COPYRIGHT AND USE OF THIS THESIS

This thesis must be used in accordance with the provisions of the Copyright Act 1968.

Reproduction of material protected by copyright may be an infringement of copyright and copyright owners may be entitled to take legal action against persons who infringe their copyright.

Section 51 (2) of the Copyright Act permits an authorized officer of a university library or archives to provide a copy (by communication or otherwise) of an unpublished thesis kept in the library or archives, to a person who satisfies the authorized officer that he or she requires the reproduction for the purposes of research or study.

The Copyright Act grants the creator of a work a number of moral rights, specifically the right of attribution, the right against false attribution and the right of integrity.

You may infringe the author’s moral rights if you:

- fail to acknowledge the author of this thesis if you quote sections from the work
- attribute this thesis to another author
- subject this thesis to derogatory treatment which may prejudice the author’s reputation

For further information contact the University’s Copyright Service.

sydney.edu.au/copyright
PARACETAMOL TOXICITY: INFLUENCE OF AGEING, FRAILTY, RESVERATROL AND SIRT1

Alice Elizabeth Kane

Ageing and Pharmacology Laboratory
Kolling Institute of Medical Research
Sydney Medical School
University of Sydney

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

March 2016
DECLARATION

The analyses and results presented in this thesis are my own work, achieved under the supervision of Professor Sarah Hilmer, Professor David Le Couteur and Associate Professor Victoria Cogger, unless otherwise acknowledged.

Signed: Alice Elizabeth Kane BSc(Adv) Hons I

Date: 3/8/15
ABSTRACT

This thesis investigates changes to paracetamol toxicity susceptibility and mechanisms in ageing, frailty and non-acute paracetamol exposures. It investigates whether the current paracetamol hepatotoxicity therapy, N-acetyl cysteine (NAC), is protective in non-acute paracetamol exposures, and explores the potential for interventions aimed at increasing lifespan and healthspan to provide novel mechanisms of paracetamol hepatotoxicity protection. Furthermore this thesis investigates the potential of both paracetamol, and lifespan and healthspan interventions in delaying or preventing frailty.

Chapter one of this thesis will summarise the relevant literature on paracetamol toxicity prevalence mechanisms and therapies; pharmacokinetic and pharmacodynamic changes with age and frailty in particular in regards to paracetamol; animal models of ageing, frailty and toxicity; and interventions to slow ageing and frailty, and the role they may play in paracetamol toxicity.

Chapter two looks at changes in acute paracetamol toxicity with old age and frailty in mice. There is increased use of the analgesic paracetamol in old age, and extensive age-related physiological changes that may affect paracetamol pharmacokinetics and pharmacodynamics. Despite this there are limited human studies and few animal studies on the changes to acute paracetamol hepatotoxicity risk and mechanisms in old age and in frailty. The changing risk of paracetamol hepatotoxicity with age has not been looked at in an ageing mouse model, and has not been explored with frailty in an animal model.
Chapter three explores changes to paracetamol toxicity with age and frailty in mouse models of chronic and sub-acute paracetamol exposure. It also looks at whether NAC is protective against chronic and sub-acute paracetamol exposure, and how this is altered with age. It is clear that in old age there is a different type of clinical over-exposure to paracetamol, with older people more likely to have chronic and accidental exposures rather than acute over-exposures. The risk of toxicity, with age and frailty, to these types of paracetamol exposures, is unknown. Furthermore, there is very little evidence for the use of the current clinical therapy for paracetamol hepatotoxicity, NAC, in non-acute paracetamol exposures.

Chapter four investigates pharmacological manipulation of the sirtuin 1 (SIRT1) pathway with resveratrol treatment as a potentially protective mechanism against paracetamol toxicity in three models: primary hepatocytes, young mice with concurrent resveratrol and paracetamol dosing, and young and old mice pre-exposed to resveratrol, then treated with paracetamol. There is a need for new paracetamol toxicity therapies, as we can see from the lack of evidence for the use of NAC in non-acute over-exposures, and the prevalence of patients who still develop acute liver failure from paracetamol toxicity. The lifespan and healthspan increasing interventions provide novel targets that can be investigated as potential paracetamol toxicity protective therapeutics.

Chapter five examines whether genetic manipulation of the SIRT1 pathway through transgenic mouse models is protective against paracetamol toxicity in young and old mice. Chapter four provides inconclusive results in regards to resveratrol protection against paracetamol hepatotoxicity, so chapter five explicitly addresses whether SIRT1
plays a protective role in paracetamol toxicity, with the use of young and old SIRT1 transgenic mice treated with acute paracetamol.

Finally, chapter six investigates frailty, and whether chronic paracetamol exposure, or activation of the SIRT1 pathway, through chronic resveratrol treatment, or calorie restriction, can delay frailty in ageing mice. This chapter investigates the effect of pharmaceutical and dietary interventions on a novel mouse clinical frailty index for the first time.
ACKNOWLEDGEMENTS

I would like to thank and acknowledge my supervisors Professor Sarah Hilmer, Professor David Le Couteur and Associated Professor Victoria Cogger, for their constant support, advice and feedback. In particular, I would like to thank Sarah for her encouragement, clever problem solving and endless positivity and enthusiasm for these projects, David for his insight and ideas and Vic for her lab assistance and practical advice.

I would like to thank and acknowledge Dr Rafael de Cabo and Dr Sarah Mitchell for their supervision and support whilst I was at the National Institute on Aging. In particular, I would like to thank Sarah for her patience, encouragement, support and friendship in teaching me new skills, developing project ideas, writing papers and getting results. She was also invaluable in her assistance with the mouse work for chapters 4 and 5, the frailty assessments for chapters 1 and 6 and the qPCR results.

I would like to thank and acknowledge all the people who I work with at the Kolling Institute. In particular I would like to thank everyone in the Ageing and Clinical Pharmacology team for their support and encouragement. In particular I would like to thank and acknowledge Dr Aniko Huizer-Pajkos, for all that she taught me in the laboratory during my Honours year and PhD, for completing the DNA fragmentation experiment in chapter 3 and for assisting with the hepatocyte isolation and resveratrol mice experiments in chapter 4. I would also like to thank my fellow PhD student John Mach for his encouragement, support and in particular, assistance with the enzyme activity assays (chapters 2, 3, 4 and 5), and the frailty assessment for chapters 2 and 6.
I would like to thank and acknowledge Dawn Boyer, Dawn Nines and Kristan Gavin for their assistance with all animal work completed at the NIA, and for the frailty assessments for chapter 6. I would like to thank Gelarah Abulwerdi for running the SIRT1 and resveratrol diet western blots for chapters 4 and 5, Dr Catriona McKenzie for completing the histological analysis, Dr Lindsay Wu for his assistance with the concurrent resveratrol western blots (Chapter 4), and Dr Brett Jones for his expertise and advice on paracetamol toxicity and the liver. I would also like to thank Professor Susan Howlett for her advice, feedback and assistance on the frailty interventions project (Chapter 6) and Dr Jillian Patterson for her assistance with statistical analysis.

I would like to thank and acknowledge the financial support I received during my PhD. Thank you to the NHMRC for the biomedical postgraduate scholarship, the Northern Clinical School, University of Sydney for the PhD top-up scholarship, the Geoff and Elaine Penney Fund for on-going support, the Australian Association of Gerontology (AAG) for the RM Gibson Scientific Research Fund Grant in 2012, the Australian Society of Clinical and Experimental Pharmacologists (ASCEPT) for the IUPHAR Travel Grant in 2014, the Royal North Shore Hospital for the Beryl and Jack Jacobs International Travel Award in 2013, the Sydney Medical School for a Travelling Fellowship in 2013 and the Royal North Shore Hospital Scientific Staff Council for a Study Fellowship in 2013.

Finally I would like to thank my parents, Lauren and John, my sister Hannah and my partner Sam, for their constant love, support and encouragement.
PUBLICATIONS RESULTING FROM THE WORK IN THIS THESIS


PUBLICATIONS PREPARED FROM THE WORK IN THIS THESIS


induced hepatotoxicity in young and old mice, Toxicology and Applied Pharmacology,
In Preparation

OTHER PUBLICATIONS


GRANTS AWARDED TO SUPPORT THE WORK IN THIS THESIS
ABSTRACTS PRESENTED AT CONFERENCES


Kane A, Huizer-Pajkos A, Cogger V, Le Couteur DG & Hilmer SN. Resveratrol does not protect against paracetamol-induced cell death in mouse primary hepatocytes. 20th IAGG World Congress of Gerontology and Geriatrics - Poster Presentation, Seoul, South Korea, 23rd-27th June 2013.

Kane A, Huizer-Pajkos A, Cogger V, Le Couteur DG & Hilmer SN. Resveratrol does not protect against paracetamol-induced cell death in mouse primary hepatocytes. 8th International Congress of Toxicology - Poster Presentation, Seoul, South Korea, 30th June-4th July 2013.

CONTENTS

1 INTRODUCTION ........................................................................................................ 1

1.1 INTRODUCTION ......................................................................................................... 1

1.2 PARACETAMOL USE AND TOXICITY ......................................................................... 3

1.2.1 Paracetamol Use and Toxicity in Australia and Internationally ..................... 3

1.2.2 Pharmacokinetics of Paracetamol Toxicity ..................................................... 4

1.2.3 Pharmacodynamics of Paracetamol Hepatotoxicity ........................................ 6

1.2.4 Clinical Assessment of Paracetamol Toxicity .................................................. 9

1.2.5 Current treatment: N-Acetyl Cysteine ............................................................ 10

1.2.6 Risk Factors for Paracetamol Hepatotoxicity ............................................. 15

1.2.7 Toxic Exposures: Intentional vs. Unintentional, Acute vs. Chronic .............. 16

1.2.8 Animal Models of Paracetamol Toxicity ........................................................ 17

1.3 AGEING AND FRAILTY ............................................................................................ 19

1.3.1 Animal Models of Ageing and Frailty ............................................................ 21

1.3.2 Pharmacokinetic, Pharmacodynamic and Toxicological Changes with Ageing and Frailty ........................................................................................................ 27

1.3.3 Changes in the Pharmacology and Toxicology of Paracetamol in Ageing and Frailty............................................................................................................. 30

1.4 INTERVENTIONS TO SLOW AGEING AND FRAILTY ............................................. 34

1.4.1 Lifespan and Healthspan Interventions ......................................................... 34

1.4.2 Calorie Restriction and the Ageing-Related Molecular Pathways .............. 35

1.4.3 Calorie Restriction Mimetics ......................................................................... 39
3 CHRONIC AND SUB-ACUTE PARACETAMOL EXPOSURE IN YOUNG AND OLD MICE, AND THE ROLE OF N-ACETYL CYSTEINE ................. 78

3.1 INTRODUCTION ........................................................................................................... 78

3.2 METHODS ................................................................................................................... 80

3.2.1 Animals ................................................................................................................... 80

3.2.2 Frailty Index Assessment ..................................................................................... 81

3.2.3 Chronic and Sub-Acute Paracetamol Treatment of Mice, and Tissue Collection ......................................................................................................................... 81

3.2.4 Serum Biochemistry to Assess Liver and Renal Function ..................................... 85

3.2.5 Liver Histology ...................................................................................................... 85

3.2.6 Biochemical Examination of CYP2E1 in Frozen Liver Samples ......................... 86

3.2.7 Biochemical Examination of Glutathione and DNA Fragmentation in Frozen Liver Samples .................................................................................................................. 86

3.2.8 Statistics ................................................................................................................ 86

3.3 RESULTS .................................................................................................................... 87

3.3.1 Animal Characteristics ......................................................................................... 87

3.3.2 Assessment of Toxicity with Chronic Paracetamol Exposure ............................ 93

3.3.3 Assessment of Toxicity with Sub-acute Paracetamol Exposure ......................... 97

3.3.4 Assessment of Toxicity with Paracetamol Exposure Plus N-Acetyl Cysteine 97

3.3.5 Serum Paracetamol Levels, Liver Glutathione Levels and CYP2E1 Activity 98

3.3.6 Correlation between Frailty and Paracetamol Toxicity Outcomes .................... 101

3.4 DISCUSSION ............................................................................................................. 103
4 INVESTIGATION OF RESVERATROL AS A POTENTIAL THERAPY TO
PROTECT AGAINST PARACETAMOL HEPATOTOXICITY .......................110

4.1 INTRODUCTION.................................................................................................................110

4.2 METHODS .................................................................................................................................113

4.2.1 Cell Culture Methods Optimisation ..............................................................113

4.2.2 Animals.........................................................................................................................115

4.2.3 Hepatocyte Isolation and Treatment ..............................................................116

4.2.4 Cell Viability Assays ............................................................................................118

4.2.5 Concurrent Resveratrol and Paracetamol Treatment of Animals ..........118

4.2.6 Dietary Resveratrol Treatment of Animals ...................................................120

4.2.7 Serum Biochemistry ..........................................................................................120

4.2.8 Histology..................................................................................................................121

4.2.9 Biochemical Examination of Frozen Liver Samples .....................................121

4.2.10 Biochemical Examination of Hepatocytes .....................................................122

4.2.11 Western Blots ........................................................................................................122

4.2.12 Statistics ..................................................................................................................124

4.3 RESULTS..............................................................................................................................125

4.3.1 Primary Hepatocyte Results .................................................................125

4.3.2 Concurrent Resveratrol and Paracetamol Treatment in Mice Results ....128

4.3.3 Dietary Pre-Treatment with Resveratrol Results ........................................132

4.4 DISCUSSION.......................................................................................................................140
5 THE ROLE OF SIRT1 IN PARACETAMOL HEPATOTOXICITY IN YOUNG AND OLD MICE ................................. 147

5.1 INTRODUCTION ........................................................................................................ 147

5.2 METHODS .............................................................................................................. 149

5.2.1 Animals .......................................................................................................... 149

5.2.2 Paracetamol Treatment and Tissue Collection ............................................. 150

5.2.3 Serum Biochemistry to Assess Liver and Renal Function ......................... 150

5.2.4 Liver Histology ............................................................................................. 151

5.2.5 Biochemical Examination of Enzymes and Glutathione in Frozen Liver Samples .......................................................... 151

5.2.6 Western Blots ............................................................................................... 152

5.2.7 mRNA Expression Measurement with qPCR ............................................. 153

5.2.8 Statistics ....................................................................................................... 153

5.3 RESULTS ............................................................................................................... 153

5.3.1 Animal and Genotype Details .................................................................... 153

5.3.2 Assessment of Paracetamol Toxicity ............................................................ 157

5.3.3 Assessment of Factors Influencing Paracetamol Pharmacokinetics ......... 161

5.3.4 mRNA expression of Inflammatory Markers ............................................. 164

5.3.5 mRNA expression of Apoptosis and Mitochondrial Function Markers ..... 165

5.4 DISCUSSION .......................................................................................................... 168

6 FRAILTY: EFFECT OF PARACETAMOL, RESVERATROL, CALORIE RESTRICTION AND MOUSE STRAIN ................................................................. 174
LIST OF TABLES

**TABLE 1.1** Changes to factors and mechanisms involved in paracetamol toxicity pathway with factors that may increase or decrease the risk of paracetamol toxicity: N-acetyl cysteine treatment, ageing, frailty, resveratrol treatment and calorie restriction. ‘-’ indicates this effect is not applicable, ‘?’ indicates this effect is not yet known, ‘↑’ indicates the effect is an increase, ‘↓’ indicates the effect is a decrease. ALF = Acute Liver Failure, ALT = Alanine Aminotransferase, CYP450 = Cytochrome P450, NQO1 = NAD(P)H:quione oxidoreductase 1, NAPQI = N-acetyl-P-benzoquinone imine, AMPK = 5’ Adenosine monophosphate-activated protein kinase, SIRT1 = Sirtuin 1

**TABLE 1.2** Animal models of frailty: details of the animal types, assessments, clinically equivalent scales, validation, interventional results, strengths and limitations of the currently developed mouse models of frailty. IL = Interleukin.

**TABLE 1.3** Animal studies of resveratrol as a therapeutic to protect against paracetamol toxicity: Consideration of strain, paracetamol and resveratrol dose, route of administration and vehicle, timing of dosing, outcomes measured and study conclusions. ALT = Alanine aminotransferase, LDH = Lactate dehydrogenase, TNF = Tumor necrosis factor, MDA = 3,4-methylenedioxymphetamine, IL = Interleukin, CYP = Cytochrome, GSH = Glutathione, SIRT1 = Sirtuin 1

**TABLE 2.1** Characteristics and serum biochemistry results for young (7.3±0.3 months) and old (18.9±2.3 months) male C57BL/6 mice from cohort 1, 6
HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL OR SALINE. DATA EXPRESSED AS MEAN (SD). *P<0.05 COMPARED TO CORRESPONDING SALINE TREATED GROUP, # P<0.05 COMPARED TO CORRESPONDING YOUNG GROUP. ALP=ALKALINE PHOSPHATASE, GGT=GAMMA GLUTAMYLTRANSFERASE.

**TABLE 2.2** CHARACTERISTICS AND SERUM BIOCHEMISTRY RESULTS FOR YOUNG (10.0±0.0 MONTHS) AND OLD (23.7±0.0 MONTHS) MALE C57BL/6 MICE FROM COHORT 2, 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL OR SALINE.. DATA EXPRESSED AS MEAN (SD). *P<0.05 COMPARED TO CORRESPONDING SALINE TREATED GROUP. ALP= ALKALINE PHOSPHATASE, GGT= GAMMA GLUTAMYLTRANSFERASE.

**TABLE 3.1** CHRONIC AND SUB-ACUTE PARACETAMOL DOSING TIMELINE

**TABLE 3.2** ANIMAL CHARACTERISTICS AND SERUM BIOCHEMISTRY RESULTS FOR YOUNG MALE C57BL/6 MICE FED EITHER A CONTROL DIET OR PARACETAMOL CONTAINING (1.33G/KG FEED) DIET FOR 6 WEEKS, THEN TREATED WITH 3 DAYS OF SALINE OR PARACETAMOL (250MG/KG X 3/DAY) PLUS A SINGLE OR DOUBLE DOSE OF N-ACETYL CYSTEINE (NAC) (1200MG/KG) FOR THE PARACETAMOL DIET GROUP. SERUM AND LIVER COLLECTED 3 HOURS AFTER THE FINAL PARACETAMOL/SALINE DOSE.*P<0.05 COMPARED TO CONTROL DIET; SALINE GROUP #P<0.05 COMPARED TO CORRESPONDING SALINE GROUP WITH TUKEY’S HSD POST-HOC TEST. ALP=ALKALINE PHOSPHATASE, GGT= GAMMA GLUTAMYLTRANSFERASE, NAC=N-ACETYL CYSTEINE, NS=NON-SIGNIFICANT

**TABLE 3.3** ANIMAL CHARACTERISTICS AND SERUM BIOCHEMISTRY RESULTS FOR OLD MALE C57BL/6 MICE FED EITHER A CONTROL DIET OR PARACETAMOL CONTAINING (1.33G/KG FEED) DIET FOR 6 WEEKS, THEN TREATED WITH 3 DAYS OF SALINE OR PARACETAMOL (250MG/KG X 3/DAY) PLUS A SINGLE OR DOUBLE DOSE OF N-ACETYL...
Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Serum and liver collected 3 hours after the final paracetamol/saline dose. ALP= alkaline phosphatase, GGT= gamma glutamyltransferase, NAC=N-acetyl cysteine.

**Table 4.1** Optimisation of cell culture model of paracetamol toxicity.
DMEM= Dulbecco’s modified eagle medium, MTT= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, FCS= Fetal calf serum, DMSO= dimethyl sulfoxide, CYP2E1 = cytochrome 2E1.

**Table 4.2** Pilot experiments to determine the acute paracetamol dose necessary to induce consistent severe hepatotoxicity in young male C57BL/6 mice sourced from the Kearns facility. ALT = alanine aminotransferase.

**Table 4.3** Weights and serum biochemistry results for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle, resveratrol (RSV, 30mg/kg in corn oil), or N-acetyl cysteine (NAC, 1200mg/kg). Serum and liver collected 6 hours after dosing. Data expressed as mean (SD). *p<0.05 compared to all saline treated groups. ALP= alkaline phosphatase, GGT= gamma glutamyltransferase.

**Table 4.4** Weights and serum biochemistry results for young (6.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver and serum collected after 6 hours. Data expressed as mean (SD). *p<0.05 compared to corresponding
TABLE 4.5  **Weights and Serum Biochemistry Results for Old (18.0±0.0 Months) Male C57BL/6 Mice Exposed to Either Control Diet or Resveratrol Diet (RSV, 1.33g/kg Feed) for 3-4 Months, Then Treated with Paracetamol (300mg/kg via Oral Gavage) or Saline. Liver and Serum Collected After 6 Hours. Data Expressed as Mean (SD). *P<0.05 Compared to Corresponding Saline Treated Group. ALP= Alkaline Phosphatase, GGT= Gamma Glutamyltransferase.**

---

TABLE 5.1  **Weights and Serum Biochemistry Results for Young and Old Male Wild-Type C57BL/6, Liver Specific SIRT1 Overexpressor, and Liver Specific SIRT1 Knock Out Mice Treated with 300mg/kg Paracetamol (Para; via Oral Gavage) or Saline. Mice Were Euthanized, and Liver and Serum Collected After 6 Hours. Data Expressed as Mean (SD). ALP= Alkaline Phosphatase, GGT= Gamma Glutamyltransferase.**

---

TABLE 6.1  **The Percentage of Scores by Raters 1 and 3, for Cohort 1 Male C57BL/6 and DBA/2J Mice Fed Either an AL Diet, or a 40% CR Diet From Age 6 Months (19±2 Months Age), That Fell Into Each Score Category (0, 0.5, 1) for Each Item of the Frailty Index.**

---

TABLE 6.2  **The Percentage of Scores by Raters 1 and 3, for Cohort 1 Female C57BL/6 and DBA/2J Mice Fed Either an AL Diet, or a 40% CR Diet (19±1 Months Age, N=9-11) From Age 6 Months, That Fell Into Each Score Category (0, 0.5, 1) For Each Item of the Frailty Index.**

---

TABLE 6.3  **The Percentage of Scores by Raters 1 and 3, for Cohort 2 Old (24±0 Months) Male C57BL/6 Mice Fed Either a Control Diet (AIN-93G), or AN
AIN-93G DIET SUPPLEMENTED WITH RESVERATROL (RSV) (100MG/KG MOUSE/DAY) (N=16 SD, N=9 RSV,) FOR 6 MONTHS FROM AGE 19 MONTHS, THAT FELL INTO EACH SCORE CATEGORY (0, 0.5, 1) FOR EACH ITEM OF THE FRAILTY INDEX .................................. 192

**Table 6.4** Inter rater correlation of frailty index scores for male and female old (19±2 months) DBA/2 and C57BL/6 mice from cohort 1, between each combination of 2 raters (2-way random, consistency, average intraclass correlation coefficient, with 95% confidence intervals). ... 195
LIST OF FIGURES

FIGURE 1.1 PHARMACOKINETICS AND PHARMACODYNAMICS OF PARACETAMOL HEPATOTOXICITY. CYP2E1 = CYTOCHROME 2E1, NAPQI = N-ACETYL-P-BENZOQUINONE IMINE

FIGURE 1.2 INTERACTION OF FOUR OF THE PATHWAYS IMPLICATED IN THE LIFESPAN AND HEALTHSPAN INCREASING EFFECTS OF CALORIE RESTRICTION (SOURCE: SOLON-BIET ET AL. (2015)). mTOR= MAMMALIAN TARGET OF RAPAMYCIN, AMPK= 5' ADENOSINE MONOPHOSPHATE-ACTIVATED PROTEIN KINASE, SIRT1= SIRTUIN 1, IGF1= INSULIN-LIKE GROWTH FACTOR-1, NAMPT= NICOTINAMIDE PHOSPHORIBOSYLTRANSFERASE, PGC1α= PEROxisome PROliferator-activated RECEPTOR GAMMA COACTIVATOR 1, FOXO= Foxhead box PROTEIN, p53= Tumor PROTEIN 53, AKT= PROTEIN KINASE B

FIGURE 2.1 SERUM ALANINE AMINOTRANSFERASE (ALT) CONCENTRATIONS FOR YOUNG (COHORT 1, 7.3±0.3 MONTHS; COHORT 2 = 10±0.0 MONTHS) AND OLD (COHORT 1, 18.9±2.3 MONTHS; COHORT 2, 23.7±0.0 MONTHS) MALE C57BL/6 MICE FROM COHORT 1 (A) OR COHORT 2 (B), 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (GREY BARS) OR SALINE (BLACK BARS). DATA EXPRESSED AS MEAN±SEM. ONE-WAY ANOVA COHORT 1, F(3,43)=10.37 P =0.00. ONE-WAY ANOVA COHORT 2, F(3,26)=9.21 P =0.00. * =P<0.05 COMPARED TO CORRESPONDING SALINE TREATED GROUP WITH TUKEY’S HSD POST-HOC TEST. N=6-15 PER GROUP

FIGURE 2.2 LIVER NECROSIS (AS A PERCENTAGE OF LIVER TISSUE) FOR YOUNG (COHORT 1, 7.3±0.3 MONTHS; COHORT 2 = 10±0.0 MONTHS) AND OLD (COHORT 1, 18.9±2.3 MONTHS; COHORT 2, 23.7±0.0 MONTHS) MALE C57BL/6 MICE FROM COHORT 1 (A)
OR COHORT 2 (B), 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (GREY BARS) OR SALINE (BLACK BARS). DATA EXPRESSED AS MEAN±SEM. ONE-WAY ANOVA COHORT 1, F(3,43)=5.47 p=0.003. ONE-WAY ANOVA COHORT 2, F(3,24)=20.69 p=0.00. *=p<0.05 COMPARED TO CORRESPONDING SALINE TREATED SIRT1 ACTIVATION DOES NOT PROVIDE AN ALTERNATIVE PROTECTIVE MECHANISMGROUP WITH TUKEY’S HSD POST-HOC TEST. N=6-15 PER GROUP........ 62

**FIGURE 2.3** REPRESENTATIVE HAEMOTOXYLIN AND EOSIN STAINED LIVER HISTOLOGY IMAGES FOR YOUNG (COHORT 1, 7.3±0.3 MONTHS; COHORT 2 = 10±0.0 MONTHS) AND OLD (COHORT 1, 18.9±2.3 MONTHS; COHORT 2, 23.7±0.0 MONTHS) MALE C57BL/6 MICE 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL OR SALINE. IMAGES TAKEN AT 100X. .......................................................... 63

**FIGURE 2.4** CYTOCHROME P450 (CYP)2E1 ACTIVITY IN THE LIVERS OF YOUNG (COHORT 1, 7.3±0.3 MONTHS; COHORT 2 = 10±0.0 MONTHS) AND OLD (COHORT 1, 18.9±2.3 MONTHS; COHORT 2, 23.7±0.0 MONTHS) MALE C57BL/6 MICE FROM COHORT 1 (A) OR COHORT 2 (B), 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (GREY BARS) OR SALINE (BLACK BARS). DATA EXPRESSED AS MEAN±SEM. ONE-WAY ANOVA COHORT 1, F(3,40)=4.64 p=0.007. ONE-WAY ANOVA COHORT 2, NON-SIGNIFICANT. *=p<0.05 COMPARED TO CORRESPONDING SALINE TREATED GROUP WITH TUKEY’S HSD POST-HOC TEST. N=6-15 PER GROUP ....................... 64

**FIGURE 2.5** TOTAL LIVER GLUTATHIONE (GSH) FOR YOUNG (COHORT 1, 7.3±0.3 MONTHS; COHORT 2 = 10±0.0 MONTHS) AND OLD (COHORT 1, 18.9±2.3 MONTHS; COHORT 2, 23.7±0.0 MONTHS) MALE C57BL/6 MICE FROM COHORT 1 (A) OR COHORT 2 (B), 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (GREY BARS) OR SALINE (BLACK BARS). DATA EXPRESSED AS MEAN±SEM. ONE-WAY ANOVA COHORT 1, NON-SIGNIFICANT. ONE-WAY ANOVA COHORT 2, F(3,27)=42.97 p=0.00. *=p<0.05
FIGURE 2.6 NAD(P)H:QUINONE OXIDOREDUCTASE 1 (NQO1) ACTIVITY FOR YOUNG (7.3±0.3 MONTHS) AND OLD (18.9±2.3 MONTHS) MALE C57BL/6 MICE FROM COHORT 1, 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (GREY BARS) OR SALINE (BLACK BARS). DATA EXPRESSED AS MEAN±SEM. ONE-WAY ANOVA, F(3,40)=5.33 P =0.004. #=P<0.05 COMPARED TO CORRESPONDING YOUNG GROUP WITH TUKEY’S HSD POST-HOC TEST. N=10-15 PER GROUP..................................................................................................66

FIGURE 2.7 mRNA EXPRESSION OF INFLAMMATORY MARKERS; (A) TUMOR NECROSIS FACTOR(TNF)-A (B) INTERLEUKIN (IL)-1B (C) IL-10 AND (D) NUCLEAR FACTOR KAPPA-LIGHT-CHAIN-ENHANCER OF ACTIVATED B CELLS (NF-κB) FOR YOUNG (7.3±0.3 MONTHS) AND OLD (18.9±2.3 MONTHS) MALE C57BL/6 MICE FROM COHORT 1, 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (GREY BARS) OR SALINE (BLACK BARS). DATA EXPRESSED AS MEAN NORMALIZED TO YOUNG WILD-TYPE SALINE GROUP ±SEM. ONE-WAY ANOVA TNF-A, F(3,36)=6.28 p=0.002. ONE-WAY ANOVA IL-1B, F(3,36)=3.09 p=0.04. ONE-WAY ANOVA IL-10, F(3,36)=4.72 p=0.008. ONE-WAY ANOVA NF-κB, F(3,36)=4.03 p=0.015. *=P<0.05 COMPARED TO CORRESPONDING SALINE TREATED GROUP, #=P<0.05 COMPARED TO CORRESPONDING YOUNG GROUP WITH TUKEY’S HSD POST-HOC TEST. N= 3-10 PER GROUP .................................................................................................................67

FIGURE 2.8 mRNA EXPRESSION OF (A) CASPASE 3 (B) BAX (C) PEROxisome proliferator-activated receptor gamma COActivator 1 (PGC1)-A AND (D) PGC1-B FOR YOUNG (7.3±0.3 MONTHS) AND OLD (18.9±2.3 MONTHS) MALE C57BL/6 MICE FROM COHORT 1, 6 HOURS AFTER TREATMENT WITH 300MG/KG
PARACETAMOL (grey bars) or saline (black bars). Data expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA Caspase 3, non-significant. One-way ANOVA BAX, F(3,36)=5.46 p=0.004. One-way ANOVA PGC1-α, non-significant. One-way ANOVA PGC1-β, F(3,36)=4.21 p=0.013. *p<0.05 compared to corresponding saline treated group, #p<0.05 compared to corresponding young group with Tukey’s HSD post-hoc test. N=3-10 per group .............................................................. 69

**Figure 2.9** Correlation between frailty index and (A) serum alanine aminotransferase (ALT), (B) percentage liver necrosis, (C) liver cytochrome P450 (CYP)2E1 activity, (D) total liver glutathione (GSH), (E) serum total protein, (F) serum albumin, (G) liver weight (as % of body weight) and (H) serum alkaline phosphatase (ALP), for old (23.7±0.0 months) male C57BL/6 mice from cohort 2, 6 hours after treatment with 300mg/kg paracetamol (grey) or saline (black). N=6-8 per group .............. 71

**Figure 3.1** Serum alanine aminotransferase (ALT) concentration and proportion of each group with detectable liver necrosis and inflammation for young (A, C, respectively, 4±0.3 months) and old (B, D, respectively, 26.8±0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-Acetyl Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Serum and liver collected 3 hours after the final paracetamol/saline dose. Serum ALT data expressed as mean ± SEM. One-way ANOVA young mice ALT, F(7,44)=5.35 p=0.00. One-way ANOVA
OLD MICE ALT, F(7,38)=3.77 p=0.003. *p<0.05 compared to corresponding saline-treated group with Tukey’s HSD post-hoc test. N=4-8 per group

**FIGURE 3.2** DNA Fragmentation in liver homogenate from young (4 ± 0.3 months) and old (26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline. Liver collected 3 hours after the final saline dose. Data expressed as % relative to young control diet ± SEM. N=4-8 per group

**FIGURE 3.3** Representative haemotoxylin and eosin stained liver histology images for young (4 ± 0.3 months) and old (26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single dose of N-acetyl cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Liver collected 3 hours after the final paracetamol/saline dose. Images taken at 100-200X

**FIGURE 3.4** Paracetamol serum concentrations for young (A, 4 ± 0.3 months) and old (B, 26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-acetyl cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Serum collected 3 hours after the final paracetamol/saline dose. One-way ANOVA young mice, F(7,31)=10.96 p=0.00. One-way ANOVA old mice, non-significant. Data are expressed as mean ± SEM. *p<0.05 compared to control diet+paracetamol group with Tukey’s HSD post-hoc test. N=4-8 per group
**Figure 3.5** Total liver glutathione (GSH) concentrations for young (A, 4 ± 0.3 months) and old (B, 26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-acetyl cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Liver collected 3 hours after the final paracetamol/saline dose. Data are expressed as mean ± SEM. One-way ANOVA young mice, F(7,46)=11.91 p=0.00. One-way ANOVA old mice, F(7,37)=17.46 p=0.00. *p<0.05 compared to control diet+saline group, #p<0.05 compared to control diet+paracetamol group, ^p<0.05 compared to paracetamol diet+paracetamol group, ~p<0.05 compared to paracetamol diet+paracetamol+NAC group with Tukey’s HSD post-hoc test. N=4-8 per group. 

**Figure 3.6** Liver cytochrome (CYP)2E1 activity for young (A, 4 ± 0.3 months) and old (B, 26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-acetyl cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Liver collected 3 hours after the final paracetamol/saline dose. One-way ANOVA young mice, non-significant. One-way ANOVA old mice, F(7,23)=3.08 p=0.019. Data are expressed as mean ± SEM. *p<0.05 compared to control diet+saline group, #p<0.05 compared to control diet+paracetamol group, !p<0.05 compared to paracetamol diet+saline group. N=4-8 per group.
FIGURE 3.7 Correlation between frailty index and (A) alanine aminotransferase (ALT), (B) necrosis, (C) cytochrome (CYP)2E1 activity, (D) total liver glutathione (GSH), (E) serum total protein, (F) serum albumin, (G) liver weight (as % body weight), and (H) serum alkaline phosphatase (ALP) for old (26.8±0.5 months) male C57BL/6 mice treated with either control diet for 6 weeks and 3 days of saline (control group, black), paracetamol (1.33g/kg feed) diet for 6 weeks and 3 days of saline (chronic group, light grey) or control diet for 6 weeks and 3 days of paracetamol, then (250mg/kg x 3/day) (sub-acute group, dark grey). Liver and serum collected 3 hours after the final paracetamol/saline dose. N=4-7 per group .................................

FIGURE 4.1 Primary mouse hepatocyte viability as a percentage of Dulbecco’s modified eagle medium (DMEM) control (A, B), and total hepatocyte glutathione (GSH) (C) with ethanol (0.5%), N-acetyl cysteine (NAC, 20mM) and/or resveratrol (0.5% ethanol vehicle, 1-100µM) alone or plus paracetamol (20mM) treatment after 24 hours. Primary hepatocytes isolated from 4 month old male C57BL/6 mice via collagenase perfusion. Viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All data are presented as mean ± SEM of N=3-9 animals per group. #p<0.05 compared to DMEM treatment only, *p<0.05 compared to ethanol treatment group ..............

FIGURE 4.2 Representative images of primary mouse hepatocytes treated with (A) vehicle (0.5% ethanol), (B) 20mM paracetamol + 0.5% ethanol, (C) 20mM N-acetyl cysteine (NAC, in 0.5% ethanol), (D) 25µM resveratrol (in 0.5% ethanol), (E) 20mM NAC + 20mM paracetamol + 0.5% ethanol or (F)
25μM Resveratrol (in 0.5% ethanol) + 20mM Paracetamol, for 24 hours, fixed and stained with propidium iodide. Red arrows indicate nuclear PI staining. Primary hepatocytes isolated from 4 month old male C57BL/6 mice via collagenase perfusion. Images taken at 200x magnification on a Leica DM LB 100T microscope with attached Leica DFC480 digital camera (Leica, Sydney, Australia).

**Figure 4.3** Serum alanine aminotransferase (ALT) concentration for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle, resveratrol (RSV, 30mg/kg in corn oil), or N-acetyl cysteine (NAC, 1200mg/kg). Serum collected 6 hours after dosing. Data expressed as mean±SEM. *p<0.10 compared to saline+corn oil group. N=3-4 per group.

**Figure 4.4** Total liver glutathione (GSH) (A) and liver cytochrome (CYP)2E1 activity (B) for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle, resveratrol (RSV, 30mg/kg in corn oil), or N-acetyl cysteine (NAC, 1200mg/kg). Liver collected 6 hours after dosing. Data expressed as mean±SEM. *p<0.05 compared to saline+corn oil, saline+N-acetyl cysteine, saline+resveratrol and paracetamol+N-acetyl cysteine groups. N=3-4 per group.

**Figure 4.5** Western blots for 5’ adenosine monophosphate-activated protein kinase (AMPK), phosphorylated AMPK, and loading control 14-3-3 for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle.
OIL VEHICLE OR RESVERATROL (RSV, 30MG/KG IN CORN OIL). LIVER COLLECTED 6 HOURS AFTER DOSING.

**Figure 4.6** Phosphorylated 5' Adenosine Monophosphate-Activated Protein Kinase (PAMPK)/AMPK Protein Expression Ratio for Young (3.7±0.6 MONTHS) Male C57BL/6 Mice Treated with 700MG/KG Paracetamol (via Oral Gavage) or Saline Control, and Concurrent Corn Oil Vehicle or Resveratrol (RSV, 30MG/KG in Corn Oil). Liver collected 6 hours after dosing. Data expressed as mean±SEM normalised to saline-treated control. N=2-3 per group.

**Figure 4.7** Serum Alanine Aminotransferase (ALT) Concentration and Percentage of Liver with Necrosis for Young (A, C; 6.0±0.0 MONTHS) and Old (B, D; 18.0±0.0 MONTHS) Male C57BL/6 Mice Exposed to Either Control Diet or Resveratrol Diet (RSV, 1.33G/KG Feed) for 3-4 MONTHS, then Treated with Paracetamol (300MG/KG via Oral Gavage) or Saline. Liver and Serum Collected after 6 hours. Data expressed as mean±SEM. One-way ANOVA Young ALT F(3,20)=12.00 p=0.00. One-way ANOVA Old ALT, F(3,24)=4.96 p=0.009. One-way ANOVA Young Necrosis F(3,21)=14.974 p=0.00. One-way ANOVA Old Necrosis, F(3,22)=13.14 p=0.00. *p<0.05 compared to Corresponding Saline Group #p<0.05 compared to Control Diet+Paracetamol Group with Tukey’s HSD post-hoc test. N=4-8 per group.

**Figure 4.8** Representative Haemotoxylin and Eosin Stained Liver Histology Images for Young (6.0±0.0 MONTHS) and Old (18.0±0.0 MONTHS) Male C57BL/6 Mice Exposed to Either Control Diet or Resveratrol Diet (RSV, 1.33G/KG Feed) for 3-4 MONTHS, then Treated with Paracetamol (300MG/KG via Oral
FIGURE 4.9  Liver total glutathione (GSH) levels and cytochrome (CYP)2E1 activity for young (A, C; 6.0±0.0 months) and old (B, D; 18.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver collected after 6 hours. Data expressed as mean±SEM. One-way ANOVA young GSH F(3,22)=47.33 p=0.00. One-way ANOVA old GSH, F(3,24)=29.07 p=0.00. One-way ANOVA young CYP2E1, non-significant. One-way ANOVA old CYP2E1, non-significant. *p<0.05 compared to corresponding saline group. N=4-8 per group with Tukey’s HSD post-hoc test.

FIGURE 4.10  SIRTuin 1 (SIRT1), phosphorylated 5’ adenosine monophosphate-activated protein kinase (PAMPK)/AMPK ratio and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein expression for young (A, C, E; 6.0±0.0 months) and old (B, D, F; 18.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver collected after 6 hours. Data expressed as mean±SEM, normalised to saline-treated young control. One-way ANOVA young SIRT1, non-significant. One-way ANOVA old SIRT1, F(3,19)=7.14 p=0.003. One-way ANOVA young PAMPK/AMPK, F(3,22)=21.92 p=0.00. One-way ANOVA old PAMPK/AMPK, F(3,19)=13.71 p=0.00. One-way ANOVA young NF-κB, F(3,22)=2.48 p=0.092. One-way ANOVA old NF-κB, non-significant. *p<0.05 compared to
CORRESPONDING SALINE GROUP, #p<0.05 COMPARED TO CONTROL DIET SALINE GROUP WITH Tukey’S HSD post-hoc test. N=3-6 per group. .......................... 139

**FIGURE 5.1** LIVER SIRTUIN 1 (SIRT1) mRNA EXPRESSION (A), AND PROTEIN EXPRESSION (B), FOR YOUNG AND OLD MALE C57BL/6 WILD-TYPE (WT), LIVER-SPECIFIC SIRT1 OVER-EXPRESSOR (OE) AND LIVER-SPECIFIC SIRT1 KNOCK-OUT (KO) MICE, 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (VIA ORAL GAVAGE) OR SALINE. DATA EXPRESSED AS MEAN RELATIVE TO YOUNG WILD TYPE CONTROL±SEM. ONE-WAY ANOVA SIRT1 mRNA, F(5,26)=4.66 p=0.004. ONE-WAY ANOVA SIRT1 PROTEIN, F(5,27)=4.27 p=0.005. *=p<0.05 COMPARED TO CORRESPONDING AGE WILD-TYPE WITH TuKEY’S HSD POST-HOC TEST. N=3-10 PER GROUP FOR mRNA, N=5-6 PER GROUP FOR WESTERN BLOT (SHOWN ON GRAPH). ..157

**FIGURE 5.2** SERUM ALANINE AMINOTRANSFERASE (ALT) CONCENTRATIONS FOR YOUNG AND OLD MALE C57BL/6 WILD-TYPE (WT), LIVER-SPECIFIC SIRT1 OVER-EXPRESSOR (OE) AND LIVER-SPECIFIC SIRT1 KNOCK-OUT (KO) MICE, 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (VIA ORAL GAVAGE) OR SALINE. DATA EXPRESSED AS MEAN±SEM. ONE-WAY ANOVA, F(11,85)=5.38 p=0.000. *=p<0.05 COMPARED TO CORRESPONDING SALINE TREATED GROUP WITH TuKEY’S HSD POST-HOC TEST. N=4-15 PER GROUP. .................................................................... 158

**FIGURE 5.3** PREVALENCE OF NECROSIS (AS A PERCENTAGE OF EACH GROUP) FOR YOUNG AND OLD MALE C57BL/6 WILD-TYPE (WT), LIVER-SPECIFIC SIRT1 OVER-EXPRESSOR (OE) AND LIVER-SPECIFIC SIRT1 KNOCK-OUT (KO) MICE, 6 HOURS AFTER TREATMENT WITH 300MG/KG PARACETAMOL (VIA ORAL GAVAGE) OR SALINE. DATA EXPRESSED AS % OF GROUP. *=p<0.05 COMPARED TO CORRESPONDING SALINE TREATED GROUP. N=4-15 PER GROUP. .............................................................................. 159
**Figure 5.4** Representative Haemotoxylin and Eosin stained liver histology images for young and old male C57BL/6 wild-type, liver-specific SIRT1 over-expressor and liver-specific SIRT1 knock-out mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. Images taken at 100X.

**Figure 5.5** Cytochrome (CYP)2E1 activity (A) and CYP2E1 mRNA expression (B) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. For activity graph, data is expressed as mean ±SEM; for mRNA graph, data is expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA CYP2E1 activity, F(11,83)=2.62 p=0.007. One-way ANOVA CYP2E1 mRNA, F(11,67)=4.91 p=0.000. *p<0.05 compared to saline treated group group with Tukey’s HSD post-hoc test. n=4-15 per group for activity, n=3-10 per group for mRNA.

