52.9  | 559.79                             | 2126.55 | 2826.92 | 905.22  | 939.66                             | 51.85 | 90.99  | 75.49  | 2072.77                                | 136.92 | 286.98 | 344.65 |
| 52              | WT       | PBS       | 116.7                             | 43.3  | 667.26                             | 2029.61 |         | 1613.23 | 1177.33                            | 77.61 | 104.61 | 82.10  | 2230.44                                | 179.40 | 343.46 | 221.07 |
| 53              | WT       | Rotenone  | 147.6                             | 56.0  | 1172.59                            | 1585.73 | 77.10   | 928.99  | 469.32                             | 45.06 | 53.52  | 39.66  | 2507.48                                | 228.40 | 259.84 | 246.45 |
| 54              | MTKO     | Rotenone  | 211.9                             | 114.3 | 2140.78                            | 1495.97 | 1004.03 | 688.99  | 481.59                             | 61.47 | 96.00  | 71.66  | 1035.47                                | 167.72 | 302.05 | 391.92 |

| Assigned number | Genotype | Treatment | State 3 Respiration (pmon/min/μg) |       | complex I activity (nmol/min/UCII) |         |         |         | complex II activity (nmol/min/UCS) |       |        |       | complex II+III activity (nmol/min/UCS) |        |        |        |
|-----------------|----------|-----------|-----------------------------------|-------|------------------------------------|---------|---------|---------|------------------------------------|-------|--------|-------|----------------------------------------|--------|--------|--------|
| 55              | MTKO     | PBS       | 319.0                             | 85.7  | 726.05                             | 2278.96 | 1546.82 | 1330.96 | 246.93                             | 52.70 | 97.75  | 55.54 | 892.11                                 | 203.44 | 268.87 | 566.90 |
| 56              | WT       | Rotenone  | 209.5                             | 104.5 | 877.80                             | 1477.34 | 4299.57 | 1341.92 | 363.11                             | 58.66 | 107.04 | 73.68 | 929.30                                 | 206.82 | 385.81 | 485.77 |
| 57              | WT       | Rotenone  | 197.6                             | 87.1  | 370.53                             | 1446.30 | 1241.80 | 918.93  | 582.50                             | 61.11 | 81.05  | 75.92 | 1237.52                                | 178.59 | 504.49 | 489.69 |
| 58              | WT       | PBS       | 235.7                             | 74.1  | 353.42                             | 1898.67 | 3769.30 | 1222.52 | 538.04                             | 54.97 | 87.38  | 62.14 | 1042.39                                | 244.17 | 435.03 | 460.94 |
| 59              | MTKO     | Rotenone  | 95.2                              | 75.7  | 522.96                             | 1575.01 | 2332.79 | 964.40  | 514.95                             | 68.69 | 125.88 | 79.24 | 2704.85                                | 171.90 | 454.67 | 197.59 |
| 60              | MTKO     | Rotenone  | 104.8                             | 70.2  | 1074.71                            | 1787.90 | 928.87  | 636.77  | 429.15                             | 65.09 | 88.15  | 70.64 | 2231.35                                | 129.41 | 315.52 | 227.40 |
| 61              | MTKO     | Rotenone  | 85.2                              | 57.1  | 599.56                             | 1961.67 | 1273.05 | 703.17  | 454.21                             | 68.15 | 62.33  | 79.00 | 2170.02                                | 176.61 | 320.82 | 289.88 |
| 62              | WT       | Rotenone  | 114.3                             | 84.0  | 281.85                             | 1134.21 | 1175.28 | 1100.01 | 564.86                             | 76.83 | 48.11  | 86.86 | 2522.60                                | 146.15 | 398.17 | 159.61 |
| 63              | WT       | Rotenone  | 100.0                             | 73.6  | 247.65                             | 1655.50 | 2450.24 | 792.28  | 504.86                             | 50.16 | 62.42  | 77.67 | 2712.60                                | 141.74 | 323.60 | 238.73 |
| 64              | WT       | Rotenone  | 91.2                              | 56.7  | 258.65                             | 1453.96 |         | 1005.26 | 458.81                             | 54.33 | 77.37  | 74.43 | 2751.49                                | 146.40 | 374.34 | 285.63 |