**Figure 5.6** NAD(P)H:quinone oxidoreductase 1 (NQO1) activity (A) and NQO1 mRNA expression (B) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. For activity graph, data is expressed as mean ±SEM; for mRNA graph, data is expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA NQO1 activity, F(11,83)=3.09 p=0.002. One-way ANOVA NQO1 mRNA, F(11,71)=6.75 p=0.000. *p<0.05 compared to saline treated group, #p<0.05 compared to young treated group, $p<0.05 compared to young saline group with Tukey’s
HSD post-hoc test. $N=4-15$ per group for activity, $N=3-10$ per group for mRNA.

**Figure 5.7** Total liver glutathione (GSH) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. Data is expressed as mean ± SEM. $N=4-15$ per group.

**Figure 5.8** mRNA expression of inflammatory markers; (A) Tumor necrosis factor (TNF)-α, (B) Interleukin (IL)-1β, (C) IL-10 and (D) Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mRNA expression for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice treated with saline or paracetamol (300mg/kg via oral gavage). Data expressed as mean normalized to young wild-type saline group ± SEM. One-way ANOVA TNFα, $F(11,71)=4.08$ $p=0.000$. One-way ANOVA IL-1β, $F(11,71)=1.71$ $p=0.09$. One-way ANOVA IL-10, $F(11,71)=2.66$ $p=0.008$. One-way ANOVA NF-κB, $F(11,71)=2.51$ $p=0.011$. *$p<0.05$ compared to saline treated group, #$p<0.05$ compared to young treated group with Tukey’s HSD post-hoc test. $N=3-10$ per group.

**Figure 5.9** mRNA expression of Caspase 3 (A) and BAX (B) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice treated with saline or paracetamol (300mg/kg via oral gavage). Data expressed as mean normalized to young wild-type saline group ± SEM. One-way ANOVA BAX, $F(11,71)=2.64$ $p=0.008$. One-way ANOVA Caspase 3, non-significant.
*=p<0.05 COMPARED TO SALINE TREATED GROUP, #=p<0.05 COMPARED TO YOUNG TREATED GROUP WITH TUKEY’S HSD POST-HOC TEST. N=3-10 PER GROUP. 

**FIGURE 5.10** mRNA EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA COACTIVATOR 1 (PGC1)-α (A) AND PGC1-β (B) FOR YOUNG AND OLD MALE C57BL/6 WILD-TYPE (WT), LIVER-SPECIFIC SIRT1 OVER-EXPRESSOR (OE) AND LIVER-SPECIFIC SIRT1 KNOCK-OUT (KO) MICE TREATED WITH SALINE OR PARACETAMOL (300MG/KG VIA ORAL GAVAGE). DATA EXPRESSED AS MEAN NORMALIZED TO YOUNG WILD-TYPE SALINE GROUP ±SEM. THREE-WAY (AGE, SIRT1, TREATMENT) ANOVA PGC1-α – TREATMENT GROUP F(1,60)=4.01 p=0.05. THREE-WAY ANOVA PGC1-β – TREATMENT GROUP F(1,60)=4.06 p=0.048, AGE GROUP F(1,60)=4.91 p=0.03. *=p<0.05 COMPARED TO SALINE TREATED GROUP, #=p<0.05 COMPARED TO YOUNG TREATED GROUP. N=3-10 PER GROUP.

**FIGURE 5.11** mRNA EXPRESSION OF SIRTUIN 1 (SIRT1) FOR YOUNG AND OLD MALE C57BL/6 WILD-TYPE (WT), LIVER-SPECIFIC SIRT1 OVER-EXPRESSOR (OE) AND LIVER-SPECIFIC SIRT1 KNOCK-OUT (KO) MICE TREATED WITH SALINE OR PARACETAMOL (300MG/KG VIA ORAL GAVAGE). DATA EXPRESSED AS MEAN NORMALIZED TO YOUNG WILD-TYPE SALINE GROUP ±SEM. ONE-WAY ANOVA, F(11,71)=4.94 p=0.000. *=p<0.05 COMPARED TO SALINE TREATED GROUP. N=3-10 PER GROUP.

**FIGURE 6.1** FRAILTY INDEX SCORES FOR OLD (26.8±0.5 MONTHS) MALE C57BL/6 MICE PRE AND POST SIX WEEKS OF BEING FED EITHER A CONTROL DIET, OR A DIET SUPPLEMENTED WITH PARACETAMOL (APPROXIMATELY 100MG/KG MOUSE/DAY, ‘PARA’). DATA IS PRESENTED AS MEAN ± 25 AND 75% QUARTILES. N=14-40 PER GROUP.
FIGURE 6.2 Frailty index scores for (A) male C57BL/6 and DBA/2J mice fed either an AL diet, or a 40% CR diet from age 6 months (19±2 months age, N=8-9) and (B) female C57BL/6 and DBA/2J mice fed either an AL diet, or a 40% CR diet (19±1 months age, N=9-11) from age 6 months. Data is presented as mean ± 25 and 75% quartiles. One-way ANOVA female mice, non-significant. One-way ANOVA male mice, F(3,30)=4.84 p=0.007. M= male; F= female; C57= C57BL/6; DBA= DBA/2J; AL= AD LIBITUM; CR= CALORIE RESTRICTED; * P < 0.05 with Tukey’s HSD post-hoc test. ............................................. 184

FIGURE 6.3 Frailty index scores for male C57BL/6 mice fed either a standard AIN-93G diet (SD), or an AIN-93G diet supplemented with resveratrol (RSV) (100mg/kg mouse/day) (N=16 SD, N=9 RSV, 24±0 months age) for 6 months from age 19 months. Data is presented as mean ± 25 and 75% quartiles. * P < 0.05. ........................................................................................... 185

FIGURE 6.4 The correlation between frailty index and latency to fall for (A1) rotarod, (A2) wire hang, and (A3) cage top functional testing in old (19±2 months) male C57BL/6 and DBA/2 mice (N=14) and the correlation between frailty index and latency to fall for (B1) rotarod, (B2) wire hang, and (B3) cage top functional testing in old (19±1 months) female C57BL/6 and DBA/2 mice (N=21). None of the correlations showed a significant association (P>0.05). ................................................................. 194

FIGURE 6.5 The percentage agreement of scoring for each item of the frailty index for all old male and female C57BL/6 and DBA/2J mice in cohort 1 across all 4 raters (black), and across raters 1 and 3 (grey). The dotted line shows a percentage agreement of 75% ......................................................... 196
LIST OF ABBREVIATIONS

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
3,4-methylenedioxyamphetamine (MDA)
5' adenosine monophosphate-activated protein kinase (AMPK)
Acute liver failure (ALF)
Adenosine monophosphate (AMP)
Adenosine triphosphate (ATP)
Alanine aminotransferase (ALT)
Alkaline phosphatase (ALP)
Analysis of covariance (ANCOVA)
Analysis of variance (ANOVA)
Aspartate aminotransferase (AST)
Bicinchoninic acid (BCA)
Bovine serum albumin (BSA)
c-Jun N-terminal kinase (JNK)
Cytochrome P450 (CYP450)
Damage-Associated Molecular Patterns (DAMPS)
Deoxyribonucleic acid (DNA)
Dichlorophenolindophenol (DCPIP)
Dimethyl sulfoxide (DMSO)
Dithiothreitol (DTT)
Drug induced liver injury (DILI)
Dulbecco’s modified eagle medium (DMEM)
Ethylene glycol tetraacetic acid (EGTA)
Ethylenediaminetetraacetic acid (EDTA)
Fetal calf serum (FCS)
Food and Drug Administration (FDA)
Foxhead box protein (FOXO)
Gamma glutamyltransferase (GGT)
Gastrointestinal tract (GIT)
Glutathione (GSH)
Hanks Balanced Salt Solution with Calcium (HBSS/+Ca²⁺)
Hanks Balanced Salt Solution without Calcium (HBSS/- Ca²⁺)
High mobility group box-1 (HMGB1)
High-performance liquid chromatography (HPLC)
Honest significance difference (HSD)
Hydrochloride (HCl)
Insulin-like growth factor-1 (IGF-1)
Interferon (IFN)
Interleukin (IL)
Intraclass correlation coefficient (ICC)
Keratin-18 (K18)
Lactate dehydrogenase (LDH)

Lipopolysaccharide (LPS)

Liver function tests (LFTs)

Liver sinusoidal endothelial cells (LSECs)

Liver-specific SIRT1 knock-out mice (SIRT1 KO)

Liver-specific SIRT1 over expressor mice (SIRT1 OE)

Mammalian target of rapamycin (mTOR)

Metaphosphoric acid (MPA)

microRNA (miR)

Mitochondrial permeability transition (MPT)

N-acetyl cysteine (NAC)

N-acetyl-p-benzoquinone imine (NAPQI)

NAD(P)H:quinone oxidoreductase 1 (NQO1)

National Institute on Aging (NIA)

Nicotinamide adenine dinucleotide (NAD+)

Nicotinamide phosphoribosyltransferase (NAMPT)

Non-alcoholic fatty liver disease (NAFLD)

Non-steroidal anti-inflammatory drugs (NSAIDS)

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

Pacific Laboratory Medicine Services (PaLMS)

Peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1)
Phosphate-buffered saline (PBS)

Phosphatidylinositol-3-kinase (PI(3)K)

Phosphorylated 5' adenosine monophosphate-activated protein kinase (pAMPK)

Propidium iodide (PI)

Protein kinase B (Akt)

Radioimmunoprecipitation (RIPA)

Resveratrol (RSV)

Sirtuin (SIRT)1

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Standard error of the mean (SEM)

Survey of Health, Ageing and Retirement in Europe (SHARE)

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

Triethanolamine (TEAM)

Tumor necrosis factor (TNF)

Tumor protein 53 (p53)

United Kingdom (UK)

United States (US)
LIST OF APPENDICES

APPENDIX 1 - FRAILTY INDEX ITEMS 242

APPENDIX 2 – PRIMER SEQUENCES 247
Chapter 1: Introduction

1 INTRODUCTION

1.1 Introduction

Paracetamol (acetaminophen) is a commonly used analgesic, especially in old age. The older population has a high prevalence of pain, and paracetamol is often the first-line analgesic prescribed to treat this pain (Makris et al. 2014; Nikles et al. 2005). Overexposure to paracetamol can cause acute liver toxicity (Larson 2007). Susceptibility to and mechanisms of paracetamol hepatotoxicity may change in old age, and in frailty, because of changes in pharmacokinetics, pharmacodynamics and homeostatic responses, although there are limited clinical and animal studies (Sarah J Mitchell, Kane, et al. 2011; Schmidt 2005). While the overall change in risk of toxicity in old age remains uncertain, there is evidence that there is a change in the type of exposure to paracetamol in old age. Older patients are more likely to have chronic and non-intentional exposures, rather than the acute and intentional exposures that are common in the younger population (Kane et al. 2012). The two most common reasons for accidental paracetamol overdose are poorly controlled pain (Michna et al. 2012), and the use of multiple paracetamol-containing medications (Civan et al. 2014), and both of these risk factors are common in the older population.
The increased rate of non-acute paracetamol overexposures in the older population is particularly problematic as the effectiveness of the current clinical paracetamol hepatotoxicity therapy, N-acetyl cysteine (NAC), in non-acute paracetamol exposures is unknown (Daly et al. 2008). Furthermore, clinical evidence has shown that there is a need for new paracetamol toxicity therapies, as there are patients, especially older people and those who present late to hospital or have staggered ingestions that still develop acute liver failure from paracetamol exposure and are unlikely to benefit from NAC (Craig et al. 2011; Ferner et al. 2011).

There has been an increasing body of research in the recent years investigating ageing mechanisms and pathways, and interventions that increase longevity. Two of these interventions are calorie restriction (the reduction in the daily intake of calories, without nutritional deficiencies), and the calorie-restriction ‘mimetic’, resveratrol (Bordone & Guarente 2005; Baur et al. 2006; Weindruch et al. 1986). The mechanisms of these interventions are still not fully understood but two of the main targets involved are the sirtuin (SIRT)1 and 5' adenosine monophosphate-activated protein kinase (AMPK) pathways (Le Couteur et al. 2012). There is increasing evidence to show that these ageing-related pathways also play a role in response to stresses, such as over-exposure to paracetamol (Michan & Sinclair 2007). There is some evidence from animal studies that resveratrol, and calorie restriction, protect against paracetamol toxicity (Harper et al. 2006; Masubuchi et al. 2009; Du et al. 2015). Exploration of these potentially protective targets, especially in old age, may provide novel insight into mechanisms of paracetamol toxicity development and new potentially protective therapies for all ages.
Chapter 1: Introduction

Frailty is an increasingly important concept in ageing research, with a recent shift of focus on the development of interventions to extend healthspan and delay frailty, rather than increase lifespan (Kirkland & Peterson 2009). The recent development of validated animal assessments of frailty (Whitehead et al. 2014; Liu et al. 2013), enables the effect of interventions, such as resveratrol, calorie restriction or exercise, on delaying or preventing frailty to be assessed in ageing animal models. Furthermore it allows the assessment of changes in vulnerability to stressors, such as paracetamol toxicity, to be assessed with both ageing and frailty in animal models.

1.2 Paracetamol Use and Toxicity

1.2.1 Paracetamol Use and Toxicity in Australia and Internationally
Paracetamol, also known as acetaminophen, is a widely used analgesic and antipyretic (Burke et al. 2009). It is believed to be safe and effective at therapeutic doses, but has serious overdose consequences, particularly hepatotoxicity (Larson 2007) with paracetamol hepatotoxicity being the most common cause of acute liver failure (ALF) in the United States (US) and the United Kingdom (UK) (Craig et al. 2011; Ostapowicz et al. 2002).

In Australia, paracetamol is one of the most commonly used medications (Morgan et al. 2011; Sood et al. 2013), and also the most common cause of acute liver failure (Gow et al. 2004), although the rates of liver failure appear to be less than in the UK (Hiles et al. 2015; Sood et al. 2013). A study of paracetamol overdoses at a regional Australian hospital found there were approximately 23 cases per 100,000 people each year (Ayonrinde et al. 2005), whilst a study of paracetamol poisoning and adverse events in Victoria, found there were 39-46 cases per 100,000 people per year in 2001 to 2006, and
Chapter 1: Introduction

26 deaths related to paracetamol toxicity over the seven year study period (Sood et al. 2013). One to two percent of patients with paracetamol overdose have associated nephrotoxicity (Boutis & Shannon 2001).

Paracetamol is available as both an over-the-counter medication, and a prescription medication for conditions including osteoarthritis and low back pain (Graham et al. 2013). It is available in several formulations both alone (as tablets, liquids, suppositories, slow-release) and in combinations with other medications including aspirin, non-steroidal anti-inflammatory drugs (NSAIDS), opioids, pseudoephedrine and antihistamines (MIMS 2013). The maximum recommended therapeutic dose of paracetamol in Australia is four grams per 24 hours (Larson 2007; Lancaster et al. 2014). The Food and Drug Administration (FDA) recently reduced the maximum recommended therapeutic daily dose to 3.25g in the US (Lee 2010).

1.2.2 Pharmacokinetics of Paracetamol Toxicity

Paracetamol is predominantly metabolised in the liver via the phase II conjugation pathways, with the glucuronide (55%) and sulphate (30%) conjugates the two predominant metabolites, which are excreted in the urine and bile (Burke et al. 2009). A small amount of paracetamol (5%) is also oxidised by the cytochrome P450 (CYP450) enzymes of the liver to the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) (Holt & Ju 2006). The most important isoform responsible for this CYP450 mediated metabolism is CYP2E1, but CYP3A4 and CYP1A2 are also involved (Lee et al. 1997). At therapeutic doses of paracetamol, NAPQI is immediately conjugated with hepatic glutathione (GSH) which de-toxifies it and allows it to be excreted via the kidneys (Mitchell et al. 1973). NAPQI may also be reduced by NAD(P)H:quinone oxidoreductase 1 (NQO1) back into paracetamol (Moffit et al. 2007). Following an
overdose of paracetamol, there is saturation of the conjugation metabolism pathways and GSH depletion (Larson 2007). Build-up of the toxic NAPQI occurs which can result in liver cell damage, predominately centrilobular necrosis (Jollow et al. 1973) (Figure 1.1).

Renal toxicity can also occur, following paracetamol overdose. The mechanisms of this are not well understood but may involve renal CYP2E1 enzymes or nephrotoxic glutathione-conjugates (Mazer & Perrone 2008). The focus of this thesis is paracetamol-induced hepatotoxicity.

**Figure 1.1** Pharmacokinetics and Pharmacodynamics of Paracetamol Hepatotoxicity.

CYP2E1 = Cytochrome 2E1, NAPQI = N-acetyl-p-benzoquinone imine
1.2.3 Pharmacodynamics of Paracetamol Hepatotoxicity
The mechanisms of paracetamol-induced liver injury involve the initial formation of the toxic metabolite, NAPQI, the mitochondrial dysfunction associated with this metabolite, the induction of cell death, and an inflammatory response (Jaeschke et al. 2012).

1.2.3.1 Role of Mitochondria in Paracetamol Hepatotoxicity
Mitochondria play a central role in the development of paracetamol hepatotoxicity in both mice and humans (McGill et al. 2012). Mouse studies have shown that NAPQI covalently binds to cellular proteins, particularly in the mitochondria (Jollow et al. 1973; Myers et al. 1995) and results in induction of mitochondrial oxidant stress (Jaeschke 1990) and the formation of peroxynitrate (Hinson et al. 1998). These reactive oxygen and nitrogen species cause mitochondrial protein and deoxyribonucleic acid (DNA) damage, and activate c-Jun N-terminal kinase, JNK, resulting in its phosphorylation and translocation to the mitochondria, which amplifies the oxidant stress (Saito, Zwingmann, et al. 2010; Hanawa et al. 2008). This oxygen and peroxynitrite stress results in permeabilization of the outer membrane of the mitochondria either by the formation of mitochondrial permeability transition (mPT) pores at low levels of stress, or by another mPT independent mechanism with higher levels of chemical stress (Loguidice & Boelsterli 2011). This ultimately causes inhibition of respiration, depletion of adenosine triphosphate (ATP) and a decrease in membrane potential (Masubuchi et al. 2005; Kon et al. 2004). This process results in the release of proteins such as apoptosis-inducing factor, endonuclease G and cytochrome C from the mitochondria, which translocate to the nucleus and cause nuclear DNA fragmentation (Jaeschke & Bajt 2006). These processes trigger necrotic or apoptotic cell death (Jaeschke et al. 2012). A similar process is believed to occur in humans, although there is only indirect evidence such as reduced serum glutathione after paracetamol treatment (Lauterburg & Mitchell 1987) and the detection of
paracetamol-protein adducts (Muldrew et al. 2002; Davern et al. 2006) and mitochondrial and nuclear DNA fragments in serum of patients after paracetamol overdose (Mcgill et al. 2012).

Autophagy is a cellular process by which the cytoplasm and organelles are sequestered into vesicles and delivered to the lysosome, where they are broken down and the macromolecules recycled (Glick et al. 2010). There has been recent evidence of a role of autophagy in paracetamol hepatotoxicity (Ni et al. 2012). Although the exact mechanisms are still not known, it is thought that the production of reactive oxygen species can induce autophagy (Ding et al. 2010; Y. Chen et al. 2008), which acts to remove mitochondria damaged by NAPQI. Further pharmacological induction of autophagy was protective against paracetamol hepatotoxicity (Ni et al. 2012), and loss of autophagy increases paracetamol induced hepatotoxicity (Igusa et al. 2012).

1.2.3.2 Necrosis and Apoptosis in Paracetamol Toxicity
Cell death in paracetamol toxicity is predominantly caused by cell necrosis, characterised by nuclear breakdown, release of cell contents and enzymes and cell swelling (Gujral et al. 2002).

The extent to which apoptotic cell death contributes to the development of paracetamol hepatotoxicity is controversial (Malhi et al. 2006). Paracetamol treatment involves factors including the translocation of Bax and Bid (death proteins involved in the apoptosis pathway) to the nucleus (Adams et al. 2001; Bajt et al. 2008), the release of cytochrome C from the mitochondria (El-Hassan et al. 2003), DNA fragmentation (Cover et al. 2005) and positive cell terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining (Lawson et al. 1999), which are historically
Chapter 1: Introduction

characteristic of apoptosis. Jaeschke et al. (2012) claim that none of these parameters are in fact specific for apoptosis, and there is no evidence of caspase activation (Mcgill et al. 2012; Adams et al. 2001) probably due to ATP depletion (Antoine et al. 2010) so question the role of apoptosis in paracetamol toxicity. However, a recent clinical observational study, found that hepatic apoptosis was seen in the early stages of paracetamol induced acute liver failure (as ATP levels were not completely depleted), and serum levels of apoptosis-associated markers (caspase-cleaved CK-18) were associated with poor outcomes (Possamai et al. 2013).

1.2.3.3 Inflammatory Response in Paracetamol Toxicity

The formation of NAPQI, and the subsequent mitochondrial damage is an important initiating factor for paracetamol induced liver injury. It has been suggested, however, that it is the inflammatory immune response, and the balance between the protective and toxic signalling processes of the cells involved in this response that determines the severity and progression of liver injury (Antoine et al. 2008). Studies suggest that hepatocyte stress or death, as a result of the reactive metabolite induced damage, causes the release of signals (known as Damage-Associated Molecular Patterns, DAMPS) that stimulate activation of the innate immune cells of the liver (Holt & Ju 2006; Jaeschke et al. 2011). These cells produce pro-inflammatory cytokines and mediators such as tumor necrosis factor TNF-α, interleukin IL-1β and interferon IFN-γ (Ishida et al. 2002; Bourdi et al. 2002; Gardner et al. 2003). Other mediators released by these immune cells are protective and anti-inflammatory such as IL-10 (Bourdi et al. 2002) and IL-6 (Masubuchi et al. 2003). Kupffer cells and neutrophils can also generate reactive oxygen species (Jaeschke & Smith 1997; Liu et al. 1995). However there is much disagreement between the studies and the exact role of each of the cell types and mediators in drug induced liver injury (DILI) generally, as well as in paracetamol-
induced liver injury, have yet to be fully determined (Holt & Ju 2006; Jaeschke et al. 2012)

1.2.4 Clinical Assessment of Paracetamol Toxicity
An acute paracetamol overdose is defined as a single dose of 10-15g in adults (Larson 2007). Clinically, acute paracetamol overdose is associated with three main stages. The first lasts for approximately 24 hours and involves non-specific gastrointestinal symptoms such as nausea, vomiting and abdominal pain with minimal elevation in serum liver enzyme concentrations. The second stage, from 24-72 hours, involves few clinical symptoms although there is release of enzymes from the liver (the mechanism of which is actually unknown), most notably elevation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations. The third stage develops in the next 24 hours (72-96 hours) and the symptoms and outcomes vary from full recovery to death depending on the severity of the liver damage (Larson 2007; Lancaster et al. 2014).

In a clinical setting, if there is suspected paracetamol toxicity, blood tests are performed including a paracetamol level, liver function tests (LFTs) and coagulation profile to assess the degree of liver damage (Daly et al. 2008). Clinical signs of paracetamol toxicity include elevated AST and ALT serum concentrations (up to many 1000s U/L in cases of extreme paracetamol-induced liver toxicity), as well as abnormal results for the other LFTs (Larson 2007) and high serum concentrations of paracetamol. Peak serum concentrations after therapeutic doses do not usually exceed 130μmol/L (20 mg/L) (Daly et al. 2008; Graham et al. 2013).
Chapter 1: Introduction

Although these blood tests can provide some evidence of the extent of the liver damage, better indicators of prognosis at presentation and during treatment, are needed to guide treatment choice (Ferner et al. 2011; Rumack & Bateman 2012). Antoine et al. (2013), identified serum microRNA(miR)-122 (a highly liver specific mRNA), high mobility group box-1 (HMGB1, a marker of necrosis) and keratin-18 (K18, a marker of necrosis) measured at first presentation to hospital, as better predictors of acute liver injury, than serum ALT or paracetamol concentration. Other potential biomarkers of paracetamol induced liver damage are reviewed by McGill & Jaeschke (2014).

1.2.5 Current treatment: N-Acetyl Cysteine

The current first line therapy for paracetamol hepatotoxicity is N-acetyl-cysteine (NAC). NAC prevents liver toxicity by increasing glutathione stores as well as by acting as a glutathione substitute that itself binds to NAPQI to de-toxify it (Corcoran, Racz, et al. 1985), although recent evidence has also shown it can also protect by maintaining mitochondrial energy metabolism (Saito, Zwingmann, et al. 2010). Table 1.1 outlines the changes to factors involved in the paracetamol toxicity pathway with NAC treatment. Clinically, the Rumack-Matthew nomogram is used to guide the treatment of acute paracetamol overdose provided time since overdose is known (Rumack & Matthew 1975). Those with serum paracetamol concentrations above the treatment line will be administered NAC, a glutathione precursor, orally or intravenously (Larson 2007). Cases in the UK of patients with levels below this treatment line not being given NAC, and developing acute liver failure, has led the UK to decrease this treatment threshold by approximately 50% (Lancaster et al. 2014; Bateman 2015). The treatment regimen for NAC is complex, requiring body weight adjustments and several different intravenous infusions, and errors are common (Ferner et al. 2001). NAC can also cause side-effects in up to 45% of patients, ranging from nausea to anaphylaxis (Kerr et al. 2005), and the risk of these reactions is inversely
related to serum paracetamol levels (Waring et al. 2008). As such it is important to optimise NAC treatment and not use it in patients for whom it will be ineffective or unnecessary (Bateman 2015).

NAC guidelines are designed for protection against a single high dose of paracetamol and there is little evidence to guide risk assessment and optimal treatment for staggered over-exposures, chronic exposures or repeated supra-therapeutic exposures (Daly et al. 2008; Ferner et al. 2011). One animal study found that several weeks of NAC pre-treatment protected against toxicity induced by twice-weekly paracetamol dosing (Chen et al. 2012), but post-treatment has not been studied. Patients with these types of paracetamol exposures, especially late presentation to hospital or repeated paracetamol ingestion, are also more at risk of adverse outcomes including liver failure (Craig et al. 2012; Daly et al. 2008; Ferner et al. 2011).

The presence of side effects, high incidence of treatment errors, and the lack of evidence to guide NAC treatment in non-acute exposures had led to much research into other potential therapeutics to prevent or treat paracetamol hepatotoxicity. These include many natural product extracts with anti-inflammatory or anti-oxidant capacities (Jaeschke et al. 2011; Zhang 2015; Imaeda & Watanabe 2009), compounds which act on the liver sinusoidal endothelial cells (Lin et al. 2010), that provide mitochondrial protection (Reid et al. 2005) and compounds that prevent the promotion of oxidant stress in the hepatocytes (Saito, Yan, et al. 2010). The potential for longevity and ageing-related interventions to protect against paracetamol toxicity will be further explored in section 1.4.5.
Table 1.1 Changes to factors and mechanisms involved in paracetamol toxicity pathway with factors that may increase or decrease the risk of paracetamol toxicity: N-Acetyl cysteine treatment, Ageing, Frailty, Resveratrol treatment and Calorie Restriction. ‘-’ indicates this effect is not applicable, ‘?’ indicates this effect is not yet known, ‘↑’ indicates the effect is an increase, ‘↓’ indicates the effect is a decrease. ALF=Acute Liver Failure, ALT= Alanine aminotransferase, CYP450=cytochrome P450, NQO1= NAD(P)H:quinone oxidoreductase 1, NAPQI= N-acetyl-p-benzoquinone imine, AMPK= 5' adenosine monophosphate-activated protein kinase, SIRT1= Sirtuin 1

<table>
<thead>
<tr>
<th>Paracetamol Use and Toxicity-Related Mechanisms</th>
<th>Change with factors explored in this thesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-Acetyl cysteine</td>
</tr>
<tr>
<td>Paracetamol Use</td>
<td>Common</td>
</tr>
<tr>
<td>- Type of Exposure</td>
<td></td>
</tr>
<tr>
<td>Acute, sub-acute and chronic</td>
<td>No evidence for use in non-acute exposure (Daly et al. 2008)</td>
</tr>
</tbody>
</table>

Pharmacokinetics:
<table>
<thead>
<tr>
<th>CYP450 activity</th>
<th>Produces toxic metabolite, NAPQI</th>
<th>(Ahmad et al. 2013)</th>
<th>(Mach et al. 2014) or (Hunt et al. 1998)</th>
<th>?</th>
<th>(Wang et al. 2015; Wu et al. 2013)</th>
<th>(Chilakapati et al. 2007; Alterman et al. 1993)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NQO1 activity</td>
<td>Converts NAPQI to paracetamol</td>
<td>?</td>
<td>(Mach et al. 2014; Chen et al. 1994)</td>
<td>?</td>
<td>(Hsieh et al. 2006)</td>
<td>(De Cabo et al. 2004)</td>
</tr>
<tr>
<td>Pharmacodynamics:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrosis</td>
<td>Predominant mode of cell death</td>
<td>(Corcoran, Racz, et al. 1985; Rumack &amp; Matthew 1975)</td>
<td>(Rikans &amp; Moore 1988)</td>
<td>?</td>
<td>(Du et al. 2015; Wang et al. 2015)</td>
<td>?</td>
</tr>
<tr>
<td>Mitochondrial Function</td>
<td>Inhibition of respiration and a decrease in membrane potential.</td>
<td>(Saito, Zwingmann, et al. 2010)</td>
<td>(Gonzalez-Freire et al. 2015)</td>
<td>(Mohler et al. 2014; Moore et al. 2010)</td>
<td>(Baur &amp; Sinclair 2006; Lagouge et al. 2006; Du et al. 2015)</td>
<td>(López-Lluch et al. 2006; Martin-Montalvo &amp; Cabo 2012)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Age-related Mechanisms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- AMPK expression/activation</td>
<td>Recent studies suggest reduces.</td>
<td>?</td>
<td>↑ (Mulligan et al. 2005) or ↓ (Salminen &amp; Kaarniranta 2012; Reznick et al. 2007)</td>
<td>?</td>
<td>↑ (Price et al. 2012; Baur &amp; Sinclair 2006)</td>
<td>↑ (Cantó &amp; Auwerx 2011; Greer et al. 2007)</td>
</tr>
<tr>
<td>- SIRT1 expression/activation</td>
<td>Unknown.</td>
<td>?</td>
<td>Tissue dependent ↓ or ↔ (Le Couteur &amp; Sinclair 2010; Ferrara et al. 2008)</td>
<td>↓ or ↔ (Le Couteur et al. 2011; Kumar et al. 2014)</td>
<td>↑ (Hubbard et al. 2013)</td>
<td>↑ (Cohen et al. 2004) although not in all tissues/experiments (Le Couteur et al. 2012)</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.2.6 Risk Factors for Paracetamol Hepatotoxicity
There are several factors that are believed to increase the risk of paracetamol toxicity, mostly due to glutathione depletion, or through the induction of CYP2E1. Alcoholism, and/or the regular consumption of large amounts of alcohol, are thought to increase the risk of paracetamol toxicity by both inducing CYP450 enzymes, and reducing total liver glutathione levels (Whitcomb & Block 1994; McClain et al. 1980; Myers et al. 2008), although the true nature of this risk remains controversial (Graham et al. 2013; Dart et al. 2010). Malnutrition and fasting are believed to increase the risk of paracetamol toxicity as a result of decreased liver glutathione (Kondo et al. 2012; Whitcomb & Block 1994). Early studies have implicated obesity with an increased risk of paracetamol toxicity with obesity (Corcoran & Wong 1987), although this was probably attributable to weight-based dosing as recent studies have shown either no change or a decreased risk of toxicity in obesity (Rutherford et al. 2006; Radosevich et al. 2013). However, non-alcoholic fatty liver disease (NAFLD) has been associated with increased risk of paracetamol toxicity due to increased CYP2E1 activity (Kucera et al. 2012; Tarantino et al. 2007; Michaut et al. 2014). Long-term use of other medications that induce CYP450 enzymes may also increase paracetamol toxicity risk, including isoniazid, phenobarbital, St Johns Wort and rifampicin (Ferner et al. 2011). Several genetic polymorphisms in enzymes of the paracetamol metabolism pathway have also been associated with increased paracetamol toxicity risk (Zhao & Pickering 2011; Court et al. 2013). Interestingly, changes in the gut microbiome have also been shown to have a role in paracetamol toxicity (Possamai et al. 2015).

The effect of increased age and frailty on paracetamol toxicity risk is unclear, and current literature is summarised in section 1.3.3.4 and in Table 1.1. Unintentional
Chapter 1: Introduction

overdose and staggered exposure to paracetamol have also been associated with increased risk of toxicity and are further discussed in section 1.2.7.

1.2.7 Toxic Exposures: Intentional vs. Unintentional, Acute vs. Chronic

Intentional paracetamol overdoses are the most common type of exposure, and thus are the most commonly studied. With this type of exposure, provided a patient presents to hospital in a timely manner, and is given NAC, the rates of mortality and need for liver transplant are low (Larson 2007). The rates of unintentional overdoses are increasing, however, and are particularly problematic as they are potentially preventable, and associated with increased risk of adverse outcomes (Craig et al. 2011). It is reported that 45-55% of paracetamol overdoses in the US are unintentional (Larson et al. 2005; Bower et al. 2007; Michna et al. 2010), and Australian studies have found the rates of unintentional overdoses are between 5% (Ayonrinde et al. 2005) and 15% (Sood et al. 2013). Sood et al. (2013) also identified that patients with unintentional overdoses were more likely to develop acute liver failure, and although only 1% of overdoses in their study resulted in death, 34% of the deaths were due to accidental exposures. The two main causes of unintentional overdose appear to be the unknowing use of multiple paracetamol containing medications (Civan et al. 2014), or an increase in the use of paracetamol containing medications for poorly managed pain (Michna et al. 2012).

Cases of hepatotoxicity due to acute paracetamol exposures are also the most common, and extensively studied. However there are many other types of paracetamol exposure including chronic therapeutic exposure, chronic supra-therapeutic exposure and staggered overexposures that have been less extensively researched. There has been recent evidence emerging of mild liver damage in some people with therapeutic levels of paracetamol, since a clinical trial found that 31-44% of healthy volunteers taking the
recommended therapeutic paracetamol dose (4g/day) for 14 days had increased serum ALT activity (Watkins et al. 2006). A systematic review of observational studies in the UK suggested a considerable degree of paracetamol toxicity especially at the upper end of standard analgesic doses (Roberts et al. 2015). Blieden et al. (2014) report that long-term paracetamol overdose due to chronic pain is very common in the US, with up to 6% of adults prescribed more than four grams of paracetamol per day. A case study (Lane et al. 2002) and a large-scale retrospective hospital study (Civan et al. 2014), also highlight the increased risk of chronic paracetamol over-exposure in patients taking multiple paracetamol-containing medications. Alarmingly, patients with these types of chronic or staggered exposures to paracetamol are not only common, but also appear to be at higher risk of severe adverse outcomes. Two studies in the UK found that compared to acute over-exposures, staggered over-exposures to paracetamol were associated with an increased risk of liver failure (Craig et al. 2012; Ferner et al. 2011), and a Spanish study showed that patients with chronic paracetamol exposure compared to acute were much more at risk for hepatotoxicity and acute liver failure (Tong et al. 2015).

1.2.8 Animal Models of Paracetamol Toxicity
Animal models of paracetamol toxicity have been extensively developed and studied, since the 1970s (Jollow et al. 1973; Mitchell et al. 1973). Many of the studies which led to crucial knowledge about the pharmacokinetics and pharmacodynamics of acute paracetamol toxicity, discussed in sections 1.2.2 and 1.2.3 above, were completed in animal models. Acute paracetamol toxicity animal models are commonly used as generalised DILI models to look at potentially protective therapeutics (Jaeschke et al. 2011), and gain increased understanding of DILI mechanisms (Jaeschke et al. 2012).
Chapter 1: Introduction

Rats and mice are the most commonly used models of paracetamol toxicity, although there has been recent evidence for the use of rabbits and zebrafish (Zubairi et al. 2014; Maciejewska-Paszek et al. 2007). Rats and mice are usually fasted overnight before acute paracetamol treatment to normalise the liver glutathione levels (Price et al. 1987). A recent study suggested that mice are a more clinically relevant model of paracetamol toxicity than rats, due to similar susceptibility to toxicity and similar mechanisms of damage, particularly mitochondrial dysfunction and JNK activation (McGill et al. 2012). However, there has been recent concern about differences in paracetamol toxicity study outcomes even in mice of the same strain and genetic background (Bourdi et al. 2011; Jaeschke et al. 2012). Mossanen & Tacke (2015) draw attention to the importance of paracetamol solution preparation, fasting period, routes of administration, sex and housing conditions on paracetamol toxicity outcomes. There have been several studies that have found differences in paracetamol-induced liver and kidney toxicity in male compared to female mice, with male mice having greater susceptibility (Hoivik et al. 1995; Hu et al. 1993; Mohar et al. 2014). Other studies have suggested that differences in the gut microbiome may explain some differences in susceptibility to paracetamol toxicity in genetically identical mice, due to changes in metabolism and inflammatory response (Possamai et al. 2015; Kubes & Mehal 2012). Furthermore, some studies have identified genetic differences between strains of mice believed to be genetically identical, due to genetic drift in the colonies at different breeding facilities, creating substrains (Bourdi et al. 2011; Zurita et al. 2011; Mekada et al. 2009). In particular, Bourdi et al. (2011) found that C57BL/6 mice from the Jackson laboratory, compared to the National Institutes of Health laboratories, were genetically different substrains (as assessed by Nnt genotyping), and had, as yet, unexplained differences in susceptibility to paracetamol toxicity, which affected the interpretation of results about
Chapter 1: Introduction

the role of JNK2 in paracetamol toxicity. Thus care must be taken in interpreting results, and comparing outcomes across mouse studies of paracetamol toxicity, particularly in regards to mouse substrains.

Two rat studies, and one mouse study, showed that low-dose paracetamol administered daily did not cause liver toxicity in the absence of other risk factors (Yisarakun et al. 2014; Kondo et al. 2012; de Meijer et al. 2012). Several rat and BALB/c mouse studies have found that pre-treatment with non-toxic doses of paracetamol for 4-8 days before exposure to a high dose of paracetamol can protect against hepatotoxicity, through reduction in CYP2E1 activity, and increased glutathione levels among other mechanisms (Ghanem et al. 2009; O’Brien et al. 2000; Shayiq et al. 1999). However, another rat study found that pre-treatment 24 hours earlier with a moderate dose of paracetamol in fact increased the degree of hepatotoxicity to a secondary paracetamol exposure, due to increased CYP2E1 activity (Kim et al. 2009)

1.3 Ageing and Frailty

Older people are generally considered to be those aged 65 years and older. The older population is increasing, and is in fact the fastest growing group of the population (Louria 2005; Christensen et al. 2009). Ageing is the major risk factor for many diseases including arthritis, cancer and dementia, and is also associated with an increased use of drugs and increased risk of adverse drug reactions (Hajjar et al. 2007; McLean & Le Couteur 2004a). Ageing is also associated with both polypharmacy (the use of more than five drugs), frailty (Morgan et al. 2011; Hilmer et al. 2007; Collard et al. 2012) and physiological changes with ageing that affect pharmacokinetics and pharmacodynamics (section 1.3.2).
Chapter 1: Introduction

Frailty is a state of high vulnerability for adverse broader health outcomes, and a reduced capacity to react to stressors (Collard et al. 2012). It is a geriatric syndrome, associated with decreased muscle mass and quality (known as sarcopenia), and altered hormonal and inflammatory functions (Mohler et al. 2014; Evans et al. 2010; Fried et al. 2009; Hubbard et al. 2009). Frailty increases the risk of falls, dependency, disability, institutionalisation, hospitalisation and mortality (Clegg et al. 2013; Mohler et al. 2014; Romero-Ortuno 2013), and affects drug use patterns, pharmacokinetics, pharmacodynamics and toxicity (Hilmer et al. 2007; Hubbard et al. 2013). Ten to fifteen percent of community dwelling Australians aged over 65 years are frail (Blyth et al. 2008; Hyde et al. 2010), and a systematic review found an overall worldwide prevalence of 10.7% (with a range of 4-59% depending on the population) in those aged 65 and over (Collard et al. 2012). The prevalence of frailty increases with age, and it is estimated that 25-50% of those aged over 85 years are frail (Collard et al. 2012; Clegg et al. 2013). There is very little current understanding about the molecular basis of frailty, its development and its mechanistic association with ageing, although there is increasing evidence that the physiological and functional changes in frailty are distinct from the changes seen with normal ageing, and other associated comorbidities (Mohler et al. 2014). The most common hypotheses about the pathophysiology of frailty are inflammatory cytokine dysregulation (Collerton et al. 2012; Walston et al. 2008), and mitochondrial dysregulation (Mohler et al. 2014), although it is likely that frailty involves a combination of physiological factors (Hubbard & Theou 2012).

Clinically, frailty can be assessed using a number of scales with varying degrees of difficulty and clinical applicability (de Vries et al. 2011). Two of the mostly commonly used frailty assessments are the phenotype model and the frailty index. The phenotype model was developed by Fried et al. (2001) based on data from the Cardiovascular
Chapter 1: Introduction

Health Study. It defines frailty as the presence of three or more criteria including weight loss, exhaustion, weakness, slow walking speed, and low physical activity (Fried et al. 2001). It conceptualises frailty as a functional phenotype, and defines patients are non-frail, pre-frail or frail. The frailty index was developed by Rockwood and colleagues and measures the proportion of accumulated deficits in a person, and focuses on the number of deficits rather than the precise nature of the deficits (Mitnitski et al. 2002; Rockwood & Mitnitski 2007). The frailty index conceptualises frailty as a multidimensional syndrome, defined by deficits accumulated over the lifetime (Hubbard & Theou 2012). It calculates frailty index as a continuous variable between 0 and 1, however cut-offs have been established to define patients as frail or non-frail (Rockwood et al. 2011; Song et al. 2010). There is no international consensus on the best definition for frailty or best assessment for frailty identification clinically (Rodríguez-Mañas et al. 2013).

1.3.1 Animal Models of Ageing and Frailty

Animal models of ageing have been used for decades to investigate both the underlying mechanisms of ageing, and interventions to increase lifespan and healthspan (Mitchell et al. 2015). Studies of ageing mechanisms and lifespan interventions in humans are limited by our long lifespans, ethical issues and extensive genetic and environmental diversity. The relatively short lifespans, reduced genetic diversity, control of environmental factors and the ability to tests a variety of outcomes including stress responses, metabolic outcomes, behavioural and functional assessments and serum, urine and tissue analysis make animal models invaluable tools in the study of ageing (Mitchell et al. 2015).
C57BL/6 mice are the classical inbred mouse strain commonly used for ageing research due to the wealth of phenotypic and genotypic information available (source: http://phenome.jax.org/), and similar age-related changes as are seen in humans including reduced activity and vascular, bone and inflammation changes (Ingram 2000; Sindler et al. 2011; Rowlatt et al. 1976; Turturro et al. 2002). C57BL/6 mice live for approximately 26-30 months, and they are responsive to interventions that extend lifespan such as calorie restriction and pharmaceuticals (Goodrick 1975; Forster et al. 2003). Other strains of mice are also often studied in ageing research as models of different ageing progressions. For example the DBA/2J substrain is considered to be short-lived, with studies showing median lifespan for males to be 23-25 months (Forster et al. 2003; Yuan et al. 2011). DBA/2J mice have also been shown to be unresponsive to interventions that extend lifespan, such as calorie restriction (Forster et al. 2003). Inbred mouse strains, due the control of both genetic and environmental factors, allow conclusions to be made about outcomes and interventions in small sample sizes. Outbred mouse strains have more genetic diversity, and as such may be considered more representative of the human population, but have limitations in assessing outcomes (Mitchell et al. 2015). Sex can also have a large impact on outcomes of both inbred and outbred mouse strains and should be carefully considered in ageing research projects (Mitchell et al. 2015; Yuan et al. 2009; Kanfi et al. 2012; Strong et al. 2008). There are also a wide variety of transgenic mouse models of either premature ageing, for example Werner syndrome and Cockayne syndrome mice (Lebel & Leder 1998; Scheibye-Knudsen et al. 2012), or delayed ageing, for example Growth Hormone receptor knockout mice and Snell dwarf mice (Flurkey et al. 2001; Hsieh et al. 2002).

Rats have also been used as ageing models, usually inbred Fischer 344 rats (Do Carmo & Cuello 2013), but their use in ageing studies is limited as they are usually less
Chapter 1: Introduction

convenient and cost-effective than mice, and transgenic models are less available (Mitchell et al. 2015). Other less common models of ageing include naked mole rats, which have preserved health for most of their lifespan (Jarvis 1981; Liang et al. 2010), fish (Gerhard 2007), birds (Holmes & Ottinger 2005) and non-human primates (Colman et al. 2010; Mattison et al. 2012). The use of non-human primates in ageing studies, although perhaps the most appropriate for translation of research into humans, is limited by their long lifespans as well as the associated ethical and cost issues (Mitchell et al. 2015).

There has been a recent focus on animal models to study frailty in the context of mouse ageing (Howlett & Rockwood 2014). The current mouse models of frailty are summarised in Table 1.2. Interleukin (IL)-10 knock-out mice were developed as a colitis model, but were identified as showing a frailty phenotype including inflammation, reduced muscle strength (Walston et al. 2008), impaired cardiac and vascular function (Sikka et al. 2013) and increased mortality (Ko et al. 2012). Although not specifically a model of naturally occurring frailty in old age, these mice have significant potential in studies of the molecular mechanisms of frailty (Akki et al. 2014). Liu and colleagues developed a mouse frailty index based on the clinical phenotype model which assesses a mouse as frail based on its grip strength, walking speed, physical activity and endurance (Liu et al. 2013). As with the clinical phenotype models a mouse is defined as non-frail, mildly frail or frail according to their performance in these assessments. They used this phenotype-based model to investigate the effects of exercise on frailty, and found that voluntary exercise reversed frailty in old mice (Graber et al. 2014). Recently, a mouse frailty index which utilizes 31 simply assessed clinical measures to assess frailty in C57BL/6J mice was developed (Whitehead et al. 2014; Feridooni et al. 2014). The validity of this scale was correlated against human
Chapter 1: Introduction

frailty index data from the Survey of Health, Ageing and Retirement in Europe (SHARE) and was found to have good agreement. As with the human frailty index assessments, this tool allows the calculation of a frailty index score for a mouse on a continuous variable from 0 to 1, whereby a higher score indicates a greater degree of frailty. As these assessment tools are recently developed, long-term studies with outcomes such as mortality have not been conducted, and these frailty assessment tools have not yet been validated against one another. These tools could be invaluable in assessing the effect of any intervention on the important clinical outcome of frailty in an animal model, or could be used to assess the effect of frailty on outcomes.
**Table 1.2** Animal models of frailty: details of the animal types, assessments, clinically equivalent scales, validation, interventional results, strengths and limitations of the currently developed mouse models of frailty. IL=interleukin.

<table>
<thead>
<tr>
<th>Article</th>
<th>Name of model</th>
<th>Animal details</th>
<th>Frailty Assessment details</th>
<th>Clinical basis for model development</th>
<th>Validation and Interventional Studies?</th>
<th>Strengths</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walston et al. (2008)</td>
<td>IL-10 knockout mice</td>
<td>IL-10&lt;sup&gt;−/+&lt;/sup&gt; mice on a C57BL/6 background</td>
<td>-</td>
<td>Inflammation in Frailty</td>
<td>Decline in strength and increase in inflammation compared to age-matched controls.</td>
<td>Can be used to explore biological mechanisms of frailty</td>
<td>Is not a model of frailty in natural ageing, and requires specific housing conditions.</td>
</tr>
<tr>
<td>Parks et al. (2012)</td>
<td>Mouse Frailty Index</td>
<td>Male and female C57BL/6 mice</td>
<td>31 health-related variables including activity levels, hemodynamic measures, body composition and metabolic status</td>
<td>Frailty Index (Mitnitski et al. 2002; Kulminski et al. 2008)</td>
<td>Correlation with age-related declines in cardiac myocytes.</td>
<td>Assesses across a variety of health parameters.</td>
<td>Invasive procedures eg large blood samples, and specialised equipment</td>
</tr>
<tr>
<td>Whitehead et al. (2014)</td>
<td>Mouse Clinical Frailty Index</td>
<td>Male and female C57BL/6 mice</td>
<td>31 ‘clinical’ items (see appendix 1)</td>
<td>Frailty Index (Mitnitski et al. 2002; Kulminski et al. 2008)</td>
<td>Same exponential function as human data from SHARE trial. Good inter-rater reliability (Feridooni et al. 2014)</td>
<td>Convenient, fast, non-invasive measures</td>
<td>Does not include a functional assessment aspect.</td>
</tr>
<tr>
<td>Study</td>
<td>Scoring System</td>
<td>Mouse Strain</td>
<td>Functional Assessment</td>
<td>Sarcopenia in Frailty</td>
<td>Age-related Declines</td>
<td>Combined Score</td>
<td>Notes</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------</td>
<td>------------------</td>
<td>-----------------------</td>
<td>-----------------------</td>
<td>----------------------</td>
<td>----------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>Graber et al.</td>
<td>C57BL/6 Neuromuscular healthspan scoring system</td>
<td>Male C57BL/6 mice</td>
<td>Rotarod and grip strength, in vitro muscle contractility.</td>
<td>Sarcopenia in Frailty</td>
<td>Age-related declines in individual outcomes.</td>
<td>Combined score reduces individual variability within groups.</td>
<td>Invasive procedure and time-consuming measurements.</td>
</tr>
<tr>
<td>Liu et al. (2013)</td>
<td>Frailty Phenotype Index</td>
<td>Male C57BL/6 mice</td>
<td>Rotarod, grip strength, voluntary wheel running and derived endurance score</td>
<td>Frailty Phenotype (Fried et al. 2001)</td>
<td>Prevalence same as humans of equivalent age. Voluntary exercise intervention reversed frailty (Graber et al. 2014)</td>
<td>Non-invasive assessments. Provides a cut-off to assess mice as frail or non-frail.</td>
<td>Time-consuming measurements, and includes derived factors.</td>
</tr>
</tbody>
</table>
1.3.2 Pharmacokinetic, Pharmacodynamic and Toxicological Changes with Ageing and Frailty

As older people are the most common users of medications (Hajjar et al. 2007), and usually excluded from clinical trials (Nair 2002; Le Quintrec et al. 2005), it is important to understand the potential changes in drug pharmacokinetics and pharmacodynamics with age and frailty, in order to optimise drug treatment in all older adults (Hilmer et al. 2007). Furthermore, adverse drug reactions are increased in older people, and are often more severe than in younger patients (Walker & Wynne 1994; Atkin et al. 1999), probably due to both pharmacokinetic and pharmacodynamic changes in old age (Routledge et al. 2004) as well as an increase in polypharmacy (the use of 5 or more medications) in old age (Veehof et al. 1999; Gnjidic et al. 2012). Furthermore falls, cognitive impairment and functional decline as a result of drug exposure are increased in old age (Agostini et al. 2004; Leipzig 1999; Moore & Keeffe 1999), and are usually not considered adverse drug reactions (Hilmer et al. 2007).

The most significant pharmacological change with increasing age is increased heterogeneity of responses to medications (McLachlan et al. 2009). General changes in absorption, distribution, metabolism and elimination of drugs in older adults have also been described (Klotz 2009; McLean & Le Couteur 2004b; Mangoni & Jackson 2004). There are age related changes in drug absorption from the gastrointestinal tract (GIT), although the effect of this on drug dosing is thought to be minimal (McLean & Le Couteur 2004b). The distribution of drugs can be effected by changes in body composition that occur in old age. Most notably, in old age there is an increase in percentage body fat, and a decrease in total body water which results in an increase in the volume of distribution of lipophilic drugs, and a decrease in the volume of distribution of water soluble drugs (Klotz 2009). Clearance describes the rate of
elimination of a drug from the blood (provided metabolism is not saturated). Hepatic clearance is affected by many factors including liver size, liver blood flow and metabolism. There is a decrease of up to 35% in liver blood flow and size from young adulthood to old age (Wynne et al. 1989). Furthermore, the liver sinusoidal endothelial cells (LSECs) undergo ultra-structural changes with old age, termed pseudocapillarisation which may affect the transfer of oxygen, drugs and other substrates between the sinusoidal blood and the hepatocytes (Le Couteur et al. 2001; Le Couteur et al. 2008). The implications of this age-related reduction in hepatic clearance are increased bioavailability for drugs with a significant first-pass effect, and reduced clearance of drugs with significant hepatic metabolism, which may result in increased risk of adverse drug reactions, or for pro-drugs requiring metabolic activation, may cause reduced efficacy in old age (Hilmer et al. 2007). Phase II drug metabolism was originally thought to not change in healthy ageing (Wynne et al. 1990; Summerbell et al. 1993), although recent studies suggest there is a decrease (Butler & Begg 2008). Although in old age there is a decrease in the total hepatic CYP450 content, there is no decrease in the in vitro activity of most of the CYP450 enzymes (Hilmer et al. 2007). Studies have shown that in old age there is reduced renal function, particularly glomerular filtration rate, that affects the renal clearance of some drugs (Mangoni & Jackson 2004) although other studies claim that renal clearance does not change in old age in the absence of diseases such as hypertension, chronic heart disease or kidney disease (McLean & Le Couteur 2004b). Pharmacodynamic changes with age result from general age-related physiological changes in the target organ systems and can result in either increased or decreased sensitivity to drug responses (Hilmer et al. 2007; McLean & Le Couteur 2004b; Bowie & Slattum 2007). For example, changes in the beta-adrenergic receptors in old age can result in reduced response to beta blockers (Abernethy et al. 1987), but physiological changes in old age can result in increased
Chapter 1: Introduction

sensitivity to sedatives such as benzodiazepines (Hilmer et al. 2007; Greenblatt et al. 2004).

Pharmacokinetic and pharmacodynamic changes in frailty have not been extensively studied, and very little is known about drug dosing optimisation or toxicity risk in frailty (Hubbard et al. 2013). Frailty is associated with polypharmacy, independently of ageing, so understanding changes to medication effects and toxicity is especially important in the frail older population, due to their extensive exposure to medicines (Gnjidic et al. 2013; Gnjidic et al. 2012). From ageing studies we also know that the risk of adverse drug reactions in old age, increases in those populations more likely to be frail such as those who are in residential aged care. Twenty percent of community dwelling older adults have experienced adverse drug reactions compared to fifty percent of those living in residential care facilities (Wilcox et al. 1994; Somers et al. 2010). The heterogeneity in physiology and morbidity with old age, is further increased in frailty, resulting in even more unpredictable responses to medicines (McLachlan et al. 2009; Fried et al. 2001). Johnston et al. (2014) identified only ten studies of drug pharmacokinetic and pharmacodynamics changes with frailty, although some general changes with frailty can be predicted from known physiological changes. It has been suggested that changes in drug absorption from the gastrointestinal tract that are mildly seen in old age, might be more significant in frailty (Hubbard et al. 2013). Frailty is also associated with increased body fat and reduced lean body mass, which may affect the volume of distribution of lipophilic drugs (Hubbard et al. 2010). There is a reduction in serum albumin in frailty, that may effect the protein binding of acidic drugs such as phenytoin or warfarin, and result in an increase in availability of unbound drug in the serum (Hubbard et al. 2013). It is also known that there is a reduction in phase II metabolism in frailty, but not healthy ageing, for some drugs (Hubbard et al. 2013),
Chapter 1: Introduction

including paracetamol and metoclopramide (Wynne et al. 1990; Wynne et al. 1993). There is also reduced activity of esterases, enzymes responsible for the metabolism of drugs such as aspirin, procaine and anaesthetics, in frailty, but not healthy ageing (Summerbell et al. 1990; Hubbard et al. 2008). Frailty is also often associated with renal impairment and kidney diseases, so there may be reduced renal clearance in frailty (Hubbard et al. 2013). The clearance of gentamicin, an antibiotic predominantly excreted by glomerular filtration in the kidneys, is reduced in frail compared to non-frail older people (Hilmer et al. 2011; Johnston et al. 2014).

1.3.3 Changes in the Pharmacology and Toxicology of Paracetamol in Ageing and Frailty

Changes to factors involved in the paracetamol toxicity pathway including use, pharmacokinetics, pharmacodynamics and toxicity outcomes, with ageing and frailty are summarised in Table 1.1.

1.3.3.1 Paracetamol Use in Ageing and Frailty

Paracetamol use is prevalent in the older and frail populations. Paracetamol is one of the most commonly prescribed, and used, drugs among older Australians (Pearson et al. 2007). Seventy five percent of older Australian participants in the Pearson et al. (2007) study received at least one paracetamol prescription over the 6 year study period, and it is suggested that 20% of Australians over 50 use daily simple analgesics including paracetamol (Morgan et al. 2011). It is the first line analgesic for many age-related diseases including osteoarthritis and chronic pain (Makris et al. 2014; Toms 2008; Nikles et al. 2005; Jordan et al. 2003). Pain is common in the older population (Blyth et al. 2001; Thomas et al. 2007), and frailty has been associated with increased pain, and analgesic, including paracetamol, use in studies from Finland, the US and Australia (Koponen et al. 2013; Blyth et al. 2008; Kemp et al. 2005). Pain in the older population is also often under-treated (Horgas & Tsai 1998; Won et al. 2004), and it is important to
understand the pharmacokinetic and pharmacodynamics changes in paracetamol exposure in old age and frailty to be able to optimise the efficacy and safety of analgesics in this population.

1.3.3.2 Paracetamol Pharmacokinetics in Ageing and Frailty

Absorption of paracetamol, does not appear to change with increasing age, although the changing body composition in old age may result in a decrease in volume of distribution for water-soluble drugs such as paracetamol (Klotz 2009). Clearance of paracetamol from the liver appears to be reduced in old age due to both reduced transfer of paracetamol across the liver sinusoidal endothelium in old age (Mitchell, Huizer-Pajkos, et al. 2011) and generally reduced liver flow and size in old age (Wynne et al. 1989). The activity of CYP2E1, the enzyme responsible for the production of toxic NAPQI does not appear to change in old age (Hunt et al. 1990). However, a clinical study of therapeutic paracetamol after major surgery found that there was an increased shift towards this pathway of metabolism in old age (Pickering et al. 2011). Hepatic glutathione, important for the detoxification of NAPQI, is decreased with ageing in animals (Hazelton & Lang 1980) and human hepatic glutathione and the serum levels of its associated enzymes are reportedly decreased in ageing human blood samples (Erden-Inal et al. 2002). NQO1 activity is also found to increase in old age (Mach et al. 2014; Chen et al. 1994; Kamzalov & Sohal 2004).

Paracetamol pharmacokinetic changes with frailty have not been extensively studied. Early studies suggested that there was reduced paracetamol clearance via the phase II metabolic pathways in frail older patients, compared to fit older patients (Wynne et al. 1990), and a recent study saw older surgical patients had reduced clearance of iv paracetamol compared to a younger patient group (Liukas et al. 2011). An observational
Chapter 1: Introduction

study of very old inpatients with polypharmacy and seven day paracetamol exposure, found that the pharmacokinetics of paracetamol did not change over seven days and there was no accumulation of paracetamol (Bannwarth et al. 2001).

1.3.3.3 Paracetamol Pharmacodynamic in Ageing and Frailty
As the pharmacodynamics of paracetamol are still not fully understood, the changes with age and frailty have not been extensively studied (summarised in Table 1.1). It is known that there is impairment of mitochondrial function with old age (reviewed in (Gonzalez-Freire et al. 2015), which results in increased reactive oxygen species production which can cause damage at high levels, but at low levels may results in increased stress resistance and, in fact, contribute to longevity (Owusu-Ansah et al. 2013; Martin-Montalvo & Cabo 2012). There is also an age-related reduction in mitochondrial number and volume (Short et al. 2005; Conley et al. 2000). The effect of these age-related mitochondrial changes on paracetamol pharmacodynamics in not known. Ageing is associated with alteration of the liver intrinsic death pathway signalling (Mach et al. 2015) and increased apoptosis (Molpeceres et al. 2007; Zhang & Herman 2002). Changes in apoptosis in ageing may contribute to other liver diseases (Wang 2014), although the effect on drug induced liver injury is not well understood. There is also an age-related decline in autophagy (Cuervo et al. 2005), which is induced as a primarily protective mechanism following paracetamol toxicity. There are significant changes to the immune system with age (Starr & Saito 2014; Miller 2012) including immunosenescence which is defined as an age-related decline in the immune response to external stimuli, such as bacteria and viruses (Aw et al. 2007). More importantly for paracetamol toxicodynamics, there are also changes to the innate immune response in old age termed inflammaging, in which there is increased low grade inflammation and circulating cytokine levels, as well as dysregulation of innate
Chapter 1: Introduction

immune cell responses (Franceschi et al. 2000; Walston et al. 2002; Franceschi et al. 2007; Cohen 2003). In animal studies, in particular, there is increased response of both pro and anti-inflammatory cytokines including IL-10 and TNF-alpha, in response to stimuli in old compared to young animals (Tateda et al. 1996; Starr et al. 2013; Okamura et al. 2012; Saito et al. 2003), although in humans there appears to be no initial difference in cytokine induction but a prolonged response after activation by stressors such as sepsis or lipopolysaccharide (LPS) (Starr & Saito 2014). Further mitochondrial, oxidative stress or inflammatory dyregulation in frailty (Mohler et al. 2014; Fried et al. 2001; Walston et al. 2008; Serviddio et al. 2009; Wu et al. 2009) may affect paracetamol pharmacodynamics and toxicodynamics in frailty.

1.3.3.4 Paracetamol Toxicity in Ageing and Frailty
The consequences of the above-described changes with ageing on the hepatotoxicity of paracetamol are not fully understood, and were recently reviewed (Mitchell, Kane, et al. 2011). There are few clinical studies of paracetamol toxicity in ageing and frailty. One study found that although the majority of paracetamol induced hepatotoxicity occurs in young adults, the majority of deaths associated with paracetamol exposure occur in old age (Schmidt 2005). Although, another study of hospitalised fit and frail older patients found that frail older people taking therapeutic paracetamol for five days had higher blood paracetamol concentrations than younger patients, without associated increased liver enzymes (Mitchell et al. 2011)

An Australian study identified that the older population have a different clinical presentation of paracetamol toxicity to younger patients, with older people more likely to have chronic low-dose exposure or accidental over-exposure to paracetamol, than acute or intentional exposures (Kane et al. 2012). The increase in both medication use,
Chapter 1: Introduction

and chronic pain in the older population may contribute to this finding as the two main causes of unintentional overdose are thought to be multiple paracetamol containing medications, or poorly managed pain (Civan et al. 2014; Michna et al. 2012). Another Australian study specifically looked at the ability of arthritis patients to recognise paracetamol-containing medications, found that older people were often not able to do this (Ellis et al. 2015). Malnutrition is a risk factor for paracetamol toxicity, as it can lower glutathione liver stores, and is common in old age and frailty (Hickson 2006; Kaiser et al. 2010).

There have been several animal studies, using Fischer 344 rat studies that have shown a decreased risk of acute paracetamol toxicity in old age (Rikans & Moore 1988), due to a reduction in CYP2E1 activity (Mach et al. 2014) or reduced transfer into the liver (Mitchell, Huizer-Pajkos, et al. 2011). Studies in Sprague Dawley rats, however, found an increased susceptibility to toxicity in old rats (Tarloff et al. 1996). This may have been related to increased dosing due to weight-gain in this ageing model (Mach et al. 2014). The only mouse studies investigating the effect of old age on paracetamol toxicity, did not look at direct toxicity outcomes, but suggested reduced glutathione capacity in old age (Chen et al. 1990; Al-Turk & Stohs 1981).

1.4 Interventions to Slow Ageing and Frailty

1.4.1 Lifespan and Healthspan Interventions
There has been much research in the last 20 years about interventions to increase lifespan and promote longevity (Le Couteur et al. 2012; Kenyon 2010; Sinclair & Guarante 2006; Sebastiani et al. 2012). As old age is the major risk factor for many diseases including arthritis, cancer and dementia, it was hypothesised that by delaying the overall ageing process we could delay all age-related diseases, rather than targeting
Chapter 1: Introduction

specific diseases (Olshansky et al. 2007). Even more recently, there has been a focus on finding interventions that can increase a person’s healthspan, rather than their lifespan (Kirkland & Peterson 2009). It is increasingly being recognised that it is important to not just increase longevity but also to delay frailty, increase function and maintain independence and quality of life (Curtis & Cabo 2013). Healthspan is a term that can be used to describe this outcome as how long a person lives healthily with reasonable quality of life (Curtis & Cabo 2013).

1.4.2 Calorie Restriction and the Ageing-Related Molecular Pathways

The most extensively studied intervention in increasing lifespan is calorie restriction. The mechanisms of this intervention have been extensively studied, especially in animal models, and several main molecular pathways involved in the ageing process have been identified and investigated as targets for interventions to delay lifespan and healthspan.

Calorie restriction involves the ingestion of a fully nutritious diet with a reduced amount of calories (50-80% of ad libitum diet), and was first studied in the 1920s (Le Couteur et al. 2012). It was the first laboratory intervention shown to extend life span in many species including yeast, fruit flies, worms and mammals (Bordone & Guarente 2005; Speakman & Mitchell 2011), as well as reduce the risk of age-related diseases in mammals (Weindruch et al. 1986; Everitt et al. 2006). Recent work has shown that the macronutrient balance, rather than just the restriction of calories, is also important for the lifespan and healthspan outcomes (Solon-Biet et al. 2014; Solon-Biet et al. 2015). There have been several molecular pathways implicated in the lifespan and healthspan increasing effects of calorie restriction. Four of the most commonly researched pathways are insulin-like growth factor-1 (IGF-1), sirtuins, mammalian target of
rapamycin (mTOR), and 5' adenosine monophosphate-activated protein kinase (AMPK) (Le Couteur et al. 2012) (Figure 1.2).

**Figure 1.2** Interaction of four of the pathways implicated in the lifespan and healthspan increasing effects of calorie restriction (source: Solon-Biet et al. (2015)). mTOR= Mammalian target of rapamycin, AMPK= 5' adenosine monophosphate-activated protein kinase, SIRT1= Sirtuin 1, IGF1= Insulin-like growth factor-1, NAMPT= Nicotinamide phosphoribosyltransferase, PGC1α= Peroxisome proliferator-activated receptor gamma coactivator 1, FOXO= Foxhead box protein, p53= Tumor protein 53, AKT= Protein kinase B.

Calorie restriction causes reduced glucose, insulin, IGF-1 and increased insulin sensitivity (Anisimov 2003; Argentino et al. 2005). The downregulation of these pathways in calorie restriction is believed to cause some of the beneficial healthspan
Chapter 1: Introduction

and lifespan effects, although the exact mechanisms are still not known (Speakman & Mitchell 2011). It may be related to oxidative stress (Lambert & Merry 2004) or autophagy (Bergamini et al. 2003). One of the main identified targets of IGF-1 is the phosphatidylinositol-3-kinase/protein kinase B (PI(3)K/Akt) pathway, which is important for cell growth and survival (Dudek et al. 1997; Rommel et al. 2001).

The sirtuins are a class of nicotinamide adenine dinucleotide (NAD+) dependent deacylases (Wagner & Hirschey 2014) that regulate proteins related to longevity, energy metabolism and cell survival (Michan & Sinclair 2007). These proteins have a range of downstream deacylase target proteins including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Rodgers et al. 2005; Gerhart-Hines et al. 2007) and foxhead box protein O/tumor protein 53 (FOXO/p53) (Giannakou et al. 2004; Hori et al. 2013). PGC-1α is a transcriptional co-activator which regulates mitochondrial biogenesis and function (Liang & Ward 2006), FOXO proteins are transcription factors that are phosphorylated by factors such as Akt signalling, and involved in the apoptosis pathways, stress resistance and gluconeogenesis (Hori et al. 2013; Calnan & Brunet 2008), and p53 is tumor suppressor protein, also involved in apoptosis (Fridman & Lowe 2003). SIRT1 is believed to be activated in calorie restriction (Cohen et al. 2004), although SIRT1 expression is not increased with calorie restriction in all tissues or all studies (Le Couteur et al. 2012). However, SIRT1 knock out mice have a decreased life span that is not extended by calorie restriction, and SIRT1 over-expressing mice have similar effects to calorie restriction, implying a critical role of SIRT1 in calorie restriction mechanisms (Boily et al. 2008; Bordone et al. 2007). Important roles of SIRT3 and 6 have also been identified in calorie restriction (Kanfi et al. 2012; Shi et al. 2005), with SIRT3 playing a particular role in the
Chapter 1: Introduction

mitochondrial antioxidant response system (Someya et al. 2011). Studies have shown interactions between SIRT1 and the other ageing-related pathways including mediation of insulin sensitivity (Sun et al. 2007) and inhibition of mTOR signalling (Ghosh et al. 2010).

mTOR is a specific protein that forms a family of kinase complexes responsive to stress, nutrients and growth factors, with the defining feature that they bind the drug rapamycin, which allosterically inhibits their kinase activity (Speakman & Mitchell 2011). The mTOR complexes can be broadly grouped into mTORC1 and mTORC2 based on their binding partners. mTORC1 is the complex involved in autophagy, and is believed to be important in ageing. It acts as a nutrient sensor (Zoncu et al. 2011), that inhibits autophagy and promotes protein synthesis, cell growth and division (Fingar et al. 2004; Wang 2006) in the presence of nutrients, amino acids and glucose, and has the opposite effect when nutrients levels are low (Harrison et al. 2009). Autophagy is thus induced by inhibition of mTOR, and the process generates nutrients during fasting. The mechanisms by which mTOR acts to increase lifespan and healthspan are not clear but may involve a prevention of an age-related decline in autophagy (Bergamini et al. 2003; Johnson et al. 2013), the promotion of mitochondrial function via PGC1-alpha with mTOR inhibition (Cunningham et al. 2007), an anti-inflammatory action (Johnson et al. 2013) or the preservation of stem cell function (Yilmaz et al. 2012; Chen et al. 2009). There are also clear studies to show the interaction of the mTOR pathway with the other ageing pathways, and it has been hypothesised that mTOR may be the central nutrient sensing pathway, through which the IGF-1 and AMPK pathways act (Le Couteur et al. 2012; Johnson et al. 2013).
Chapter 1: Introduction

AMPK is an enzyme, (a trimer with 3 subunits) that is allosterically activated by adenosine monophosphate (AMP), and is also regulated by upstream kinases and other factors (Speakman & Mitchell 2011). It is essentially a sensor of the energy status of a cell, so is activated (phosphorylated) by increased AMP, and reduced adenosine triphosphate (ATP), as is seen with calorie restriction (Edwards et al. 2010; Cantó & Auwerx 2011). AMPK activation results in phosphorylation of downstream target protein PGC-1α (Cantó & Auwerx 2009; Wan et al. 2014), and an increase in expression of nicotinamide phosphoribosyltransferase (NAMPT), which is an enzyme involved in regulating NAD+ content (Brandauer et al. 2013; Fulco et al. 2009). The AMPK pathway is changed in old age (Salminen & Kaarniranta 2012), and the mechanism by which AMPK acts to increase lifespan and healthspan is also not completely understood. AMPK activation results in the promotion of fat oxidation, and a reduction in fatty acid synthesis in the liver, and a shift from carbohydrate to fat metabolism (Hardie 2011). There is also interaction of AMPK with the other ageing pathways, including inhibition of mTOR, promotion of glycolysis and activation of SIRT1 (in certain conditions) (Price et al. 2012).

1.4.3 Calorie Restriction Mimetics

Calorie-Restriction mimetics are drugs with the potential to replicate the beneficial effects of calorie restriction, without the need for actual restriction of calories (Le Couteur et al. 2012). Many drugs have been developed targeting the four main pathways of calorie restriction, although many act across several of these pathways (Le Couteur et al. 2012; Athar et al. 2009).
Chapter 1: Introduction

The IGF-1/Insulin pathway was targeted with biguanides such as metformin, phenformin and pioglitazone, that are insulin sensitisers (Tosca et al. 2010; Anisimov 2003). Metformin, in particular was shown to cause lifespan extension (Martin-Montalvo et al. 2013), but there are some side effect concerns. Rapamycin acts to inhibit the mTOR proteins and thus induce autophagy (Ballou & Lin 2008). It has been shown to extend lifespan in mouse studies (Harrison et al. 2009; Miller et al. 2011) although there are immunosuppressant side effect concerns, which would limit its clinical use.

Resveratrol, a polyphenol found in grapes, was shown to be an activator of SIRT1 in a large scale screening study (Howitz et al. 2003). Resveratrol has been shown to extend lifespan in high-fat diet-fed mice (Baur et al. 2006), and increase healthspan but not longevity in standard diet-fed mice (Pearson et al. 2008; Miller et al. 2011) and Werner mice (Labbé et al. 2011). Resveratrol has also been shown to play a protective role in many age-related diseases including diabetes (Palsamy & Subramanian 2009), cancer (Jang et al. 1997), cardiovascular disease (Rimbaud et al. 2011) and many neurological conditions (Yu et al. 2012). Resveratrol is known to have a variety of protective effects including effects on DNA repair, apoptosis, inflammation, insulin secretion, mitochondrial biogenesis (Baur & Sinclair 2006; Lagouge et al. 2006; B. P. Hubbard et al. 2013; King et al. 2005; Park et al. 2012). The mechanism of resveratrol’s action is controversial (Borra et al. 2005; Kaeberlein et al. 2005) and it is likely there are several relevant targets. A recent study clearly showed that one of resveratrol’s mechanisms of action is direct allosteric SIRT1 agonism (Hubbard et al. 2013). Resveratrol may also activate AMPK via phosphorylation at Thr172 (Baur et al. 2006; Ido et al. 2015; Suchankova et al. 2009), although AMPK activation by resveratrol appears to be dependent on SIRT1 at low doses, but independent of SIRT1 at high doses (Price et al.
Chapter 1: Introduction

2012). The protein complex, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which regulates DNA transcription in response to stimuli such as stress, is thought to be inhibited by resveratrol (Ren et al. 2013). Other SIRT1 agonists have also been developed and investigated, such as SIRT1720 (Mitchell et al. 2014), as resveratrol has a relatively low bioavailability (Baur & Sinclair 2006; Walle 2011) and activates a variety of targets (Speakman & Mitchell 2011).

Although it was definitely not developed as a calorie restriction mimetic, and its mechanism in this capacity has not been investigated there is some evidence to show that paracetamol may have beneficial healthspan effects. Mouse studies have shown that paracetamol was able to normalise glucose homeostasis in mouse models of diabetes (Shertzer et al. 2008; Kendig et al. 2008), and a study of rat aorta showed that paracetamol protected against age-related effects (Rice et al. 2012). Furthermore, AMPK activation has been seen to be affected by paracetamol in other studies (Saberi et al. 2014; Kim et al. 2015) demonstrating a potential effect of paracetamol on these ageing-related pathways.

1.4.4 Interventions to Delay Frailty
The increasing focus on improving healthspan and quality of life, rather than lifespan, with interventions has also led to an increased focus on interventions to delay frailty. Possible interventions that have been investigated include exercise training (strength, endurance and balance training) (Theou et al. 2011; Cadore et al. 2013), dietary interventions (protein, calories, amino acids, fish oil, vitamins) (Mohler et al. 2014), reduced polypharmacy (Tjia et al. 2013), and some pharmaceuticals, usually hormones or drugs to target muscle wastage (Neto et al. 2013; Muller et al. 2006; Kenny et al. 2010; Srinivas-Shankar et al. 2010; Hennessey et al. 2001).
A systematic review of exercise interventions for frailty, assessed by physical function, found that of 19 completed trials, overall exercise was associated with improved gait speed and improved short physical performance battery, but there was no conclusive effect on balance, endurance, and activities of daily living functional mobility (Giné-Garriga et al. 2014). Another review of specific frailty interventions identified eight high quality studies, and found that exercise interventions improved outcomes in 5/6 studies, hormone replacement therapy had no effect and multifactorial interventional studies were inconclusive (Lee et al. 2012). A third review of randomised control trials of frailty interventions, although frailty was not necessarily the primary outcome, found 38 studies, with overall small sample sizes and short follow-up. Overall the studies found that exercise training was beneficial, although often the benefits were lost once the program stopped, and that some pharmaceutical and dietary interventions improved bone mineral density and lean body mass (Bibas et al. 2014). An Australian study of a multifactorial personally-targeted intervention randomised control trial with the frailty phenotype and mobility as the primary outcomes found a 15% difference between intervention and control groups (Cameron et al. 2013), and are following this up with a pre-frailty interventional study (Cameron et al. 2015). There have also been a variety of interventional studies aimed at improving sarcopenia, a distinct feature of frailty (Walston 2012). These are usually nutritional and exercise interventions (Fiatarone et al. 1994; Timmerman et al. 2012; Aagaard et al. 2010).

Mouse studies of frailty interventions are very limited. Graber et al. (2014) used the mouse frailty phenotype assessment to show that voluntary exercise reversed frailty in old mice. Another mouse study showed that losartan, an angiotensin II antagonist,
improved activity, inflammation and oxidative stress in old mice, although it didn’t assess frailty as a specific outcome (Lin et al. 2014). The recently developed mouse frailty assessment tools (Liu et al. 2013; Whitehead et al. 2014) provide an ideal opportunity to investigate the effect of a wide range of interventions on frailty as an outcome in mouse models.

1.4.5 Ageing Mechanisms as Potential Targets to Reduce Paracetamol Toxicity

1.4.5.1 Calorie Restriction and Paracetamol Toxicity
Calorie restriction involves several mechanisms that may also be involved in the paracetamol toxicity pathways (summarised in Table 1.1). With calorie restriction there is increased NQO1 activity (De Cabo et al. 2004), potentially increased phase II metabolism (Leakey et al. 1989) and increased glutathione capacity (as calorie restriction involves a reduction in calories, but not a loss of nutritional balance) (Cho et al. 2003; Mune et al. 1995), which may all be pharmacokinetic protective mechanisms against paracetamol toxicity. Furthermore, calorie restriction is associated with reduced oxidative stress (Lambert & Merry 2004; Yu & Chung 2001; Lin et al. 2004), increased mitochondrial function/biogenesis (López-Lluch et al. 2006; Martin-Montalvo et al. 2011), decreased inflammation (Ye & Keller 2010; J. Wang et al. 2013) and increased autophagy (Harrison et al. 2009; Morselli et al. 2010; Kume et al. 2010), which may also contribute towards paracetamol toxicity protection.

Two animal studies have found that calorie restriction protects the liver against paracetamol hepatotoxicity. Harper et al. (2006) found that young BALBc X C57BL/6 mice fed a calorie restriction diet for 8 months had reduced ALT and lactate dehydrogenase (LDH) levels after toxic paracetamol dosing, compared to those fed *ad libitum*. Another study found that a methionine-deficient diet resulted in similar
Chapter 1: Introduction

protection from paracetamol hepatotoxicity in mice (Miller et al. 2005), although again ALT and LDH were the only measured outcomes. A human study found that patients on a calorie restricted diet receiving two grams of paracetamol daily for five days had no change in paracetamol metabolism or liver function tests, compared to control patients (Schenker et al. 2001).

This potentially protective role of calorie restriction is an intriguing finding since, given that calorie restriction reverses many of the effects of ageing, and ageing is associated with changes that may protect against paracetamol toxicity, it may have been expected that there would be increased risk of paracetamol hepatotoxicity with calorie restriction. The mechanisms by which calorie restriction and ageing protect against paracetamol hepatotoxicity are not well understood and better understanding of the mechanisms of this protection would be beneficial. The changes with calorie restriction and ageing to factors involved in paracetamol hepatotoxicity are summarised in Table 1.1.

1.4.5.2 Resveratrol and Paracetamol Toxicity
Resveratrol has a variety of potentially protective effects upon factors involved in the paracetamol toxicity pathway (summarised in Table 1.1). Resveratrol has been seen to reduce CYP2E1 activity (Wu et al. 2013), induce NQO1 activity (Hsieh et al. 2006) and induce phase II metabolic enzymes (Cao & Li 2004), which may all provide pharmacokinetic protection against paracetamol toxicity. Furthermore, resveratrol has shown anti-oxidant effects (Yu et al. 2012), improvements in mitochondrial function (Baur et al. 2006; Lagouge et al. 2006; Hubbard et al. 2013), anti-inflammatory effects (Yoshizaki et al. 2010) and induction of autophagy (Yu et al. 2012) which may also contribute to a protective effect against paracetamol toxicity. The effect of increased apoptosis with resveratrol on paracetamol toxicity is unknown and may be either
beneficial or contribute to toxicity development (Lin et al. 2011). Resveratrol has also been found to be protective against many other causes of hepatotoxicity such as hydrogen peroxide (Yang et al. 2005), tert-butyl hydroperoxide (Rubiolo & Vega 2008), ethanol (Bujanda et al. 2006) and carbon tetrachloride (Dani et al. 2008; Rivera & Shibayama 2008; Vitaglione et al. 2009).

Several animal studies have investigated the potential protection from resveratrol against paracetamol toxicity (summarised in Table 1.3). Sener et al. (2006) showed concurrently administered paracetamol and resveratrol in BALB/c mice of both sexes, attenuated paracetamol-induced increases in ALT and TNF-α at 4 hours, and attributed this to its anti-oxidant effects in preventing depletion of glutathione, and accumulation of 3,4-methylenedioxymethamphetamine (MDA) (a marker of lipid peroxidation). Masubuchi et al. (2009) found similar results when resveratrol was given to CD-1 mice, one and six hours after paracetamol, and serum tested for ALT and TNF-α at eight hours, however protection from hepatotoxicity was not seen at 24 hours. Du et al. (2015) found similar protective results when C57BL/6 mice were dosed with resveratrol 1.5 hours after paracetamol. They attributed the protective effect to antioxidant effects and an inhibition of mitochondrial dysfunction (Du et al. 2015). Only one study has looked at resveratrol dosing before paracetamol dosing, in which they treated C57BL/6 mice twice daily for three days with resveratrol then a single dose of paracetamol (Wang et al. 2015). They also saw protection, and attributed this to reduced CYP2E1 activity, and an induction of hepatocyte proliferation (Wang et al. 2015). Noticeably, the strain used, timing, dose, route of administration and resveratrol vehicle and outcome measurements varied significantly across these studies. Furthermore only one of these studies investigated the downstream effects of resveratrol, and confirmed its molecular
Chapter 1: Introduction

action in these animal models (Wang et al. 2015). Thus, confirmation and further examination of the mechanisms of the protective role of resveratrol against paracetamol toxicity would be beneficial.
Table 1.3 Animal studies of resveratrol as a therapeutic to protect against paracetamol toxicity: Consideration of strain, paracetamol and resveratrol dose, route of administration and vehicle, timing of dosing, outcomes measured and study conclusions. ALT= Alanine aminotransferase, LDH= Lactate dehydrogenase, TNF= Tumor necrosis factor, MDA= 3,4-methylenedioxymphetatine, IL= interleukin , CYP= cytochrome, GSH= glutathione, SIRT1 = sirtuin 1

<table>
<thead>
<tr>
<th>Study</th>
<th>Animal Model details</th>
<th>Paracetamol Dose, and Route of Administration</th>
<th>Resveratrol Dose, Vehicle and Route of Administration</th>
<th>Timing</th>
<th>Outcomes</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sener et al. (2006)</td>
<td>Male and female BALB/c mice, age not reported</td>
<td>900mg/kg via intraperitoneal injection</td>
<td>30mg/kg, vehicle and route of administration not reported</td>
<td>Concurrent resveratrol and paracetamol. Tissue collected at 4 hours.</td>
<td>ALT, LDH, TNF-α, GSH, MDA</td>
<td>Resveratrol protected against paracetamol toxicity via antioxidant actions.</td>
</tr>
<tr>
<td>Masubuchi et al. (2009)</td>
<td>Male 8-9 week CD-1 mice</td>
<td>300mg/kg via intraperitoneal injection</td>
<td>75mg/kg in 10ml/kg 60% polyethylene glycol/saline via intraperitoneal injection</td>
<td>Resveratrol dosed 1 and 6 hours post paracetamol. Tissue collected at 8 and 24 hours.</td>
<td>ALT, TNF-α, IL-6</td>
<td>Resveratrol protected at 8 but not 24 hours via anti-inflammatory actions.</td>
</tr>
<tr>
<td>Wang et al. (2015)</td>
<td>Male 6-8 week C57BL/6 mice</td>
<td>400mg/kg via intraperitoneal injection</td>
<td>25-100mg/kg in 0.5% sodium carboxymethyl cellulose, given orally</td>
<td>Resveratrol pre-dosed every 12 hours, 7 times. Paracetamol dosed after final resveratrol dose. Tissue collected at 6 hours.</td>
<td>ALT, CYP2E1 activity, GSH, SIRT1</td>
<td>Resveratrol protected against paracetamol toxicity via reduced CYP2E1 activity and increased hepatocyte proliferation.</td>
</tr>
<tr>
<td>Du et al. (2015)</td>
<td>Male 8-12 week C57BL/6 mice (and primary hepatocytes)</td>
<td>300mg/kg via intraperitoneal injection</td>
<td>50mg/kg in 40% DMSO via intraperitoneal injection</td>
<td>Resveratrol dosed 1.5 hours post paracetamol. Tissue collected at 0, 6, 24 and 48 hours.</td>
<td>ALT, LDH, GSH, endonuclease release, nitrotyrosine staining</td>
<td>Resveratrol protected against paracetamol toxicity via antioxidant effects and inhibition of Endonuclease release from mitochondria.</td>
</tr>
</tbody>
</table>
1.4.5.3 SIRT1 and Paracetamol Toxicity
It is hypothesised that the mechanism of protection against paracetamol toxicity with calorie restriction and resveratrol may be SIRT1 activation. SIRT1 allosteric activation is one of the central actions of resveratrol (Hubbard et al. 2013), and one paracetamol and resveratrol study showed that SIRT1 expression was increased with resveratrol treatment in paracetamol toxicity (Wang et al. 2015). Furthermore, SIRT1 activation is associated with a variety of potentially protective mechanisms, and is thought to play an important role not only in longevity and delaying age-related changes, but also in regulation of response to stressors and nutrients (Michan & Sinclair 2007; Raynes et al. 2013). In particular, SIRT1 has been shown to play a role in the regulation of inflammation (Purushotham et al. 2009; Yoshizaki et al. 2010), apoptosis (Yamakuchi et al. 2008) and mitochondrial function (Price et al. 2012), important factors in the development of paracetamol hepatotoxicity. Clarification of the role of SIRT1 in paracetamol toxicity may provide further insight into the mechanisms of paracetamol toxicity, and allow the identification of novel targets for protective therapeutics.

1.5 Aims and Hypotheses of the Thesis
The objective of this project was to improve the safety of paracetamol use for patients of all ages. The aim of this thesis was to investigate the impact of old age and frailty on the risk, exposure-type and mechanisms of paracetamol toxicity. Furthermore, this thesis aimed to investigate whether the current paracetamol hepatotoxicity therapy, NAC, is effective in old age and in non-acute exposure to paracetamol; and to test whether the age-related interventions of resveratrol and activation of the SIRT1 pathway may provide novel potential therapeutic targets. Finally, this thesis aimed to look at the effect of paracetamol and these potential age-related therapies on frailty.
Chapter 1: Introduction

It was hypothesised that there would be changes in susceptibility to, and mechanisms of, paracetamol toxicity with old age and frailty. It was also hypothesised that NAC would not be effective in protecting against paracetamol hepatotoxicity in non-acute exposure situations. It was hypothesised that the interventions of resveratrol and SIRT1 activation would protect against paracetamol hepatotoxicity, although this protection may be different across age groups. Finally it was hypothesised that paracetamol, resveratrol and calorie restriction would delay frailty in old mice.
2 ACUTE PARACETAMOL TOXICITY IN OLD AND/OR FRAIL MICE

2.1 Introduction
Paracetamol is a commonly used analgesic that is usually well tolerated but can have severe toxicity following acute or chronic overdose. The pharmacokinetic and toxicological mechanisms of an acute over-exposure to paracetamol in young adults have been well established (see chapter 1.2). Following an acute overdose of paracetamol, there is saturation of the conjugation metabolism pathways and glutathione depletion (Larson 2007). This results in build-up of the toxic NAPQI, which can result in liver cell damage (Jollow et al. 1973). The mechanisms of how cell damage occurs involve covalent binding of NAPQI to cytoplasmic and mitochondrial proteins, oxidative stress (Jaeschke 1990), an inflammatory response (Antoine et al. 2008), and ultimately the stimulation of cell death, either apoptosis or necrosis (Jaeschke et al. 2012).
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

With old age, and frailty, there are both pharmacokinetic and pharmacodynamic changes that occur which may affect the pharmacology and toxicology of paracetamol (Summarised in section 1.3.4). Despite these significant physiological changes with old age and frailty, the increased intake of drugs, including paracetamol, in old age and frailty (Pearson et al. 2007; Koponen et al. 2013; Blyth et al. 2008), and the higher prevalence of adverse drug reactions in old age (Burgess et al. 2005), there are very limited studies on the changing risks of toxicity with paracetamol in old age and frailty. A study of hospitalised fit and frail older patients found that frail older people taking therapeutic paracetamol for five days had higher blood paracetamol concentrations than younger patients, without associated increased liver enzymes (Mitchell et al. 2011). A study of paracetamol self-poisoning in Denmark, found that although the majority of paracetamol induced hepatotoxicity occurs in young adults, the majority of deaths associated with paracetamol exposure occur in old age (Schmidt 2005).