Table 4: Processed data points of CIII and CIV activity analyses

| Assigned number | Genotype | Treatment | complex III activity (nmol/min/UCS) |        |         |         | complex IV activity (nmol/min/UCS) |        |        |        |
|-----------------|----------|-----------|-------------------------------------|--------|---------|---------|------------------------------------|--------|--------|--------|
|                 |          |           | Liver                               | Heart  | Muscle  | Brain   | Liver                              | Heart  | Muscle | Brain  |
| 1               | MTKO     | PBS       | 1682.73                             | 287.27 | 1607.14 | 741.21  | 1561.24                            | 199.92 | 683.95 | 453.65 |
| 2               | MTKO     | PBS       | 2277.14                             |        | 1275.70 | 848.79  | 1520.23                            | 237.60 | 842.17 | 420.50 |
| 3               | WT       | PBS       | 2404.59                             | 230.30 | 1108.90 | 773.44  | 2153.92                            | 224.24 | 804.21 | 417.01 |
| 4               | WT       | PBS       | 2030.06                             | 291.04 | 935.37  | 574.56  | 1614.34                            | 270.33 | 575.47 | 377.59 |
| 5               | WT       | Rotenone  | 3261.19                             | 232.67 | 1114.09 | 696.42  | 2120.72                            | 240.23 | 784.34 | 467.31 |
| 6               | MTKO     | Rotenone  | 1868.29                             |        | 1143.12 | 742.90  | 861.71                             | 320.80 | 533.74 | 450.92 |
| 7               | MTKO     | Rotenone  | 2464.24                             | 235.49 | 1159.47 | 748.33  | 2053.46                            | 254.72 | 684.76 | 434.89 |
| 8               | MTKO     | PBS       | 1928.92                             | 304.67 | 1104.58 | 826.85  | 734.04                             | 286.45 | 611.26 | 475.65 |
| 9               | WT       | Rotenone  | 2615.76                             | 208.06 | 936.12  | 921.20  | 2867.02                            | 203.32 | 639.53 | 497.30 |
| 10              | WT       | Rotenone  | 2207.13                             | 269.10 | 1076.12 | 1764.83 | 2163.35                            | 228.08 | 618.55 | 705.73 |
| 11              | WT       | PBS       | 2090.25                             |        | 1075.92 | 1902.43 | 1688.33                            | 239.24 | 593.40 | 825.64 |

| Assigned number | Genotype | Treatment | complex III activity (nmol/min/UCS) |        |         |         | complex IV activity (nmol/min/UCS) |        |        |        |
|-----------------|----------|-----------|-------------------------------------|--------|---------|---------|------------------------------------|--------|--------|--------|
| 11              | WT       | PBS       | 2090.25                             |        | 1075.92 | 1902.43 | 1688.33                            | 239.24 | 593.40 | 825.64 |
| 12              | MTKO     | PBS       | 2489.19                             |        | 1126.90 | 1740.11 | 2068.48                            | 226.75 | 753.72 | 785.28 |
| 13              | MTKO     | PBS       | 3283.99                             | 244.90 | 1495.10 | 966.37  | 2234.34                            | 253.13 | 529.26 | 458.80 |
| 14              | MTKO     | Rotenone  | 3417.56                             |        | 1479.48 | 1010.19 | 2181.90                            | 184.19 | 858.12 | 435.73 |
| 15              | WT       | PBS       | 3132.12                             | 243.00 | 1420.04 | 1187.79 | 1842.66                            | 267.25 | 536.36 | 471.37 |
| 16              | WT       | PBS       | 2889.62                             | 237.57 | 1280.27 | 1385.67 | 1889.56                            | 240.06 | 575.05 | 417.43 |
| 17              | WT       | Rotenone  | 2920.51                             | 282.16 | 1308.77 | 850.87  | 2178.12                            | 269.46 | 652.61 | 473.99 |
| 18              | MTKO     | Rotenone  | 2928.52                             | 125.51 | 1123.19 | 861.65  | 1799.14                            | 200.66 | 492.53 | 439.20 |