There have been several animal studies, using Fischer 344 rats that have shown a decreased risk of acute paracetamol toxicity in old age (Rikans & Moore 1988), due to a reduction in CYP2E1 activity (Mach et al. 2014) or reduced transfer into the liver (Mitchell, Huizer-Pajkos, et al. 2011). Studies in Sprague Dawley rats, however, found an increased susceptibility to toxicity in old rats (Tarloff et al. 1996). This may have been related to weight-based dosing in the context of obesity in this ageing model resulting in relatively higher doses (Mach et al. 2014). A recent paper, suggested that mice are a more clinically relevant model of paracetamol toxicity than rats, due to similar susceptibility and mechanisms of damage (McGill et al. 2012). The only mouse studies investigating the effect of old age on paracetamol toxicity, did not look at direct toxicity outcomes, but suggested reduced glutathione capacity in old age (Chen et al.
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

1990; Al-Turk & Stohs 1981). This study seeks to establish for the first time the risk of paracetamol toxicity in old age and frailty in mice.

The recent development of frailty assessment tools for animals, allows the study of not only age-related changes, but also frailty-related changes in animals models (Liu et al. 2013; Whitehead et al. 2014). Liu et al. (2013) developed and validated a mouse frailty index based on the clinical phenotype model of frailty that assesses a mouse as frail based on its grip strength, walking speed, physical activity and endurance. This assessment is non-invasive, and has been used to investigate the effect of exercise intervention on frailty (Graber et al. 2014), however the functional assessments may be time-consuming. Whitehead et al. (2014) developed and validated a mouse frailty index based on the deficit accumulation model of frailty, which assesses a mouse as frail based on 31 clinical measures. Although this assessment includes no functional measures, it is non-invasive and fast, so easily included as part of a larger study protocol. This tool has not yet been used to look at changes in drug toxicity or mechanisms with frailty.

This chapter aims to assess changes to acute paracetamol toxicity risk and mechanisms in old age and frailty in C57BL/6 mice. Understanding the risk of paracetamol hepatotoxicity in old age and frailty is essential for optimising the treatment of pain in the older population.
2.2 Methods

2.2.1 Animals
Two cohorts of young and old male C57BL/6 mice were used to test acute paracetamol toxicity. Cohort 1 was made up of young (age=7.3±0.3 months, n=23) and old (age=18.9±2.3 months, n=25) male C57BL/6 mice, aged inhouse at the National Institute on Aging (NIA, Baltimore, MD). Mice were fed a 2018 Teklad Global 18% Protein Rodent diet (Harlan laboratories). A second cohort of young (age=10±0.0 months, n=12) and old (age=23.7±0.02 months, n=16) male C57BL/6 mice were also aged at the NIA (Baltimore, MD). They were fed a standard AIN-93G diet (Harlan laboratories). The median lifespan of C57BL/6 mice is 26-30 months. These two cohorts of mice were also used as the control groups for other studies of paracetamol toxicity interventions and mechanisms (see chapter four and five of this thesis).

Mice were group housed in cages of 4 with ad libitum access to food and water. Animal rooms were maintained on a 12 hr light/dark cycle at 20-22°C, and 30-70% humidity. Animals were randomly assigned to treatment or control groups prior to the treatment day. All animal protocols were approved by the Animal Care and Use Committee of the National Institute on Aging (429-TGB-2017 and 405-TGB-2016).

2.2.2 Frailty Index Assessment
The Mouse Clinical Frailty Index, is a simple, noninvasive tool for the assessment of frailty in mice. A frailty index score is calculated for each mouse using a 31 item frailty index (Whitehead et al. 2014) of in vivo assessments, plus mouse weight and temperature (items explained in detail in Appendix 1). Young (12 week) C57BL/6J and DBA/2J weight and temperature means and standard deviations were obtained from the Jacksons Laboratory (http://jaxmice.jax.org/support/weight/000664.html).
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

Old mice from cohort two were assessed for frailty, after randomization to treatment group, the day before being dosed with paracetamol. Frailty testing was carried out by two raters. The raters were blinded to the scores assigned by the other rater. The final reported frailty index scores were calculated from the mean of the two rater’s scores.

2.2.3 Paracetamol Treatment and Tissue Collection
Animals were fasted overnight (16 hours) to reduce and normalise glutathione levels, then dosed with 300mg/kg paracetamol (Panadol Color-free Baby Drops, 100mg/ml, GlaxoSmithKline, Australia) or saline vehicle via oral gavage between 8 and 10am. This paracetamol dose was chosen as a standard dose to induce toxicity in C57BL/6 mice (McGill et al. 2012). Food was returned to the mice two hours after dosing. Six hours after dosing mice were anaesthetized with an i.p injection of ketamine (75 mg/kg, DVR Pharmacy, Bethesda MD) and xylazine (10 mg/kg, DVR Pharmacy Bethesda MD). A midline laparotomy was performed and blood taken from the Inferior Vena Cava. The portal vein was then cannulated with a 21G intravenous catheter (BD, Sydney, Australia) through which the liver was perfused in-situ at 1–1.5 mL/min/g of liver with oxygenated Krebs-Henseleit bicarbonate buffer (95% O₂–5% CO₂, 37°C). Sections of the liver were snap frozen in liquid nitrogen for subsequent enzyme activity assays, protein and RNA extraction and fixed in 10% neutral formalin for subsequent histopathological analysis.

2.2.4 Serum Biochemistry to Assess Liver and Renal Function
Blood was stored at 4°C for up to two hours before separating the serum by centrifugation at 10,000g for 10 minutes. Serum was stored in aliquots at -80°C. Serum liver function tests (total protein, albumin, bilirubin, alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), alanine aminotransferase (ALT)), for both cohorts,
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

and creatinine, for cohort one, were measured by a National Association of Testing Authorities accredited hospital laboratory, PaLMS (Pacific Laboratory Medicine Services) at Royal North Shore Hospital (Sydney, Australia) using an Architect i1000SR immunoassay analyser (Abbott Diagnostics, IL, USA). Vitamin B6 is added to the ALT activity assay to accommodate for the age related decline in this vitamin, an integral cofactor for the assay (Krishnamurthy et al. 1967; Bordoni et al. 1998).

2.2.5 Liver Histology

Fixed liver tissue was embedded in paraffin, 5µm sections were cut on a microtome and mounted on slides. Slides were stained with Haemotoxylin and Eosin in the National Association of Testing Authorities accredited hospital laboratory of the Pathology department of Royal Prince Alfred Hospital, Sydney, Australia. Histopathology was scored by an anatomical pathologist, who was blinded to the age and treatment of the samples. Necrosis was scored as a percentage of the liver tissue on each slide. Images were taken on an Olympus BX51 microscope connected to an Olympus DP26 camera (Olympus, Sydney, Australia).

2.2.6 Biochemical Examination of Enzymes and Glutathione in Frozen Liver Samples

Liver microsomes were isolated, according to the method of Shoaf et al. (1987). Liver samples were homogenised with a glass homogeniser, in ice cold buffer (0.25M sucrose, 0.1M Tris-Hydrochloride (HCl), pH 7.4), then centrifuged at 9000g for 20 minutes at 4°C. The supernatant was collected, then spun at 100,000g for one hour, at 4°C. The supernatant was collected (cytosolic fraction), the cell pellet re-suspended in buffer, then centrifuged at 100,000g for 30 minutes. The supernatant was discarded, and the microsomes re-suspended in 0.1M Tris-HCl (pH 7.4) with 20% (v/v) glycerol (Sigma Aldrich, #G7757), before being aliquoted and stored at -80°C.
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

CYP2E1 activity measured in the microsomes using absorption of p-aminophenol, converted from aniline via aniline hydroxylase activity, as described by Roberts et al. (1995) and Mach et al. (2014). Microsomal samples were made up to a concentration 0.325μg/μl in 0.1M Tris-HCl (pH 7.4) with 20% (v/v) glycerol. 61.57ul of microsomal solution for each sample was then added to 24.56ul reaction mixture (6mM MgCl₂, 1.2 mM nicotinamide adenine dinucleotide phosphate (NADP), 9.8mM Isocitric acid, 8mM aniline hydrochloride, 2% (v/v) glycerol, 0.46% (w/v) KCl, 18mM Tris-HCl pH 7.4), and 13.89ul of isocitric dehydrogenase (1/10 diluted for a total activity of 0.2U). An equal volume of 10% trichloroacetic acid (TCA, Sigma Aldrich #522082) was added to the solution to stop the reaction after either 0 or 30 minutes. The samples were then centrifuged at 1000g for 15 minutes, and the supernatant added, along with the standards (0-100nM p-aminophenol in 79.57mM Tris-HCl pH 7.4) to a 96 well plate with an equal volume of 1% phenol (in 0.1M NaOH), and ½ volume of 10% Na₂CO₃. Plates were incubated in the dark for 23 hours (the time required for consistent colour development) then absorbance read at 629nm.

NQO1 activity was measured in the cytosol as described by Aleksunes et al. (2006) in which NQO1 enzymatic activity is measured over 1 minute using the reduction of dichlorophenolindophenol (DCPIP) at 595 nm with NADPH as the reducing cofactor. NQO1 activity was determined at 27°C in a reaction mixture containing liver cytosol, 200 μmol/L NADPH, 40μmol/L DCPIP, 25 mmol/L Tris–HCl buffer pH 7.4, and 0.7 mg/mL bovine serum albumin (BSA). Parallel reactions were conducted with 20 μmol/L of selective NQO1 inhibitor dicumarol. The rates of dicumarol-sensitive NQO1
activity were determined by the difference between uninhibited and dicumarol-inhibited rates.

Liver concentrations of total glutathione were determined with a Glutathione Assay kit (Cayman Chemicals, #703002), according to the manufacturers instructions. Tissue was homogenised in phosphate-buffered saline (PBS) supplemented with 1mM ethylenediaminetetraacetic acid (EDTA), then centrifuged at 10,000g for 15 minutes at 4°C, and the supernatant collected. Samples were deproteinated by adding an equal volume of metaphosphoric acid (MPA, 1g/10ml), incubating at room temperature for 5 minutes, centrifuging samples at 2000g for 2 minutes, and collecting the supernatant. Triethanolamine (TEAM) was added to each sample at 50ul/ml, then samples were diluted 1/50 in 2-(N-morpholino)ethanesulfonic acid (MES) buffer, and added with standards to a 96 well plate (50ul). 150 ul of assay cocktail (MES buffer plus cofactor mixture, enzyme mixture, and beta dystrobrevin (DTNB) was added, the plates incubated in the dark, and absorbance measured at 405nm after 25 minutes.

2.2.7 mRNA Expression Measurement with qPCR
For livers of mice from cohort 1 only, RNA was extracted from liver tissue using the Trizol reagent (Invitrogen, Carlsbad CA), according to the manufacturer’s instructions. Briefly, tissue was homogenized in Trizol reagent. Chloroform was added, the sample centrifuged, and the aqueous phase collected. RNA was precipitated with isopropanol, the sample centrifuged and the supernatant discarded. The RNA pellet was washed with 70% ethanol then re-dissolved in nuclease-free water, and the concentration determined. Complementary DNA (cDNA) was synthesized from the extracted RNA using a Reverse Transcription Kit (Applied Biosystems, #4368814) and in a BioRad thermocycler (PTC-200, CA, USA). PCR plates were then loaded in triplicate with
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

sample, appropriate primers and SYBR Green Mastermix (Qiagen #330523). Plates were run in the 7300 Real-Time PCR System (Applied Biosystems). Primers used were CYP2E1, NQO1, TNF-α, IL-1β, IL-10, NF-κB, Caspase 3, BAX, PGC-1α, PGC-1β and normalised to the housekeeper GAPDH (sequences in Appendix 2).

2.2.8 Statistics
Data are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated. Differences between mean values across groups were calculated with one-way or two-way analysis of variance (ANOVA) with Tukey’s honest significance difference (HSD) post-hoc test where appropriate. The prevalence of necrosis in each treated group compared to the saline group was compared with Chi squared. The calculation of mRNA expression was performed by the 2-ΔΔCT method. Spearman correlation coefficients (r) were used to analyse the correlation between Frailty Index and other outcomes, and linear regression was used to calculate slopes of best fit for each treatment group. Analysis of covariance (ANCOVA) was used to compare the regression slopes between paracetamol or saline treated groups, with frailty index as a covariate. Data analysis was completed using the statistics program SPSS (Version 21.0, SPSS Inc., Chicago, Illinois, USA) and GraphPad Prism (Version 6.04, GraphPad Software, La Jolla California USA).

2.3 Results

2.3.1 Assessment of Paracetamol Toxicity
Table 2.1 and

Table 2.2 show mouse and liver weights and serum liver function test and creatinine results, for all mouse groups for cohorts 1 and 2. There was no difference across any group, for either cohort of mice, for total protein, albumin, ALP, GGT or liver weight (as percentage of body weight). For cohort 1 young and old mice, and cohort 2 young
mice, groups treated with paracetamol had higher bilirubin serum concentrations (p=0.004), than those treated with saline. In cohort 1, but not cohort 2, the old mice weighed significantly more than the young mice (p<0.001). Creatinine was measured for cohort 1 mice, and there was no change in serum creatinine level with either age or paracetamol treatment (Table 2.1).

**Table 2.1** Characteristics and serum biochemistry results for young (7.3±0.3 months) and old (18.9±2.3 months) male C57BL/6 mice from cohort 1, 6 hours after treatment with 300mg/kg paracetamol or saline. Data expressed as mean (SD). *p<0.05 compared to corresponding saline treated group, # p<0.05 compared to corresponding young group. ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase.
Table 2.2 Characteristics and serum biochemistry results for young (10.0±0.0 months) and old (23.7±0.0 months) male C57BL/6 mice from cohort 2, 6 hours after treatment with 300mg/kg paracetamol or saline. Data expressed as mean (SD). *p<0.05 compared to corresponding saline treated group. ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase.

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
<th>One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline n=6</td>
<td>Paracetamol n=6</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>50.2 (1.6)</td>
<td>49.6 (3.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>27.0 (1.1)</td>
<td>28.0 (1.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>2.3 (0.5)</td>
<td>6.8 (3.0)*</td>
<td>F(3,25)=9.39, p=0.000</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>62.0 (5.2)</td>
<td>70.0 (13.9)</td>
<td>NS</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>34.9 (3.1)</td>
<td>35.2 (2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>3.7 (0.4)</td>
<td>5.8 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Food intake, last week of diet (g/g mouse/day)</td>
<td>0.07 (0.01)</td>
<td>0.07 (0.01)</td>
<td>NS</td>
</tr>
</tbody>
</table>

All paracetamol treated groups, in both cohorts, had increased mean ALT (Figure 2.1) and increased hepatic necrosis on histology (Figure 2.2) when compared to their corresponding saline treated groups (p<0.05), except for young mice in cohort 1, where there was only a trend towards increased necrosis (p=0.11). Two-way ANOVA showed that there was no age effect, nor any age-treatment interaction effect on the degree of hepatotoxicity as measured by serum ALT concentrations or the mean percentage of liver necrosis, in either cohort of mice.

Alice Kane - March 2016
Figure 2.1 Serum alanine aminotransferase (ALT) concentrations for young (cohort 1, 7.3±0.3 months; cohort 2 = 10±0.0 months) and old (cohort 1, 18.9±2.3 months; cohort 2, 23.7±0.0 months) male C57BL/6 mice from cohort 1 (A) or cohort 2 (B), 6 hours after treatment with 300mg/kg paracetamol (grey bars) or saline (black bars). Data expressed as mean±SEM. One-way ANOVA cohort 1, F(3,43)=10.37 p=0.00. One-way ANOVA cohort 2, F(3,26)=9.21 p=0.00. *=p<0.05 compared to corresponding saline treated group with Tukey’s HSD post-hoc test. n=6-15 per group.

Figure 2.2 Liver necrosis (as a percentage of liver tissue) for young (cohort 1, 7.3±0.3 months; cohort 2 = 10±0.0 months) and old (cohort 1, 18.9±2.3 months; cohort 2, 23.7±0.0 months) male C57BL/6 mice from cohort 1 (A) or cohort 2 (B), 6 hours after treatment with 300mg/kg paracetamol (grey bars) or saline (black bars). Data expressed
as mean±SEM. One-way ANOVA cohort 1, F(3,43)=5.47 p=0.003. One-way ANOVA cohort 2, F(3,24)=20.69 p=0.00. *=p<0.05 compared to corresponding saline treated SIRT1 activation does not provide an alternative protective mechanism group with Tukey’s HSD post-hoc test. n=6-15 per group

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort 1 - Saline</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Cohort 1 - Paracetamol</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Cohort 2 - Saline</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Cohort 2 - Paracetamol</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 2.3** Representative Haemotoxylin and Eosin stained liver histology images for young (cohort 1, 7.3±0.3 months; cohort 2 = 10±0.0 months) and old (cohort 1,
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

18.9±2.3 months; cohort 2, 23.7±0.0 months) male C57BL/6 mice 6 hours after treatment with 300mg/kg paracetamol or saline. Images taken at 100X.

2.3.2 Assessment of Factors Influencing Paracetamol Pharmacokinetics
Activity of microsomal CYP2E1, the enzyme that metabolises paracetamol to its hepatotoxic metabolite, NAPQI, was significantly decreased for old cohort 1 mice treated with paracetamol, compared to saline control (Figure 2.4) whilst this effect was only a trend for young cohort 1 mice, and for both age groups of cohort 2 mice (p=0.10). Two-way ANOVA showed an overall significant effect of only treatment for both cohorts (cohort 1 p=0.001, cohort 2 p=0.015) (Figure 2.4).

**Figure 2.4** Cytochrome P450 (CYP)2E1 activity in the livers of young (cohort 1, 7.3±0.3 months; cohort 2 = 10±0.0 months) and old (cohort 1, 18.9±2.3 months; cohort 2, 23.7±0.0 months) male C57BL/6 mice from cohort 1 (A) or cohort 2 (B), 6 hours after treatment with 300mg/kg paracetamol (grey bars) or saline (black bars). Data expressed as mean±SEM. One-way ANOVA cohort 1, F(3,40)=4.64 p=0.007. One-way ANOVA cohort 2, non-significant. *=p<0.05 compared to corresponding saline treated group with Tukey’s HSD post-hoc test. n=6-15 per group
Total liver glutathione (GSH), which binds and detoxifies NAPQI, was not significantly reduced with acute high dose paracetamol treatment in young or old mice in cohort 1 (p=0.05) (Figure 2.5). For cohort 2 mice, glutathione was significantly decreased with paracetamol treatment in both young and old mice (p=0.002, p<0.001) (Figure 2.5). Additionally older mice in cohort 2 had higher total liver GSH levels, than younger mice, for both saline and paracetamol treated mice, with two-way ANOVA showing a significant effect of both age and treatment individually (p<0.001), as well as a significant age x treatment effect (p=0.036).

**Figure 2.5** Total liver glutathione (GSH) for young (cohort 1, 7.3±0.3 months; cohort 2 = 10±0.0 months) and old (cohort 1, 18.9±2.3 months; cohort 2, 23.7±0.0 months) male C57BL/6 mice from cohort 1 (A) or cohort 2 (B), 6 hours after treatment with 300mg/kg paracetamol (grey bars) or saline (black bars). Data expressed as mean±SEM. One-way ANOVA cohort 1, non-significant. One-way ANOVA cohort 2, F(3,27)=42.97 p=0.00. *=p<0.05 compared to corresponding saline treated group. #=p<0.05 compared to corresponding young group with Tukey’s HSD post-hoc test. n=6-15 per group
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

The activity of cytosolic NQO1, which transforms NAPQI back to paracetamol was found to be increased with age (p<0.001) but unchanged with paracetamol treatment (Figure 2.6) for cohort 1, and was not assessed for cohort 2.

![Figure 2.6](image)

**Figure 2.6** NAD(P)H:quinone oxidoreductase 1 (NQO1) activity for young (7.3±0.3 months) and old (18.9±2.3 months) male C57BL/6 mice from cohort 1, 6 hours after treatment with 300mg/kg paracetamol (grey bars) or saline (black bars). Data expressed as mean±SEM. One-way ANOVA, F(3,40)=5.33 p=0.004. #=p<0.05 compared to corresponding young group with Tukey’s HSD post-hoc test. n=10-15 per group

2.3.3 mRNA expression of Inflammatory, Apoptosis and Mitochondrial Function Markers

Livers from cohort 1 mice were analysed for changes in mRNA expression of: pro-inflammatory cytokines TNF-α and IL-1β; anti-inflammatory cytokine IL-10; the cytokine-production regulating protein complex, NF-κB; the apoptosis marker Caspase 3; apoptosis promoting protein BAX; and the mitochondrial regulator proteins, PGC1-α and PGC1-β, with age and paracetamol treatment. Figure 2.7 shows that with paracetamol treatment there is an increase in liver mRNA expression of the inflammatory markers TNF-α, IL-1β, IL-10 and NF-κB for old, but not young mice (p<0.05). Two-way ANOVA only identified a significant age x treatment interaction effect for TNF-α (p=0.024), with a greater increase in TNF-alpha seen after acute high
dose paracetamol in old than in young mice. There was no change in the ratio of pro-inflammatory to anti-inflammatory cytokine expression (TNF-α/IL-10), for any age or treatment group.

**Figure 2.7** mRNA expression of inflammatory markers; (A) Tumor necrosis factor(TNF)-α (B) Interleukin (IL)-1β (C) IL-10 and (D) Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) for young (7.3±0.3 months) and old (18.9±2.3 months) male C57BL/6 mice from cohort 1, 6 hours after treatment with 300mg/kg paracetamol (grey bars) or saline (black bars). Data expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA TNF-α, F(3,36)=6.28 p=0.002. One-way ANOVA IL-1β, F(3,36)=3.09 p=0.04. One-way ANOVA IL-10, F(3,36)=4.72 p=0.008. One-way ANOVA NF-κB, F(3,36)=4.03
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

p=0.015. *=p<0.05 compared to corresponding saline treated group, #=p<0.05 compared to corresponding young group with Tukey’s HSD post-hoc test. n= 3-10 per group

There is no change in apoptosis marker, Caspase 3 mRNA expression with age or paracetamol treatment. BAX mRNA expression is increased in old mice treated with paracetamol, compared to saline controls, and young mice treated with paracetamol (p<0.05) (Figure 2.8) mRNA expression of mitochondrial regulator protein, PGC1-α does not change with age or treatment, whilst PGC1-β mRNA expression is increased in old mice treated with paracetamol only (p=0.013). Two-way ANOVA shows a significant age x treatment interaction effect for BAX and PGC1-β (p=0.027, p=0.023), with a greater response to treatment seen in old age.
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

Figure 2.8 mRNA expression of (A) Caspase 3 (B) BAX (C) Peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1)-α and (D) PGC1-β for young (7.3±0.3 months) and old (18.9±2.3 months) male C57BL/6 mice from cohort 1, 6 hours after treatment with 300mg/kg paracetamol (grey bars) or saline (black bars). Data expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA Caspase 3, non-significant. One-way ANOVA BAX, F(3,36)=5.46 p=0.004. One-way ANOVA PGC1-α, non-significant. One-way ANOVA PGC1-β, F(3,36)=4.21 p=0.013. *=p<0.05 compared to corresponding saline treated group, #=p<0.05 compared to corresponding young group with Tukey’s HSD post-hoc test. n= 3-10 per group

2.3.4 Correlation between Frailty and Paracetamol Toxicity Outcomes
To assess whether frailty affected the risk or mechanisms of paracetamol hepatotoxicity, we investigated the correlation between frailty index and associated outcomes, for paracetamol and saline treated old mice from cohort 2 (Figure 2.9).
There was no correlation between frailty and serum ALT, percentage of hepatic necrosis, microsomal CYP2E1 activity or total liver GSH for paracetamol or saline treated mice (p<0.05). There was a significant negative correlation between serum total protein, and serum albumin, and frailty index (r=-0.963, p=0.000 and r=-0.871, p=0.011) for paracetamol treated mice (Figure 2.9). An ANCOVA showed no significant difference between the regression slopes for either outcome (serum protein or albumin) for treatment group, when adjusted for frailty index as a covariate, implying that this correlation is probably not specific to the paracetamol treatment group, and rather an overall correlation with frailty.
Figure 2.9 Correlation between frailty index and (A) serum alanine aminotransferase (ALT), (B) percentage liver necrosis, (C) liver cytochrome P450 (CYP)2E1 activity, (D) total liver glutathione (GSH), (E) serum total protein, (F) serum albumin, (G) liver weight (as % of body weight) and (H) serum alkaline phosphatase (ALP), for old
(23.7±0.0 months) male C57BL/6 mice from cohort 2, 6 hours after treatment with 300mg/kg paracetamol (grey) or saline (black). n=6-8 per group

2.4 Discussion
This study, which is the first to look at the effect of age on paracetamol toxicity in mice, found that there was no effect of age on susceptibility to acute paracetamol toxicity, as assessed by liver necrosis and serum ALT concentration. This is in contrast to the previous studies of acute paracetamol hepatotoxicity in rats, demonstrating increased risk in Sprague-Dawley rats with age (Tarloff et al. 1996) and decreased risk in old age in Fischer 344 rats (Rikans & Moore 1988; Mach et al. 2014). There was no change in CYP2E1 activity in old mice compared to young, from any cohort. Interestingly, in old age rats have a reduction in CYP2E1 activity (Mach et al. 2014), and this provides one mechanism for the observed interspecies difference in toxicity risk. Old rats, but not young, were also found to have an increase in creatinine with paracetamol treatment, indicating potential increased risk of paracetamol induced nephrotoxicity in old rats, which was not seen for old mice in the current study (Mach et al. 2014; Beierschmitt et al. 1986).

Recent research has identified mice as a more clinically relevant model of paracetamol toxicity, due to similar susceptibility to, and mechanisms of, toxicity in mice and humans, but not rats (McGill et al. 2012). This implies that the current finding of no change in hepatotoxicity risk due to acute paracetamol exposure in old age in mice, may be translatable to humans. Research confirming this finding in a clinical setting, may enable the optimisation of the use of paracetamol for pain relief in the older population, as a well-tolerated analgesic at therapeutic doses, with no increased risk of hepatotoxicity in old age. However, it must be noted that older patients are more likely
to have chronic, staggered or accidental over-exposures to paracetamol, rather than single acute over-exposures (Kane et al. 2012), and the effect of these types of paracetamol over-exposures on the risk of hepatotoxicity in old age has not been established. This will be explored in chapter 3.

Total liver GSH concentration was decreased with paracetamol treatment in cohort 2, with a trend towards a decrease in cohort 1. Previous studies have shown that total liver glutathione is maximally reduced at 0.5-1 hour post acute paracetamol treatment, and steadily recovers back to baseline over the following hours (Mitchell et al. 1973; McGill et al. 2012). Our results are consistent with these findings. In cohort 2 only, young mice had significantly lower GSH concentrations than old mice, although this did not translate into a difference in degree of toxicity. Previous animal studies have seen a reduction or no change in total liver GSH concentrations with old age (Mune et al. 1995; Mach et al. 2014; Suh et al. 2004), although none of these studies were in C57BL/6 mice. Vogt & Richie (1993) found that a decrease in liver GSH was only seen at 29 months of age in mice, so the old mice in our study may have been too young to have significant decreases in liver GSH concentration.

This study found increased basal NQO1 activity in old mice compared to young, as has been seen in other animal studies (Mach et al. 2014; Kamzalov & Sohal 2004; Chen et al. 1994). Increased NQO1 activity may be a protective mechanism against paracetamol toxicity, and may explain the lack of increased risk to paracetamol toxicity in old age in our study. This study also saw an increased inflammatory response to acute paracetamol in the old mice compared to the young. Interestingly, this increased response was seen for both pro- and anti-inflammatory cytokines, with no change in the ratio of pro/anti-
inflammatory cytokines, and did not translate into a change in degree of paracetamol toxicity. This increased inflammatory response in old age is consistent with previous ageing animal studies of inflammatory response to stressors (Starr et al. 2013; Okamura et al. 2012; Tateda et al. 1996; Saito et al. 2003), although the relevance in human studies remains to be determined (Starr & Saito 2014).

Another interesting age related paracetamol effect observed in this study was changes to the mRNA expression levels of BAX and PGC1-β in old mice, but not young, after paracetamol treatment. Changes in the expression of PGC1 proteins have been associated with ageing in several studies (Dillon et al. 2012), and studies have seen age-related changes in apoptosis associated proteins (Mach et al. 2015), and increased apoptotic response to other stressors in age (Turnbull et al. 2004). Further exploration of age-related changes in the role of the mitochondria and apoptosis, as well as other related mechanisms such as autophagy, in paracetamol toxicity would be interesting, and may provide targets for therapeutic interventions to prevent or treat paracetamol toxicity.

This study was the first to investigate the effect of frailty on paracetamol toxicity in an animal model. There was no overall effect of frailty on the degree of paracetamol toxicity from acute exposure, as assessed with serum ALT and prevalence of necrosis. The lack of correlation between frailty index and microsomal CYP2E1 activity or total liver GSH provides a mechanism for this finding. The single clinical study on paracetamol exposure in frailty, suggested that with exposure to therapeutic doses of paracetamol, frailer older patients may have been less at risk of toxicity (Mitchell, Kane, et al. 2011). Although, this initial finding implies that it is safe to use similar doses of
paracetamol in frail, older patients, as in young, further research is needed to clarify the effect of frailty on paracetamol toxicity clinically, in order to optimise pain treatment in these patients.

The lack of an effect on the degree of paracetamol toxicity with frailty was unexpected, as frailty is often recognised as a reduced capacity to respond to stressors (Collard et al. 2012). This negative finding may be a result of experimental design. It is possible that the time-frame of our experiment was too short to see a reduced capacity to recover with frailty, and it would be interesting to look at a longer time-frame after paracetamol insult in future studies. It would also be interesting to look at a wider range of paracetamol toxicity-associated outcomes such as inflammatory cytokine response, and the association with frailty. It is also possible that with a different mouse assessment tool, especially one that involved functional assessments (Liu et al. 2013) or biomarkers, we may have seen a more significant correlation between frailty and degree of hepatotoxicity. It would be interesting, in future studies, to look at both mouse frailty assessment tools (Whitehead et al. 2014; Liu et al. 2013), in an acute paracetamol toxicity model. Clinically, the patients identified as frail with the frailty index, are not always identified as frail with the phenotype approach that includes measures of functional performance, and vice versa (Rockwood et al. 2007; Kulminsiki et al. 2008), and it would be interesting to see if this was also true in mice. It would also be interesting, with larger mouse numbers, to dichotomise the mice into frail and non-frail groups, by using either the Liu et al. (2013) assessment, or introducing a cut-off point for the Whitehead et al. (2014) scale as has been done clinically (Rockwood et al. 2011; Song et al. 2010), and assessing changes in paracetamol outcomes in frail compared to non-frail mice.
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

There was a significant correlation of frailty index with serum protein and albumin levels in the paracetamol treated old mice, but not the saline treated. However, an ANCOVA showed no difference in the regression slopes for these 2 groups. Thus it is hard to conclude whether this was an effect of frailty on these outcomes with paracetamol treatment, or just an overall effect of frailty. Interestingly, several clinical studies have identified low albumin serum levels as a potential frailty biomarker (Schalk et al. 2004; Kitamura et al. 2012). It would be interesting, in future studies, to look at the association between frailty index and other markers associated with frailty clinically, such as inflammatory cytokines IL-6 or TNF-alpha (Hubbard et al. 2009).

Limitations of this study include the use of two separate cohorts of mice with some baseline differences (diet, weights, ages), smaller numbers than a large single cohort study would have provided, and the fact that data was not collected on all outcomes for each cohort. Furthermore, as only mRNA data is available for some outcomes, these findings should be confirmed with protein expression experiments. Strengths of this study include the novelty of being the first study to investigate the effect of frailty and age on paracetamol toxicity in the clinically relevant model of ageing mice, and the quality of our data, with the use of validated assays to assess enzyme activities and qPCR to assess mRNA expression.

Overall, the experiments in this chapter found that there is no difference in the degree of hepatotoxicity induced by acute paracetamol toxicity in old age or frailty in mice. There were some age related changes in the paracetamol toxicity mechanistic pathway including potentially protective increased liver GSH concentrations in one mouse.
Chapter 2: Acute Paracetamol Toxicity in Old and/or Frail Mice

cohort, potentially protective increased NQO1 activity, an increased pro and anti inflammatory response to paracetamol with uncertain impact and potentially protective increased mRNA expression of BAX and PGC1β with paracetamol treatment in old age. However, none of these changes translated into detectable changes in risk of hepatotoxicity. Frailty related changes included a negative correlation between frailty index and serum protein and albumin concentrations. The next chapter will explore changes with age and frailty to chronic and sub-acute paracetamol exposure, and the effectiveness of the current therapy, NAC, in protecting against these exposure types.
3 **CHRONIC AND SUB-ACUTE PARACETAMOL EXPOSURE IN YOUNG AND OLD MICE, AND THE ROLE OF N-ACETYLCYSTEINE**

3.1 Introduction
The majority of paracetamol toxicity studies have explored single, acute doses, and there have been limited studies on the risk of paracetamol toxicity from chronic or repeated paracetamol exposure. Chapter two of this thesis showed that in mice, there was no difference in the degree of hepatotoxicity induced by acute paracetamol toxicity in old age or frailty. However, older people are more likely to have chronic low-dose exposure or accidental over-exposure to paracetamol, than acute exposures (Kane et al. 2012), and the changing risk of toxicity to these types of paracetamol exposures in old age and frailty has not been investigated.
Chapter 3: Chronic and Sub-acute Paracetamol Exposure in Young and Old Mice, And The Role of N-Acetyl Cysteine

In humans, two studies found that staggered over-exposures to paracetamol were associated with an increased risk of liver failure (Craig et al. 2012; Ferner et al. 2011). Another found that 31-44% of patients taking the recommended therapeutic paracetamol dose (4g/day) for 14 days had increased serum ALT concentrations, an indicator of liver damage (Watkins et al. 2006). A case study (Lane et al. 2002) and a large-scale retrospective hospital study (Civan et al. 2014), highlight the increased risk of chronic paracetamol over-exposure in patients taking multiple paracetamol-containing medications. As older patients are more likely to be taking multiple medications including over the counter medications (Qato et al. 2008), there is the possibility that they are at increased risk of chronic high exposure due to multiple paracetamol-containing medications or other medications that increase the risk of paracetamol-induced hepatotoxicity.

There have been few animal studies of non-acute paracetamol exposure, and these are summarised in section 1.2.8. More research is needed to clarify the effect of species/strain, timing, and dose on the effect of paracetamol pre-treatment on susceptibility to toxicity. There have been no animal studies on chronic or sub-acute paracetamol exposure in old age.

Current treatment of paracetamol overdose to prevent hepatotoxicity is the glutathione precursor N-acetyl-cysteine (NAC) (Corcoran, Racz, et al. 1985). NAC guidelines are designed for protection from a single high dose of paracetamol and there is little evidence to guide risk assessment and optimal treatment for staggered over-exposures, chronic exposure or repeated supra-therapeutic exposures (Daly et al. 2008). One animal study found that several weeks of NAC pre-treatment protected against toxicity.
induced by twice-weekly paracetamol dosing (Chen et al. 2012). However this study does not model the clinically relevant situation, in which a patient would have post rather than pre-treatment with NAC. There have been no studies, to our knowledge on the effectiveness of NAC in old age. As older patients are more likely to have the types of exposures for which the NAC treatment evidence is lacking (Kane et al. 2012), optimisation of treatment for staggered or chronic paracetamol exposures, would be particularly of benefit to the older population.

This chapter models these clinically relevant paracetamol exposure situations in mice. The study aim was to examine the effect of chronic paracetamol and sub-acute paracetamol exposure in both young and old mice, and investigate whether NAC was effective at preventing paracetamol toxicity induced by chronic and sub-acute exposure.

3.2 Methods

3.2.1 Animals
Young (4.0±0.3 months, n=59) or old (26.8±0.5 months n=56) male C57BL/6 mice were obtained from, and housed at the Kearns Facility (Sydney, NSW). The median lifespan of C57BL/6 mice is 26-30 months The mouse numbers chosen for this study allowed for n=6 per group. This number was chosen based on previous work by Miller et al (2005), to account for expected variability and to detect a clinically significant difference in the main outcome of serum ALT. Unfortunately, the loss of more old mice than expected before the experimental timeframe required the use of n=4 for some old mouse groups.Mice were group housed in cages of 1-5 with ad libitum access to food (Rat and Mouse Premium Breeder Diet, Gordon’s Specialty Stockfeeds, NSW, Australia) and water. Animal rooms were maintained on a 12 hr light/dark cycle at 20-
22°C, and 30-70% humidity. Animals were randomly assigned to diet groups by cage, and then individually randomly assigned to treatment groups prior to the treatment day. Mice were euthanized if moribund, and where possible necropsied. Animal protocols were approved by the Animal Care Ethics Committee at Royal North Shore Hospital.

3.2.2 Frailty Index Assessment
The Mouse Clinical Frailty Index was used to assess frailty as detailed in section 2.2.2. Young (12 week) weight and temperature means and standard deviations were obtained from the mean of young male C57BL/6 mice (n=5) from the Kearns Facility (weight=26.0± 0.8 grams, temperature=31.6± 1.0°C).

All old mice were assessed for frailty, the day before being randomised to a diet group. Frailty testing was carried out by 2 raters. The raters were blinded to the scores assigned by the other rater. The final reported frailty index scores were calculated from the mean of the 2 rater’s scores.

3.2.3 Chronic and Sub-Acute Paracetamol Treatment of Mice, and Tissue Collection
Mice were randomized at 4.0±0.3 months of age (young, n=59) or 26.8±0.5 months of age (old, n=56) to either a control diet (Standard Meat Free Mouse and Rat Feed, 20 % Protein, 60% Carbohydrate, 5% Fat, Specialty Feeds, Australia), or control diet supplemented with paracetamol at a concentration of 1.33g/kg feed, both diets fed ad libitum. All mice were fed control diet for two weeks prior to the experimental period to adjust to the new diet. Body weight and food intake for all mice were monitored weekly. Mice remained on their respective diets for a further 6 weeks.
Chapter 3: Chronic and Sub-acute Paracetamol Exposure in Young and Old Mice, And The Role of N-Acetyl Cysteine

Following six weeks of their control or experimental diet, mice were given, via oral gavage, sub-acute paracetamol or saline treatment. Mice were dosed an additional paracetamol dose (250mg/kg, Panadol Color-free Baby Drops, 100mg/ml, GlaxoSmithKline, Australia), or saline, three times per day for two days. The mice were gavaged at 8am, 1pm and 6pm. On the third day the mice were fasted at 6am, then gavaged two more doses of paracetamol (250mg/kg), or saline, at 8am and 1pm. For the paracetamol diet group only, immediately following the final sub-acute paracetamol dose, mice were also dosed, via oral gavage, NAC (1200mg/kg in saline, pH=7.0, Sigma-Aldrich, MO, USA) or saline. At 3pm these mice were given a second NAC dose (1200mg/kg), or saline, via oral gavage. This created eight treatment groups for each age group: control diet+saline, control diet+paracetamol, paracetamol diet+saline, paracetamol diet+paracetamol, paracetamol diet+paracetamol+NAC, paracetamol diet+paracetamol+2xNAC, paracetamol diet+paracetamol+2xNAC.

For all mice, three hours after the final sub-acute paracetamol dose (4pm), each mouse was anaesthetized with an i.p. injection of ketamine (75 mg/kg, Cenvet Australia, #K1500) and xylazine (10 mg/kg, Cenvet Australia, #X5010). A midline laparotomy was performed and blood taken from the Inferior Vena Cava (IVC). The portal vein was then cannulated with a 21G intravenous catheter (BD, Sydney, Australia) through which the liver was perfused in-situ at 1–1.5 mL/min/g of liver with oxygenated Krebs-Henseleit bicarbonate buffer (95% O₂–5% CO₂, 37°C) to remove the blood. Sections of the liver were snap frozen in liquid nitrogen for subsequent enzyme activity assays and fixed in 10% neutral buffered formalin for subsequent histopathological analysis. See Table 3.1 for the experimental timeline.
Chapter 3: Chronic and Sub-acute Paracetamol Exposure in Young and Old Mice, And The Role of N-Acetyl Cysteine

The chronic paracetamol daily dose was chosen based on a previous study that determined this dose caused analgesia in mice (Minville et al. 2011), and a weight-based calculation of the equivalent of an average person taking twice the recommended daily dose (8g/80kg person/day = 100mg/kg/day). Pilot studies were conducted in young C57BL/6 mice to determine the minimum sub-acute paracetamol dose that would cause an ALT over three times the upper limit of normal in 50% of mice. Six mice fed a paracetamol-containing diet for six weeks, were treated with either 50mg/kg (n=2), 100mg/kg (n=2) or 250mg/kg (n=2) paracetamol via oral gavage three times per day for three days. Only one of the mice, who was dosed 250mg/kg, had ALT levels that were more than three times the upper limit of normal so this dose was chosen to ensure there was sufficient detectable hepatotoxicity.
Table 3.1 Chronic and Sub-acute Paracetamol Dosing Timeline

<table>
<thead>
<tr>
<th>DIET</th>
<th>Weeks 0-2</th>
<th>Weeks 2-8</th>
<th>Week 9, Day 1 8am, 1pm, 6pm</th>
<th>Week 9, Day 2 8am, 1pm, 6pm</th>
<th>Week 9, Day 3 6am</th>
<th>Week 9, Day 3 8am</th>
<th>Week 9, Day 3 1pm</th>
<th>Week 9, Day 3 3pm</th>
<th>Week 9, Day 3 4pm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Diet</td>
<td>Control Diet</td>
<td>Control Diet</td>
<td>Control Diet</td>
<td>Control Diet</td>
<td>Control Diet</td>
<td>Control Diet</td>
<td>Control Diet</td>
<td>Control Diet</td>
<td>Control Diet</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sub-acute Paracetamol, or Saline, Dosing (250mg/kg)</td>
<td>Sub-acute Paracetamol, or Saline, Dosing (250mg/kg)</td>
<td>Mice fasted</td>
<td>Sub-acute Paracetamol, or Saline, Dosing (250mg/kg)</td>
<td>Sub-acute Paracetamol, or Saline, Dosing (250mg/kg)</td>
<td>-</td>
<td>Mice euthanized, serum/liver collected</td>
</tr>
<tr>
<td>Paracetamol Diet</td>
<td>Control Diet</td>
<td>Paracetamol Diet (1.33mg/kg feed)</td>
<td>Sub-acute Paracetamol, or Saline, Dosing (250mg/kg)</td>
<td>Sub-acute Paracetamol, or Saline, Dosing (250mg/kg)</td>
<td>Mice fasted</td>
<td>Sub-acute Paracetamol, or Saline, Dosing (250mg/kg)</td>
<td>Sub-acute Paracetamol, or Saline, Dosing (250mg/kg) + NAC or saline Dosing (1200mg/kg)</td>
<td>NAC or Saline Dosing (1200mg/kg)</td>
<td>Mice euthanized, serum/liver collected</td>
</tr>
</tbody>
</table>

Alice Kane - March 2016
3.2.4 Serum Biochemistry to Assess Liver and Renal Function
Blood was stored and serum extracted as detailed in section 2.2.4, serum liver function tests (total protein, albumin, bilirubin, alkaline phosphatase (ALP), gamma glutamyltransferase (GGT)), creatinine and serum paracetamol concentrations were measured by a National Association of Testing Authorities accredited hospital laboratory, PaLMS (Pacific Laboratory Medicine Services) at Royal North Shore Hospital (Sydney, Australia) using an Architect i1000SR immunoassay analyser (Abbott Diagnostics, IL, USA). The lower limit of the paracetamol serum concentration test was 20μmol/L. Vitamin B6 is added to the ALT activity assay to accommodate for the age related decline in this vitamin, an integral cofactor for the assay (Krishnamurthy et al. 1967; Bordoni et al. 1998).

3.2.5 Liver Histology
Liver samples for all mice, and spleens and macroscopically abnormal tissue from old mice with macroscopically suspected cancer, were assessed for histopathology. Fixed tissue was embedded in paraffin, 5µm sections were cut on a microtome and mounted on slides. Slides were stained with Haemotoxylin and Eosin in the National Association of Testing Authorities accredited hospital laboratory of the Pathology department of Royal Prince Alfred Hospital, Sydney, Australia. Histopathology was scored by an anatomical pathologist (CM), who was blinded to the treatment of the samples. Inflammation was scored as either present or not, based on the presence of lymphocytes, histiocytes and neutrophils. Necrosis was scored as a percentage of the liver tissue on each slide, which was then converted to a dichotomous variable (present or not present) for analysis. Images were taken on an Olympus BX51 microscope connected to an Olympus DP26 camera (Olympus, Sydney, Australia).
3.2.6 Biochemical Examination of CYP2E1 in Frozen Liver Samples
Liver microsomes were isolated according to the method of Shoaf et al. (1987), and CYP2E1 activity was measured according to the method of Roberts et al. (1995) and Mach et al. (2014), as detailed in section 2.2.6.

3.2.7 Biochemical Examination of Glutathione and DNA Fragmentation in Frozen Liver Samples
Liver concentrations of total glutathione were determined with a Glutathione Assay kit (Cayman Chemicals, #703002) as detailed in section 2.2.6.

DNA fragmentation was determined using an enzyme-linked immunosorbent assay (ELISA) Cell Death detection kit (#11544675001, Roche, Switzerland) according to the manufacturer’s specifications. In brief, frozen liver was homogenised in 5 times the volume of incubation buffer to liver weight. The homogenate was then centrifuged at 13,000 g at 4°C for 20 min. The supernatant was diluted 250-fold before following the rest of the ELISA protocol.

3.2.8 Statistics
Data are expressed as mean ± SEM unless otherwise indicated. Differences between mean values across treatment and age groups were calculated with one- two- or three-way ANOVA with Tukey’s HSD post-hoc test where appropriate. Chi-squared tests were used for comparisons of proportions across groups. Spearman correlation coefficients (r) were used to analyse the correlation between Frailty Index and other outcomes, and linear regression was used to calculate slopes of best fit for each treatment group. Analysis of covariance (ANCOVA) was used to compare the regression slopes between paracetamol or saline treated groups, with frailty index as a covariate. Data analysis was completed using the statistics program SPSS (Version
3.3 Results

3.3.1 Animal Characteristics
Weights, food consumed and serum biochemistry results for each mouse group are shown in Table 3.2 and Table 3.3. One-way ANOVA across the eight treatment groups for young and old mice, with Tukey’s HSD post-hoc testing, showed no difference in animal weights, food consumed or any serum biochemistry results across any of the old groups. For the young mice, compared to control diet-saline dosed controls, there was a significant increase in liver weight (as a percentage of body weight), bilirubin and total protein, in some paracetamol treated groups. The paracetamol doses received in the diet ranged from 167 to 188 mg/kg/day. Several old mice had suspected cancer, as assessed via histology (Table 3.3). These mice were not excluded from analysis, in order to mimic a true model of an aged population with multimorbidity, and these mice were randomly spread across the treatment groups.

Several mice died or were moribund, and thus euthanized, during the experiment. One young mouse on paracetamol diet, died after two days of sub-acute paracetamol dosing (suspected severe paracetamol-induced liver toxicity, serum ALT concentration of 32545 U/L with 80% liver necrosis). Three old mice on control diet, and three old mice on paracetamol diet died before the subacute dosing was started (unable to be necropsied). Three old mice fed paracetamol diet, died after starting the sub-acute dosing regimen, though they were receiving saline, and are not suspected of paracetamol-induced liver toxicity (one unable to be necropsied; one suspected kidney...
failure with serum creatinine of 348 µmol/L and ALT of 63 U/L; one macroscopically suspected liver cancer with ALT of 78 U/L).
Table 3.2 Animal characteristics and serum biochemistry results for young male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-Acetyl Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Serum and liver collected 3 hours after the final paracetamol/saline dose.*p<0.05 compared to control diet; saline group #p<0.05 compared to corresponding saline group with Tukey’s HSD post-hoc test. ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase, NAC=N-Acetyl Cysteine, NS=Non-significant

<table>
<thead>
<tr>
<th>Age at sac (months)</th>
<th>5.7 (0.0)</th>
<th>5.6 (0.1)</th>
<th>5.8 (0.1)</th>
<th>5.5 (0.1)</th>
<th>5.8 (0.1)</th>
<th>5.5 (0.1)</th>
<th>5.3 (0.1)</th>
<th>5.3 (0.0)</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol dose, week 6 of diet (mg/kg mouse/day)</td>
<td>-</td>
<td>-</td>
<td>174.6 (5.7)</td>
<td>187.8 (10.8)</td>
<td>173.4 (5.1)</td>
<td>167.3 (4.4)</td>
<td>187.1 (3.1)</td>
<td>187.6 (3.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight pre-subacute dosing (g)</td>
<td>29.6 (0.6)</td>
<td>28.8 (0.6)</td>
<td>30.7 (0.7)</td>
<td>28.4 (0.8)</td>
<td>28.1 (0.6)</td>
<td>30.6 (0.6)</td>
<td>28.2 (0.7)</td>
<td>27.5 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight post-subacute dosing (g)</td>
<td>27.6 (0.6)</td>
<td>25.0 (0.9)</td>
<td>28.2 (0.4)</td>
<td>24.9 (0.7)</td>
<td>25.8 (1.2)</td>
<td>27.3 (0.6)</td>
<td>26.3 (0.7)</td>
<td>24.7 (0.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Food eaten, day 2 of subacute dosing (g/mouse/day)</td>
<td>2.2 (0.2)</td>
<td>2.1 (0.3)</td>
<td>2.6 (0.4)</td>
<td>2.6 (0.2)</td>
<td>2.5 (0.3)</td>
<td>1.9 (0.1)</td>
<td>2.3 (0.5)</td>
<td>2.3 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Variable</td>
<td>Control Diet</td>
<td>Paracetamol Diet</td>
<td>One-way</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------</td>
<td>------------------</td>
<td>-----------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>3.8 (0.1) 4.3 (0.3)</td>
<td>3.7 (0.2) 4.6 (0.2)</td>
<td>4.3 (0.2) 4.8 (0.1)*</td>
<td>3.9 (0.2) 4.8 (0.3)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>44.7 (0.9) 49.3 (1.3)</td>
<td>45.5 (2.2) 49.5 (1.5)</td>
<td>44.0 (1.9) 50.7 (1.0)*</td>
<td>49.1 (1.2) 53.7 (1.4)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>24.3 (0.8) 26.4 (0.7)</td>
<td>25.0 (1.0) 26.3 (0.8)</td>
<td>25.2 (0.2) 26.3 (0.3)</td>
<td>26.0 (0.7) 28.0 (0.6)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>1.4 (0.3) 3.2 (0.3)*</td>
<td>3.0 (0.4) 5.0 (1.1)*</td>
<td>- 3.6 (0.2)*</td>
<td>2.7 (0.3) 3.5 (0.2)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>99.7 (9.4) 96.7 (6.9)</td>
<td>112.5 (4.2) 93.8 (12.3)</td>
<td>104.6 (6.9) 110.6 (5.2)</td>
<td>110.4 (2.2) 110.8 (6.7)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>3.0 (0.0) 3.0 (0.0)</td>
<td>3.0 (0.0) 3.0 (0.0)</td>
<td>3.0 (0.0) 3.0 (0.0)</td>
<td>3.0 (0.0) 3.0 (0.0)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>32.0 (1.7) 32.5 (2.9)</td>
<td>29.5 (1.7) 30.7 (0.7)</td>
<td>27.0 (0.0) 30.2 (0.5)</td>
<td>28.4 (1.1) 29.0 (0.6)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.3** Animal characteristics and serum biochemistry results for old male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-Acetyl Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Serum and liver collected 3 hours after the final paracetamol/saline dose. ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase, NAC=N-Acetyl Cysteine.
<table>
<thead>
<tr>
<th></th>
<th>Saline (n=6)</th>
<th>Paracetamol (n=7)</th>
<th>Saline (n=6)</th>
<th>Paracetamol (n=7)</th>
<th>Saline + NAC (n=6)</th>
<th>Paracetamol + NAC (n=7)</th>
<th>Saline + 2 x NAC (n=4)</th>
<th>Paracetamol + 2 x NAC (n=4)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at sac (weeks)</strong></td>
<td>28.6 (0.0)</td>
<td>28.6 (0.0)</td>
<td>28.6 (0.0)</td>
<td>28.5 (0.0)</td>
<td>28.6 (0.0)</td>
<td>28.6 (0.0)</td>
<td>26.9 (0.1)</td>
<td>27.1 (0.2)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Paracetamol dose, week 6 of diet (mg/kg mouse/day)</strong></td>
<td>-</td>
<td>-</td>
<td>140.6 (9.4)</td>
<td>126.9 (5.8)</td>
<td>133.1 (3.7)</td>
<td>139.6 (6.9)</td>
<td>151.8 (13.9)</td>
<td>159.5 (9.8)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Weight pre- subacute dosing (g)</strong></td>
<td>29.6 (2.2)</td>
<td>28.3 (2.2)</td>
<td>32.0 (1.5)</td>
<td>34.2 (0.7)</td>
<td>32.0 (0.4)</td>
<td>32.1 (0.6)</td>
<td>31.6 (1.4)</td>
<td>28.4 (2.2)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Weight post- subacute dosing (g)</strong></td>
<td>28.6 (2.0)</td>
<td>26.5 (1.6)</td>
<td>32.0 (1.6)</td>
<td>30.6 (0.9)</td>
<td>29.9 (0.3)</td>
<td>28.6 (0.5)</td>
<td>30.6 (1.3)</td>
<td>26.1 (1.6)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Food eaten, day 2 of subacute dosing (g/mouse/day)</strong></td>
<td>2.8 (0.3)</td>
<td>2.2 (0.3)</td>
<td>2.8 (0.6)</td>
<td>1.5 (0.4)</td>
<td>1.7 (0.3)</td>
<td>1.8 (0.3)</td>
<td>2.5 (0.5)</td>
<td>2.7 (0.6)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Liver weight (% body weight)</strong></td>
<td>4.1 (0.3)</td>
<td>4.5 (0.2)</td>
<td>4.4 (0.2)</td>
<td>4.6 (0.2)</td>
<td>6.7 (1.4)</td>
<td>4.8 (0.4)</td>
<td>4.2 (0.2)</td>
<td>5.1 (0.2)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Total protein (g/L)</strong></td>
<td>48.6 (1.7)</td>
<td>50.0 (1.7)</td>
<td>49.4 (1.1)</td>
<td>47.8 (2.1)</td>
<td>48.2 (1.1)</td>
<td>48.8 (1.1)</td>
<td>51.3 (0.8)</td>
<td>52.3 (2.2)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Albumin (g/L)</strong></td>
<td>23.8 (0.7)</td>
<td>23.7 (1.4)</td>
<td>23.2 (0.5)</td>
<td>23.1 (1.1)</td>
<td>23.0 (0.4)</td>
<td>23.3 (0.5)</td>
<td>25.5 (0.3)</td>
<td>25.0 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Bilirubin (µmol/L)</strong></td>
<td>2.2 (0.5)</td>
<td>2.7 (0.6)</td>
<td>2.0 (0.4)</td>
<td>2.6 (0.5)</td>
<td>3.2 (0.5)</td>
<td>3.6 (0.8)</td>
<td>2.5 (0.3)</td>
<td>3.0 (0.4)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ALP (U/L)</strong></td>
<td>99.6 (12.7)</td>
<td>100.8 (12.2)</td>
<td>90.6 (4.7)</td>
<td>115.6 (17.4)</td>
<td>173.7 (75.8)</td>
<td>115.9 (21.1)</td>
<td>146.3 (21.6)</td>
<td>144.3 (7.6)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>GGT (U/L)</strong></td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.7 (0.7)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Alice Kane - March 2016
<table>
<thead>
<tr>
<th>Creatinine (µmol/L)</th>
<th>46.3 (15.3)</th>
<th>24.7 (2.2)</th>
<th>35.7 (4.2)</th>
<th>37.7 (6.2)</th>
<th>31.7 (1.2)</th>
<th>30.5 (1.2)</th>
<th>40.8 (7.8)</th>
<th>40.5 (13.2)</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspected cancer (n, %)</td>
<td>1 (16.7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (16.7)</td>
<td>1 (14.3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
</tbody>
</table>
3.3.2 Assessment of Toxicity with Chronic Paracetamol Exposure

Chronic dietary paracetamol exposure did not result in an increase in serum ALT in young or old mice (Figure 3.1A and B). To determine whether there was low-grade biomolecular liver damage that serum ALT did not reveal, we investigated the level of DNA fragmentation in the livers of young and old, control or paracetamol diet, saline treated mice groups (Figure 3.2). There was no increase in DNA fragmentation with dietary paracetamol exposure in young or old mice (young p=0.91, old p=0.72). For those mice receiving sub-acute saline, with the paracetamol diet compared to the control diet, there was no significant difference in the proportion of young mice with necrosis (p=0.27) (Figure 3.1C), and a trend towards an increase in the proportion of young and old mice with inflammation (young p=0.14, old p=0.12) (Figure 3.1C and D). The average percentage of the scored liver with necrosis was 4.4±12.5 % across chronic paracetamol exposed groups.
Figure 3.1 Serum alanine aminotransferase (ALT) concentration and proportion of each group with detectable liver necrosis and inflammation for young (A, C, respectively, 4 ± 0.3 months) and old (B, D, respectively, 26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-Acetyl Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Serum and liver collected 3 hours after the final paracetamol/saline dose. Serum ALT data expressed as mean ± SEM. One-way ANOVA young mice ALT, F(7,44)=5.35 p=0.00. One-way ANOVA old mice ALT, F(7,38)=3.77 p=0.003. *p<0.05 compared to corresponding saline-treated group with Tukey’s HSD post-hoc test. n=4-8 per group
**Figure 3.2** DNA Fragmentation in liver homogenate from young (4 ± 0.3 months) and old (26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline. Liver collected 3 hours after the final saline dose. Data expressed as % relative to young control diet ± SEM. n=4-8 per group.
Figure 3.3 Representative Haemotoxylin and Eosin stained liver histology images for young (4 ± 0.3 months) and old (26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single dose of N-Acetyl Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Liver collected 3 hours after the final paracetamol/saline dose. Images taken at 100-200X.
3.3.3 Assessment of Toxicity with Sub-acute Paracetamol Exposure

All young mouse groups treated with sub-acute paracetamol had increased serum ALT concentrations compared to their corresponding saline-treated groups (p<0.05, Figure 3.1A). For old mice, sub-acute paracetamol treatment significantly increased ALT compared to the corresponding saline treated group only for the paracetamol diet+paracetamol group and paracetamol diet+paracetamol+2xNAC group (p<0.05). There was a trend towards increased serum ALT with sub-acute paracetamol treatment in the other old groups compared to saline controls (control diet+paracetamol group p=0.09, paracetamol diet+paracetamol+NAC group p=0.19) (Figure 3.1B). Histological grading also showed an increase in the proportion of mice with necrosis and inflammation, for all sub-acute paracetamol treated groups compared to the saline-treated groups (young necrosis p=0.002, inflammation p=0.001; old necrosis p=0.001, inflammation p=0.06). The average percentage of the scored liver with necrosis was 24.0±24.9 % across subacute paracetamol exposed groups. A three-way ANOVA of age, diet and treatment group of all mice, showed that treatment was the only significant factor associated with serum ALT concentration, with all age and diet interaction terms non-significant, implying that neither the age nor diet group of the mice affected the degree of sub-acute paracetamol-induced toxicity.

3.3.4 Assessment of Toxicity with Paracetamol Exposure Plus N-Acetyl Cysteine

In our study, neither treatment with a single nor double dose of NAC was able to reduce the degree of toxicity induced by sub-acute paracetamol dosing. A two-way ANOVA for each age group, considering only those mice on the paracetamol diet, of sub-acute paracetamol/saline treatment and NAC treatment, showed that the interaction term between sub-acute treatment and NAC, for ALT serum concentration, was not significant (Figure 3.1A and B). There was also no reduction in the proportion of
necrosis or inflammation seen in the groups treated with either a single or double dose of NAC plus paracetamol, compared to paracetamol alone (young necrosis $p=0.72$, inflammation $p=0.69$; old necrosis $p=0.23$, inflammation $p=0.23$).

3.3.5 Serum Paracetamol Levels, Liver Glutathione Levels and CYP2E1 Activity

We also assessed the serum paracetamol levels for all young (Figure 3.4A) and old (Figure 3.4B) mouse groups treated with sub-acute paracetamol or saline. In all saline treated mice, even those receiving chronic paracetamol in the diet, paracetamol serum levels were below the detectable limit of the test. A one-way ANOVA across treatment groups for each mouse group, identified a significant increase in paracetamol serum level for young mice treated with sub-acute paracetamol plus NAC, compared to those treated with sub-acute paracetamol only (Figure 3.4A) ($p=0.036$).

![Figure 3.4](image)

**Figure 3.4** Paracetamol serum concentrations for young (A, $4 \pm 0.3$ months) and old (B, $26.8 \pm 0.5$ months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or...
paracetamol (250mg/kg x 3/day) plus a single or double dose of N-Acetyl Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Serum collected 3 hours after the final paracetamol/saline dose. One-way ANOVA young mice, $F(7,31)=10.96\ p=0.00$. One-way ANOVA old mice, non-significant. Data are expressed as mean ± SEM. *$p<0.05$ compared to control diet+paracetamol group with Tukey’s HSD post-hoc test. $n=4-8$ per group.

Total liver glutathione levels were reduced with sub-acute paracetamol treatment in control fed old mice ($p=0.01$), but not young mice ($p=0.55$) (Figure 3.5). This reduction in total liver glutathione levels was not seen for old mice fed a paracetamol diet followed by sub-acute paracetamol (Figure 3.4). In both young and old mice, there was a significant increase in GSH levels with both a single and double dose of NAC, in combination with sub-acute paracetamol treatment, compared to sub-acute paracetamol treatment alone, for mice on either a control or paracetamol diet (Figure 3.5).
Chapter 3: Chronic and Sub-acute Paracetamol Exposure in Young and Old Mice, And The Role of N-Acetyl Cysteine

Figure 3.5 Total liver glutathione (GSH) concentrations for young (A, 4 ± 0.3 months) and old (B, 26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-Acetyl Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Liver collected 3 hours after the final paracetamol/saline dose. Data are expressed as mean ± SEM. One-way ANOVA young mice, F(7,46)=11.91 p=0.00. One-way ANOVA old mice, F(7,37)=17.46 p=0.00. *p<0.05 compared to control diet+saline group, #p<0.05 compared to control diet+paracetamol group, ^p<0.05 compared to paracetamol diet+paracetamol group, ~p<0.05 compared to paracetamol diet+paracetamol+NAC group with Tukey’s HSD post-hoc test. n=4-8 per group.

Old mice treated with saline+NAC, paracetamol+NAC or paracetamol+2xNAC had significantly lower CYP2E1 activity levels than old mice treated with saline only (on either control or paracetamol diet). There was no significant change in CYP2E1 activity for young mice with paracetamol or NAC dosing (Figure 3.6), although a similar trend towards reduced CYP2E1 levels with NAC treatment was seen for some groups.
Figure 3.6 Liver cytochrome (CYP)2E1 activity for young (A, 4 ± 0.3 months) and old (B, 26.8 ± 0.5 months) male C57BL/6 mice fed either a control diet or paracetamol containing (1.33g/kg feed) diet for 6 weeks, then treated with 3 days of saline or paracetamol (250mg/kg x 3/day) plus a single or double dose of N-Acetyl Cysteine (NAC) (1200mg/kg) for the paracetamol diet group. Liver collected 3 hours after the final paracetamol/saline dose. One-way ANOVA young mice, non-significant. One-way ANOVA old mice, F(7,23)=3.08 p=0.019. Data are expressed as mean ± SEM. *p<0.05 compared to control diet+saline group, #p<0.05 compared to control diet+paracetamol group, !p<0.05 compared to paracetamol diet+saline group. n=4-8 per group

A three-way ANOVA of age, diet and treatment group showed that there was no significant diet or age effect on paracetamol serum level, total hepatic GSH levels or CYP2E1 activity.

3.3.6 Correlation between Frailty and Paracetamol Toxicity Outcomes
To assess whether frailty affected the risk or mechanisms of chronic or subacute paracetamol hepatotoxicity, we investigated the correlation between frailty index and
associated outcomes, for old control diet+saline, paracetamol diet+saline (chronic) and control diet+paracetamol (subacute) mice (Figure 3.7).
Chapter 3: Chronic and Sub-acute Paracetamol Exposure in Young and Old Mice, And The Role of N-Acetyl Cysteine

Figure 3.7 Correlation between frailty index and (A) alanine aminotransferase (ALT), (B) necrosis, (C) cytochrome (CYP)2E1 activity, (D) total liver glutathione (GSH), (E) serum total protein, (F) serum albumin, (G) liver weight (as % body weight), and (H) serum alkaline phosphatase (ALP) for old (26.8±0.5 months) male C57BL/6 mice treated with either control diet for 6 weeks and 3 days of saline (control group, black), paracetamol (1.33g/kg feed) diet for 6 weeks and 3 days of saline (chronic group, light grey) or control diet for 6 weeks and 3 days of paracetamol, then (250mg/kg x 3/day) (sub-acute group, dark grey). Liver and serum collected 3 hours after the final paracetamol/saline dose. n=4-7 per group.

There was no correlation between frailty and serum ALT, percentage of hepatic necrosis, microsomal CYP2E1 activity, total liver GSH and serum total protein for saline, chronic paracetamol or sub-acute paracetamol treated mice (Figure 3.7). There were significant strong negative correlations between frailty index and serum albumin ($r=-0.80, p=0.05$) and ALP ($r=-0.92, p=0.01$) for sub-acute paracetamol treated old mice only. There was a positive correlation between frailty index and liver weight ($r=0.96, p=0.04$) for chronic paracetamol treated old mice only. An ANCOVA showed no significant difference between the regression slopes for these outcomes (ALP, albumin, liver weight %) for treatment group, when adjusted for frailty index as a covariate.

3.4 Discussion
In this chapter, three clinically relevant paracetamol exposure situations were successfully modelled in young and old mice: chronic exposure to therapeutic levels of paracetamol; sub-acute exposure to supra-therapeutic doses of paracetamol over three days (most often an accidental overdose due to confusion with multiple paracetamol
containing medications or dose escalation for unrelieved pain); and a combination of these two exposures. We also tested whether NAC administered after sub-acute dosing to mimic the human clinical situation, would protect against hepatotoxicity. We found no effect of age or frailty on susceptibility to paracetamol toxicity from chronic or sub-acute exposure to paracetamol in mice. We also found that chronic low-dose paracetamol exposure did not cause hepatotoxicity, as measured by serum ALT. Three days of sub-acute exposure caused significant hepatotoxicity, and neither a single nor double dose of NAC protected against this toxicity in young or old mice.

In chapter 2 of this thesis it was found there was no change in the susceptibility of mice to paracetamol toxicity induced by acute paracetamol exposure in old age or frailty. As older patients are more likely to have non-acute exposures to paracetamol, the current chapter explored the effect of age and frailty on paracetamol toxicity risk to chronic or sub-acute paracetamol exposures. We found no effect of age of frailty on susceptibility to paracetamol toxicity from these types of exposures. Research confirming these findings in a clinical setting, would enable the optimisation of the use of chronic or repeated dose paracetamol for pain relief in the older population, as a relatively well-tolerated analgesic, with no increased risk of toxicity in old age. Consistent with the results reported in chapter 2, in the current study there was no overall effect of frailty on the degree of paracetamol toxicity, microsomal CYP2E1 activity or total liver GSH, although there was a significant correlation of frailty index with serum ALP and albumin concentrations in the sub-acute paracetamol treated mice, which again may be attributed simply to frailty (Schalk et al. 2004; Kitamura et al. 2012). It would be interesting to look at the effect of a longer time-frame after paracetamol insult, a more functional mouse frailty assessment tool (Liu et al. 2013) or the dichotomisation of mice.
into frail and non-frail groups on the correlation between frailty and degree of hepatotoxicity after non-acute exposures.

Chronic paracetamol exposure in the diet for six weeks did not cause clinically detectable hepatotoxicity in young or old mice. There was no elevation in serum ALT, or DNA fragmentation, in the paracetamol diet group compared to those fed a control diet. Interestingly, however, several samples in the paracetamol diet fed groups, without sub-acute paracetamol treatment, had histological evidence of necrosis and/or inflammation. Although statistically, this was only a trend compared to the control diet group, it does suggest that the paracetamol diet may have caused some low-level damage in some mice which is not measurable in the circulation, at least not after 6 weeks of treatment. Perhaps future studies modeling clinically therapeutic levels of paracetamol ingestion, may need to use higher daily doses of paracetamol, or a longer treatment period, in order to detect circulating levels of liver toxicity markers. Previous animal studies that have dosed once daily, via intraperitoneal injection or oral gavage, for 30-99 days with 75-300mg/kg showed no evidence of toxicity as assessed by liver function tests (de Meijer et al. 2012; Yisarakun et al. 2014; Kondo et al. 2012). Our study, which modelled a more clinically relevant exposure to paracetamol at regular intervals over a full day, as it was consumed in the diet, rather than once daily, appears to confirm these findings. Paracetamol serum levels were below the detectable level for paracetamol diet groups in the current study, despite the mice consuming daily paracetamol doses of up to 188mg/kg/day. A more sensitive method of testing serum paracetamol levels, such as high-performance liquid chromatography (HPLC), may enable the detection of serum paracetamol levels in future studies. Although the clinical method of paracetamol serum testing is reliable and accurate for the high paracetamol
levels seen in toxicity, it may be limited in detecting changes at lower serum levels. The lower limit of the Abbott Architect method used in the current study was 20 µmol/L, and the lower limit of HPLC protocols to measure serum paracetamol can be 0.4µmol/L (Jensen et al. 2004).

Sub-acute paracetamol exposure over three days caused significant hepatotoxicity in young and old mice, as measured by biochemistry and histology. However, there was no clear combinatorial toxicity effect of paracetamol exposure in the diet plus sub-acute paracetamol exposure, although dietary pre-exposure to paracetamol certainly did not protect against subsequent sub-acute exposure in this study, as has been seen with higher dose pre-treatment in other studies (Ghanem et al. 2009; O’Brien et al. 2000; Shayiq et al. 1999). Perhaps the daily dose received in the current study was not enough to induce the protective pharmacokinetic changes that were seen in the previous studies, as we saw no change in total glutathione or CYP2E1 activity with chronic paracetamol treatment. Interestingly, despite no combined effect on the degree of paracetamol toxicity, it appeared that, in the old mice only, paracetamol diet prevented the reduction in total liver glutathione seen with sub-acute paracetamol treatment and control diet (Figure 3.4). However, this potentially protective effect did not translate into reduced toxicity in this group of old mice.

The pre-treatment protective effect seen in the previous studies (Ghanem et al. 2009; O’Brien et al. 2000; Shayiq et al. 1999), was also not seen with the sub-acute paracetamol treatment in the current study. Although the mice were receiving doses of 250mg/kg three times per day for three days, we did not see the protective pharmacokinetic changes seen with pre-treatment as reported by other studies. In our
Chapter 3: Chronic and Sub-acute Paracetamol Exposure in Young and Old Mice, 
And The Role of N-Acetyl Cysteine

study, total liver glutathione concentration was unchanged with sub-acute paracetamol 
treatment in most groups, which is consistent with previous acute paracetamol studies 
for this time frame (Mitchell et al. 1973; McGill et al. 2012). CYP2E1 activity was not 
changed with sub-acute paracetamol treatment in any group. Previous acute studies have 
found CYP2E1 activity to be reduced with 400mg/kg after four hours in mice (Snawder 
et al. 1994). In the studies of multiple paracetamol dose treatment, Shayiq et al. (1999) 
found that eight days of increasingly high daily doses of paracetamol treatment in 
BALB/c mice resulted in increased total liver glutathione levels, and reduced CYP2E1 
activity, which in term contributed to protection against toxicity from a larger acute 
dose on the ninth day. However it is important to note that genetic background can 
affect the outcomes, as Shayiq et al. (1999) saw 100% mortality 24 hours after a dose of 
500mg/kg paracetamol in BALB/c mice, whilst the C57BL/6 mice in the current study 
received 750mg/kg per day for three days with zero mortality. The other multiple 
paracetamol dose studies were conducted in rats, which also have altered paracetamol 
pharmacokinetics and toxicology compared to C57BL/6 mice (McGill et al. 2012), and 
found protection from toxicity with four days of low dose pre-treatment (O’Brien et al. 
2000; Ghanem et al. 2009), but increased toxicity with one moderate pre-treatment dose 
18 hours before a second dose (Kim et al. 2009). These results demonstrate that time-
frame, dose, species and genetic background (strain) can significantly affect 
paracetamol pharmacokinetics and toxicity, and more research is needed to clarify the 
effect of multi-day paracetamol dosing on susceptibility to hepatotoxicity in all ages.

NAC did not protect against hepatotoxicity induced by sub-acute paracetamol exposure 
in young or old mice. As seen in chapter 4 of this thesis (Figure 4.3), and in previous 
studies (James et al. 2003; Daly et al. 2008), concurrent dosing of acute paracetamol
and NAC does protect against hepatotoxicity. NAC treatment did result in increased total liver glutathione concentrations in all mice, and decreased CYP2E1 activity, in old mice. These potentially protective pharmacokinetic changes, did not translate into protection in this study. It is likely that with three days of dosing, the paracetamol-induced liver damage has progressed too far for these mechanisms to be protective. The high prevalence of inflammation in the sub-acute paracetamol treated groups would imply that the damage has progressed beyond the early stages of covalent protein NAPQI binding damage, and into the induction of inflammatory damage (Holt & Ju 2006). A recent mouse study found that with delayed treatment, NAC also did not protect against acute paracetamol induced hepatotoxicity (Soeda et al. 2014). NAC treatment also resulted in increased serum paracetamol levels in the current study, which implies reduced absorption of paracetamol into the liver. Perhaps, with concurrent paracetamol and NAC gavaging, there was also reduced NAC absorption that may have contributed to the lack of protection seen in this study. This finding of a lack of protection from NAC against sub-acute paracetamol dosing has important clinical implications in treating different types of paracetamol exposures. Our study indicates that for those who have taken staggered doses of paracetamol over several days, NAC may not be effective at preventing paracetamol hepatotoxicity. This finding is particularly important for older patients, as they are more likely to have the type of exposures, as explored here, against which NAC does not protect.

In conclusion, this study found that, as we saw with acute paracetamol exposure in chapter 2, there is no effect of age or frailty on susceptibility to chronic or subacute paracetamol toxicity in mice. This chapter also found that in young and old mice, sub-acute paracetamol exposure causes severe hepatotoxicity, which NAC does not reverse.
Chapter 3: Chronic and Sub-acute Paracetamol Exposure in Young and Old Mice, And The Role of N-Acetyl Cysteine

Furthermore, chronic low-level paracetamol exposure does not cause toxicity in young or old mice. Although it is re-assuring that chronic therapeutic paracetamol exposure does not cause toxicity, atleast in a mouse model, the clinical implications of NAC not protecting against sub-acute paracetamol induced toxicity are alarming, and highlight the need to develop new treatments for sub-acute paracetamol induced toxicity. The potential of interventions that delay ageing, as novel therapeutics to protect against paracetamol toxicity is explored in chapter four.
4 INVESTIGATION OF RESVERATROL AS A POTENTIAL THERAPY TO PROTECT AGAINST PARACETAMOL HEPATOTOXICITY

4.1 Introduction
The current clinically used therapy for paracetamol over-exposure, N-acetyl-cysteine (NAC), prevents liver toxicity by increasing glutathione stores to bind and de-toxify NAPQI (Corcoran, Racz, et al. 1985). Despite well-developed NAC treatment guidelines, there are patients who still develop liver failure from paracetamol toxicity, particularly those who present late or have repeated paracetamol ingestion (Craig et al. 2012; Daly et al. 2008; Ferner et al. 2011). In chapter three of this thesis, it was found that NAC does not protect against toxicity induced by sub-acute paracetamol exposure in young or old mice. Thus there is a need for new therapies to treat paracetamol toxicity.
toxicity from all types of exposures, and in all age groups. This chapter explores the potential for the healthspan increasing intervention, resveratrol, to protect against paracetamol hepatotoxicity.

Resveratrol is a polyphenol, often named a calorie-restriction mimetic drug, as it has been shown to mimic some of the beneficial effects of calorie restriction. Resveratrol’s mechanism of action is direct allosteric activation of the NAD-dependent deacetylase sirtuin-1 (SIRT1) enzyme (Hubbard et al. 2013), an enzyme which plays a role in longevity and regulation of response to stressors and nutrients (Michan & Sinclair 2007; Raynes et al. 2013). Resveratrol may also activate 5’ adenosine monophosphate-activated protein kinase (AMPK), an enzyme crucial for cellular energy homeostasis (Baur et al. 2006; Suchankova et al. 2009; Ido et al. 2015; Hardie 2011). A recent study has proposed that activation of these two pathways of resveratrol is dose-dependent, whereby low dose resveratrol directly activates SIRT1, which in turn results in activation of AMPK, and high-dose resveratrol activates AMPK independently of SIRT1 (Price et al. 2012). The protein complex, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which regulates DNA transcription in response to stimuli such as stress, is thought to be inhibited by resveratrol (Ren et al. 2013).

Resveratrol may have a variety of potentially protective effects upon factors involved in the paracetamol toxicity pathway including the potential to reduce CYP2E1 activity (Wu et al. 2013), induce NQO1 activity (Hsieh et al. 2006) and induce phase II metabolic enzymes (Cao & Li 2004), as well as causing anti-oxidant effects (Yu et al. 2012), improvements in mitochondrial function (Baur et al. 2006; Lagouge et al. 2006;
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

Hubbard et al. 2013), anti-inflammatory effects (Yoshizaki et al. 2010) and the induction of autophagy (Yu et al. 2012).

Several animal studies have investigated the potential protection with resveratrol from paracetamol toxicity and are summarised in section 1.4.5.2. Noticeably the mouse strain, paracetamol and resveratrol timing, doses, vehicles and routes of administration as well as outcome measurements varied significantly across these studies (detailed in Table 1.3). Furthermore only one of these studies investigated the downstream effects of resveratrol and confirmed its molecular action in these animal models (Wang et al. 2015). Thus confirmation and further examination of the mechanisms of the protective role of resveratrol against paracetamol toxicity would be beneficial. The potentially protective effects of resveratrol in old age versus young have also not been explored, and are particularly important given that older people are more at risk of the type of over-exposures that the current therapy does not protect against. Furthermore, there are changes to SIRT1 and AMPK in old age, which may affect the protective mechanisms of resveratrol (Salminen & Kaarniranta 2012).

This study aimed to investigate the potential protection of resveratrol against paracetamol toxicity in three models of paracetamol toxicity: primary mouse hepatocytes, young animals concurrently exposed to resveratrol and paracetamol, and young and old animals pre-treated with resveratrol in their diet then exposed to paracetamol.
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

4.2 Methods

4.2.1 Cell Culture Methods Optimisation

Optimisation of the cell culture model for paracetamol toxicity required some troubleshooting. The conditions tested, with justification and results, are summarised in Table 4.1. The final conditions used for the experimental results presented in this chapter are explained in more detail in section 4.2.3.

Table 4.1 Optimisation of cell culture model of paracetamol toxicity. DMEM= Dulbecco’s modified eagle medium, MTT= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, FCS= Fetal calf serum, DMSO= dimethyl sulfoxide, CYP2E1 = cytochrome 2E1.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Conditions</th>
<th>Justification</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2 cells, obtained from the Kolling Institute</td>
<td>DMEM medium with 10% FCS, 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-glutamine</td>
<td>Establishing confluent cells</td>
<td>Established confluent cells</td>
</tr>
<tr>
<td>HepG2 cells, obtained from the Kolling Institute</td>
<td>As above plus 4mM or 10mM Paracetamol</td>
<td>Tested toxicity of cells with MTT</td>
<td>Establishing toxicity model</td>
</tr>
<tr>
<td>HepG2 cells, obtained from the Kolling Institute</td>
<td>Removed 10% FCS from media, added 10mM paracetamol</td>
<td>Tested toxicity with MTT</td>
<td>Establishing toxicity model</td>
</tr>
<tr>
<td>HepG2 cells, obtained from the Kolling Institute</td>
<td>Removed 10% FCS from media, added 10mM paracetamol</td>
<td>Tested toxicity of cells with LDH assay in media</td>
<td>Testing different toxicity outcome measurement</td>
</tr>
<tr>
<td>Study</td>
<td>Experimental Details</td>
<td>Method</td>
<td>Results</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>HepG2 cells, obtained from Concord Hospital</td>
<td>As above</td>
<td>Testing different source of HepG2 cells</td>
<td>An increase in cell survival with paracetamol treatment – opposite to what expect - suspect paracetamol is interfering with the LDH assay (Xu et al. 2003)</td>
</tr>
<tr>
<td>HepG2 cells, obtained from Concord Hospital</td>
<td>As above, plus 20mM paracetamol</td>
<td>Establishing toxicity model</td>
<td>20mM paracetamol treatment reduced survival by approximately 30%, with a lot of variability</td>
</tr>
<tr>
<td>HepG2 cells, obtained from Concord Hospital</td>
<td>As above, plus 0.5% DMSO and 50µM Resveratrol</td>
<td>Testing potential resveratrol protection</td>
<td>DMSO as vehicle protects against paracetamol toxicity - CYP2E1 inhibitor (Jaeschke et al. 2011)</td>
</tr>
<tr>
<td>HepG2 cells, obtained from Concord Hospital</td>
<td>As above, plus 0.03% ethanol and 50µM Resveratrol</td>
<td>Testing potential resveratrol protection, and establishing toxicity model</td>
<td>Ethanol as vehicle does not affect paracetamol toxicity. Reduction in viability with paracetamol treatment not consistently seen – HepG2 cells have too little CYP2E1 activity to induce toxicity (Zhuge et al. 2003).</td>
</tr>
<tr>
<td>Primary Hepatocytes isolated with Liberase (Roche, #5401119001)</td>
<td>DMEM medium with 10% FCS, 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-glutamine, plus added 20mM Paracetamol, 0.03% ethanol and 50µM Resveratrol. Tested toxicity of cells with MTT</td>
<td>Testing new cell type – primary hepatocytes</td>
<td>Cell viability very low for all conditions – unsuccessful hepatocyte isolation</td>
</tr>
<tr>
<td>Primary Hepatocytes isolated with Liberase (Roche, #5401119001)</td>
<td>As above, but washed cells with Percoll to isolate only viable hepatocytes, plated at 0.03x10^6 cells/48 well and did not treat cells</td>
<td>Optimising isolation method for primary hepatocytes</td>
<td>Cell viability of control cells much better (greater than 90%), but amount of cells isolated is low (over-digestion of the liver) and cells overcrowded and unevenly distributed in wells</td>
</tr>
</tbody>
</table>
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

<table>
<thead>
<tr>
<th>Primary Hepatocytes isolated with Liberase (Roche, #5401119001)</th>
<th>As above, plated at 0.015x10^6 cells/48 well</th>
<th>Optimising isolation method for primary hepatocytes</th>
<th>Not enough cell numbers isolated and viability still low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Hepatocytes isolated with Collagenase (Type IV, Sigma, C1539)</td>
<td>As above, with no percoll wash and added 20mM Paracetamol, 0.03% ethanol, 25nM NAC and 50µM Resveratrol. Tested toxicity of cells with MTT</td>
<td>Testing new hepatocyte isolation method</td>
<td>Cell viability 90% with media, and isolated lots of cells (liver not over-digested). A lot of variability in results with MTT assay</td>
</tr>
<tr>
<td>Primary Hepatocytes isolated with Collagenase (Type IV, Sigma, C1539)</td>
<td>As above, but plated cells in 24 well plates with 4 wells per condition, or 96 well plates with 6 wells per condition at 0.025 x 106 cells/cm^2 to reduce variability</td>
<td>Establishing toxicity model</td>
<td>Optimised conditions used for rest of the hepatocyte experiments</td>
</tr>
</tbody>
</table>

4.2.2 Animals
For the hepatocyte isolation and concurrent resveratrol experiment, male C57BL/6 mice were obtained from the Kearns Facility (Sydney, NSW) (hepatocyte experiment age=4.0±0.6 months n=10; concurrent experiment age=3.7±0.6 months, n=19). They were fed Rat and Mouse Premium Breeder Diet (Gordon’s Specialty Stockfeeds, NSW, Australia). These mouse numbers were chosen to allow n=3 per mouse group, as these were pilot studies to assess whether a costly and limited group of old mice should be used for these experiments.

For the resveratrol pre-treatment experiments, male C57BL/6 mice were aged at the National Institute on Aging (NIA, Baltimore, MD) (age=18.1±0.02 months, n=25), and young C57BL/6 mice were obtained from the NIA (age=6±0.0 months, n=23). The median lifespan of C57BL/6 mice is 26-30 months. They were fed standard feed (2018X 18% Protein Diet, Harlan Teklad, Indianapolis IN). The mouse numbers
chosen for this study allowed for n=6 per group. This number was chosen based on previous work by Miller et al (2005), to account for expected variability and to detect a clinically significant difference in the main outcome of serum ALT. Unfortunately, the loss of more old mice than expected before the experimental timeframe required the use of n=4-5 for some old mouse groups.

All mice were group housed in cages of 4-5 with ad libitum access to food and water. Animal rooms were maintained on a 12 hr light/dark cycle at 20-22°C, and 30-70% humidity. Animals were randomly assigned to treatment or control groups prior to the treatment day. All animal protocols were approved by the Animal Care and Use Committee of the National Institute on Aging (429-TGB-2017 and 405-TGB-2016) or the Animal Care Ethics Committee at Royal North Shore Hospital.

4.2.3 Hepatocyte Isolation and Treatment
Primary hepatocytes were isolated from male 4-month-old C57BL/6 mice by collagenase perfusion (Seglen 1976). Mice were anaesthetized with an i.p injection of ketamine (75 mg/kg, Cenvet Australia, #K1500) and xylazine (10 mg/kg, Cenvet Australia, #X5010). A midline laparotomy was performed and 150U Heparin (Pfizer, #2112105) injected into the Inferior Vena Cava. The portal vein was then cannulated with a 23G intravenous catheter (Becton Dickinson, Sydney, Australia) through which the liver was perfused in-situ at 1–1.5 mL/min/g of liver with Hanks Balanced Salt Solution without Ca²⁺ (HBSS/- Ca²⁺, 37°C, Life Technologies #14170161), to clear the liver of blood. The liver was then perfused with a solution of 0.67mg/ml Collagenase IV (Sigma #C5139) and 5% fetal calf serum (FCS, Gibco #10082-147) in Hanks Balanced Salt Solution with Calcium (HBSS/+Ca²⁺, 37°C, Life Technologies #14025134) to digest the liver. The liver was quickly removed with sterile surgical equipment.
transferred to a biohood, and macerated in a sterile petri dish with approximately 15mls
of the collagenase solution supplemented with 0.04μL/ml DN’ase (Invitrogen, #18047019). The solution was incubated for 10 minutes in humidified 5% CO₂, 95% air
at 37°C, then filtered through a 100μm filter. The solution was centrifuged for 5 minutes
at room temperature at 70g, before removing the supernatant, and re-suspending the cell
pellet in approximately 45 mls of dulbecco’s phosphate-buffered saline (DPBS)/+Ca²⁺
(Invitrogen, #14040182). This wash step was repeated three times. Cells were
resuspended in approximately 3mls of dulbecco’s modified eagle medium (DMEM)
(low-glucose, phenol-red free, Sigma Aldrich #D5921), supplemented with 10% FCS,
2mM L-glutamine (Invitrogen), 100U/ml penicillin and 0.1mg/ml streptomycin
(Invitrogen). Isolated cell viability was determined by trypan blue exclusion assay,
immediately after isolation, and only cell preparations with viability greater than 80%
were plated. Hepatocytes were plated on type 1 collagen coated 96 or 24-well culture
dishes (Life Technologies, #A1142802), at a density of 0.025 x 10⁶ cells/cm², in
supplemented medium. Hepatocytes were incubated in humidified 5% CO₂, 95% air at
37°C for 2.5 hours, then the culture medium was changed to FCS-free supplemented
medium and cells incubated overnight (16 hours).