| 19              | MTKO     | Rotenone  | 3475.83                             | 263.29 | 1074.65 | 850.93  | 2383.28                            | 337.06 | 496.26 | 427.43 |
| 20              | MTKO     | PBS       | 2599.83                             | 286.53 | 1136.97 | 807.57  | 1502.60                            | 272.98 | 457.32 | 442.29 |
| 21              | WT       | Rotenone  | 2299.16                             |        | 1017.64 | 758.60  | 1349.55                            | 185.84 | 582.74 | 450.41 |
| 22              | WT       | Rotenone  | 3262.31                             | 194.71 | 1239.89 | 885.79  | 1774.78                            | 324.42 | 557.55 | 457.74 |
| 23              | WT       | PBS       | 3440.12                             | 314.17 | 1360.82 | 698.79  | 1709.88                            | 251.03 | 551.02 | 446.94 |
| 24              | MTKO     | PBS       | 3466.41                             | 289.71 | 1440.77 | 863.60  | 1826.88                            | 300.17 | 361.07 | 410.65 |
| 25              | MTKO     | PBS       | 2230.47                             | 278.82 | 443.98  | 572.51  | 1653.63                            | 124.56 | 592.86 | 464.20 |
| 26              | MTKO     | Rotenone  | 2222.12                             | 358.61 | 542.79  | 587.44  | 1952.53                            | 193.88 | 955.11 | 458.17 |
| 27              | WT       | PBS       | 3008.91                             | 269.31 | 580.39  | 619.76  | 2661.93                            | 232.01 | 714.54 | 410.48 |
| 28              | WT       | PBS       | 2096.32                             | 285.13 | 536.38  | 428.64  | 2120.72                            | 303.79 | 733.15 | 440.39 |
| 29              | WT       | Rotenone  | 1923.40                             | 311.15 | 428.15  | 465.80  | 2500.30                            | 267.29 | 670.88 | 420.96 |
| 30              | MTKO     | PBS       | 2334.09                             | 266.72 | 371.88  | 615.42  | 2142.48                            | 214.17 | 519.01 | 486.03 |
| 31              | MTKO     | Rotenone  | 2330.72                             | 239.57 | 329.04  | 484.26  | 2058.28                            | 238.50 | 691.50 | 366.24 |
| 32              | MTKO     | Rotenone  | 2751.76                             | 209.95 | 454.04  | 486.82  | 2523.03                            | 240.52 | 611.38 | 398.87 |
| 33              | WT       | PBS       | 2456.10                             | 218.91 | 456.80  | 579.76  | 1721.26                            | 297.45 | 689.86 | 420.74 |
| 34              | WT       | Rotenone  | 2991.39                             | 266.17 | 473.98  | 817.18  | 2649.19                            | 291.41 | 509.19 | 458.82 |
| 35              | WT       | Rotenone  | 2403.51                             | 276.09 | 532.15  | 635.58  | 2163.86                            | 305.14 | 653.06 | 422.28 |
| 36              | MTKO     | PBS       | 2271.76                             | 310.90 | 363.46  | 679.16  | 1304.46                            | 313.11 | 633.36 | 424.45 |
| 37              | MTKO     | PBS       | 2660.41                             | 261.20 | 386.53  | 764.10  | 2394.11                            | 295.67 | 603.17 | 482.77 |
| 38              | MTKO     | Rotenone  | 2338.88                             | 324.70 | 612.42  | 595.49  | 2332.86                            | 332.32 | 715.80 | 485.02 |
| 39              | WT       | PBS       | 2401.86                             | 263.79 | 449.73  | 735.66  | 2061.19                            | 311.45 | 580.02 | 561.85 |
| 40              | WT       | PBS       | 2625.13                             | 275.18 | 589.11  | 370.21  | 2079.09                            | 301.95 | 707.55 | 281.71 |

| Assigned number | Genotype | Treatment | complex III activity (nmol/min/UCS) |        |        |        | complex IV activity (nmol/min/UCS) |        |         |        |
|-----------------|----------|-----------|-------------------------------------|--------|--------|--------|------------------------------------|--------|---------|--------|
| 41              | WT       | Rotenone  | 2854.68                             | 404.18 | 654.33 | 326.57 | 1633.38                            | 371.72 | 791.72  | 447.95 |
| 42              | MTKO     | PBS       | 2750.73                             | 338.74 | 470.84 | 558.33 | 1438.00                            | 333.42 | 441.71  | 422.80 |