Paracetamol toxicity was induced in the hepatocytes by adding 20mM paracetamol in
DMEM, either alone or in combination with the treatments. The treatments were
resveratrol (1-100μM in 0.5% Ethanol, Sigma #R5010), NAC (20mM in 0.5% ethanol,
Sigma #A9165) or vehicle (0.5% ethanol). Hepatocytes were plated in 6 separate wells
per condition for 96 well plates, and 4 wells per condition for 24 well plates.
4.2.4 Cell Viability Assays
Hepatocyte survival was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 24 hours post paracetamol administration. Media containing MTT (0.001g /ml, Sigma Aldrich #M5655) was added to the hepatocytes, and they were incubated at 37°C for 1 hr 15 mins. The supernatant was collected, 250ul propanol added to cells, and cells shaken at room temperature for 20 mins. 100ul from each sample was added to an eppendorf containing 450ul propanol and 35ul NaOH. The absorbance of each sample was read at 595nm. Cell viability was calculated as a mean % of DMEM control for each condition on each 96 well plate and 2-10 hepatocyte isolations and treatments were completed for each condition.

Hepatocyte survival was confirmed with propidium iodide staining. Cells were fixed with 4% paraformaldehyde for two hours, 24 hours after paracetamol administration. Cells were then washed with PBS, incubated with a 1µg/µl solution of propidium iodide (Invitrogen #P3566), and then visualized on an inverted fluorescence microscope (Leica DM LB 100T microscope with attached Leica DFC480 digital camera, Sydney, Australia). As propidium iodide is membrane impermeant in viable cells, cells with strong nuclear staining were identified as non-viable.

4.2.5 Concurrent Resveratrol and Paracetamol Treatment of Animals
Male C57BL/6J mice from the Kearns facility were fasted overnight (16 hours), then dosed with 700mg/kg paracetamol or saline vehicle via oral gavage between 8am and 10am. Immediately following this mice were dosed with a second oral gavage of either resveratrol (30mg/kg in corn oil), corn oil (vehicle, Sigma Aldrich, #C8267), or NAC (1200mg/kg in saline). Food was returned to the mice after dosing. Six hours after dosing mice were anaesthetized with an i.p injection of ketamine (75 mg/kg, Cenvet...
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

Australia, #K1500) and xylazine (10 mg/kg, Cenvet Australia, #X5010). A midline laparotomy was performed and blood taken from the Inferior Vena Cava. The portal vein was then cannulated with a 21G intravenous catheter (BD, Sydney, Australia) through which the liver was perfused \textit{in-situ} at 1–1.5 mL/min/g of liver with oxygenated Krebs-Henseleit bicarbonate buffer (95% O₂–5% CO₂, 37°C). Sections of the liver were snap frozen in liquid nitrogen for subsequent enzyme activity assays, protein extraction and fixed in 10% neutral formalin for subsequent histopathological analysis.

This paracetamol dose is higher (700mg/kg) than the dose used to induce acute hepatotoxicity in experiments done on mice sourced from the NIA (300mg/kg) (See sections 2.2.3, 4.2.6, 5.2.2). Pilot experiments were conducted in young male C57BL/6 mice, sourced from the Kearns facility, to determine the dose at which there was consistent (seen in all treated mice) severe hepatotoxicity (as assessed by serum ALT levels >5 times the upper limit of normal = 275U/L). The number of mice assessed at each dose, and the resultant ALT results are shown in Table 4.2. The dose of 700mg/kg (Panadol Color-free Baby Drops, 100mg/ml, GlaxoSmithKline, Australia) given via oral gavage, with euthanasia at 6 hours, gave consistent severe hepatotoxicity results, and was used for the rest of the experiments.

\textbf{Table 4.2} Pilot experiments to determine the acute paracetamol dose necessary to induce consistent severe hepatotoxicity in young male C57BL/6 mice sourced from the Kearns facility. ALT = Alanine aminotransferase.
### 4.2.6 Dietary Resveratrol Treatment of Animals

Male C57BL/6J mice from the NIA were randomized at 6 months of age (young group) or 18 months of age (old group) to either a standard AIN-93G diet (SD; Dyets, Inc, Bethlehem PA) (young n=12, old n=16), or an AIN-93G diet supplemented with resveratrol (RSV) at a concentration of 1.33g/kg feed, (approx. 100mg/kg/day for mice) (young n=11, old n=9), both diets fed *ad libitum*. Mice remained on their respective diets for 3-4 months, at which time they were fasted overnight (16 hours), then dosed with 300mg/kg paracetamol (Panadol Color-free Baby Drops, 100mg/ml, GlaxoSmithKline, Australia) or saline vehicle via oral gavage. This paracetamol dose was chosen as a standard dose to induce toxicity in C57BL/6 mice (McGill et al. 2012). Six hours after treatment serum and tissue were collected as described above. Body weight and food intake for all mice was monitored biweekly whilst on the experimental diets.

### 4.2.7 Serum Biochemistry

Blood was stored and serum extracted as detailed in section 2.2.4, serum liver function tests (total protein, albumin, bilirubin, alkaline phosphatase (ALP), gamma...
glutamyltransferase (GGT)) and serum paracetamol concentrations were measured by a National Association of Testing Authorities accredited hospital laboratory, PaLMS (Pacific Laboratory Medicine Services) at Royal North Shore Hospital (Sydney, Australia) using an Architect i1000SR immunoassay analyser (Abbott Diagnostics, IL, USA). The lower limit of the paracetamol serum concentration test was 20μmol/L. Vitamin B6 is added to the ALT activity assay to accommodate for the age related decline in this vitamin, an integral cofactor for the assay (Krishnamurthy et al. 1967; Bordoni et al. 1998).

4.2.8 Histology
Fixed liver tissue was embedded in paraffin, 5μm sections were cut on a microtome and mounted on slides. Slides were stained with Haemotoxylin and Eosin in the National Association of Testing Authorities accredited hospital laboratory of the Pathology department of Royal Prince Alfred Hospital, Sydney, Australia. Histopathology was scored by an anatomical pathologist, who was blinded to the age and treatment of the samples. Necrosis was scored as a percentage of the liver tissue on each slide. Images were taken on an Olympus BX51 microscope connected to an Olympus DP26 camera (Olympus, Sydney, Australia).

4.2.9 Biochemical Examination of Frozen Liver Samples
Liver microsomes were isolated according to the method of Shoaf et al. (1987) and CYP2E1 activity was measured according to the method of Roberts et al. (1995) and Mach et al. (2014), as detailed in section 2.2.6.
Liver concentrations of total glutathione were determined with a Glutathione Assay kit (Cayman Chemicals, #703002) as detailed in section 2.2.6.

4.2.10 Biochemical Examination of Hepatocytes
Hepatocyte concentrations of total glutathione were determined with a Glutathione Assay kit (Cayman Chemicals, #703002), as detailed above, 24 hours after paracetamol treatment. Cells for each treatment (4 wells of a 24 well plate) were prepared according to the manufacturer’s instructions. Cells were scraped off plates in media and the supernatant and cell suspension for each condition collected into an eppendorf. The cells were centrifuged for 10mins at 1500g at 4°C and the supernatant discarded. The cells were re-suspended in PBS supplemented with 1mM EDTA, then frozen at -80°C for 30 mins, and thawed at room temperature for 30 minutes. Cells were then homogenised, centrifuged at 10,000g for 15 minutes at 4°C and the supernatant collected. Standards and samples were added to a 96 well plate (50ul), then 150 ul of assay cocktail (MES buffer plus cofactor mixture, enzyme mixture, and DTNB) was added and the plates incubated in the dark with absorbance measured at 405nm after 25 minutes.

4.2.11 Western Blots
AMPK and phosphorylated AMPK (pAMPK) protein levels were measured in liver from the concurrent resveratrol and paracetamol experiment mice. Liver was homogenized with lysis buffer (radioimmunoprecipitation (RIPA) buffer with protease inhibitor (complete EDTA-free, Roche, #05892953001), 100uM Na₃Vo₄ and 50mM NaF) then centrifuged at 10,000g for 10 minutes at 4°C and the supernatant collected. Protein levels in the cell lysate were measured with a Pierce bicinchoninic acid (BCA) Protein Assay Kit (Life Technologies, #23225). Samples were then diluted into a 0.75 mg/mL concentration with 25% Laemmli buffer and 10% dithiothreitol (DTT). 15µg of
proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and then transferred to nitrocellulose membranes. Membranes were blocked for one hour in 5% skim milk in 0.1% Tween-20 tris-buffered saline (TBS-T). Membranes were washed in TBS-T, and incubated overnight at 4ºC in primary antibody solution (5% bovine serum albumin (BSA), TBS-T). Primary antibodies used were AMPK (1:1000, Cell Signaling, #CS2532) p-AMPK (1:1000, cell signalling, #CS2531), and 14-3-3 (1:1000 cell signalling, #8312). Membranes were washed and incubated for one hour in secondary antibody solution (5% skim milk in TBS-T, rabbit secondary antibody 1:10,000, cell signalling, #7074P2). Membranes were washed and analysed on a ImageQuant LAS 4000 machine (GE Healthcare Life Sciences). Densitometry was performed using ImageJ (v 1.47) and 14-3-3 was used as the loading control.

SIRT1, AMPK, p-AMPK, NFκB and p-NFκB protein levels were measured in liver from the resveratrol diet experiment mice. Liver was homogenised in lysis buffer (RIPA buffer supplemented with EDTA and ethylene glycol tetraacetic acid (EGTA) (Boston BioProducts), protease inhibitor cocktail (Sigma-Aldrich #P8340), phosphatase inhibitor Cocktail 2 and 3 (Sigma-Aldrich #P5726 and #P0044), and Trichostatin A (Sigma-Aldrich #T1952)) then centrifuged at 14,000g for 15 minutes at 4ºC, and the supernatant collected. Protein concentration was quantified using the Quickstart Bradford assay (Bio-Rad, #500-0201). 30µg of protein was separated by SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA for one hour at room temperature, and then incubated overnight at 4ºC with the primary antibody in 5% milk. Primary antibodies used were SIRT1 (1:1000, Sigma, #S5196), AMPK (1:1000, Cell Signaling, #CS2532),
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

p-AMPK (1:1000, Cell Signaling, #CS2531), NFκB (1:2000, Abcam, #ab32536;), p-NFκB (1:1000, Cell Signaling, #CS3031s). Membranes were washed, then incubated in secondary antibody solution (5% skim milk in PBS-T, rabbit secondary antibody 1:5,000, GE healthcare, Pascataway, NJ) for one hour at room temperature. The membranes were washed then visualization of the immunoreactive bands was performed using the ECL Prime Western blotting detection system (GE Healthcare, Pascataway, NJ). Densitometry was performed using ImageJ (v 1.47). Ponceau S (Sigma Aldrich, St Louis MO) was used as a loading control and data of each group was normalized to the young control diet fed saline treated group.

4.2.12 Statistics
Data are expressed as mean ± SEM unless otherwise indicated. Differences between mean values across groups for hepatocyte and concurrent resveratrol/paracetamol experiments were calculated with a Kruskal-Wallis non-parametric test, then Mann-Whitney U-tests where appropriate, due to non-normal distribution of the data. Differences between mean values across groups for resveratrol diet experiments were calculated with one- two- or three- way ANOVA with Tukey’s HSD post-hoc test or t-tests where appropriate. The prevalence of necrosis in each treated group compared to the corresponding saline group was compared with Chi squared tests. Data analysis was completed using the statistics program SPSS (Version 21.0, SPSS Inc., Chicago, Illinois, USA) and GraphPad Prism (Version 6.04, GraphPad Software, La Jolla California USA).
4.3 Results

4.3.1 Primary Hepatocyte Results
Treatment of primary mouse hepatocytes with 20mM paracetamol resulted in a reduced cell viability of 60.7±6.2% compared to treatment with media alone (p<0.001). The addition of 0.5% ethanol (resveratrol vehicle) to the media did not reduce cell viability, and 0.5% ethanol plus 20mM paracetamol treatment resulted in similar cell viability to 20mM paracetamol alone. As such, 0.5% ethanol was used as the control for the rest of the experiments. Concurrent treatment with 20mM NAC and 20mM paracetamol (with 0.5% ethanol), maintained cell viability at control levels (Figure 4.1A).

Treatment of primary mouse hepatocytes with 1-100μM resveratrol did not change cell viability compared to control (Figure 4.1B). Concurrent treatment with 20mM paracetamol and 1-100μM resveratrol resulted in no increase in cell viability compared to treatment with 20mM paracetamol, in 0.5% ethanol, alone (Figure 4.1B).

Hepatocyte total glutathione concentration was reduced by approximately 35% with 20mM paracetamol treatment compared to control (p=0.05). Compared to control, glutathione concentration was also decreased in cells with 20mM paracetamol plus 5, 25 and 100uM resveratrol (p<0.05), whilst treatment with 20mM paracetamol plus 20mM NAC did not result in decreased cell glutathione concentration (Figure 4.1B).
Figure 4.1 Primary mouse hepatocyte viability as a percentage of dulbecco’s modified eagle medium (DMEM) control (A, B), and total hepatocyte glutathione (GSH) (C) with ethanol (0.5%), N-acetyl cysteine (NAC, 20mM) and/or resveratrol (0.5% ethanol vehicle, 1-100µM) alone or plus paracetamol (20mM) treatment after 24 hours. Primary hepatocytes isolated from 4 month old male C57BL/6 mice via collagenase perfusion. Viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All data are presented as mean ± SEM of n=3-9 animals per group. #p<0.05 compared to DMEM treatment only, *p<0.05 compared to ethanol treatment group.

The cell viability results as measured by the MTT assay were confirmed with propidium iodide (PI) staining. 20mM paracetamol treatment resulted in increased nuclear PI.
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

staining, than treatment with vehicle (0.5% ethanol) alone. Treatment with 20mM NAC, either alone or with paracetamol, did not result in nuclear PI staining. Increased nuclear PI staining was also seen with 25uM resveratrol plus paracetamol treatment, but not with 25uM resveratrol treatment alone (Figure 4.2).

Figure 4.2 Representative images of primary mouse hepatocytes treated with (A) Vehicle (0.5% ethanol), (B) 20mM Paracetamol + 0.5% ethanol, (C) 20mM N-acetyl cysteine (NAC, in 0.5% ethanol), (D) 25uM Resveratrol (in 0.5% ethanol), (E) 20mM
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

NAC + 20mM Paracetamol + 0.5% ethanol or (F) 25uM Resveratrol (in 0.5% ethanol) + 20mM Paracetamol, for 24 hours, fixed and stained with propidium iodide. Red arrows indicate nuclear PI staining. Primary hepatocytes isolated from 4 month old male C57BL/6 mice via collagenase perfusion. Images taken at 200X magnification on a Leica DM LB 100T microscope with attached Leica DFC480 digital camera (Leica, Sydney, Australia).

4.3.2 Concurrent Resveratrol and Paracetamol Treatment in Mice Results
Weights and serum biochemistry results for each mouse treatment group are shown in Table 4.3. A Kruskal-Wallis test across the six treatment groups, with Mann-Whitney post-hoc testing, showed no difference in weights, or total protein, albumin, ALP or GGT results across any of the groups. Serum bilirubin was significantly increased in paracetamol+corn oil treated mice compared to all saline treated groups (p=0.006).

Table 4.3 Weights and serum biochemistry results for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle, resveratrol (RSV, 30mg/kg in corn oil), or N-acetyl cysteine (NAC, 1200mg/k). Serum and liver collected 6 hours after dosing. Data expressed as mean (SD). *p<0.05 compared to all saline treated groups. ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>Paracetamol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment 2</td>
<td>Corn Oil</td>
<td>NAC</td>
</tr>
<tr>
<td>n=3</td>
<td>n=3</td>
<td>n=3</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>46 (2.8)</td>
<td>48.7 (2.1)</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>25.5 (2.1)</td>
<td>27.3 (1.5)</td>
</tr>
</tbody>
</table>
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

<table>
<thead>
<tr>
<th>Bilirubin (umol/L)</th>
<th>2.0 (0.0)</th>
<th>2.3 (0.6)</th>
<th>1.3 (0.6)</th>
<th>22.5 (4.2)*</th>
<th>4.3 (0.6)</th>
<th>12.0 (3.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/L)</td>
<td>124.0 (25.5)</td>
<td>124.3 (6.4)</td>
<td>131.0 (9.5)</td>
<td>157 (16.9)</td>
<td>133 (15.7)</td>
<td>192.7 (95.4)</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>21.7 (0.5)</td>
<td>23.1 (1.2)</td>
<td>23.5 (1.0)</td>
<td>23.2 (2.8)</td>
<td>24.1 (0.6)</td>
<td>23.6 (1.1)</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>5.7 (0.1)</td>
<td>5.4 (0.3)</td>
<td>5.6 (0.4)</td>
<td>5.0 (0.4)</td>
<td>5.1 (0.2)</td>
<td>4.7 (0.3)</td>
</tr>
</tbody>
</table>

A Kruskal-Wallis non-parametric test shows a significant difference across treatment groups for serum ALT (p=0.024) (Figure 4.3). Treatment with acute paracetamol resulted in a trend towards increased ALT serum concentration, compared to saline treated controls (p=0.06), whilst concurrent NAC treatment maintained ALT serum concentration at control levels (Figure 4.3). Concurrent resveratrol treatment also resulted in a trend towards increased ALT serum concentration, compared to saline treated controls (p=0.08). Although the post-hoc statistics were only trending towards significance, due to small sample size, the degree of protection against paracetamol toxicity seen with NAC treatment and the lack of protection with resveratrol treatment, is convincing.

Histopathological analysis showed that paracetamol+corn oil treatment resulted in detectable necrosis six hours after treatment in one mouse, and one mouse in the paracetamol+resveratrol group showed mild detectable inflammation.
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

Figure 4.3 Serum alanine aminotransferase (ALT) concentration for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle, resveratrol (RSV, 30mg/kg in corn oil), or N-acetyl cysteine (NAC, 1200mg/k). Serum collected 6 hours after dosing. Data expressed as mean±SEM. *=p<0.10 compared to saline+corn oil group. n= 3-4 per group.

Total liver glutathione was reduced in the paracetamol+corn oil, and paracetamol+resveratrol groups compared to all other groups (p=0.008) (Figure 4.4A). There was no difference in CYP2E1 activity across any treatment group (Figure 4.4B).
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

**Figure 4.4** Total liver glutathione (GSH) (A) and liver cytochrome (CYP)2E1 activity (B) for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle, resveratrol (RSV, 30mg/kg in corn oil), or N-acetyl cysteine (NAC, 1200mg/k). Liver collected 6 hours after dosing. Data expressed as mean±SEM. *=p<0.05 compared to saline+corn oil, saline+N-acetyl cysteine, saline+resveratrol and paracetamol+N-acetyl cysteine groups. n= 3-4 per group.

Western blots for pAMPK and AMPK protein expression for mice treated with paracetamol or saline and corn oil or resveratrol are shown in Figure 4.5. Although it appears that there was a trend towards an increase in the ratio of pAMPK to AMPK expression in the paracetamol groups compared to their corresponding saline treated groups, this was not significant (Figure 4.6).

**Figure 4.5** Western blots for 5' adenosine monophosphate-activated protein kinase (AMPK), phosphorylated AMPK, and loading control 14-3-3 for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle or resveratrol (RSV, 30mg/kg in corn oil). Liver collected 6 hours after dosing.
**Figure 4.6** phosphorylated 5' adenosine monophosphate-activated protein kinase (pAMPK)/AMPK protein expression ratio for young (3.7±0.6 months) male C57BL/6 mice treated with 700mg/kg paracetamol (via oral gavage) or saline control, and concurrent corn oil vehicle or resveratrol (RSV, 30mg/kg in corn oil). Liver collected 6 hours after dosing. Data expressed as mean±SEM normalised to saline-treated control. n=2-3 per group.

4.3.3 Dietary Pre-Treatment with Resveratrol Results

Weights, food consumed and serum biochemistry results for each mouse treatment and diet group are shown in Table 4.4 for young mice and Table 4.5 for old mice. A one way ANOVA across the four treatment groups for each age group, showed no difference in weights, or total protein, albumin or GGT results across any of the groups. For both young diet groups, and the old control diet group, bilirubin was significantly increased with paracetamol treatment compared to saline treatment (Table 4.4 and Figure 4.4). For old resveratrol diet mice only, ALP was significantly increased with paracetamol treatment compared to saline treatment (Figure 4.4). A three-way ANOVA of diet, treatment and age showed a significant age*treatment interaction effect only for serum bilirubin.
Table 4.4 Weights and serum biochemistry results for young (6.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver and serum collected after 6 hours. Data expressed as mean (SD). *p<0.05 compared to corresponding saline treated group. ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase.

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>Resveratrol Diet</th>
<th>One way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline n=6</td>
<td>Paracetamol n=6</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>50.2 (1.6)</td>
<td>49.6 (3.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>27.0 (1.1)</td>
<td>28.0 (1.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Bilirubin (umol/L)</td>
<td>2.3 (0.5)</td>
<td>6.8 (3.0)*</td>
<td>F(3,19)=3.49, p=0.04</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>62.0 (5.2)</td>
<td>70.0 (13.9)</td>
<td>NS</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>34.9 (3.1)</td>
<td>35.2 (2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>3.7 (0.4)</td>
<td>5.8 (0.7)*</td>
<td>F(3,22)=25.88, p=0.00</td>
</tr>
<tr>
<td>Food eaten (g)</td>
<td>2.4 (0.0)</td>
<td>2.4 (0.0)</td>
<td>F(3,22)=21.24, p=0.00</td>
</tr>
<tr>
<td>RSV dose consumed (mg/kg mouse/day)</td>
<td>-</td>
<td>85.8 (11.1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4.5 Weights and serum biochemistry results for old (18.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver and serum collected after 6 hours. Data expressed as mean (SD). *p<0.05
compared to corresponding saline treated group. ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase.

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th></th>
<th>Resveratrol Diet</th>
<th>One way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Paracetamol</td>
<td>Saline</td>
<td></td>
</tr>
<tr>
<td>n=8</td>
<td>n=8</td>
<td>n=4</td>
<td>n=5</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>55.8 (11.0)</td>
<td>51.2 (4.8)</td>
<td>46.5 (3.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>27.8 (5.4)</td>
<td>27.6 (2.1)</td>
<td>24.3 (2.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Bilirubin (umol/L)</td>
<td>1.4 (0.7)</td>
<td>4.0 (2.4)*</td>
<td>1.0 (0.0)</td>
<td>F(3,23)=5.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=0.008</td>
<td></td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>65.3 (21.6)</td>
<td>82.2 (16.8)</td>
<td>59.8 (4.0)</td>
<td>F(3,23)=4.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>101.2 (29.8)*</td>
<td>p=0.018</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>3.0 (0.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>37.1 (6.9)</td>
<td>41.2 (4.2)</td>
<td>35.8 (3.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>5.2 (3.3)</td>
<td>4.8 (0.7)</td>
<td>4.0 (0.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Food eaten (g)</td>
<td>2.2 (0.1)</td>
<td>2.4 (0.0)</td>
<td>2.3 (0.0)</td>
<td>F(3,22)=9.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.6 (0.2)*</td>
<td>p=0.001</td>
</tr>
<tr>
<td>RSV dose consumed (mg/kg mouse/day)</td>
<td>-</td>
<td>-</td>
<td>82.7 (9.9)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>82.2 (12.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Serum ALT, and percentage of liver necrosis, was significantly increased in young paracetamol-treated mice of both diet groups, compared to saline treated mice (Figure 4.7A, C). For the old mice, serum ALT and percentage of liver necrosis, were only significantly increased with paracetamol treatment in mice on the control diet, not in those on the resveratrol diet (Figure 4.7B, D). In old mice, a two-way ANOVA of diet and treatment showed a significant diet*treatment effect on percentage of liver necrosis (p=0.000). A three-way ANOVA of age, diet and treatment showed that age, treatment
and an age x treatment interaction effect were related to serum ALT and percentage of liver necrosis.

**Figure 4.7** Serum alanine aminotransferase (ALT) concentration and percentage of liver with necrosis for young (A, C; 6.0±0.0 months) and old (B, D; 18.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver and serum collected after 6 hours. Data expressed as mean±SEM. One-way ANOVA young ALT F(3,20)=12.00 p=0.00. One-way ANOVA old ALT, F(3,24)=4.96 p=0.009. One-way ANOVA young necrosis F(3,21)=14.974 p=0.00. One-way ANOVA old necrosis, F(3,22)=13.14 p=0.00. *p<0.05 compared to corresponding saline group #p<0.05 compared to control diet+paracetamol group with Tukey’s HSD post-hoc test. n=4-8 per group.
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Diet + Saline</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Control Diet + Paracetamol</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Resveratrol Diet + Saline</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Resveratrol Diet + Paracetamol</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 4.8** Representative Haemotoxylin and Eosin stained liver histology images for young (6.0±0.0 months) and old (18.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver collected after 6 hours. Images taken at 100X.
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

Total liver GSH was decreased in young and old mice treated with paracetamol compared to saline, for both control and resveratrol diets (Figure 4.9A, B). A three-way ANOVA of diet, treatment and age showed a significant effect of age, treatment and an age x treatment interaction effect on total liver GSH (p=0.000). There was no significant change in CYP2E1 activity for young or old mice of either diet group with paracetamol treatment compared to saline treatment, although there was a trend towards a decrease in young control diet mice (p=0.10) (Figure 4.9C, D).

Figure 4.9 Liver total glutathione (GSH) levels and cytochrome (CYP)2E1 activity for young (A, C; 6.0±0.0 months) and old (B, D; 18.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver collected after 6 hours. Data expressed as mean±SEM. One-way ANOVA young GSH F(3,22)=47.33 p=0.00. One-way ANOVA old GSH, F(3,24)=29.07 p=0.00. One-way
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

ANOVA young CYP2E1, non-significant. One-way ANOVA old CYP2E1, non-significant. *p<0.05 compared to corresponding saline group. n=4-8 per group with Tukey’s HSD post-hoc test.

Western blot results for SIRT1, pAMPK/AMPK ratio and NF-κB expression in young and old mice are shown in Figure 4.10. SIRT1 mRNA expression was increased with resveratrol diet compared to control diet, for saline treated old but not young mice (Figure 4.10A, B). The ratio of phosphorylated AMPK to AMPK, indicating activation of AMPK, was increased with resveratrol diet compared to control diet for saline treated young but not old mice (Figure 4.10C, D). Paracetamol treatment reduced SIRT1 mRNA expression in old resveratrol diet mice treated with paracetamol compared to saline (Figure 4.10B), and increased the ratio of phosphorylated AMPK to AMPK, in both old diet groups (Figure 4.10D).
Figure 4.10 Sirtuin 1 (SIRT1), phosphorylated 5' adenosine monophosphate-activated protein kinase (pAMPK)/AMPK ratio and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein expression for young (A, C, E; 6.0±0.0 months) and old (B, D, F; 18.0±0.0 months) male C57BL/6 mice exposed to either control diet or resveratrol diet (RSV, 1.33g/kg feed) for 3-4 months, then treated with paracetamol (300mg/kg via oral gavage) or saline. Liver collected after 6 hours. Data expressed as mean±SEM, normalised to saline-treated young control. One-way ANOVA young SIRT1, non significant. One-way ANOVA old SIRT1, F(3,19)=7.14 p=0.003. One-way
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

ANOVA young pAMPK/AMPK, F(3,22)=21.92 p=0.00. One-way ANOVA old pAMPK/AMPK, F(3,19)=13.71 p=0.00. One-way ANOVA young NF-κB, F(3,22)=2.48 p=0.092. One-way ANOVA old NF-κB, non-significant. *p<0.05 compared to corresponding saline group, #p<0.05 compared to control diet saline group with Tukey’s HSD post-hoc test. n=3-6 per group.

4.4 Discussion

This study found that resveratrol did not protect against paracetamol hepatotoxicity in three different models: primary hepatocytes; concurrently treated young mice; and young and old mice pre-treated with resveratrol in the diet. Paracetamol toxicity was induced in each model, and the positive control, NAC, protected against toxicity. Changes in downstream resveratrol targets could not be confirmed in the concurrent model, implying a pharmacokinetic explanation for the lack of protection. The mechanisms behind the lack of resveratrol protection in the pre-treatment study appear to be age-differential and require further research. Overall, these results suggest that resveratrol is not a good candidate as a novel therapeutic to protect against paracetamol toxicity.

In the primary hepatocyte model, paracetamol toxicity was induced in the cells at a level similar to other rat studies (Lewerenz et al. 2003), but not to the same extent as seen in other mouse studies (Jemnitz et al. 2008). In the current study, cells are washed prior to all tests, so only those cells still attached to the cell culture plates are measured for outcomes. This may bias the results towards those cells that are viable enough not to become unattached, and underestimate the toxic effects we observed. The reduction in viability we did see with paracetamol, however, was completely reversed by adding

Alice Kane - March 2016
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

NAC, the current clinical treatment for paracetamol toxicity and thus a good positive control, concurrently to the media, indicating the validity of our model.

Concurrent paracetamol treatment with any resveratrol dose did not increase hepatocyte viability compared to paracetamol alone. It is possible, that the lack of protection seen in this model was in fact due to a lack of permeability of the hepatocytes to resveratrol, and this should be confirmed in further studies with measurement of downstream resveratrol target protein expression, to confirm whether or not resveratrol was able to enter the hepatocytes in cell culture. It is also possible, however, that these findings suggest that the protective effects of resveratrol against paracetamol toxicity observed in animal studies (Sener et al. 2006; Du et al. 2015) may not be due to the drug acting directly on hepatocytes. Some of the protective properties of resveratrol seen in other studies, such as anti-inflammatory actions, reducing neutrophil infiltration, acting on the endothelium, and preventing platelet aggregation (Pace-Asciak et al. 1995; Yoshizaki et al. 2010), would not be observed in isolated hepatocyte experiments. In order to answer these questions the experiments were continued in in vivo toxicity models.

Concurrent treatment of young C57BL/6 mice with high dose paracetamol and resveratrol also did not result in protection against paracetamol induced hepatotoxicity. As with our primary hepatocytes, we were able to establish a good model of paracetamol hepatotoxicity assessed by increased serum ALT and bilirubin and decreased total liver GSH with paracetamol treatment, which is consistent with other mouse studies (Mitchell et al. 1973; McGill et al. 2012). The positive control, NAC, concurrently given, was able to completely protect against liver toxicity, but resveratrol, concurrently given with paracetamol, had no protective effect on serum ALT or total
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

liver GSH. In this in vivo model, it is possible the lack of protection may be due to the pharmacokinetics of resveratrol. Resveratrol has a very short half life, and undergoes significant phase II metabolism (Yu et al. 2012; Vitrac et al. 2003; Marier et al. 2002), implying that a single dose may be metabolised too quickly to exert downstream protective changes. Western blot analysis of liver tissue in this study showed no change for resveratrol treated mice, compared to saline treated mice, in the ratio of phosphorylated AMPK to total AMPK protein expression, a known target of resveratrol (Baur et al. 2006; Suchankova et al. 2009; Ido et al. 2015; Hardie 2011) which would support this hypothesis. Previous mouse studies that did see protection against paracetamol toxicity with resveratrol treatment, gave resveratrol 1-1.5 hours post paracetamol treatment, and one study administered resveratrol at two time-points, indicating that delayed treatment or more doses may be necessary for protection (Du et al. 2015; Masubuchi et al. 2009). All previous studies also gave resveratrol via a different route of administration (intraperitoneal injection), and in different vehicles (Masubuchi et al. 2009; Sener et al. 2006; Wang et al. 2015; Du et al. 2015) to the current study which may affect absorption, bioavailability and metabolism (Walle 2011). Interestingly, one study saw protection against hepatotoxicity at eight hours after paracetamol treatment (and two hours after the final resveratrol dose), but not after 24 hours, so perhaps continued dosing of resveratrol is necessary to maintain the protective effects, or resveratrol may delay but not prevent toxicity (Masubuchi et al. 2009). Two of the previous studies that saw protection against paracetamol toxicity with resveratrol treatment were conducted in different mouse strains (BALB/c mice, (Sener et al. 2006) and CD1 mice, (Masubuchi et al. 2009)), which are known to be less susceptible to paracetamol toxicity, and have a different mechanism of toxicity response to C57BL/6 mice. Further studies that measured resveratrol and its metabolites in the serum and
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

liver after concurrent dosing with paracetamol, would determine whether pharmacokinetics may explain the lack of protection seen in the current study.

The final experiment in this study looked at the effect of long-term dietary pre-treatment of both young and old mice with resveratrol. Six weeks of exposure to resveratrol, also did not protect young or old mice from an acute dose of paracetamol. In old mice there was not a statistically significant increase in serum ALT with paracetamol treatment for resveratrol diet mice, and there was a statistically significant interaction between diet and treatment for percentage necrosis. These statistical results may imply that in old mice, resveratrol diet was associated with less hepatotoxicity from paracetamol, compared to control diet. However the clinical significance of this is negligible as the mean serum ALT for this mouse group was still almost 5000U/L, well above the upper limit of normal.

As in our other models, in the study of long-term dietary pretreatment with resveratrol, paracetamol toxicity was induced as demonstrated by histology, increased serum ALT and decreased liver GSH. It was found that resveratrol in the diet, does cause downstream target changes in protein expression, implying that resveratrol is entering, and exerting some effect in the liver, but still not protecting against paracetamol toxicity. This negative result may be due to a limited number of outcome measures and it would be beneficial to measure a wider range of paracetamol toxicity-associated outcomes such as inflammatory cytokine response. Measurement of resveratrol in the serum or liver to confirm the ingestion of the drug from the diet would also be beneficial in understanding the lack of protection from resveratrol against paracetamol toxicity seen in this model. Interestingly, however, there was a differential effect of

Alice Kane - March 2016
resveratrol exposure on protein expression across the age groups. In young mice there was an increased pAMPK/AMPK protein expression ratio with resveratrol, but no change to SIRT1 protein expression. In old mice, resveratrol caused increased SIRT1 protein expression, but no change in activation of AMPK as assessed via the pAMPK/AMPK ratio. Previous studies have shown reduced AMPK pathway responsiveness in old age (Salminen & Kaarniranta 2012) and age-dependent effects of resveratrol on other liver protein markers (Tung et al. 2015). Studies have also identified differential activation of these two pathways in response to different resveratrol doses (Price et al. 2012). Low dose resveratrol directly activates SIRT1, as seen in our old mice, while high-dose resveratrol activates AMPK independently of SIRT1, as seen in our young mice (Price et al. 2012). Pharmacokinetic changes in old age, such as potentially reduced transfer of resveratrol across the aged liver sinusoidal endothelium (Le Couteur et al. 2001; Le Couteur et al. 2008) may partly explain this finding. Previous studies have shown that activation of AMPK is protective against paracetamol toxicity (King et al. 2015), but this was not seen for young mice in the current study. Wang et al. (2015) dosed young mice twice per day for three days with resveratrol, to investigate the protective effects of resveratrol against paracetamol toxicity. They found increased SIRT1 protein expression with resveratrol treatment alone, which was slightly decreased with resveratrol plus paracetamol treatment, as we saw in the old mice only in the current study (Wang et al. 2015). Future research needs to look at other downstream protein markers involved in these pathways to understand the mechanisms of these age-related differences.

Interestingly, an increase in the pAMPK/AMPK protein expression ratio was seen in paracetamol treated old mice in the pre-exposure experiment, and there was a trend
Chapter 4: Investigation of Resveratrol as a Potential Therapy to Protect Against Paracetamol Hepatotoxicity

towards this in the concurrently treated mice. Previous studies have found either no activation of AMPK in mice treated with low-dose paracetamol (King et al. 2015), or a down regulation in pAMPK with high dose paracetamol (Saberi et al. 2014). Saberi et al (2014) used a lower paracetamol dose and shorter time-frame than in the current study, and this may explain the different results. The AMPK pathway is known to be associated with cell stress (Salminen & Kaarniranta 2012) so the increase seen in our study may be due to the severe cell toxicity induced by paracetamol.

Limitations of this study include the technical difficulties with establishing our cell culture model, which highlight the need to carefully consider cell type and source, vehicles, media and the type of outcome measurement for all cell culture experiments, as small changes in methodologies and conditions can significantly impact results, as has been seen in other in vitro studies (Clement et al. 2001). There were also some baseline differences in the two C57BL/6 mouse cohorts used in this study including different locations of breeding and housing and different base diets, requiring the use of different paracetamol doses to induce toxicity. It is possible that the C57BL/6 mice sourced from the Kearns facility compared to those sourced from the NIA are genetically different sub-strains due to genetic drift in the colonies, and as such have differences in susceptibility to paracetamol toxicity (Bourdi et al. 2011; Zurita et al. 2011; Mekada et al. 2009). This warrants further investigation. Another limitation in this study is the lack of absolute measures of resveratrol levels in the serum and tissue, or other downstream target outcomes such as inflammatory or oxidative response measures, which would be beneficial for future experiments, and allow better understanding of the mechanisms behind the lack of protection seen with resveratrol in this study. Furthermore, the negative result seen in this chapter is based on a limited
number of outcome measures, and it would be beneficial to confirm these findings by looking. The strength of this study is the use of three models of paracetamol and resveratrol exposure, with positive controls where possible.

Overall, this study induced paracetamol toxicity in three different models, and showed that resveratrol does not protect against paracetamol toxicity in mice, even with six weeks of dietary pre-exposure. Dietary exposure to resveratrol was found to increase pAMPK/AMPK protein expression in young mice, and caused increased SIRT1 protein expression in old mice. High dose paracetamol was also found to increase the ratio of pAMPK/AMPK protein expression in the liver. The role of SIRT1 in paracetamol toxicity will be further explored in chapter 5 in a study of young and old transgenic SIRT1 mice treated with paracetamol.
5 THE ROLE OF SIRT1 IN PARACETAMOL HEPATOTOXICITY IN YOUNG AND OLD MICE

5.1 Introduction
The results presented in chapter three of this thesis demonstrate the inability of NAC to protect against non acute paracetamol toxicity, and there is also clinical evidence of patients who still develop liver failure from paracetamol toxicity, particularly those who present late or have repeated paracetamol ingestion (Craig et al., 2011; Daly et al., 2008; Ferner et al., 2011). These findings highlight the need to develop new therapies and evidence to guide treatment of different clinical presentations of paracetamol hepatotoxicity, other than acute single high dose exposure. This is particularly crucial for the older population who are more at risk of liver failure (Schmidt 2005) and who are more likely to have non-acute exposures to paracetamol (Kane et al. 2012).
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

Animal studies have shown that calorie restriction (CR) (Harper et al. 2006) and the CR-mimetic resveratrol (Sener et al. 2006), protect against paracetamol toxicity, although the mechanisms are not well understood. These interventions are believed to, either directly or indirectly, activate the NAD-dependent deacetylase sirtuin-1 (SIRT1) pathway (B. P. Hubbard et al. 2013; Cohen et al. 2004; Lagouge et al. 2006). SIRT1 is thought to play an important role not only in longevity and delaying age-related changes, but also in regulation of response to stressors and nutrients (Michan & Sinclair 2007; Raynes et al. 2013). In particular, it has been shown to play a role in the regulation of inflammation (Purushotham et al. 2009; Yoshizaki et al. 2010), apoptosis (Wang et al. 2006; Yamakuchi et al. 2008) and mitochondrial function (Price et al. 2012); all important factors in the development of paracetamol hepatotoxicity. Thus it is hypothesised that it is activation of the lifespan-related SIRT1 pathway that may provide a protective mechanism against paracetamol toxicity.

In chapter four of this thesis we saw that resveratrol did not protect against paracetamol toxicity in three different in vitro and in vivo models. Differences in drug vehicles, route and timing of administration and mouse species between previous studies (Sener et al. 2006; Masubuchi et al. 2009; Du et al. 2015; Wang et al. 2015) and our work, suggest a potential role of resveratrol pharmacokinetics in explaining the different results. Future studies of absolute measures of resveratrol levels in the serum and tissue, or other downstream target outcomes such as inflammatory or oxidative response would allow for the mechanisms behind the lack of protection to be understood. However, in order to unambiguously address the original hypothesis of whether SIRT1 plays an underlying protective role in paracetamol toxicity, we can use young and old SIRT1 transgenic mice treated with acute paracetamol.
The aim of this study is to investigate the role of SIRT1 in paracetamol hepatotoxicity, in young and old mice. Understanding the potentially protective role of the SIRT1 pathway in paracetamol hepatotoxicity, and the effect of age on this protection, could provide insight into new potential protective therapeutics, for all ages.

5.2 Methods

5.2.1 Animals
Male liver-specific SIRT1 knock-out mice (SIRT1 KO) were developed by crossing Albumin Cre-Lox C57BL/6 mice with floxed SIRT1Δex4 mice (Chen et al. 2008). Liver-specific SIRT1 over expressor mice (SIRT1 OE) were developed by crossing Albumin Cre-Lox C57BL/6 mice with SIRT1 transgenic mice (Price et al. 2012). Young SIRT1 KO (age=6.7±0.0 months, n=13) and SIRT1OE mice (age=6.8±0.0 months, n=11) were bred at the NIA (Baltimore, MD). Old SIRT1 KO (age=18.4±2.1 months, n=11) and SIRT1OE mice (age=20.3±1.7 months, n=8) were bred and aged at the NIA (Baltimore, MD). Young (age=7.3±0.3 months, n=23) and old (age=18.9±2.3 months, n=25) male control littermates, and C57BL/6 wild-type mice were also bred and aged at the NIA (Baltimore, MD). Animals were randomly assigned to treatment or control groups prior to the treatment day. The mouse numbers were determined by a sample of convenience as the transgenic mice were excess from another experiment that were re-assigned to this study. However we believe the mouse numbers were still powered to detect a clinically significant change in our primary outcome of serum ALT.
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

Mice were group housed in cages of four with *ad libitum* access to food and water. Mice were fed a 2018 Teklad Global 18% Protein Rodent diet (Harlan laboratories). Animal rooms were maintained on a 12 hr light/dark cycle at 20-22°C, and 30-70% humidity. All animal protocols were approved by the Animal Care and Use Committee of the National Institute on Aging (429-TGB-2017 and 405-TGB-2016).

5.2.2 Paracetamol Treatment and Tissue Collection

Animals were fasted overnight (16 hours), then dosed with 300mg/kg paracetamol (Panadol Color-free Baby Drops, 100mg/ml, GlaxoSmithKline, Australia) or saline vehicle via oral gavage between 8 and 10am. This paracetamol dose was chosen as a standard dose to induce toxicity in C57BL/6 mice (McGill et al. 2012). Food was returned to the mice two hours after dosing. Six hours after dosing mice were anaesthetized with an *i.p.* injection of ketamine (75 mg/kg, DVR Pharmacy, Bethesda MD) and xylazine (10 mg/kg, DVR Pharmacy Bethesda MD). A midline laparotomy was performed and blood taken from the Inferior Vena Cava. The portal vein was then cannulated with a 21G intravenous catheter (Becton Dickinson, Sydney, Australia) through which the liver was perfused *in-situ* at 1–1.5 mL/min/g of liver with oxygenated Krebs-Henseleit bicarbonate buffer (95% O₂–5% CO₂, 37°C). Sections of the liver were snap frozen in liquid nitrogen for subsequent enzyme activity assays, protein and RNA extraction and fixed in 10% neutral formalin for subsequent histopathological analysis.

5.2.3 Serum Biochemistry to Assess Liver and Renal Function

Blood was stored and serum extracted as detailed in section 2.2.4, serum liver function tests (total protein, albumin, bilirubin, alkaline phosphatase (ALP), gamma glutamyltransferase (GGT)) and creatinine were measured by a National Association of Testing Authorities accredited hospital laboratory, PaLMS (Pacific Laboratory...
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

Medicine Services) at Royal North Shore Hospital (Sydney, Australia) using an Architect i1000SR immunoassay analyser (Abbott Diagnostics, IL, USA). Vitamin B6 is added to the ALT activity assay to accommodate for the age related decline in this vitamin, an integral cofactor for the assay (Krishnamurthy et al. 1967; Bordoni et al. 1998).

5.2.4 Liver Histology
Fixed liver tissue was embedded in paraffin and 5µm sections were cut on a microtome and mounted on slides. Slides were stained with Haemotoxylin and Eosin in the National Association of Testing Authorities accredited hospital laboratory of the Pathology department of Royal Prince Alfred Hospital, Sydney, Australia. Histopathology was scored by an anatomical pathologist, who was blinded to the age, SIRT1 group and treatment of the samples. Necrosis was scored as a percentage of the liver tissue on each slide, which was then converted to a dichotomous variable (present or not present) for analysis. Images were taken on an Olympus BX51 microscope connected to an Olympus DP26 camera (Olympus, Sydney, Australia).

5.2.5 Biochemical Examination of Enzymes and Glutathione in Frozen Liver Samples
Liver microsomes were isolated according to the method of Shoaf et al. (1987), and CYP2E1 activity was measured according to the method of Roberts et al. (1995) and Mach et al. (2014), as detailed in section 2.2.6.

NQO1 activity was measured in the cytosol as described by Aleksunes et al. (2006), and detailed in section 2.2.6.
Liver concentrations of total glutathione were determined with a Glutathione Assay kit (Cayman Chemicals, #703002) as detailed in section 2.2.6.

5.2.6 Western Blots
SIRT1 protein levels were measured in liver from all mice. Liver was homogenised in lysis buffer (RIPA buffer supplemented with EDTA and EGTA (Boston BioProducts), protease inhibitor cocktail (Sigma-Aldrich #P8340), phosphatase inhibitor Cocktail 2 and 3 (Sigma-Aldrich #P5726 and #P0044), and Trichostatin A (Sigma-Aldrich #T1952)) then centrifuged at 14,000g for 15 minutes at 4°C, and the supernatant collected. Protein concentration was quantified using the Quickstart Bradford assay (Bio-Rad, #500-0201). 30μg of protein was separated by SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA for one hour at room temperature, and then incubated overnight at 4°C with the primary antibody SIRT1 (1:1000, Sigma, #S5196) in 5% BSA. Membranes were washed, then incubated in secondary antibody solution (5% skim milk in TBS-T, mouse secondary antibody 1:5,000, GE Healthcare, Piscataway, NJ) for one hour at room temperature. The membranes were washed then visualization of the immunoreactive bands was performed using the ECL Prime Western blotting detection system (GE Healthcare, Piscataway, NJ). Densitometry was performed using ImageJ (v 1.47). Ponceau S was used as a loading control and data of each group was normalized to the young wild-type saline treated group.
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

5.2.7 mRNA Expression Measurement with qPCR
RNA was extracted, and qPCR run to assess mRNA expression of CYP2E1, NQO1, TNF-α, IL-1β, IL-10, NF-κB, Caspase 3, BAX, PGC-1α, PGC-1β (primer sequences in Appendix 2), as detailed in section 2.2.7.

5.2.8 Statistics
Data are expressed as mean ± SEM unless otherwise indicated. Differences between mean values across groups were calculated with one-way ANOVA with Tukey’s HSD post-hoc test, or a 2 or 3 way ANOVA to compare the effect of age, treatment and SIRT1 groups. The prevalence of necrosis in each treated group compared to the saline group was compared with Chi squared. The calculation of mRNA expression was performed by the 2-ΔΔCT method. Data analysis was completed using the statistics program SPSS (Version 21.0, SPSS Inc., Chicago, Illinois, USA) and GraphPad Prism (Version 6.04, GraphPad Software, La Jolla California USA).

5.3 Results

5.3.1 Animal and Genotype Details
Weights and ages for all mouse groups are shown in Table 5.1. Numbers per group are as stated in table unless specified elsewhere. Five old mice had evidence of lymphoma (n=3 wild-type, n=2 SIRT1 over-expressors).
Table 5.1 Weights and serum biochemistry results for young and old male wild-type C57BL/6, liver specific SIRT1 overexpressor, and liver specific SIRT1 knock out mice treated with 300mg/kg paracetamol (para; via oral gavage) or saline. Mice were euthanized, and liver and serum collected after 6 hours. Data expressed as mean (SD). ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase.

<table>
<thead>
<tr>
<th></th>
<th>Young Wild-Type</th>
<th>Old Wild-Type</th>
<th>Young Over-Expressor</th>
<th>Old Over-Expressor</th>
<th>Young Knock-Out</th>
<th>Old Knock-Out</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline n=10</td>
<td>Para n=13</td>
<td>Saline n=10</td>
<td>Para n=15</td>
<td>Saline n=4</td>
<td>Para n=7</td>
</tr>
<tr>
<td></td>
<td>Saline n=5</td>
<td>Para n=6</td>
<td>Saline n=6</td>
<td></td>
<td>Saline n=6</td>
<td>Para n=6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (months)</strong></td>
<td>7.2 (0.3)</td>
<td>7.5 (0.0)</td>
<td>19.1 (1.8)</td>
<td>18.8 (2.7)</td>
<td>20.5 (1.8)</td>
<td>22.1 (4.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.8 (0.0)</td>
<td>6.8 (0.0)</td>
<td>6.7 (0.0)</td>
<td>6.7 (0.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17.9 (1.9)</td>
<td>18.9 (2.4)</td>
</tr>
<tr>
<td><strong>Pre-dosing Weight (g)</strong></td>
<td>27.6 (1.7)</td>
<td>27.8 (3.3)</td>
<td>41.0 (6.7)</td>
<td>39.2 (7.2)</td>
<td>37.8 (3.1)</td>
<td>39.0 (6.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28.0 (1.5)</td>
<td>28.6 (1.8)</td>
<td>29.8 (1.7)</td>
<td>30.2 (2.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>36.6 (8.6)</td>
<td>36.3 (6.2)</td>
</tr>
<tr>
<td><strong>Total protein (g/L)</strong></td>
<td>50.00 (2.94)</td>
<td>50.67 (3.08)</td>
<td>52.30 (5.1)</td>
<td>49.83 (5.89)</td>
<td>50.80 (3.42)</td>
<td>47.00 (7.87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50.00 (2.94)</td>
<td>50.67 (3.08)</td>
<td>50.00 (2.94)</td>
<td>50.00 (2.94)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>52.30 (5.1)</td>
<td>49.83 (5.89)</td>
<td>50.80 (3.42)</td>
<td>47.00 (7.87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>55.50 (7.55)</td>
<td>53.25 (9.6)</td>
<td>47.50 (3.78)</td>
<td>41.67 (16.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>51.00 (4.24)</td>
<td>47.50 (3.45)</td>
<td>51.00 (4.24)</td>
<td>47.50 (3.45)</td>
</tr>
<tr>
<td><strong>Albumin (g/L)</strong></td>
<td>26.50 (1.27)</td>
<td>27.75 (1.82)</td>
<td>26.70 (2.55)</td>
<td>24.92 (3.94)</td>
<td>26.20 (2.59)</td>
<td>25.80 (3.63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26.20 (2.59)</td>
<td>25.80 (3.63)</td>
<td>27.75 (1.89)</td>
<td>28.25 (1.26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27.75 (1.89)</td>
<td>28.25 (1.26)</td>
<td>24.00 (2.10)</td>
<td>20.83* (7.88)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24.00 (2.10)</td>
<td>20.83* (7.88)</td>
<td>25.75 (1.71)</td>
<td>24.00 (1.71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24.00 (2.10)</td>
<td>20.83* (7.88)</td>
<td>25.75 (1.71)</td>
<td>24.00 (1.71)</td>
</tr>
<tr>
<td><strong>Bilirubin (umol/L)</strong></td>
<td>1.60 (0.52)</td>
<td>2.45 (0.52)</td>
<td>1.70 (0.48)</td>
<td>2.58 (1.08)</td>
<td>1.20 (0.45)</td>
<td>2.20 (0.45)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.20 (0.45)</td>
<td>2.20 (0.45)</td>
<td>2.00 (1.15)</td>
<td>3.50 (1.91)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.83 (2.56)</td>
<td>2.50 (1.05)</td>
<td>3.00 (3.37)</td>
<td>2.50 (2.26)</td>
</tr>
<tr>
<td><strong>ALP (U/L)</strong></td>
<td>73.00 (10.19)</td>
<td>87.00 (19.05)</td>
<td>91.10 (20.09)</td>
<td>85.25 (30.18)</td>
<td>73.80 (20.28)</td>
<td>73.50 (18.51)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>73.80 (20.28)</td>
<td>73.50 (18.51)</td>
<td>79.00 (10.2)</td>
<td>101.25 (31.58)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>79.00 (10.2)</td>
<td>101.25 (31.58)</td>
<td>66.17 (9.47)</td>
<td>62.83 (25.53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72.80 (15.11)</td>
<td>84.00 (15.94)</td>
<td>72.80 (15.11)</td>
<td>84.00 (15.94)</td>
</tr>
<tr>
<td><strong>GGT (U/L)</strong></td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.08</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.00</td>
<td>3.08</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.75</td>
<td>3.00</td>
<td>3.00</td>
<td>4.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.00</td>
<td>4.67</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Creatinine (umol/L)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.29)</td>
<td>(0.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>33.40</td>
<td>33.09</td>
<td>41.40</td>
<td>47.25</td>
<td>32.60</td>
<td>32.80</td>
</tr>
<tr>
<td>(2.76)</td>
<td>(4.06)</td>
<td>(16.08)</td>
<td>(31.19)</td>
<td>(4.04)</td>
<td>(3.63)</td>
<td>(86.84)</td>
</tr>
<tr>
<td>Liver weight</td>
<td>4.83</td>
<td>5.38</td>
<td>4.40</td>
<td>4.99</td>
<td>4.85</td>
<td>5.22</td>
</tr>
<tr>
<td>(% body weight)</td>
<td>(0.60)</td>
<td>(0.43)</td>
<td>(0.73)</td>
<td>(0.86)</td>
<td>(0.40)</td>
<td>(0.77)</td>
</tr>
</tbody>
</table>
SIRT1 mRNA expression in the liver was reduced 10-fold in both young and old KO mice, compared to wild types (p<0.001) (Figure 5.1A) confirming the SIRT1 KO phenotype. Young and old SIRT1 OE mice had a trend towards an increase in SIRT1 mRNA compared to wild types (p>0.05). A two way ANOVA showed a significant SIRT group effect on SIRT1 mRNA expression, but no age effect. Western blot analysis of SIRT1 showed an expected downward shift in the band for SIRT1 in both young and old KO mice, consistent with the KO generated by Price et al (2012), and a trend towards an increase in protein intensity for young and old SIRT1 OE mice, compared to WTs (Figure 5.1B), indicating increased protein expression.
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

**Figure 5.1** Liver sirtuin 1 (SIRT1) mRNA expression (A), and protein expression (B), for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. Data expressed as mean relative to young wild type control±SEM. One-way ANOVA SIRT1 mRNA, F(5,26)=4.66 p=0.004. One-way ANOVA SIRT1 protein, F(5,27)=4.27 p=0.005. *=p<0.05 compared to corresponding age wild-type with Tukey’s HSD post-hoc test. n=3-10 per group for mRNA, n=5-6 per group for western blot (shown on graph).

5.3.2 Assessment of Paracetamol Toxicity

Table 5.1 shows blood test results, liver weight and post-fast weights for all mouse groups. There was no difference across any group for total protein, albumin, bilirubin, ALP, GGT, creatinine or liver weight (as percentage of body weight).

All paracetamol treated groups had increased mean ALT when compared to their corresponding saline treated groups (p<0.05), except for old SIRT1 OE mice where this was not significant (p=0.15) (Figure 5.2). 71-85% of samples in all paracetamol treated groups had evidence of necrosis, with one sample in the saline treated young SIRT1 knock-out showing necrosis (Figure 5.3). The average percentage of the scored liver with necrosis was 22.0±20.6 % across all groups. A three way ANOVA showed that treatment was the only significant factor affecting serum ALT, with no significant SIRT1 or age effect on the degree of hepatotoxicity.
Figure 5.2 Serum alanine aminotransferase (ALT) concentrations for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. Data expressed as mean±SEM. One-way ANOVA, F(11,85)=5.38 p=0.000. *=p<0.05 compared to corresponding saline treated group with Tukey’s HSD post-hoc test. n=4-15 per group.
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

**Figure 5.3** Prevalence of necrosis (as a percentage of each group) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. Data expressed as % of group. *=p<0.05 compared to corresponding saline treated group. n=4-15 per group.
<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Paracetamol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Young SIRT1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Knock out</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Old SIRT1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Knock out</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Young SIRT1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Over Expressor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Old SIRT1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Over Expressor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Young SIRT1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Wild Type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Old SIRT1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Wild Type</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

Figure 5.4 Representative Haemotoxylin and Eosin stained liver histology images for young and old male C57BL/6 wild-type, liver-specific SIRT1 over-expressor and liver-specific SIRT1 knock-out mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. Images taken at 100X.

5.3.3 Assessment of Factors Influencing Paracetamol Pharmacokinetics

CYP2E1 activity was significantly reduced in paracetamol treated young and old wild-type mice, and young SIRT1 knock-out mice compared to saline controls (Figure 5.5A). CYP2E1 mRNA expression was reduced for old wild-type and old SIRT1 over-expressors treated with paracetamol, compared to saline controls (p<0.05) (Figure 5.5B).

Figure 5.5 Cytochrome (CYP)2E1 activity (A) and CYP2E1 mRNA expression (B) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

(OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. For activity graph, data is expressed as mean ±SEM; for mRNA graph, data is expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA CYP2E1 activity, F(11,83)=2.62 p=0.007. One-way ANOVA CYP2E1 mRNA, F(11,67)=4.91 p=0.000. *=p<0.05 compared to saline treated group group with Tukey’s HSD post-hoc test. n=4-15 per group for activity, n=3-10 per group for mRNA.

Saline treated old wild-type and SIRT1 OE mice had significantly greater NQO1 activity than their comparative young groups (p<0.05) (Figure 5.6A) and there was a trend towards increased NQO1 activity in all other old mouse groups compared to their comparative young groups (p>0.05). A three way ANOVA of treatment, age and SIRT1 group, showed that age was the only significant factor affecting NQO1 activity (Figure 5.6B). Interestingly, NQO1 mRNA expression was significantly increased in all paracetamol treated groups compared to their saline controls (p<0.05), but there was no SIRT1 or age effect (Figure 5.6B).
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

Figure 5.6 NAD(P)H:quinone oxidoreductase 1 (NQO1) activity (A) and NQO1 mRNA expression (B) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. For activity graph, data is expressed as mean ±SEM; for mRNA graph, data is expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA NQO1 activity, F(11,83)=3.09 p=0.002. One-way ANOVA NQO1 mRNA, F(11,71)=6.75 p=0.000. * = p<0.05 compared to saline treated group, # = p<0.05 compared to young treated group, $ = p<0.05 compared to young saline group with Tukey’s HSD post-hoc test. n=4-15 per group for activity, n=3-10 per group for mRNA.
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

Liver total GSH did not significantly change across any SIRT1, age or treatment group (Figure 5.7).

![Figure 5.7](image)

**Figure 5.7** Total liver glutathione (GSH) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice, 6 hours after treatment with 300mg/kg paracetamol (via oral gavage) or saline. Data is expressed as mean ±SEM. n=4-15 per group.

5.3.4 mRNA expression of Inflammatory Markers

The mRNA expression of the pro-inflammatory cytokine TNF-α was increased in young and old wild-type and knock-out mice treated with paracetamol compared to saline controls (p<0.05) (Figure 5.8A). A similar trend was seen for anti-inflammatory cytokine IL-10 (Figure 5.8C). Old wild-type mice treated with paracetamol had a larger increase in TNF-α and IL-10 mRNA expression than young wild-type mice treated with paracetamol (p<0.05) (Figure 5.8A, C). IL-1β mRNA expression had a trend towards an increase with paracetamol treatment compared to saline treatment in old wild-type but not young wild-type mice (Figure 5.8B). NF-κB mRNA expression was increased in old wild type and old SIRT1 knock-out mice treated with paracetamol, compared to their saline treated groups (p<0.05) whilst there was no increase in NF-κB mRNA expression with paracetamol treatment in any of the young mouse groups (Figure 5.8D).
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

Figure 5.8 mRNA expression of inflammatory markers; (A) Tumor necrosis factor (TNF)-α, (B) Interleukin (IL)-1β, (C) IL-10 and (D) Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mRNA expression for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice treated with saline or paracetamol (300mg/kg via oral gavage). Data expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA TNFα, F(11,71)=4.08 p=0.000. One-way ANOVA IL-1β, F(11,71)=1.71 p=0.09. One-way ANOVA IL-10, F(11,71)=2.66 p=0.008. One-way ANOVA NF-κB, F(11,71)=2.51 p=0.011. *=p<0.05 compared to saline treated group, #=p<0.05 compared to young treated group with Tukey’s HSD post-hoc test. n=3-10 per group.

5.3.5 mRNA expression of Apoptosis and Mitochondrial Function Markers
The mRNA expression of Caspase 3, a protein activated in apoptosis, showed no change with treatment, SIRT1 group or age. The mRNA expression of apoptosis promoting
protein, BAX, was only increased in old wild type mice and young SIRT1 OE mice treated with paracetamol, compared to saline treated controls ($p<0.05$) (Figure 5.9B).

![Figure 5.9](image)

**Figure 5.9** mRNA expression of Caspase 3 (A) and BAX (B) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice treated with saline or paracetamol (300mg/kg via oral gavage). Data expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA BAX, $F(11,71)=2.64$ $p=0.008$. One-way ANOVA Caspase 3, non-significant. *$p<0.05$ compared to saline treated group, #*$p<0.05$ compared to young treated group with Tukey’s HSD post-hoc test.. $n=3-10$ per group.
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

The only significant change in mRNA expression of the mitochondrial regulator proteins PGC1-α and PGC1-β was a decrease and increase respectively in old wild type mice treated with paracetamol, compared to old saline treated controls (p<0.05) (Figure 5.10C, D). There was no effect observed in young mice.

Figure 5.10 mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1)-α (A) and PGC1-β (B) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice treated with saline or paracetamol (300mg/kg via oral gavage). Data expressed as mean normalized to young wild-type saline group ±SEM. Three-way (Age, SIRT1, Treatment) ANOVA PGC1-α – Treatment group F(1,60)=4.01 p=0.05. Three-
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

way ANOVA PGC1-β – Treatment group F(1,60)=4.06 p=0.048, Age group F(1,60)=4.91 p=0.03. *=p<0.05 compared to saline treated group, #=p<0.05 compared to young treated group. n=3-10 per group.

In both young and old wild-type mice there was an increase in SIRT1 mRNA expression in the paracetamol treated mice compared to saline treated mice (p=0.006 young, p=0.01 old) (Figure 5.11E).

**Figure 5.11** mRNA expression of sirtuin 1 (SIRT1) for young and old male C57BL/6 wild-type (WT), liver-specific SIRT1 over-expressor (OE) and liver-specific SIRT1 knock-out (KO) mice treated with saline or paracetamol (300mg/kg via oral gavage). Data expressed as mean normalized to young wild-type saline group ±SEM. One-way ANOVA, F(11,71)=4.94 p=0.000. *=p<0.05 compared to saline treated group. n=3-10 per group.

5.4 Discussion

This comprehensive investigation utilising young and old wild type, SIRT1 knockout and SIRT1 over expressing mice demonstrates that SIRT1 does not play a protective role in paracetamol induced hepatotoxicity in our model. Furthermore this study shows
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

the induction of SIRT1 mRNA expression with high dose paracetamol treatment implying a role of the SIRT1 pathway in paracetamol hepatotoxicity.

This study found no effect of SIRT1 knock-out or overexpression on the risk of paracetamol hepatotoxicity. This finding was unexpected as previous studies of CR and resveratrol, intervention known to activate SIRT1, have shown protection against paracetamol hepatotoxicity (Harper et al. 2006; Sener et al. 2006; Du et al. 2015). This may imply that the protection from CR and resveratrol seen in previous studies, although not this thesis, is through a SIRT1 independent mechanism. Recent studies have shown SIRT1-independent activation of anti-oxidant and anti-inflammatory pathways by resveratrol in other situations (Kitada et al. 2011; Minagawa et al. 2014). Interestingly, a recent study found that SIRT3 knockout mice were protected against paracetamol-induced toxicity, and that direct deacetylation of mitochondrial protein targets by SIRT3 exacerbated hepatotoxicity (Lu et al. 2011), implying a potentially paradoxical role of the different sirtuin proteins in paracetamol toxicity. Further research into SIRT1 independent potential protective pathways against paracetamol toxicity is warranted and may help in finding new therapeutics to protect against paracetamol toxicity. It is also possible that increasing SIRT1 expression alone, without a concurrent increase in important co-factors such as NAD+, will not be protective against stressors. Recent evidence is supporting an increasing protective role for increased NAD+ levels, independent of SIRT1 activity (Cantó & Auwerx 2012). Interestingly, induction of NQO1 activity, which we see with old age in all groups in the current study, has been shown to increase cellular NAD+ levels (Kim et al. 2014). Another possibility is that the degree of toxicity induced in our model was too severe to see protection from the SIRT1-induced mechanisms. Recent studies in cancer cells have
found beneficial effects of SIRT1 activation only at sublethal doses of chemotherapeutic agents (Wang et al. 2013; Luo et al. 2001). Further research into the role of SIRT1 in protecting against lower-dose, or chronic paracetamol exposure would answer these questions.

As SIRT1 plays a central role in the lifespan-related pathways, and activation of SIRT1 has been theorized as a potential mechanism for increasing longevity with interventions such as calorie restriction or resveratrol exposure (Baur & Sinclair 2006), it was hypothesised that it would be interesting to investigate the effect of genetic manipulation of SIRT1 and paracetamol toxicity in ageing. The current study found no interaction effect of SIRT1 and age on any of the measured outcomes, and no differential effect of SIRT1 status on paracetamol toxicity in old age compared to young. This further confirms that activation of SIRT1 does not play a protective role in paracetamol toxicity in C57BL/6 mice, even in old age when SIRT1 expression and activity have been shown to be reduced (Jin et al. 2011; Ramsey et al. 2008).

SIRT1 status did not affect any of the pharmacokinetic factors measured in this study. However, in young mice there was an increase in mRNA expression of IL-1β, but no increase in TNF-α with paracetamol treatment in the SIRT1 OE mice with the opposite being observed in the KO mice. This implies a greater anti-inflammatory response to paracetamol in the SIRT1 OE mice, and a more pro-inflammatory response in the SIRT1 KO mice. A similar result was seen in another study, which found that SIRT1 overexpressor mice fed a high fat diet did not see the increase in pro-inflammatory cytokine mRNA expression levels, seen in wild type mice (Pfluger et al. 2008). It is
Chapter 5: The Role of SIRT1 in Paracetamol Hepatotoxicity in Young and Old Mice

interesting, however, that this differential inflammatory response did not translate into a different degree of paracetamol toxicity.

Despite the hypothesised therapeutic interventions of resveratrol treatment and genetic SIRT manipulation not protecting against paracetamol toxicity, there is still a need to develop new therapies to treat or prevent paracetamol hepatotoxicity. Possible targets for paracetamol toxicity therapeutics that could be investigated in future studies may include other age-related mechanisms such as increasing NQO1 activity (Mach et al. 2014), increasing autophagy via manipulation of the mTOR pathways (Ni et al. 2012) and further investigation of the potentially anti-oxidant, anti-inflammatory and NAD+ manipulating effects of the other ageing-related pathways such as AMPK (Speakman & Mitchell 2011).

In this study we observed, for the first time, an increase in SIRT1 mRNA expression levels with high-dose paracetamol treatment. Certain stressors have been shown to induce SIRT1 activity and protein expression (Raynes et al. 2013; Kwon & Ott 2008). In our study it is possible that paracetamol-induced cellular damage may be facilitating the transcription of SIRT1 mRNA. In chapter 4 of this thesis, we saw an increase in phosphorylated AMPK protein expression with paracetamol treatment, which may be related as the stress response SIRT1 and AMPK pathways are tightly linked (Price et al. 2012). It would be interesting to explore whether therapeutic paracetamol dosing also affects these pathways. One rat study suggested a potentially age-delaying role for paracetamol in the aorta (Rice et al. 2012), and another paper showed that paracetamol had a protective affect in mouse models of diabetes, the pathways of which are closely associated with the SIRT1-AMPK pathways (Shertzer et al. 2008).
Changes seen with high-dose paracetamol treatment were overall consistent with the previously published literature. Decreased CYP2E1 activity has been previously reported in mice after acute paracetamol treatment (Snawder et al. 1994). Previous studies have shown, following paracetamol treatment total glutathione is maximally reduced at 0.5-1 hour post acute treatment, and steadily recovers back to baseline over the following hours, as was seen in our study (Mitchell et al. 1973; Jaeschke et al. 2012). In the current study, NQO1 mRNA expression was increased with treatment for all groups, whereas NQO1 activity was essentially unchanged with treatment. A previous human study showed an increase in liver NQO1 activity and protein levels after paracetamol treatment (Aleksunes et al. 2006), whilst a recent mouse study showed a reduction in NQO1 protein expression 6 hours after paracetamol treatment (Fan et al. 2014). The differences between these studies may be due to time frame or species differences and more research is needed into the effect of paracetamol on NQO1 expression and activity. The effect of high-dose paracetamol treatment on the mRNA expression of pro and anti-inflammatory cytokines TNF-α and IL-10 was also consistent with previous literature (Gardner et al. 2002; Ishida et al. 2002). In this study paracetamol treatment resulted in an increase in caspase 3 mRNA expression, perhaps implying changes in the regulation of the apoptosis pathway (Boulares & Ren 2004), which a recent clinical review has shown plays a role in paracetamol toxicity (Lancaster et al. 2014).

The strength of this study is the use of transgenic mice to test the absolute role of SIRT1 in paracetamol toxicity for the first time, and the use of both young and old mice to see the effect of age. A limitation of this study is the small sample sizes for some mouse
groups which may reduce the power to draw statistical conclusions, and the moderate degree of SIRT1 overexpression in the livers of the SIRT1 transgenic mice. Furthermore as this study was conducted in male C57BL/6 mice the applicability to other animal strains and species, and clinically, still needs to be confirmed. Additionally, the qPCR results in this experiment identify some interesting downstream effects of paracetamol and the transgenic mice on mRNA expression of proteins including SIRT1, inflammatory cytokines and caspase 3, but to fully understand the mechanisms behind and consequences of these changes it would be important to also look at protein expression, enzyme activity and further downstream markers related to these proteins.

This chapter shows for the first time that SIRT1 does not play a role in protecting against paracetamol hepatotoxicity in C57BL/6 mice. This important negative finding highlights the importance of thoroughly investigating potential toxicity therapeutics in animal models, and suggests that SIRT1 may not be a good therapeutic target for protection against paracetamol toxicity. Despite SIRT1 not playing a protective role, this study did show the induction of SIRT1 mRNA expression with high dose paracetamol treatment, implying some role of SIRT1 in paracetamol toxicity development mechanisms.
6 Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

6.1 Introduction

In chapters two and three of this thesis, it was shown that frailty does not effect the risk of paracetamol toxicity in old mice. This chapter will investigate the inverse relationship, to determine whether paracetamol may affect frailty in mice. We will also examine whether the lifespan and healthspan interventions (tested for their potential paracetamol protective effects in chapters four and five), of resveratrol and calorie restriction, can affect frailty status.

Frailty is a state of high vulnerability for adverse health outcomes. The prevalence of frailty increases with increasing age, resulting in a higher risk of disability, falls,
hospitalization and mortality (Clegg et al. 2013). In humans, frailty can be assessed using a number of scales with varying degrees of difficulty and clinical applicability (de Vries et al. 2011). Two of the mostly commonly used and cited definitions are the phenotype model and the frailty index. The phenotype model defines frailty as the presence of three or more criteria including unintentional weight loss, self-reported exhaustion, weakness measured by grip strength, slow walking speed, and low physical activity (Fried et al. 2001). The frailty index (Mitnitski et al. 2002) measures the proportion of accumulated deficits in a patient, and focuses on the number of deficits rather than the precise nature of the deficits.

Validated animal models to study frailty are lacking, and are summarised in section 1.3.2. The mouse frailty index (Whitehead et al. 2014) is an important tool that could be used to assess the effect of any intervention on the important clinical outcome of frailty in an animal model.

The main factors known to affect the lifespan and healthspan of mice are strain, diet and pharmaceutical interventions. Of the inbred mouse strains, the DBA/2J substrain is considered to be short-lived, with studies showing median lifespan for males to be 23-25 months, compared to 26-30 months for male C57BL/6J mice (Goodrick 1975; Forster et al. 2003; Yuan et al. 2011). DBA/2J mice have also been shown to be unresponsive to interventions that extend lifespan, such as calorie restriction (Forster et al. 2003). C57BL/6J mice are the classical inbred mouse strains commonly used for ageing research due to the wealth of phenotypic and genotypic information available (source: http://phenome.jax.org/). Furthermore, they are responsive to interventions that extend lifespan such as calorie restriction and pharmaceuticals (Sohal & Weindruch...
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

1996; Baur et al. 2006). Calorie restriction, the daily reduction in calories without malnutrition, has long been known to increase the lifespan and healthspan of mice (Weindruch et al. 1986). Pharmaceutical agents that mimic the beneficial lifespan and/or healthspan effects of calorie restriction have also been investigated. Rapamycin and metformin have shown lifespan extension in mice on standard diets (Harrison et al. 2009; Martin-Montalvo et al. 2013), but there are some concerns about side effects. Resveratrol has shown healthspan improvements in standard diet fed mice (Pearson et al. 2008), and lifespan extension in high-fat diet mice (Baur et al. 2006). Recently, there has been an increasing focus on improving healthspan and quality of life, rather than lifespan, and assessment of frailty would provide a valuable measure of this in animal studies.

Paracetamol is commonly prescribed and used in the older population, as an analgesic (Pearson et al. 2007). The effect of therapeutic paracetamol on healthspan outcomes has not been extensively investigated. The induction of mRNA expression of SIRT1, and protein expression of phosphorylated AMPK, important proteins affected by other lifespan and healthspan interventions, was seen with acute high-dose paracetamol treatment in chapters 4 and 5 of this thesis. Furthermore, recent studies have suggested a potentially age-delaying role for paracetamol in the rat aorta (Rice et al. 2012) and in aged skeletal muscle (Wu et al. 2010), and another paper showed that paracetamol had a protective effect in mouse models of diabetes, the pathways of which are closely associated with the ageing pathways (Shertzer et al. 2008). A longevity screening study in mammalian neurons, also identified paracetamol as reducing an age-related increase in mortality rate (Lublin et al. 2011). Thus there is some evidence to suggest that
paracetamol may also be an intervention that is able to improve healthspan or delay frailty.

The primary aim of this chapter is to investigate the effect of an example of each of these effective lifespan and healthspan interventions – DBA/2J (short lived) vs. C57BL/6J (long lived) strain, calorie restriction vs. ad libitum feeding, and resveratrol vs. placebo – as well as the effect of chronic therapeutic paracetamol exposure, on the mouse frailty index (Whitehead et al. 2014) in male and female mice. The secondary aims of this chapter are to correlate the frailty index with functional outcomes in mice, and determine the inter-rater variability in assessment of frailty index.

6.2 Methods

6.2.1 Animals
Three cohorts of mice were used to assess the effect of interventions on frailty index in this study. The first cohort was made up of male C57BL/6 mice obtained from, and housed at the Kearns Facility (Sydney, NSW). Mice were group housed in cages of five. Mice were randomized at 26.8±0.5 months of age (n=56) to either a control diet (Standard Meat Free Mouse and Rat Feed, 20 % Protein, 60% Carbohydrate, 5% Fat, Specialty Feeds, Australia), or control diet supplemented with paracetamol at a concentration of 1.33g/kg feed, both diets fed ad libitum. Before beginning their experimental diet periods, the frailty index (described below) was scored for each mouse (pre-treatment). Mice remained on their respective diets for six weeks, at which time the frailty index was scored for each mouse again (post-treatment).
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

The second cohort of mice was made up of male and female C57BL/6J (n=18 males, n=21 females) and DBA/2J (n=16 males, n=19 females) mice that were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in house at the National Institute on Aging (Baltimore, MD). Mice were group housed in cages of four. At six months of age mice were randomized to either an *ad libitum* (AL) diet group, or a calorie restricted (CR; 40% of *ad libitum*) diet group for the remainder of their lives (Curtis & Cabo 2013). CR mice were fed daily at 7.30am (± 1 hour) on the floor of the cage, while AL mice received food in the hopper. Mice were fed 2918 Teklad Global 18% Protein Rodent Diet (Harlan Teklad, Indianapolis IN). At 19 months of age the frailty index was scored for each mouse. This time-frame was chosen based on a previously reported study of DBA2/J mice (Forster et al. 2003) to correlate with the time at which we see a drop in survival for calorie restricted DBA2/J mice to approximately 75%.

The third cohort was made up of male C57BL/6J mice that were obtained from the NIA Aging Colony (Charles Rivers) at 2 months of age, and then aged in house at the NIA (Baltimore, MD). Mice were fed standard feed (2018X 18% Protein Diet, Harlan Teklad, Indianapolis IN) *ad libitum* until they reached 18 months of age. These mice were then randomized to either a standard AIN-93G diet (SD; Dyets, Inc, Bethlehem PA) (n=16), or an AIN-93G diet supplemented with resveratrol (RSV) at a dose of 100mg/kg (n=9), both diets fed *ad libitum*. This timeframe was chosen to emulate the conditions of previous studies in which resveratrol showed beneficial outcomes even when started late in life (Baur et al. 2006; Pearson et al. 2008). Mice remained on their respective diet until they reached 24 months of age at which time the frailty index (described below) was scored. This time-frame was chosen as Forster and colleagues...
(Forster et al. 2003) previously found survival in *ad libitum* fed C57BL/6 mice was approximately 75% at 24 months.

Body weight and food intake for all mice was monitored biweekly, and all groups had *ad libitum* access to water. Animal rooms were maintained on a 12 hr light/dark cycle at 20-22°C, and 30-70% humidity. All animal protocols were approved by the Animal Care and Use Committee of the National Institute on Aging (429-TGB-2017 and 405-TGB-2016) or the Animal Care Ethics Committee at Royal North Shore Hospital.

6.2.2 Frailty Index Assessment
The Mouse Clinical Frailty Index was used to assess frailty as detailed in section 2.2.2. Young (12 week) C57BL/6J and DBA/2J weight and temperature means and standard deviations were obtained from the Jacksons Laboratory (http://jaxmice.jax.org/support/weight/000664.html).

For cohort one, mice were assessed for frailty, before randomization to treatment group and then after six weeks on their experimental diets. Frailty testing was carried out by two raters (raters 1 and 2) over one day. The raters were blinded to treatment group of the mice, and the scores assigned by the other rater.

For the second mouse cohort, 19 month old male DBA/2J and C57BL/6J mice were assessed by four raters (raters 1, 3, 4 and 5). Each mouse was brought to a quiet assessment room and allowed to acclimatize for 30 minutes prior to the assessment. The mouse was weighed, subcutaneous body temperature was obtained (average of two readings) using a subcutaneous implanted transponder (BDMS, Seaford, DE), and then
each mouse was scored for each item in the frailty index by each rater. The mice were scored over two days. The raters were blinded to the diet group of the mice and to the scores assigned by the other rater.

For the third mouse cohort, 24 month old male C57BL/6J mice were assessed by two raters (Raters 1 and 3). Mice were scored over one day, and the raters were blinded to the treatment group of the mice and to the score assigned by the other rater. Temperatures were not measured for these mice, so their final frailty index score was calculated for a 30 item scale only.

6.2.3 Examiners/Raters
Five raters with varying degrees of animal handling experience performed frailty assessments. Raters 1, 2 and 3 are scientists with 3-7 years of animal handling experience. Raters 4 and 5 are animal research technicians with 10 years animal handling experience.

6.2.4 Functional Assessments
At 16 months of age a subset of the cohort 1 DBA/2J (male n=5, female n=10) and C57BL/6J (male n=9, female n=11) mice had functional assessments performed (see timeline in supplemental figure 1). Motor co-ordination, balance, and endurance were assessed by the rotarod; forelimb and hindlimb strength using the cagetop test and forelimb and neuromuscular strength were assessed using the wirehang test. For all assessments mice were bought to the testing room and allowed to acclimitise for 15 minutes prior to the commencement of testing.
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

For rotator measurements the ability to run on the rotarod was measured as described previously (Mitchell et al. 2014). Mice were given a habituation trial at a constant speed of four rpm for one min before the first trial. Mice were then placed on the rotarod, which accelerated from 4 to 40 rpm over a period of 5 min, and latency to fall was recorded. A total of three trials were given on the same day, separated by 30 min rest periods. The average latency to fall was taken over all three trials.

For cagetop measurements, the ability to hang upside down from a wire screen was tested as previously described (McDonald et al. 2001). This was modified from the Kondziella's inverted screen test (Kondziella 1964). The mouse was place on the wire lid of a mouse cage which was turned upside down. The cage top was then turned until the mouse was hanging from the cage top with all four limbs grasping the bars, and the latency to fall was recorded. Each mouse was given three trials with a maximum possible latency of 60 seconds. If the mouse fell before 10 seconds, the trial was repeated two more times. The average latency to fall was taken over all three trials.

For wirehang measurements, a 55 cm wide 2-mm thick metallic wire was secured to two vertical stands approximately one metre above a thick foam pad. Mice were raised by their tail above the wire, slowly lowered until the mouse grasped the center of the wire with both front paws, then slowly lowered until the body was below the wire and released. The latency time that the mouse remained on the wire was recorded for three trials, with a maximum of 60 seconds. If the mouse fell before 10 seconds the test was repeated. If on the 3rd trial the mouse still fell before 10 seconds, the best latency out of three trials was recorded. The average latency to fall was taken over all three trials.
6.2.5 Statistics
Data are expressed as mean ± SEM unless otherwise indicated. The primary aim was assessed by taking the mean of the frailty index scores for two raters (raters 1 and 2 for cohort 1, and raters 1 and 3 for cohort 2 and 3), for each mouse. The data was assessed as normal using a D'Agostino & Pearson omnibus normality test, and thus differences between frailty index scores across groups were calculated with one-way ANOVA with Tukey’s HSD post-hoc test or t-tests where appropriate. The proportion of mice scored 0, 0.5 or 1 for an individual frailty index item, by raters 1 and 3, was compared across cohort 2 and 3 mouse groups using a Chi-squared test. To determine whether functional outcomes were correlated with frailty index scores, the Pearson correlation coefficient (r) was calculated, and linear regression was used to calculate slopes of best fit for each treatment group. Inter-rater reliability was assessed with the 2-way random, consistency, average intraclass correlation coefficient (ICC). The inter-rater reliability for specific items of the frailty index across raters was assessed by calculating the percentage of mice from cohorts 2 and 3 scored in agreement for each item across either across 2 or 4 raters. The percentage agreement was reported instead of Cohen’s or Fleiss’ kappa coefficient, as the nature of the index (only 3 possible scores, some scores used very infrequently) resulted in kappa values that severely underestimated the agreement (Mchugh 2012). Data analysis was completed using the statistics program SPSS (Version 21.0, SPSS Inc., Chicago, Illinois, USA) and GraphPad Prism (Version 6.04, GraphPad Software, La Jolla California USA).
6.3 Results

6.3.1 Effect of Paracetamol, Strain, Calorie Restriction and Resveratrol on Frailty Index Scores
Six weeks of dietary paracetamol did not affect frailty index scores in male C57BL/6 mice. There was no change in frailty index scores over the experimental period for control diet or paracetamol diet fed mice, and there was no difference in frailty index scores between control and paracetamol diet groups at either time point (Figure 6.1).

![Frailty index scores for old (26.8±0.5 months) male C57BL/6 mice pre and post six weeks of being fed either a control diet, or a diet supplemented with paracetamol (approximately 100mg/kg mouse/day, ‘Para’). Data is presented as mean ± 25 and 75% quartiles. n=14-40 per group.]

Male CR DBA/2J mice had higher frailty index scores than male CR C57BL/6J mice (p=0.01) (Figure 6.2). There was no change in frailty index scores in the male and female AL-fed DBA/2J mice, and female CR DBA/2J mice compared to sex-matched
C57BL/6J mice (Figure 6.2). In male C57BL/6J mice, CR significantly reduced the frailty index score, relative to their AL-fed counterparts (p=0.04). There was a trend towards reduced frailty index in calorie restricted female C57BL/6J, male DBA/2J and female DBA/2J mice compared to the sex and strain matched AL-fed groups (Figure 6.2). There was no significant sex-effect upon frailty index with no change in frailty index observed in all female groups compared to the diet and strain matched male groups (Figure 6.2).

Figure 6.2 Frailty index scores for (A) male C57BL/6 and DBA/2J mice fed either an AL diet, or a 40% CR diet from age 6 months (19±2 months age, n=8-9) and (B) female C57BL/6 and DBA/2J mice fed either an AL diet, or a 40% CR diet (19±1 months age, n=9-11) from age 6 months. Data is presented as mean ± 25 and 75% quartiles. One-way ANOVA female mice, non-significant. One-way ANOVA male mice, F(3,30)=4.84 p=0.007. M= male; F= female; C57= C57BL/6; DBA= DBA/2J; AL= ad libitum; CR= calorie restricted; * p < 0.05 with Tukey’s HSD post-hoc test.
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

In the third cohort of mice, 6 months of resveratrol treatment significantly reduced the frailty index in C57BL/6J mice relative to SD-fed mice (p=0.01) (Figure 6.3).

![Frailty Index Scores](image)

**Figure 6.3** Frailty index scores for male C57BL/6 mice fed either a standard AIN-93G diet (SD), or an AIN-93G diet supplemented with resveratrol (RSV) (100mg/kg mouse/day) (n=16 SD, n=9 RSV, 24±0 months age) for 6 months from age 19 months. Data is presented as mean ± 25 and 75% quartiles. * p < 0.05.

6.3.2 Effect of Strain, Calorie Restriction and Resveratrol on Specific Frailty Index Item Scores

The proportion of mice scored 0, 0.5 or 1 was determined for each sex, strain and diet group, for each item (Table 6.1 for males and Table 6.2 for females). There were some interesting strain and diet effects on the scoring of specific items. Temperature and body weight scores greater than 0 were more common in AL-fed mice of both strains and sexes, than in the CR groups. AL-fed male mice were also more likely to have rectal prolapse than the male mice on the CR diet. Alopecia was more common in the male and female DBA/2J mice, whilst loss of fur colour was more common in the C57BL/6J mice of both sexes. Male and female DBA/2J mice were much more likely to have
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

hearing loss with 100% of male mice from both diet groups scoring 0.5 or 1. Compared to both diet groups of male C57BL/6J mice, male DBA/2J mice were also more likely to have microphthalmia, a change in breathing rate and piloerection (Table 6.1). Female DBA/2J mice were more likely than female C57BL/6J mice to have kyphosis, tremor and a change in breathing rate. Distended abdomen was more common in female C57BL/6J mice than in female DBA/2J mice. Coat condition was more likely to be scored as normal in female C57BL/6J CR mice, than female ad libitum fed C57BL/6J mice or either female diet group of DBA/2J mice (Table 6.2)
Table 6.1 The percentage of scores by raters 1 and 3, for cohort 1 male C57BL/6 and DBA/2J mice fed either an AL diet, or a 40% CR diet from age 6 months (19±2 months age), that fell into each score category (0, 0.5, 1) for each item of the frailty index.