| 43              | WT       | PBS       | 2400.23                             | 261.53 | 781.06 | 654.82 | 1378.48                            | 309.05 | 1060.84 | 472.55 |
| 44              | MTKO     | Rotenone  | 2520.91                             | 212.93 | 498.40 | 499.13 | 1794.79                            | 328.87 | 723.85  | 436.52 |
| 45              | WT       | Rotenone  | 3055.35                             | 297.69 | 640.52 | 539.42 | 2693.39                            | 256.70 | 672.94  | 468.08 |
| 46              | MTKO     | Rotenone  | 2707.69                             | 268.02 | 488.66 | 612.05 | 1444.36                            | 283.91 | 715.55  | 474.90 |
| 47              | WT       | Rotenone  | 3603.40                             | 304.84 | 488.38 | 588.15 | 2523.04                            | 299.05 | 723.77  | 449.05 |
| 48              | MTKO     | PBS       | 3303.60                             | 297.82 | 496.54 | 311.85 | 2102.51                            | 322.59 | 754.09  | 297.43 |
| 49              | MTKO     | PBS       | 2945.16                             | 280.01 | 406.93 | 569.29 | 1534.06                            | 361.78 | 447.58  | 254.15 |
| 50              | MTKO     | Rotenone  | 2958.46                             | 372.73 | 393.31 | 502.02 | 2544.92                            | 655.34 | 372.43  | 288.77 |
| 51              | WT       | PBS       | 2323.35                             | 171.96 | 264.92 | 602.70 | 1827.63                            | 268.10 | 309.20  | 308.28 |
| 52              | WT       | PBS       | 2074.76                             | 275.68 | 404.02 | 547.92 | 1970.93                            | 402.65 | 41.75   | 231.37 |
| 53              | WT       | Rotenone  | 2310.71                             | 387.80 | 178.50 | 447.88 | 1797.71                            | 522.06 | 402.81  | 271.22 |
| 54              | MTKO     | Rotenone  | 1718.86                             | 302.44 | 368.09 | 667.35 | 1206.12                            | 476.65 | 514.64  | 335.84 |
| 55              | MTKO     | PBS       | 1604.69                             | 390.56 | 346.61 | 773.05 | 657.06                             | 488.88 | 323.77  | 350.20 |
| 56              | WT       | Rotenone  | 1491.01                             |        | 433.22 | 748.38 | 967.71                             | 395.65 | 392.52  | 212.01 |
| 57              | WT       | Rotenone  | 2041.94                             | 292.40 | 631.46 | 978.99 | 1258.42                            | 324.48 | 413.04  | 369.99 |
| 58              | WT       | PBS       | 1490.82                             | 375.15 | 604.88 | 670.19 | 1074.87                            | 425.94 | 484.19  | 394.17 |
| 59              | MTKO     | Rotenone  | 3070.24                             | 290.36 | 575.19 | 672.79 | 2304.76                            | 441.69 | 509.06  | 288.28 |
| 60              | MTKO     | Rotenone  | 2415.24                             | 241.95 | 417.97 | 734.93 | 1868.71                            | 421.46 | 416.49  | 294.72 |
| 61              | MTKO     | Rotenone  | 2463.98                             | 304.88 | 456.69 | 759.47 | 1981.62                            | 462.61 | 355.79  | 306.19 |
| 62              | WT       | Rotenone  | 3617.40                             | 268.70 | 410.11 | 716.95 | 2455.60                            | 373.67 | 296.39  | 296.01 |
| 63              | WT       | Rotenone  | 3360.53                             | 233.48 | 483.84 | 756.70 | 2069.44                            | 425.95 | 391.20  | 296.73 |
| 64              | WT       | Rotenone  | 3162.58                             | 246.25 | 456.00 | 728.16 | 2182.55                            | 393.17 | 443.78  | 293.30 |

In Tables 3 and 4, the processed data points (*i.e.* the means calculated for each sample, see Section 3.14) are given for respiration and RC enzyme analyses. CI activity is normalised to CII (UCII) while all other complex activities are normalised to citrate synthase(UCS). Outliers identified by Tukey's methods (Section 3.14), in a specific tissue, for a specific analysis, are highlighted in purple. Where no value is given (blank spaces), no sample was available to complete the corresponding analysis in triplicate.