<table>
<thead>
<tr>
<th>Frailty Index Item</th>
<th>Male DBA2/J Calorie Restricted</th>
<th>Male DBA2/J Ad Libitum Fed</th>
<th>Male C57BL/6J Calorie Restricted</th>
<th>Male C57BL/6J Ad Libitum Fed</th>
<th>Chi Squared P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.5</td>
<td>1</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Temperature Score</td>
<td>71.4</td>
<td>14.3</td>
<td>14.3</td>
<td>57.1</td>
<td>42.9</td>
</tr>
<tr>
<td>Body Weight Score</td>
<td>85.7</td>
<td>14.3</td>
<td></td>
<td>42.9</td>
<td>42.9</td>
</tr>
<tr>
<td>Alopecia</td>
<td>57.1</td>
<td>42.9</td>
<td></td>
<td>56.3</td>
<td>43.8</td>
</tr>
<tr>
<td>Loss of fur colour</td>
<td>100.0</td>
<td>93.8</td>
<td>6.3</td>
<td>25.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Dermatitis</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Loss of whiskers</td>
<td>100.0</td>
<td>75.0</td>
<td>12.5</td>
<td>12.5</td>
<td>90.0</td>
</tr>
<tr>
<td>Coat condition</td>
<td>35.7</td>
<td>64.3</td>
<td></td>
<td>25.0</td>
<td>68.8</td>
</tr>
<tr>
<td>Tumours</td>
<td>100.0</td>
<td>87.5</td>
<td>12.5</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>Distended abdomen</td>
<td>100.0</td>
<td>87.5</td>
<td>12.5</td>
<td></td>
<td>95.0</td>
</tr>
<tr>
<td>Kyphosis</td>
<td>28.6</td>
<td>50.0</td>
<td>21.4</td>
<td>37.5</td>
<td>31.3</td>
</tr>
<tr>
<td>Tail stiffening</td>
<td>35.7</td>
<td>50.0</td>
<td>14.3</td>
<td>12.5</td>
<td>31.3</td>
</tr>
<tr>
<td>Gait disorders</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Value_1</td>
<td>Value_2</td>
<td>Value_3</td>
<td>Value_4</td>
<td>Value_5</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Tremor</td>
<td>71.4</td>
<td>28.6</td>
<td>75.0</td>
<td>25.0</td>
<td>95.0</td>
</tr>
<tr>
<td>Forelimb grip strength</td>
<td>35.7</td>
<td>50.0</td>
<td>14.3</td>
<td>18.8</td>
<td>31.3</td>
</tr>
<tr>
<td>Body condition score</td>
<td>57.1</td>
<td>35.7</td>
<td>7.1</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Vestibular disturbance</td>
<td>78.6</td>
<td>21.4</td>
<td>81.3</td>
<td>18.8</td>
<td>50.0</td>
</tr>
<tr>
<td>Hearing loss</td>
<td>28.6</td>
<td>71.4</td>
<td>31.3</td>
<td>68.8</td>
<td>70.0</td>
</tr>
<tr>
<td>Cataracts</td>
<td>92.9</td>
<td>7.1</td>
<td>93.8</td>
<td>6.3</td>
<td>80.0</td>
</tr>
<tr>
<td>Corneal capacity</td>
<td>100.0</td>
<td>7.1</td>
<td>93.8</td>
<td>6.3</td>
<td>85.0</td>
</tr>
<tr>
<td>Eye discharge/swelling</td>
<td>78.6</td>
<td>14.3</td>
<td>7.1</td>
<td>37.5</td>
<td>56.3</td>
</tr>
<tr>
<td>Microphthalmia</td>
<td>35.7</td>
<td>7.1</td>
<td>57.1</td>
<td>56.3</td>
<td>31.3</td>
</tr>
<tr>
<td>Vision loss</td>
<td>50.0</td>
<td>14.3</td>
<td>35.7</td>
<td>31.3</td>
<td>25.0</td>
</tr>
<tr>
<td>Menace reflex</td>
<td>78.6</td>
<td>21.4</td>
<td>75.0</td>
<td>25.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malocclusions</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal prolapse</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal/penile prolapse</td>
<td>57.1</td>
<td>42.9</td>
<td>50.0</td>
<td>50.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breathing rate/depth</td>
<td>42.9</td>
<td>35.7</td>
<td>21.4</td>
<td>43.8</td>
<td>50.0</td>
</tr>
</tbody>
</table>
Table 6.2 The percentage of scores by raters 1 and 3, for cohort 1 female C57BL/6 and DBA/2J mice fed either an AL diet, or a 40% CR diet (19±1 months age, n=9-11) from age 6 months, that fell into each score category (0, 0.5, 1) for each item of the frailty index.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
<th>Value</th>
<th>Value</th>
<th>Correlation Coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distended abdomen</td>
<td>100.0</td>
<td>100.0</td>
<td>85.0</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Kyphosis</td>
<td>40.0</td>
<td>45.0</td>
<td>55.6</td>
<td>5.6</td>
<td>55.0</td>
</tr>
<tr>
<td>Tail stiffening</td>
<td>25.0</td>
<td>65.0</td>
<td>10.0</td>
<td>11.1</td>
<td>66.7</td>
</tr>
<tr>
<td>Gait disorders</td>
<td>95.0</td>
<td>5.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Tremor</td>
<td>50.0</td>
<td>50.0</td>
<td>77.8</td>
<td>22.2</td>
<td>95.0</td>
</tr>
<tr>
<td>Forelimb grip strength</td>
<td>40.0</td>
<td>60.0</td>
<td>38.9</td>
<td>38.9</td>
<td>40.0</td>
</tr>
<tr>
<td>Body condition score</td>
<td>50.0</td>
<td>35.0</td>
<td>15.0</td>
<td>44.4</td>
<td>55.6</td>
</tr>
<tr>
<td>Vestibular disturbance</td>
<td>90.0</td>
<td>10.0</td>
<td>83.3</td>
<td>16.7</td>
<td>75.0</td>
</tr>
<tr>
<td>Hearing loss</td>
<td>5.0</td>
<td>30.0</td>
<td>65.0</td>
<td>6.3</td>
<td>18.8</td>
</tr>
<tr>
<td>Cataracts</td>
<td>90.0</td>
<td>10.0</td>
<td>88.9</td>
<td>11.1</td>
<td>85.0</td>
</tr>
<tr>
<td>Corneal capacity</td>
<td>100.0</td>
<td>100.0</td>
<td>95.0</td>
<td>5.0</td>
<td>90.9</td>
</tr>
<tr>
<td>Eye discharge/swelling</td>
<td>65.0</td>
<td>30.0</td>
<td>5.0</td>
<td>44.4</td>
<td>44.4</td>
</tr>
<tr>
<td>Microphthalmia</td>
<td>75.0</td>
<td>10.0</td>
<td>15.0</td>
<td>66.7</td>
<td>11.1</td>
</tr>
<tr>
<td>Vision loss</td>
<td>35.0</td>
<td>40.0</td>
<td>25.0</td>
<td>38.9</td>
<td>44.4</td>
</tr>
<tr>
<td>Menace reflex</td>
<td>90.0</td>
<td>10.0</td>
<td>83.3</td>
<td>16.7</td>
<td>85.0</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Malocclusions</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Condition</td>
<td>Value1</td>
<td>Value2</td>
<td>Value3</td>
<td>Value4</td>
<td>Value5</td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Rectal prolapse</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Vaginal/penile prolapse</td>
<td>85.0</td>
<td>15.0</td>
<td>100.0</td>
<td>100.0</td>
<td>95.5</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Breathing rate/depth</td>
<td>65.0</td>
<td>35.0</td>
<td>38.9</td>
<td>61.1</td>
<td>80.0</td>
</tr>
<tr>
<td>Mouse grimace scale</td>
<td>80.0</td>
<td>10.0</td>
<td>10.0</td>
<td>61.1</td>
<td>33.3</td>
</tr>
<tr>
<td>Pilo erection</td>
<td>35.0</td>
<td>65.0</td>
<td>11.1</td>
<td>88.9</td>
<td>90.0</td>
</tr>
</tbody>
</table>
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

The only significant effect of resveratrol on scores of frailty index items, was an increase in the proportion of mice with an above 0 score for corneal opacity in the SD-fed group (34%) compared to the resveratrol diet group (0%, p=0.005) (Table 6.3).

Table 6.3 The percentage of scores by raters 1 and 3, for cohort 2 old (24±0 months) male C57BL/6 mice fed either a control diet (AIN-93G), or an AIN-93G diet supplemented with resveratrol (RSV) (100mg/kg mouse/day) (n=16 SD, n=9 RSV,) for 6 months from age 19 months, that fell into each score category (0, 0.5, 1) for each item of the frailty index.

<table>
<thead>
<tr>
<th>Frailty Index Score</th>
<th>Male C57BL/6J Mice with Resveratrol Diet</th>
<th>Male C57BL/6J Mice with Control Diet</th>
<th>Chi Squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight Score</td>
<td>0 0.5 1</td>
<td>0 0.5 1</td>
<td>P value</td>
</tr>
<tr>
<td>Alopecia</td>
<td>100.0</td>
<td>100.0</td>
<td>NS</td>
</tr>
<tr>
<td>Loss of fur colour</td>
<td>50.0 50.0</td>
<td>25.0 71.9 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>Dermatitis</td>
<td>100.0</td>
<td>100.0</td>
<td>NS</td>
</tr>
<tr>
<td>Loss of whiskers</td>
<td>100.0</td>
<td>100.0</td>
<td>NS</td>
</tr>
<tr>
<td>Coat condition</td>
<td>38.9 61.1</td>
<td>37.5 59.4 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>Tumours</td>
<td>100.0</td>
<td>100.0</td>
<td>NS</td>
</tr>
<tr>
<td>Distended abdomen</td>
<td>72.2 27.8</td>
<td>62.5 37.5</td>
<td>NS</td>
</tr>
<tr>
<td>Kyphosis</td>
<td>88.9 11.1</td>
<td>71.9 25.0 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>Tail stiffening</td>
<td>33.3 38.9 27.8</td>
<td>18.8 59.4 21.9</td>
<td>NS</td>
</tr>
<tr>
<td>Gait disorders</td>
<td>94.4 5.6</td>
<td>100.0</td>
<td>NS</td>
</tr>
<tr>
<td>Tremor</td>
<td>100.0</td>
<td>93.8 6.3</td>
<td>NS</td>
</tr>
<tr>
<td>Forelimb grip strength</td>
<td>33.3 44.4 22.2</td>
<td>22.6 41.9 35.5</td>
<td>NS</td>
</tr>
<tr>
<td>Body condition score</td>
<td>55.6 33.3 11.1</td>
<td>46.9 34.4 18.8</td>
<td>NS</td>
</tr>
<tr>
<td>Vestibular disturbance</td>
<td>50.0 50.0</td>
<td>31.3 56.3 12.5</td>
<td>NS</td>
</tr>
</tbody>
</table>
### Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

<table>
<thead>
<tr>
<th>Condition</th>
<th>CR</th>
<th>AL</th>
<th>NS</th>
<th>CR</th>
<th>AL</th>
<th>NS</th>
<th>CR</th>
<th>AL</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hearing loss</td>
<td>61.1</td>
<td>22.2</td>
<td>16.7</td>
<td>50.0</td>
<td>26.7</td>
<td>23.3</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cataracts</td>
<td>94.4</td>
<td>5.6</td>
<td></td>
<td>100.0</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal capacity</td>
<td>100.0</td>
<td></td>
<td></td>
<td>65.6</td>
<td>34.4</td>
<td></td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eye discharge/swelling</td>
<td>55.6</td>
<td>44.4</td>
<td></td>
<td>56.3</td>
<td>37.5</td>
<td>6.3</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microphthalmia</td>
<td>94.4</td>
<td>5.6</td>
<td></td>
<td>96.9</td>
<td>3.1</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vision loss</td>
<td>5.6</td>
<td>38.9</td>
<td>55.6</td>
<td>12.9</td>
<td>38.7</td>
<td>48.4</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menace reflex</td>
<td>55.6</td>
<td>44.4</td>
<td></td>
<td>48.4</td>
<td>41.9</td>
<td>9.7</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>94.4</td>
<td>5.6</td>
<td></td>
<td>100.0</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malocclusions</td>
<td>100.0</td>
<td></td>
<td></td>
<td>100.0</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal prolapse</td>
<td>100.0</td>
<td></td>
<td></td>
<td>100.0</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal/penile prolapse</td>
<td>100.0</td>
<td></td>
<td></td>
<td>100.0</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>100.0</td>
<td></td>
<td></td>
<td>100.0</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breathing rate/depth</td>
<td>38.9</td>
<td>61.1</td>
<td></td>
<td>18.8</td>
<td>75.0</td>
<td>6.3</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse grimace scale</td>
<td>50.0</td>
<td>50.0</td>
<td></td>
<td>34.4</td>
<td>65.6</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pilo erection</td>
<td>88.9</td>
<td>11.1</td>
<td></td>
<td>81.3</td>
<td>18.8</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 6.3.3 Correlation between Frailty Index Scores and Functional Outcomes

Figure 2 shows the correlation between frailty index scores and the functional outcome measures of latency to fall for rotarod, wire hang and cage top, for a small subset of the CR and AL-fed male and female DBA/2J and C57BL/6J mice. Standard correlation analysis showed no significant correlation between frailty index scores and any functional outcomes measured in this subset of animals.
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

Figure 6.4 The correlation between frailty index and latency to fall for (A1) rotarod, (A2) wire hang, and (A3) cage top functional testing in old (19±2 months) male C57BL/6 and DBA/2 mice (n=14) and the correlation between frailty index and latency to fall for (B1) rotarod, (B2) wire hang, and (B3) cage top functional testing in old (19±1 months) female C57BL/6 and DBA/2 mice (n=21). None of the correlations showed a significant association (p>0.05).

6.3.4 Inter-Rater Reliability
The inter rater correlation coefficient across raters 1 and 2 for all mice from cohort 1 was moderate for both pre-treatment (ICC=0.522[CI 0.181-0.721]) and post-treatment
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

(ICC=0.488[CI 0.244-0.652]) assessments. The inter rater correlation coefficient across raters 1 and 3 for all mice from cohort 2 and 3 was excellent (ICC = 0.88, 95% CI [0.80, 0.92]). The mean frailty index scores presented in the data above for cohorts 2 and 3 were determined from the scores of raters 1 and 3 as they were the only raters to assess both cohorts of mice.

In order to look at the effect of background and training of the raters on the inter rater reliability of the mouse clinical frailty index, the inter rater correlation coefficient was also calculated for the four raters of cohort 2. The inter rater correlation coefficient across the 4 raters for the mice in cohort 2 indicated moderate to excellent agreement (ICC = 0.65, 95% CI [0.48, 0.77]). The inter rater correlation coefficients between each pair of raters was calculated (Table 6.4). Interestingly, although there was excellent correlation between raters 1 and 3 (ICC = 0.88, 95% CI [0.80, 0.92]), and moderate correlation between raters 4 and 5 (ICC = 0.61, 95% CI [0.34, 0.76]), there was only poor or poor-moderate correlation between the other combinations of raters.

Table 6.4 Inter rater correlation of frailty index scores for male and female old (19±2 months) DBA/2 and C57BL/6 mice from cohort 1, between each combination of 2 raters (2-way random, consistency, average intraclass correlation coefficient, with 95% confidence intervals).

<table>
<thead>
<tr>
<th>Raters</th>
<th>1</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.88 (0.80-0.92)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.20 (-0.30-0.51)</td>
<td>-0.06 (-0.73-0.35)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.49 (0.17-0.69)</td>
<td>0.451 (0.11-0.66)</td>
<td>0.605 (0.34-0.76)</td>
<td>-</td>
</tr>
</tbody>
</table>
The average percentage agreement was calculated for each item of the frailty scale as explained in the methods section. This agreement was calculated for raters 1 and 3, and for raters 1, 3, 4 and 5, across all mice scored in cohorts 2 and 3 (Figure 6.5). The items of the scale which had the worst agreement across all raters were coat condition, pilo-erection, body condition score, hearing loss, kyphosis, tail stiffening, vision loss and forelimb grip strength, with less than 75% agreement. Between raters 1 and 2 the items with less than 75% agreement were coat condition, body condition score, vision loss, forelimb grip strength and menace reflex.

Figure 6.5 The percentage agreement of scoring for each item of the frailty index for all old male and female C57BL/6 and DBA/2J mice in cohort 1 across all 4 raters (black), and across raters 1 and 3 (grey). The dotted line shows a percentage agreement of 75%.
Chapter 6: Frailty: Effect of Paracetamol, Resveratrol, Calorie Restriction and Mouse Strain

6.4 Discussion

In this chapter we found that six weeks of dietary paracetamol exposure did not affect frailty index in mice. We also found that both calorie restriction from age 6 months, and 6 months of dietary resveratrol, significantly reduced the frailty index in old C57BL/6J male mice, compared to those fed control *ad libitum* diets. Interestingly, frailty index scores were also higher for male calorie restricted DBA/2J mice compared to age and diet-matched C57BL/6J mice.

Old C57BL/6 mice (27 months) exposed to chronic low-dose paracetamol treatment for 6 weeks showed no change in frailty index, either over time, or compared to control-diet fed mice. There is some evidence to show that paracetamol may play a role in protecting against age-related changes (Rice et al. 2012; Wu et al. 2010; Lublin et al. 2011), however these studies were investigating specific isolated tissues, rather than a systemic measure, such as the frailty index. It is possible that six weeks of paracetamol exposure was insufficient to see changes in frailty index, especially as the treatment was not started until late in the mouse lifespan, and it would be interesting in future studies to look at the effect of longer term exposure to paracetamol, started earlier in life, on healthspan and frailty outcomes.

Old C57BL/6J mice (24 months) with chronic resveratrol treatment had a lower frailty index than *ad libitum* fed mice. Long term treatment with resveratrol in mice fed a standard diet has previously been shown to delay functional decline, and increase healthspan, without increasing lifespan (Pearson et al. 2008). The mouse frailty index may provide a new and important healthspan measure to be used as an outcome in interventional longevity studies. Consistent with previous studies, we found that the
resveratrol diet group had a decreased proportion of mice with corneal opacity compared to the control diet group (Pearson et al. 2008; Baur et al. 2006).

Old male C57BL/6J mice (19 months) with calorie restriction from age six months had a lower frailty index than ad libitum fed male C57BL/6J mice. This is consistent with previous studies demonstrating that calorie restriction both delays age-related decline and increases lifespan in mice (Weindruch et al. 1986; Mattson & Wan 2005). There has, however, been some recent controversy about whether the effects of calorie restriction on mice are universally positive (Swindell 2012; Liao et al. 2010; Sohal & Forster 2014). The age of initiation of calorie restriction (Cheney et al. 1980), the mouse strain (Swindell 2012; Liao et al. 2010), and the composition of the diet (Solon-Biet et al. 2014) are increasingly being recognized as important. It would be interesting in future studies to look at the effect of calorie restriction on frailty in a non-inbred mouse strain, and at the effect of dietary composition of calorie restriction on clinical mouse frailty index.

Consistent with a previous study showing DBA/2J mice are not responsive to the lifespan extension effects of calorie restriction (Forster et al. 2003), we found that frailty scores did not significantly change with calorie restriction in male and female DBA/2J mice. Earlier studies did find there was an effect of 40% calorie restriction in DBA2/J mice on longevity (Turturro et al. 1999), and on delaying age-related pathologies (Bronson & Lipman 1991). The lack of change in frailty index scores for calorie restricted DBA/2J mice in this study would suggest that calorie restriction may not delay age-related decline, or increase the healthspan of this strain of mice. The difference in frailty scores between the short-lived DBA/2J strain and the C57BL/6J strain...
strain was not as substantial as expected. This cohort of mice were younger (19 months) than the resveratrol-fed mice (24 months) so perhaps the effects of interventions on the frailty index may be more clearly seen with increasing age. We observed less variability in frailty scores at 24 months of age in our study (coefficient of variation for male *ad libitum* fed C57BL/6J was 0.16 at 24 months and 0.30 at 19 months), which is consistent with previous findings in mice (Whitehead et al. 2014) and humans (Rockwood et al. 2005). On the other hand, it may indicate that although the DBA/2J strain are up to 6 months short-lived than the C57BL/6J strain (Yuan et al. 2011), perhaps they have a comparatively longer healthspan, as indicated by moderate frailty scores. It is also possible that the frailty index scale may need to be tailored to include strain-specific ageing deficits for use in different mouse strains. The high prevalence of factors like hearing loss in the 19 month old DBA/2J mice both in this study and in much younger mice (2-3 weeks) in other studies (Willott et al. 2005) suggest that this deficit saturates too early to be useful as a deficit in assessment of a frailty index in this strain. Early saturation has previously been identified as a reason an item should be excluded as a deficit in clinical frailty studies (Searle et al. 2008).

There were some interesting changes in the specific items of the frailty index across the strain and diet groups. Calorie restriction brought the body weight and temperature scores of both DBA/2J and C57BL/6J mice back to the ‘normal’ 12 week old mouse range, compared to the *ad libitum* fed groups. This is consistent with previous studies in which *ad libitum* feeding caused young and middle-aged mice of both strains to gain weight, and this effect was stopped by calorie restriction (Sohal & Ferguson 2009). Temperature is also known to increase with feeding and decrease with calorie restriction (Rikke & Johnson 2007) and decreased core temperature is also associated with increased longevity (Conti et al. 2006).
The correlation between the functional outcomes of latency to fall for rotarod, wire hang and cage top and the frailty index was investigated for a subset of cohort 1 C57BL/6J and DBA/2J mice. There were no significant associations between any of the measured functional outcomes and frailty index. This is consistent with human data, as clinically the patients identified as frail with the frailty index, are not always identified as frail with the phenotype approach that includes measures of functional performance, and vice versa (Rockwood et al. 2007; Kulminski et al. 2008). Furthermore, as this study was completed as part of a larger study, the functional outcome testing was done at 16 months, whilst the frailty assessment was done at 19 months. The smaller sample size and the difference in time for assessment may explain the lack of correlation, and it would be interesting to explore this further, with both functional assessment and frailty index assessment completed at the same time. Graber et al (2014) developed a composite score, called a frailty intervention assessment value that they used to evaluate the effect of exercise intervention on frailty. Future studies could calculate a similar score from functional assessments, such as those measured in the current study, to determine the effect of interventions such as paracetamol, resveratrol and calorie restriction on frailty interventions.

One of our secondary aims of this study was look at the inter-rater variability in assessment of the mouse clinical frailty index. Interestingly, we found that the professional background and baseline animal training of the raters, was a main factor in affecting the inter-rater reliability. The inter-rater reliability between the animal technicians, with extensive training and experience, and researchers, with limited training and experience, was poor, despite moderate inter-rater reliability between the
technicians and moderate to excellent inter-rater reliability between the scientists. This suggests that the selection of raters for the mouse clinical frailty index must be done carefully, and preference given to raters with the same training/background if comparisons are to be made between and across mouse groups and studies.

The percentage correlation for the specific items of the frailty index was calculated to determine if there were certain items on which raters were more likely to disagree. The items of the scale which had the worst agreement across all raters were coat condition, piloerection, menace reflex, body condition score, hearing loss, kyphosis, tail stiffening, vision loss and forelimb grip strength. These items all seem to have an element of subjectivity, where the rater is required to judge the reaction of the mouse compared to ‘normal’ rather than an objective criteria such as ‘more than 25% hair loss’ for example. Feridooni et al. published a recent study also investigating the reliability of the mouse clinical frailty index, and also identified the majority of these items as those with the most discrepancies between raters (Feridooni et al. 2014). They found that after a discussion period between raters about discrepancies in scoring, inter-rater correlation increased. The use of a training and/or discussion period, or a more detailed scoring manual may have further improved the inter rater correlation in the current study. There is currently much research into the search for frailty biomarkers clinically (Darvin et al. 2014), which could provide additional objective items for the frailty index, but would add complexity and cost to the assessment.

Strengths of this study include the novelty of reporting for the first time on the impact of strain, dietary and pharmaceutical interventions on the mouse clinical frailty index (Whitehead et al. 2014), the rigorous and blinded data collection, and quality of the
animal cohorts. Some of the limitations of this study include the lack of temperature scores for one mouse cohort, and the time and sample size differences between the functional outcome scoring and the frailty index scoring. Furthermore, the short timeframe of the paracetamol exposure group does not allow us to conclude on the potential long-term frailty effects of paracetamol. It would also be interesting, in future studies, to assess the frailty index score on the same mice over time, and to assess the association of frailty prospectively with other markers of healthspan and lifespan.

In summary, the study explored in this chapter was the first to investigate the effect of mouse strain and dietary and pharmaceutical interventions on the novel clinical frailty index in mice. Whilst chronic paracetamol exposure did not affect the frailty index, resveratrol treatment and calorie restriction reduced the frailty index in ageing male C57BL/6J mice. This chapter also investigated the inter-rater reliability of the mouse clinical frailty index across several raters of varied backgrounds, and identified some aspects of the index that could be improved. The mouse clinical frailty index provides an invaluable tool for studies in ageing mice to assess the impact of interventions on frailty, as was explored in this chapter, or the impact of frailty on response to interventions, as was explored in chapters 2 and 3.
Chapter 7: Conclusions

7 CONCLUSIONS

The objective of this thesis was to help improve the safety of paracetamol for patients of all ages by adding evidence to the body of knowledge on the changing risks of paracetamol toxicity in ageing, frailty and non-acute exposures, and the potential for lifespan and healthspan increasing interventions to provide novel mechanisms of paracetamol hepatotoxicity protection. Furthermore, this thesis aimed to investigate the potential of both paracetamol, and lifespan and healthspan interventions in delaying or preventing frailty.

Methods used to investigate these aims included: the use of C57BL/6 mice as ageing models; the assessment of frailty with the novel mouse clinical frailty index; the investigation of dietary and pharmaceutical interventions, either toxic or therapeutic, in mouse ageing models; the use of SIRT1 transgenic mice to investigate mechanisms; the use of primary cell cultures; and the assessment of outcomes using a variety of biochemical and molecular techniques including realtime quantitative PCR, western blots, enzymatic activity assays and biochemical and histological assessments.
Chapter 7: Conclusions

Overall the work presented in this thesis contributed to three main findings with significant clinical significance, and several secondary findings.

Firstly, we demonstrated that there is no increase in susceptibility to paracetamol toxicity in old age or frailty, with either acute, chronic or sub-acute paracetamol exposure. This implies that paracetamol may be a safe analgesic option for use in the older and frail population, as a well-tolerated analgesic at therapeutic doses, with no increased risk of toxicity in old age, although more research is needed to confirm these findings in the clinical setting.

Secondly, an important finding of this thesis with significant clinical implications, was that the currently used paracetamol hepatotoxicity therapy, NAC, does not protect against toxicity induced by sub-acute paracetamol exposure. This highlights the need for more research into other potential therapies to protect against or treat paracetamol hepatotoxicity induced by all exposure types. Studies in this thesis investigated the potential of the lifespan and healthspan increasing mechanism of SIRT1 activation as a paracetamol hepatotoxicity therapy, and found that neither pharmacological or genetic activation of SIRT1 was protective. This was confirmed with in vitro, concurrent and pre-dosing studies in mice, and in transgenic SIRT1 knock-out and overexpressor mice. Potential future targets for paracetamol toxicity therapeutics may include increasing NQO1 activity, increasing autophagy, or further investigation of the potentially protective effects of the other ageing-related pathways such as mTOR and AMPK.

Thirdly, this thesis found that the lifespan and healthspan increasing interventions of resveratrol and calorie restriction, but not therapeutic paracetamol, were able to delay
Chapter 7: Conclusions

frailty in aged C57BL/6 mice. This study was the first to investigate dietary and pharmaceutical interventions on the novel clinical frailty index in mice, and provides the pre-clinical evidence for testing these interventions on frailty in humans.

There were other secondary findings of the studies presented in this thesis. These included the age-related changes of increased NQO1 activity in old mice and an age-related increase in pro and anti-inflammatory cytokines, and the mRNA expression of BAX and PGC1β, in response to acute paracetamol in old mice. These are potentially paracetamol hepatotoxicity protective mechanisms that interestingly, did not translate into a protection against paracetamol hepatotoxicity in our studies.

We also found that high-dose paracetamol induced phosphorylated AMPK protein expression, and SIRT1 mRNA expression in the liver, which implies a role of these pathways in paracetamol toxicity mechanisms. This requires more research and would be interesting to investigate with therapeutic paracetamol exposure.

Furthermore, these studies showed a differential protein expression response to long-term resveratrol treatment in young compared to old mice. This finding confirms the complexity of these pathways in age and with interventions and more research is needed to understand the reasons for and implications of this finding.

Finally, the studies presented in this thesis added to the body of research using the novel mouse clinical frailty index as an effective ageing research tool. We were the first to use this index to look at both frailty as an outcome and as a potential mediator of toxicity.
Chapter 7: Conclusions

We also identified some important factors to consider when using the index, such as the background and training of the raters, and the subjectivity of some of the index items. Furthermore, we showed in mice a negative correlation between the frailty index and serum protein, albumin and ALP, low levels of which have been identified as potential frailty biomarkers in humans.

In conclusion, the work presented in this thesis used a series of mouse models, to identify that age and frailty do not increase the risk of paracetamol hepatotoxicity, even for non-acute exposures. We also showed that NAC does not protect against paracetamol toxicity induced by sub-acute exposure, and that SIRT1 activation does not provide an alternative protective mechanism. Finally, we showed that the interventions of resveratrol and calorie restriction can delay frailty in mice.

The findings of this thesis represent important contributions to the bodies of work both on optimising the safety of medication use in old age and frailty, as well as increasing the healthspan and quality of life of older people by delaying the onset of frailty. There is, however, still much work to be done in both of these important areas of research.

Older people have the most need for the beneficial effects of and are the largest users of medications, yet they are also at the most risk of adverse effects from medications. Despite this, they continue to be excluded from clinical trials, and prescribed medications and medication doses without guiding empirical evidence. Research into optimising the benefits of, and perhaps even more importantly reducing the potential risks and harms from, medications in older and frail people is hugely important, but has not to date been a priority for research. This thesis added some relevant findings on this
Chapter 7: Conclusions

issue, but this is still a lot to be done to ensure that all older people are treated appropriately and safely with medications.

The optimisation of a person’s healthspan, in order to ensure that people live happily, healthily and with high quality of life for as long as possible, should be the ultimate goal and focus of all medical research. The ethical, social and personal implications of the focus upon improving our maximum lifespan, without a focus on the quality of that lifespan are serious. Research into longevity mechanisms and interventions, should continue the recent shift of focus onto investigating person-relevant outcomes such as quality of life, independence, function, and resilience, rather than just longevity. Research into delaying the onset of frailty, such as that presented in this thesis, provides one way to contribute to this focus.

“The quality, not the longevity, of one’s life is what is important.” Martin Luther King, Jr
8 REFERENCES


Chapter 8: References


Alice Kane - March 2016
Chapter 8: References


Chapter 8: References


Chapter 8: References

is NAD(+)? Pharmacological reviews, 64(1), pp.166–87.


Alice Kane - March 2016
Chapter 8: References


Court, M., M, F., Wang, X., Peter, I., et al., 2013. The UDP-glucuronosyltransferase (UGT) 1A polymorphism rs8330 is associated with increased liver acetaminophen glucuronidation, increased UGT1a exon 5a/5b splice variant mRNA ratio, and decreased risk of unintentional acetaminophen-induced acute liver fail. *Journal of Pharmacology and Experimental Therapeutics*, 345(2), pp.297–307.


Chapter 8: References


Alice Kane - March 2016
Chapter 8: References


Chapter 8: References


Gerhart-Hines, Z., Rodgers, J.T., Bare, O., Lerin, C., et al., 2007. Metabolic control of
Chapter 8: References

muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. The EMBO journal, 26(7), pp.1913–1923.


Chapter 8: References


Chapter 8: References

326.


Chapter 8: References


Chapter 8: References


Alice Kane - March 2016
Chapter 8: References


Chapter 8: References


Liang, S., Mele, J., Wu, Y., Buffenstein, R., et al., 2010. Resistance to experimental tumorigenesis in cells of a long-lived mammal, the naked mole-rat (Heterocephalus
Chapter 8: References


Chapter 8: References


Alice Kane - March 2016
Chapter 8: References


Chapter 8: References


Chapter 8: References

Syndrome: Clinical measurements and basic underpinnings in humans and animals. Experimental Gerontology, 54, pp.6–13.


Chapter 8: References

60.


Pearson, S. a., Ringland, C., Kelman, C., Mant, a., et al., 2007. Patterns of analgesic and anti-inflammatory medicine use by Australian veterans. *Internal Medicine*
Chapter 8: References


Chapter 8: References


Chapter 8: References


Scheibye-Knudsen, M., Ramamoorthy, M., Sykora, P., Maynard, S., et al., 2012. Cockayne syndrome group B protein prevents the accumulation of damaged mitochondria by promoting mitochondrial autophagy. Journal of Experimental...
Chapter 8: References

Medicine, 209(4), pp.855–869.


Chapter 8: References


Wagner, G.R. & Hirschey, M.D., 2014. Nonenzymatic Protein Acylation as a Carbon
Chapter 8: References


Chapter 8: References


9 APPENDICES

APPENDIX 1 - FRAILTY INDEX ITEMS  242

APPENDIX 2 – PRIMER SEQUENCES  247
## Appendix 1 - Frailty Index Items

Table 2: Mouse Frailty Assessment Form

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>Date of Birth</th>
<th>Sex</th>
<th>F</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>Body surface temperature (°C)</td>
<td>Date:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Integument
- Alopecia 0 0.5 1
- Loss of fur colour 0 0.5 1
- Dermatitis 0 0.5 1
- Loss of whiskers 0 0.5 1
- Coat condition 0 0.5 1

### Physical/Musculoskeletal
- Tumours 0 0.5 1
- Distended abdomen 0 0.5 1
- Kyphosis 0 0.5 1
- Tail stiffening 0 0.5 1
- Gait disorders 0 0.5 1
- Tremor 0 0.5 1
- Forelimb grip strength 0 0.5 1
- Body condition score 0 0.5 1

### Vestibular/Auditory
- Vestibular disturbance 0 0.5 1
- Hearing loss 0 0.5 1

### Ocular/Nasal
- Cataracts 0 0.5 1
- Corneal opacity 0 0.5 1
- Eye discharge/swelling 0 0.5 1
- Microphthalmia 0 0.5 1
- Vision loss 0 0.5 1
- Menace reflex 0 0.5 1
- Nasal discharge 0 0.5 1

### Digestive/Urogenital
- Malocclusions 0 0.5 1
- Rectal prolapse 0 0.5 1
- Vaginal/uterine/penile prolapse 0 0.5 1
- Diarrhoea 0 0.5 1

### Respiratory system
- Breathing rate/depth 0 0.5 1

### Discomfort
- Mouse Grimace Scale 0 0.5 1
- Piloerection 0 0.5 1

- Temperature score:  
- Body weight score:  

**Total Score/Max Score:**  
© Susan E. Howlett, 2013
## Chapter 9: Appendices

<table>
<thead>
<tr>
<th>System/Parameter</th>
<th>Clinical assessment of deficit</th>
<th>Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Integument</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Alopecia         | Gently restrain the animal and inspect it for signs of fur loss. | 0 = normal fur density  
0.5 = <25% fur loss  
1 = >25% fur loss |
| Loss of fur colour | Note any change in fur colour from black to grey or brown. | 0 = normal colour  
0.5 = focal grey/brown changes  
1 = grey/brown fur throughout body |
| Dermatitis       | Document skin lesions.        | 0 = absent  
0.5 = focal lesions (e.g. neck, flanks, under chin)  
1 = widespread or multifocal lesions |
| Loss of whiskers | Inspect the animal for signs of a reduction in the number of whiskers. | 0 = no loss  
0.5 = reduced number of whiskers  
1 = absence of whiskers |
| Coat condition   | Inspect the animal for signs of poor grooming. | 0 = smooth, sleek, shiny coat  
0.5 = coat is slightly ruffled  
1 = unkempt and un-groomed, matted appearance |
| **Physical/  
Musculoskeletal** |                               |         |
| Tumours          | Observe the mice to look for symmetry. Hold the base of the tail and manually examine mice for visible or palpable tumours. | 0 = absent  
0.5 = <1.0 cm  
1 = >1.0 cm or multiple smaller tumours |
| Distended abdomen | Hold the mouse vertically by the base of their tail and tip backwards over your hand. Excess fluid visible as a bulge below the rib cage. | 0 = absent  
0.5 = slight bulge  
1 = abdomen clearly distended |
| Kyphosis         | Inspect the mouse for curvature of the spine or hunched posture. Run your fingers down both sides of the spine to detect abnormalities. | 0 = absent  
0.5 = mild curvature  
1 = clear evidence of hunched posture |
| Tail stiffening  | Grasp the base of the tail with one hand, and stroke the tail with a finger of the other hand. The tail should wrap freely around the finger when mouse is relaxed. | 0 = no stiffening  
0.5 = tail responsive but does not curl  
1 = tail completely unresponsive |
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Rating</th>
</tr>
</thead>
</table>
| **Gait disorders**            | Observe the freely moving animal to detect abnormalities such as hopping, wobbling, circling, wide stance and weakness. | 0 = no abnormality  
0.5 = abnormal gait but animal can still walk  
1 = marked abnormality, impairs ability to move |
| **Tremor**                    | Observe the freely moving animal to detect tremor, both at rest and when the animal is trying to climb up an incline. | 0 = no tremor  
0.5 = slight tremor  
1 = marked tremor; animal cannot climb |
| **Forelimb grip Strength**    | Hold the mouse. Allow it to grip the bars on the cage lid. Lift animal by the base of the tail to assess grip strength. | 0 = sustained grip  
0.5 = reduction in grip strength  
1 = no grip strength, no resistance |
| **Body condition score**      | Place mouse on flat surface, hold tail base and manually assess the flesh/fat that covers the sacroiliac region (back and pubic bones). | 0 = bones palpable, not prominent  
0.5 = bones prominent or barely felt  
1 = bones very prominent or not felt due to obesity |

**Vestibulocochlear/Auditory**

| Vestibular disturbance       | Hold the base of the tail and lower mouse towards a flat surface. Inspect for head tilt, spinning, circling, head tuck or trunk curling. | 0 = absent  
0.5 = mild head tilt and/or slight spin when lowered  
1 = severe disequilibrium |
| Hearing loss                 | Test startle reflex. Hold a clicker ~10 cm from mouse, sound it 3 times and record responses. | 0 = always reacts (3/3 times)  
0.5 = reacts 1/3 or 2/3 times  
1 = unresponsive (0/3 times) |
| Cataracts                    | Visual inspection of the mouse to detect opacity in the centre of the eye. | 0 = no cataract  
0.5 = small opaque spot  
1 = clear evidence of opaque lens |
| Eye discharge/swelling       | Visual inspection of the mouse to detect ocular discharge and swelling of the eyes. | 0 = normal  
0.5 = slight swelling and/or secretions  
1 = obvious bulging and/or secretions |
| Microphthalmia               | Inspect eyes. | 0 = normal size  
0.5 = one or both eyes slightly small or sunken  
1 = one or both eyes very small or sunken |
## Chapter 9: Appendices

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
<th>Scores</th>
</tr>
</thead>
</table>
| **Corneal opacity**            | Visual inspection of the mouse to superficial white spots and/or clouding of the cornea. | 0 = normal  
                                   |                                               | 0.5 = minimal changes in cornea  
                                   |                                               | 1 = marked clouding and/or spotting of cornea |
| **Vision loss**                | Lower mouse towards a flat surface. Evaluate the height at which the mouse reaches towards the surface. | 0 = reaches >8 cm above surface  
                                   |                                               | 0.5 = reaches 2-8 cm above surface  
                                   |                                               | 1 = reaches <2 cm above surface |
| **Menace reflex**              | Move an object towards the mouse’s face 3 times. Record whether the mouse blinks in response | 0 = always responds  
                                   |                                               | 0.5 = no response to 1 or 2 approaches  
                                   |                                               | 1 = no response to 3 approaches |
| **Nasal discharge**            | Visual inspection of the mouse to detect nasal discharge.                    | 0 = no discharge  
                                   |                                               | 0.5 = small amount of discharge  
                                   |                                               | 1 = obvious discharge, both nares |
| **Malocclusions**              | Grasp the mouse by the neck scruff, invert and expose teeth. Look for uneven, overgrown teeth. | 0 = mandibular longer than maxillary incisors  
                                   |                                               | 0.5 = teeth slightly uneven  
                                   |                                               | 1 = teeth very uneven and overgrown |
| **Rectal prolapse**            | Grasp the mouse by the base of the tail to detect signs of rectal prolapse.  | 0 = no prolapse  
                                   |                                               | 0.5 = small amount of rectum visible below tail  
                                   |                                               | 1 = rectum clearly visible below tail. |
| **Vaginal/uterine/penile prolapse** | Grasp the mouse by the base of the tail to detect signs of vaginal/uterine or penile prolapse. | 0 = no prolapsed  
                                   |                                               | 0.5 = small amount of prolapsed tissue visible  
                                   |                                               | 1 = prolapsed tissue clearly visible |
| **Diarrhoea**                  | Grasp the mouse and invert it to check for signs of diarrhoea. Also look for fecal smearing in home cage. | 0 = none  
                                   |                                               | 0.5 = some feces or bedding near rectum  
                                   |                                               | 1 = feces plus blood and bedding near rectum, home cage smearing |

Alice Kane - March 2016  

245
### Chapter 9: Appendices

| Breathing rate/depth | Observe the animal. Note the rate and depth of breathing as well as any gasping behaviour. | 0 = normal  
0.5 = modest change in breathing rate and/or depth  
1 = marked changes in rate/depth, gasping |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Discomfort</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| **Mouse Grimace Scale** | Note facial signs of discomfort: 1) orbital tightening, 2) nose bulge, 3) cheek bulge, 4) ear position (drawn back) or 5) whisker change (either backward or forward). | 0 = no signs present  
0.5 = 1 or 2 signs present  
1 = 3 or more signs present |
| **Piloerection** | Observe the animal and look for signs of piloerection, in particular on the back of the neck. | 0 = no piloerection  
0.5 = involves fur at base of neck  
only 1 = widespread piloerection |
| **Other** |  |
| **Temperature** | Measure surface body temperature with an infrared thermometer directed at the abdomen (average of 3 measures). Compare with reference values from sex-matched adult animals. | 0 = differs by <1 SD from reference value  
0.25 = differs by 1 SD  
0.5 = differs by 2 SD  
0.75 = differs by 3 SD  
1 = differs by >3 SD |
| **Weight** | Weigh the mouse. Compare with reference values from sex-matched adult animals. | 0 = differs by <1 SD from reference value  
0.25 = differs by 1 SD  
0.5 = differs by 2 SD  
0.75 = differs by 3 SD  
1 = differs by >3 SD |

## APPENDIX 2 – PRIMER SEQUENCES

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1</td>
<td>5’- CAG TGT CAT GGT TCC TTT GC-3’</td>
<td>5’- CAC CGA GGA ACT ACC TGA T-3’</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>5’- CGC ATG GAA CTG TTT CTG C-3’</td>
<td>5’-CAA TTG TAA CAG GGC TGA GGT C-3’</td>
</tr>
<tr>
<td>NQO1</td>
<td>5’-GAG TGC TCG TAG CAG GAT TTG-3’</td>
<td>5’-ACC CCC AGT GGT GAT AGA AAG-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’-TAC TGA ACT TCG GGG TGA TTG GTC C-3’</td>
<td>5’- CAG CCT TGT CCC TTG AAG AGA ACC-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’-CTG TGG CAG CTA CCT GTG TC-3’</td>
<td>5’-GTC ACA GAG GAT GGG CTC TT -3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>5’-GCA ACT GTT CCT GAA CTC AAT C-3’</td>
<td>5’-ATC TTG TGG GGT CCG TCA ACT -3’</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5’- CCT CTG GCG AAT GGC TTT AC-3’</td>
<td>5’-GCT ATG GAT ACT GCG GTC TGG-3’</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>5’-TGA CTG GAA AGC CGA AAC TC-3’</td>
<td>5’-ATC TTC TGG CAA GCC ATC TC-3’</td>
</tr>
<tr>
<td>BAX</td>
<td>5’-CAA GAA GCT GAG CGA GTG TCT-3’</td>
<td>5’- GTG TCC ACG TCA GCA ATC AT -3’</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>5’- CCT GGC CGA GTT CTT TGA AT-3’</td>
<td>5’-GCC AGA TTT GCT TGT TGG G-3’</td>
</tr>
<tr>
<td>PGC-1β</td>
<td>5’-TGG AAA GCC CCT GTG AGA GT-3’</td>
<td>5’-TTG TAT GGA GGT GTG GTG GG -3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CAC CAA CTG CTT AGC CCC-3’</td>
<td>5’-TCT TCT GGG TGG CAG TGA TG-3’</td>
</tr>
</tbody>
</table>